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EFECTOS DE LA MELATONINA SOBRE LA FISIOLOGÍA DE LAS CÉLULAS ESTRELLADAS PANCREÁTICAS.

MATÍAS ESTARÁS HERMOSEL

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Conformidad del director/a y codirector en su caso

Fdo. Dr. D. Antonio González Mateos

Fdo. Dr. D. José Antonio Tapia García

Esta tesis cuenta con la autorización del director y codirector de la misma y de la Comisión Académica del programa. Dichas autorizaciones constan en el Servicio de la Escuela Internacional de Doctorado de la Universidad de Extremadura.

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ABREVIATURAS

- ACh: acetilcolina
- Akt: proteína cinasa B
- AMPc: adenosin monofosfato cíclico
- AMPKα (AMP-activated protein kinase α): proteína cinasa α activada por AMP
- ARE (Antioxidant Response Elements): elementos de la respuesta antioxidante
- Bad (Bcl-2-associated death promoter): proteina del promotor de muerte asoacido a Bcl-2
- Bcl-2 (B-cell lymphoma 2): proteína del protooncogén de linfoma 2 de las células B
- CCK: colecistocinina
- CEP: células pancreáticas estrelladas.
- CP: cáncer de páncreas
- CTGF (Conective tissue growth factor): factor de crecimiento del tejido conectivo
- 2-DG: 2-deoxiglucosa
- ECAR (Extracellular acidification rate): ratio de acidificación extracelular
- ERK-1/2 (Extracellular signal-regulated kinases 1/2) o p44/42: cinasas reguladas por señales extracelulares 1/2
- ERK-5 (Extracellular signal-regulated kinase 5): cinasa regulada por señales extracelulares 5
- ERN: especies reactivas de nitrógeno
- ERO: especies reactivas de oxígeno
- FAD (flavin adenine dinucleotide): flavín adenín dinucleótido
- FCCP (Carbonylcyanide p-(trifluoro-methoxy) phenylhydrazone): Carbonilcianuro-p-trifluorometoxifenilhidrazona
- FGF (Fibroblast growth factor): factor de crecimiento fibroblástico
- GFAP (*Glial fibrilar acidic protein*): proteína ácidica fribrilar glial
- Glut (glucose tranporter): transportador de glucosa
- GMPc (Cyclic guanosine monophosphate): monofosfato de guanosina cíclico
- GPX: glutatión peroxidasa
- GSK3-β (Glycogen synthase kinase-3β): glucógeno sintasa cinasa 3-β
- GSH: glutatión reducido
- GSR: glutatión reductasa
- GSSG: glutatión oxidado
- HBSS (Hank's Balanced Salt Solution): solucioón salina balanceada de Hank

- HIF (hypoxia-inducible factor): factor inducido por la hipoxia
- HK: hexoquinasa
- HO-1: hemo oxigenasa 1
- IκB (Proteins of the inhibitory κB family): proteína inhibidora de κB
- IGF (Insulin-like growth factor): factor de crecimiento similar a la insulina)
- IKK (complejo inhibidor cinasa de IκB): complejo inhibidor cinasa de IκB
- IL-1β: interleucina-1β
- IL-6: interleucina-6
- IL-11: interleucina-11
- JAK (Janus kinase): cinasa Janus
- JNK (c-Jun N-terminal kinases): cinasas c-Jun N-terminal
- KEAP1 (Kelch-like ECH-associated protein 1): proteína 1 asociada a ECH similar a Kelch
- LDH (lactate deshidrogenase): lactato deshidrogenada
- LPS: lipopolisacárido
- MAPKs (Mitogen activated protein kinases): proteínas cinasas activadas por mitógenos.
- MCP-1 (monocyte chemoattractant protein-1): proteína quimioatrayente de monocitos 1
- MDA: malondialdehido
- MEC: matriz extracelular
- MMP: metaloproteinasa
- MT1: receptor para la melatonina 1
- MT2: receptor para la melatonina 2
- mTOR (mammalian target of rapamycin): proteína diana de rapamicina en células de mamífero
- NAD (Nicotinamide adenine dinucleotide): dinucleótido adenina nicotinamida
- NF-κB (Nuclear factor kappa-light-chain-enhancer of activated B cells): factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas
- Nfr2 (Nuclear factor erythroid 2-related factor 2): factor 2 relacionado con el factor nuclear eritroide 2
- NGF (neural growth factor): factor de crecimiento neural
- NIK (NF-κB-inducing kinase): cinasa inductora de NF-κB
- NO: óxido nítrico
- NQO-1: N-quinona oxidorreductasa 1
- OCR (oxygen consumption rate): ratio del consumo de oxígeno

- OMS: Organización Mundial de la Salud
- PBS (phosphate-buffered saline): tampón fosfato salino
- PC: pancreatitis crónica
- PDAC (Pancreatic Ductal AdenoCarcinoma): adenocarcinoma de células ductales.
- PDGF (Platelet derived growth factor): factor de crecimiento derivado de plaquetas
- PDK-1 (3-phosphoinositide-dependent protein kinase-1): proteína cinasa 1 dependiente de 3-fosfoinositido
- PFK (Phosphofructokinase): fosfofructoquinasa
- PHD (Prolyl-hydrolases protein): prolil-hidrolasas
- PI3-K (Phosphoinositide 3-kinase): fosfoinositol-3 cinasa
- PIP_2: fosfatidilinositol 4,5-bifosfato
- PIP_3: fosfatidilinositol 1,4,5-trifosfato
- PKB (protein kinases B): proteínas cinasas B
- PKB (protein kinases C): proteínas cinasas C
- PTEN (Phosphatase and tensin homolog): proteína homologa de fosfatasa y tensina o fosfatidilinositol-3,4,5-trisfosfato 3-fosfatasa
- RORα (RAR-related orphan receptor α): receptor α huerfáno relacionado con RAR
- α-sma (Alpha smooth muscle actin): alfa-actina de músculo liso
- SOD: superóxido dismutasas
- STAT (signal transducer and activator of transcription): transductor de señales y activador de la transcripción
- TAC (Total antioxidant capacity): capacidad antioxidante total
- TCA (tricarboxylic acid cycle): ciclo de los ácidos tricarboxílicos
- TGF-β1 (Transforming growth factor β1): factor de crecimiento transformante β1
- TIMP (Tisular inhibitor of metaloproteinases): inhibidor tisular de las metaloproteinasas
- TMRM: tetrametilrodamina metil éster
- TNF- α (Tumoral necrosis factor α): factor de necrosis tumoral α
- TRPC (transient receptor potential cation channels): canales cationicos de potenciales transitorios
- VEGF (Vascular endothelial growth factor): factor de crecimiento del endotelio vascular.
- VLDL (Very low density lipoprotein): lipoproteínas de muy baja densidad.

Indice

1. RESUMEN

La fibrosis pancreática es una característica común en patologías pancreáticas como la inflamación y el cáncer. Las células estrelladas pancreáticas (CEP) han sido identificadas como las principales responsables del desarrollo del estroma y se ha hipotetizado que el control de esta población celular podría ser una diana terapéutica para el manejo de estas patologías. La melatonina ha ganado atención durante los últimos años debido a sus propiedades antioxidantes, antiinflamatorias y anticancerosas. En esta Tesis Doctoral nos hemos propuesto estudiar el posible efecto anti-fibrótico de esta molécula, investigando sus efectos sobre las CEP en condiciones tanto de normoxia como de hipoxia. Además, hemos abordado las adaptaciones que sufren estas células bajo hipoxia. Hemos evaluado diferentes aspectos de la fisiología celular que incluyen la viabilidad y proliferación celular, el estado oxidativo, la función mitocondrial y el metabolismo glucolítico. Los resultados muestran que la melatonina reduce la proliferación de estas células, modulando diferentes vías de señalización como las rutas MAPKs o PI3K/Akt/mTOR. La melatonina también induce un estado prooxidante y altera la fisiología mitocondrial. Por su parte, la hipoxia también induce un ambiente prooxidante en las CEP, ante el cual las células sufren diferentes adaptaciones de la respuesta antioxidante o del metabolismo energético, lo que les permite no solamente sobrevivir sino, además, incrementar su estado proliferativo. En este contexto de hipoxia, la melatonina también reduce la proliferación evocada por la hipoxia, a través de vías que involucran la resolución del estrés oxidativo o la alteración de la función mitocondrial. A la vista de estos resultados, la melatonina podría considerarse como un posible agente terapéutico de la fibrosis pancreática.

1. ABSTRACT

Pancreatic fibrosis is a common feature in pancreatic pathologies such as inflammation and cancer. Pancreatic stellate cells (PSC) have been identified as the main responsible for the development of the stroma and it has been hypothesised that the control of this cell population could be a therapeutic target for the management of these pathologies. Melatonin has gained attention along the past years due to its antioxidant, anti-inflammatory and anticancer properties. In this PhD Thesis we have investigated the possible anti-fibrotic effect of this molecule, examining its effects on PSC either under normoxic or under hypoxic conditions. In addition, we have addressed the adaptations that these cells undergo under hypoxia. We have evaluated different aspects of cell physiology including proliferative state, oxidative state, mitochondrial function and glycolytic metabolism. The results show that melatonin reduces the viability snt the proliferation of these cells by modulating different signalling pathways such as MAPKs or PI3K/Akt/mTOR. Melatonin also induces a pro-oxidative state and impairs mitochondrial physiology. On its side, hypoxia also induces a pro-oxidant environment in PSC, in response to which cells undergo different adaptations of the antioxidant response or in the energy metabolism, that allow them not only to survive but also to increase their proliferative state. In this context of hypoxia, melatonin also reduces hypoxia-evoked proliferation, through pathways involving the resolution of oxidative stress or the altered mitochondrial function. In view of these results, melatonin could be considered as a potential therapeutic agent for pancreatic fibrosis.

2. INTRODUCCIÓN

El desarrollo de un excesivo tejido fibrótico en el páncreas es una marca patológica común entre dos de las enfermedades más prevalentes y graves que afectan a esta glándula, la pancreatitis crónica y el cáncer de páncreas. En la pancreatitis crónica, el daño en las estructuras acinares y ductales, acompañado de un estado de inflamación sostenida, conduce al desarrollo de este tejido fibrótico que desplaza al tejido funcional. Esto compromete la funcionalidad de la glándula. En el cáncer de páncreas, el tejido fibrótico tiene una doble consecuencia. La reacción desmoplásica protege a las células tumorales del ataque del sistema inmunitario, los tratamientos quimioterapéuticos y la radiación. Sin embargo, produce la encapsulación tumoral evitando la diseminación del tumor y la formación de metástasis. Hasta la fecha, no se ha desarrollado ninguna terapia eficaz para el tratamiento de estas patologías que use como diana el desarrollo de este tejido estromal.

El conocimiento sobre la fibrosis pancreática y su implicación en las patologías del páncreas experimentó un gran avance cuando se identificó a las células responsables de la formación del tejido fibrótico en la glándula, las células estrelladas pancreáticas (CEP). En condiciones normales, estado de salud o de ausencia de enfermedad, estas células se encuentran en el denominado “estado de quiescencia” y son las responsables de la regulación y el mantenimiento de la matriz extracelular del páncreas. Sin embargo, ante señales de daño liberadas por las células acinares, las células inmunitarias y/o tumorales, o por la acción de sustancias exógenas como el alcohol o el tabaco, estas células sufren un cambio fenotípico hacia un denominado “estado activado”. En este estado, se incrementa la proliferación celular y la producción de una excesiva cantidad de proteínas de matriz extracelular. Estos y otros cambios son los responsables del desarrollo del tejido fibrótico.

El establecimiento de un estado de hipoxia tisular, definida como un estado de baja disponibilidad de oxígeno, es una consecuencia, no exclusiva, del desarrollo de la fibrosis en el tejido. Para poder sobrevivir en este ambiente, las células tienen que adaptar su fisiología. Estudiar los cambios que sufren las CEP en un entorno que mimetiza al microambiente patológico, nos puede permitir encontrar nuevas dianas terapéuticas para controlar el cambio hacia el fenotipo activado. Además, la hipoxia modifica la sensibilidad de las células al tratamiento. Es por ello, que estudiar los efectos

de cualquier droga bajo modelos que imitan el estado patológico nos puede permitir conocer de manera más realista su eficacia.

La melatonina, el principal producto hormonal de la glándula pineal, ha recibido un gran interés por parte de la comunidad científica durante los últimos años. Se ha descrito su participación en diferentes procesos fisiológicos como la regulación de los ciclos de sueño y vigilia, la función reproductora, la inmunidad o la actividad del sistema gastrointestinal. Alteraciones en los ciclos de secreción de melatonina en trabajadores de turnos nocturnos se ha relacionado con el incremento en la incidencia de cáncer en esta población. Otras propiedades atribuidas a esta molécula son su capacidad antioxidante, antiinflamatoria e inmunomoduladora. Sin embargo, los efectos de esta molécula parecen ser muy dependientes del contexto celular. En células sanas demuestra un papel protector, mientras que en células transformadas desempeña un papel tóxico. Estas observaciones han permitido atribuir a la melatonina una posible función de “guardián o protector de la salud”.

En esta tesis doctoral examinaremos el efecto de la melatonina sobre la fisiología de las CEP en condiciones tanto de normoxia como de hipoxia. Nos centraremos en estudiar los efectos sobre la viabilidad y proliferación celular, y las vías moleculares implicadas en el control de estos parámetros. Además, estudiaremos los efectos sobre el estado redox y la activación de diferentes sistemas antioxidantes. Y finalmente, abordaremos las adaptaciones del metabolismo energético que pudieran tener lugar en las CEP bajo los tratamientos ensayados. Estos datos nos permitirán conocer los efectos de la melatonina como agente modulador de la fibrosis pancreática.

2. INTRODUCTION

The development of excessive fibrotic tissue in the pancreas is a common pathological hallmark in two of the most prevalent and severe diseases affecting this gland, chronic pancreatitis, and pancreatic cancer. In chronic pancreatitis, damage to the acinar and ductal structures, accompanied by a state of sustained inflammation, leads to the development of fibrotic tissue that replaces functional tissue. This compromises the functionality of the gland. In pancreatic cancer, fibrotic tissue has a dual effect. The desmoplastic reaction protects tumour cells from the attack by the immune system, chemotherapeutic treatments and radiation. However, it results in tumour encapsulation, preventing tumour dissemination and metastasis formation. To date, no effective therapy has been developed for the treatment of these pathologies pointing to the development of the stromal tissue as a target.

The understanding of pancreatic fibrosis and its involvement in pancreatic pathologies underwent a significant advance when were identified the cells responsible for the formation of fibrotic tissue in the gland, namely the pancreatic stellate cells (PSC). In the healthy pancreas, these cells exhibit a so-called “quiescent state” and are responsible for the regulation and maintenance of the extracellular matrix. However, in response to damage-signals, released by acinar cells, immune and/or tumour cells, or by the action of exogenous substances such as alcohol or tobacco, these cells undergo a phenotypic switch to a termed “activated state”. In this state, PSC exhibit increased proliferation and the production of excessive amounts of extracellular matrix proteins. These and other changes are responsible for the development of fibrotic tissue in the pancreas.

The establishment of a condition of tissular hypoxia, defined as a state of low oxygen availability, is a non-exclusive consequence of the development of fibrosis in the tissue. In order to survive in this environment, cells have to undergo adaption in their physiology. The study of the changes that PSC display in an environment that mimics the pathological microenvironment established in inflammation and/or cancer, may allow us to find new therapeutic targets to control the shift towards the activated phenotype. In addition, hypoxia changes the sensitivity of the cells to treatments. Therefore, studying the effects of any drug under the conditions that mimic the pathological state may allow us to gain a more realistic understanding of its efficacy.

Melatonin, the main hormonal product of the pineal gland, has received a high interest from the scientific community in the recent years. Its involvement in various physiological processes such as the regulation of sleep-wake cycles, reproductive function, immune response and the activity of the gastrointestinal system has been described. Alterations in melatonin secretion cycles in night-shift workers have been related with an increased incidence of cancer. Other properties attributed to this molecule comprise of its antioxidant, anti-inflammatory and immunomodulatory capacity. Interestingly, the effects of this molecule are completely dependent on the cellular context. In healthy cells. it appears to play a protective role, whereas in transformed cells it shows a toxic role. These observations have signalled melatonin as a possible "health-warden or caretaker".

In this doctoral thesis we will examine the effects of melatonin on the physiology of PSC under normoxic and under hypoxic conditions. We will focus on the effects on cell viability and proliferation, and on the molecular pathways involved in their control. In addition, we will study the effects on the red-ox state and the activation of different antioxidant systems. Finally, we will address the adaptations in energy metabolism that may occur in PSC under the treatments tested. These data will allow us to understand the effects of melatonin as a modulating agent of pancreatic fibrosis.

3. REVISIÓN BIBLIOGRÁFICA

3.1. El páncreas: estructura y función.

El páncreas es un órgano anexo del tracto gastrointestinal, localizado en la parte posterior del estómago, en posición inferior a éste y anterior a los grandes vasos sanguíneos prevertebrales (Figura 3.1.) [1]. Se trata de una glándula túbulo-acinar compuesta. Esta víscera está formada principalmente por estructuras acinares y sistemas ductales. El contenido secretado por los acinos se colecta por los conductos o ductos que recorren toda la glándula hasta verter el contenido en los grandes conductos pancreáticos. Los conductos pancreáticos convergen con el conducto colédoco, el cual comunica la vesícula biliar, el hígado y el páncreas con el intestino delgado a nivel del duodeno, en la ampolla de Water.

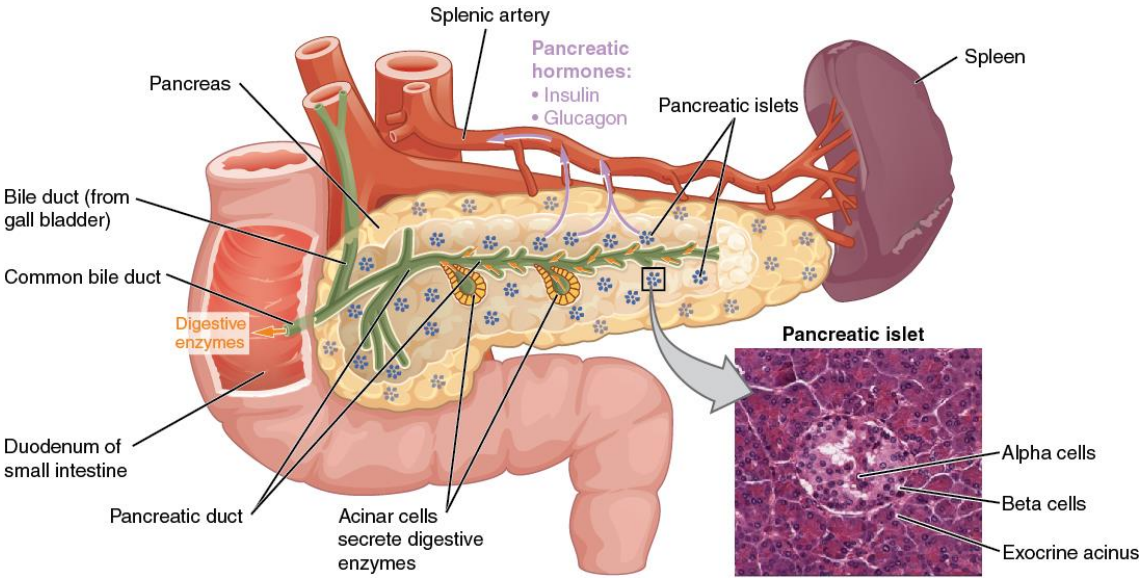

Figura 3.1. Anatomía del páncreas en humano. *Fuente: https://www.share4rare.org/es/library/pancreatic-rare-tumours/introduccion-al-pancreas*

La vascularización arterial del páncreas compete a las arterias esplénica, gastroduodenal y mesentérica superior [1]. Estos vasos mayores se ramifican en arterias o ramas menores, que irrigan toda la víscera. La sangre venosa se drena en la vena porta a través de las venas mesentérica superior y esplénica. La linfa del páncreas se drena a través de los ganglios linfáticos pancreatoduodenales, pilóricos y pancreáticos, conectados a su vez a los ganglios linfáticos regionales celiacos y mesentéricos superiores.

La inervación del páncreas corresponde al sistema nervioso autónomo [2]. Las fibras simpáticas que inervan el páncreas proceden del plexo celiaco y las fibras parasimpáticas del tronco vagal posterior. En el páncreas, las fibras parasimpáticas son más abundantes que las fibras simpáticas. La inervación parasimpática está relacionada con los procesos secretores-digestivos, mientras que la inervación simpática se ha relacionado con la sensibilidad al dolor. Estas terminaciones nerviosas mantienen sinapsis con distintas células del páncreas como las células acinares, las células de los islotes o con la musculatura de los vasos sanguíneos.

A nivel histológico [1], el páncreas puede dividirse en:

- El parénquima pancreático. Esta porción está formada por los acinos pancreáticos, los sistemas ductales y los islotes de Langerhans. Representa la parte funcional del páncreas.
- El estroma pancreático. El estroma está formado por tejido conectivo laxo a través del cual se localizan los vasos sanguíneos y linfáticos, las fibras nerviosas y los conductos secretores. La función del estroma es dar soporte al parénquima.

A nivel fisiológico, el páncreas es una glándula mixta o anficrina, es decir, tiene una función exocrina y una endocrina [3].

La función exocrina es llevada a cabo por los acinos pancreáticos y los sistemas ductales (Figura 3.2.) [4]. Los acinos pancreáticos están formados por una agrupación de células acinares cuya función es la secreción de diferentes enzimas digestivas como la tripsina, la lipasa o la amilasa, entre otras. Además, en el lumen de los acinos también se localizan otra población celular conocida como células centroacinares. Esta población celular participa, junto con las células ductales, en la secreción del componente hidroelectrolítico del jugo pancreático. El componente hidroelectrolítico es una solución hipertónica rica en iones bicarbonato, cuya función es proporcionar un pH adecuado para la actividad enzimática y amortiguar el pH ácido del jugo gástrico. Otro componente celular de los acinos pancreáticos son las células epiteliales cilíndricas glandulares. Estas células se localizan entre las células acinares y las células formadoras de los ductos y su función es secretar el mucus que protege y lubrica los conductos pancreáticos.

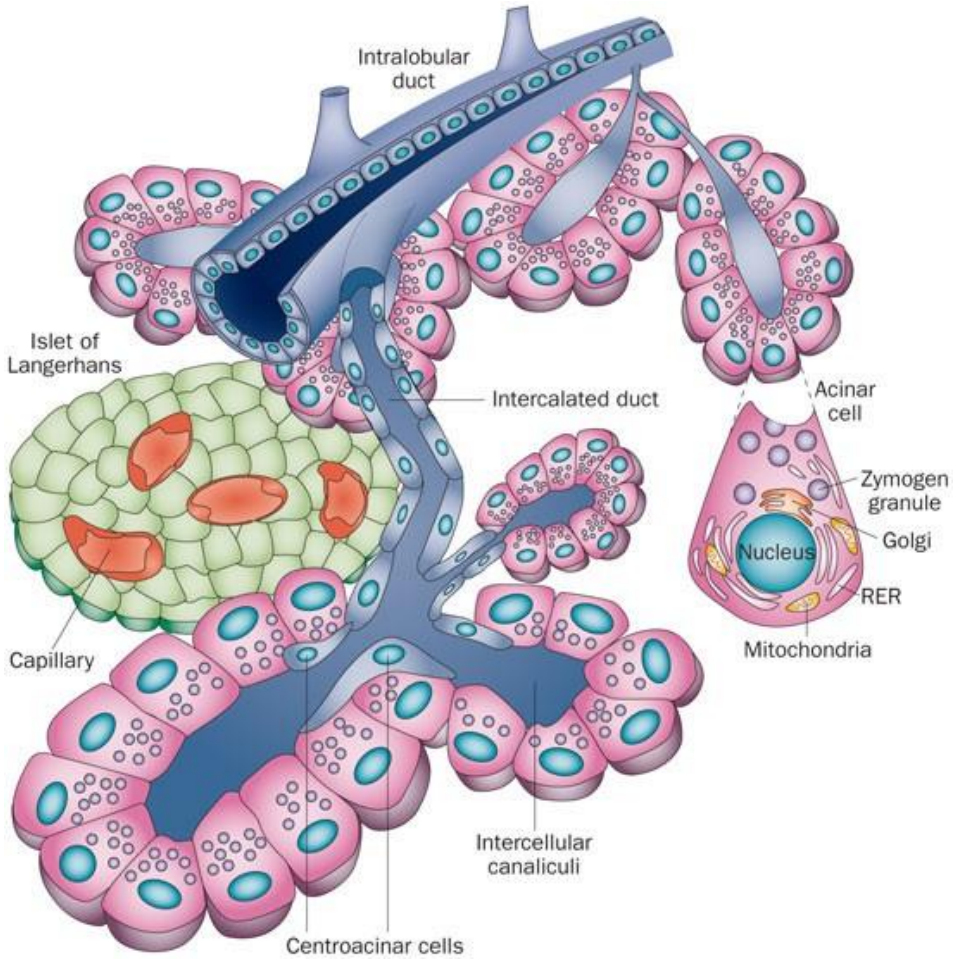

Figura 3.2. La histología del páncreas exocrino. *Los acinos pancreáticos están formado por la agrupación de células acinares encargadas de la síntesis de enzimas digestivas. Estas células están altamente especializadas y presentan una morfología muy característica. Se trata de una célula polarizada: el núcleo se encuentra en la región basal rodeado por una retículo endoplasmático muy desarrollado y en la zona apical es donde se produce la exocitosis de los gránulos que contienen los zimógenos precursores de las enzimas digestivas. Los ductos y sistemas ductales constituyen un sistema por el que circulan las secreciones producidas por las células acinares. Fuente: The role of protein synthesis and digestive enzymes in acinar cell injury* [4]

La función endocrina del páncreas es realizada por los islotes pancreáticos o de Langerhans [1]. Estos islotes representan aproximadamente el 1% del páncreas y son agrupaciones de diferentes tipos celulares localizadas entre las estructuras acinares. Estas células secretan diferentes hormonas que se transportan por el torrente circulatorio por todo el organismo. Las células que conforman los islotes son [5]:

- Células α. Son las células encargadas de la síntesis y secreción del glucagón. Se localizan en la periferia de los islotes y representan alrededor del 20% de las células de los islotes.
- Células β. Son las células mayoritarias de los islotes y se encargan de la síntesis y secreción de la insulina. Junto con el glucagón, esta hormona participa en el control hormonal de la glucemia.

- Células δ. Esta población es responsable de la síntesis y secreción de la hormona somatostatina. En el páncreas, esta hormona puede actuar inhibiendo la producción de las hormonas secretadas por las células α y β.
- Células PP o F. Estas células participan en la síntesis y secreción del polipéptido pancreático. Este péptido tiene acciones fisiológicas sobre el páncreas, regulando la producción hormonal de otras células de los islotes, y sobre el tracto gastrointestinal, controlando el vaciamiento gástrico y la motilidad intestinal.
- Otras células que conforman los islotes son las células productoras de grelina, las células productoras de serotonina (células enterocromafines), células productoras de gastrina (células G) y células que contienen pequeños gránulos de contenido desconocido (células P/D1).

3.2. La fibrosis pancreática, un proceso patológico común en la pancreatitis crónica y el cáncer pancreático

La pancreatitis crónica (PC) es un síndrome fibrótico-inflamatorio del páncreas, en el cual repetidos episodios de inflamación y destrucción del parénquima conducen a la pérdida de la funcionalidad del órgano [6]. Generalmente, el evento que desencadena el proceso inflamatorio y fibrótico en la PC es el daño inducido en el parénquima por la activación intraglandular de las enzimas digestivas proteolíticas secretadas por el páncreas. La incidencia de la PC varía entre 1,6-23 casos cada 100.000 habitantes/año [7]. Respecto a la etiología de esta enfermedad, se han descrito factores genéticos y ambientales que contribuyen al desarrollo de la PC [8]. La exposición a tóxicos como el alcohol o el tabaco son factores de riesgo para el desarrollo de PC, siendo el alcoholismo responsable del 65% de los casos de PC [9]. Otras causas menos frecuentes de desarrollo de PC son procesos obstructivos de la glándula o fenómenos autoinmunes [6]. El ambiente inflamatorio y lesivo establecido en el páncreas durante la PC incrementa el riesgo de mutaciones de las células de la glándula, siendo esta patología un factor de riesgo para el desarrollo del cáncer de páncreas [10].

Según datos de la Agencia Internacional de la Investigación del Cáncer de la OMS, el cáncer de páncreas (CP) es actualmente el séptimo tipo de cáncer más mortal [11], con una clara tendencia ascendente en este funesto ranquin. El adenocarcinoma de células ductales (PDAC) es el principal tipo de tumor del páncreas, representando el 90% de los casos [12]. En Europa, la incidencia del CP estandarizada con la edad es de 18,7 casos por 100.000 habitantes. La tasa de supervivencia a los 5 años es el 11,5%, aunque este valor depende del estadio en que se diagnostica el tumor [13]. El

diagnostico se realiza frecuentemente en etapas donde el tumor ya ha invadido los ganglios linfáticos regionales u otros órganos, siendo imposible la resección del tumor [14]. Los tratamientos quimioterapéuticos de primera elección son el FOLFIRINOX, una combinación de diferentes drogas citotóxicas, y la gemcitabina. Estos fármacos tienen una gran citotoxicidad y efectos adversos muy frecuentes y graves, lo que limita su uso y eficacia [14]. El diagnostico tardío y las pocas herramientas terapéuticas disponibles son las principales causas de la baja supervivencia del cáncer de páncreas.

La fibrosis pancreática es una característica patológica común en la pancreatitis crónica y el cáncer pancreático [12] (Figura 3.3.). El origen del tejido fibrótico es el proceso de cicatrización o curación de heridas [15]. Señales de daño liberadas por el tejido acinar dañado en la PC o por las células tumorales en el CP contribuyen a la activación de la respuesta inflamatoria, que recluta en el tejido dañado a las células inmunitarias con el fin de destruir las células muertas y dañadas. Además, estas señales de daño y los mediadores proinflamatorios liberados por las células inmunitarias actúan de manera paracrina sobre los fibroblastos residentes y células mesenquimales para transformarse en células miofibroblásticas. Estos miofibroblastos liberan factores que contribuyen a la activación de más células de este tipo y secretan proteínas de matriz extracelular. En la respuesta de cicatrización, los miofibroblastos activados son eliminados mediante apoptosis cuando se resuelve el daño que indujo su activación. Sin embargo, en el proceso fibrótico, la activación de estos miofibroblastos se sostiene tras la desaparición del estímulo y esta población continúa proliferando y secretando proteínas de matriz extracelular [16]. Por tanto, el tejido fibrótico está compuesto por un denso depósito de proteínas de matriz extracelular y por las células productoras de sus componentes.

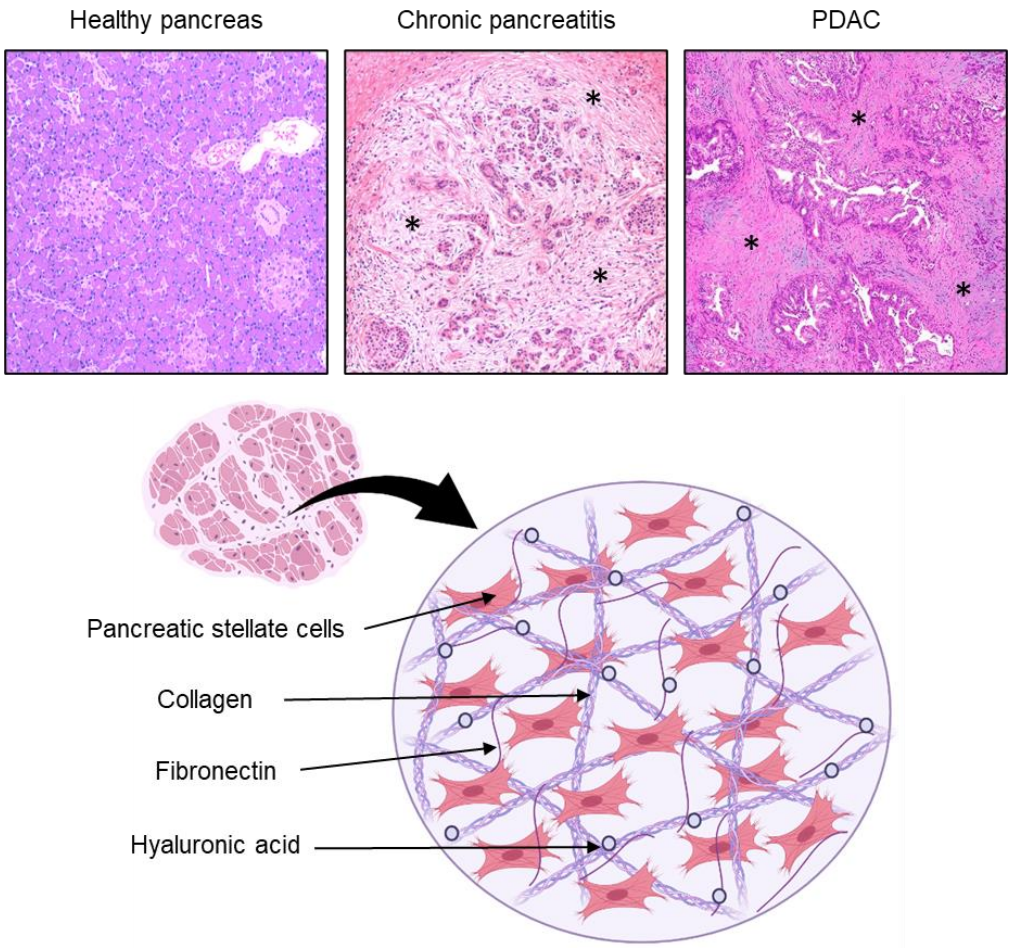

Figura 3.3. La fibrosis pancreática. *El tejido fibrótico pancreático está formado por una densa red de proteínas de matriz extracelular y por las células responsables de su secreción, las CEP. Esta característica patológica es común en la pancreatitis crónica y el cáncer de páncreas. La imagen de la histología del páncreas sano fue tomada de: http://medcell.org/histology/digestive_organs_lab/pancreas.php La imagen de la histología de la pancreatitis fue tomada de: https://basicmedicalkey.com/chronic-pancreatitis-2/ La imagen de la histología del PDAC fue tomada de: https://www.pathologyoutlines.com/topic/pancreasductal.html Imagen creada con Biorender.com*

En la pancreatitis crónica, la excesiva producción de tejido fibrótico reemplaza al tejido acinar dañado como consecuencia del proceso citolítico e inflamatorio, produciendo la pérdida de la estructura funcional del páncreas y, con ella, la función pancreática.

Respecto al cáncer pancreático, el tejido fibrótico o estroma puede suponer entre un 50-80% del volumen tumoral [17]. La formación de tejido fibrótico o reacción desmoplásica tiene consecuencias tanto negativas como positivas para el desarrollo y crecimiento del tumor [18]. El tejido fibrótico protege a las células tumorales del ataque del sistema inmunitario, impide la llegada de los quimioterapéuticos y las protege de la radiación. Sin embargo, esta encapsulación del tumor por el tejido fibrótico impide la diseminación del tumor y la formación de metástasis.

Poder controlar la excesiva formación del tejido fibrótico puede servir para establecer una herramienta terapéutica útil para el manejo de estas patologías, las cuales se sitúan

entre las principales afecciones del páncreas y tienen un riesgo muy elevado para la vida del paciente.

3.3. Las células estrelladas pancreáticas

Las células estrelladas pancreáticas (CEP) fueron descubiertas en 1982 por el equipo de Watari y colaboradores, tras examinar al microscopio muestras de tejido pancreático obtenidas de ratones tratados con altas dosis de vitamina A [19]. Estos autores describieron cómo estas células con capacidad de almacenar gotas lipídicas con alto contenido en vitamina A se localizaban distribuidas aleatoriamente en el estroma y en las zonas perivasculares del tejido pancreático. En 1998, el equipo de JM Wilson y MV Apte aisló y caracterizo a las CEP obtenidas del páncreas de ratón [20]. Estos investigadores identificaron esta población celular en secciones histológicas de páncreas sanos y establecieron un método de aislamiento y cultivo que permitió un gran avance en el conocimiento de la biología de las CEP y su implicación en los procesos patológicos.

3.3.1. Las CEP en estado de quiescencia

En condiciones fisiológicas, las CEP representan aproximadamente el 4-7% de la población celular del páncreas [21]. Estas células se localizan en la periferia de los acinos, los conductos pancreáticos y los vasos sanguíneos conformando el estroma del páncreas (Figura 3.4.). En estado de salud o condiciones fisiológicas normales, las CEP muestran unas características fenotípicas y funcionales asociadas a un estado denominado de quiescencia o no activado. Las CEP quiescentes se caracterizan por:

- Una morfología estrellada [20]. Las CEP presentan un cuerpo celular central del que emanan proyecciones citoplasmáticas dando lugar a la morfología en forma de estrella.
- La presencia de gotas lipídicas citoplasmáticas con alto contenido en Vitamina A [22]. Estas estructuras lipídicas contienen colesterol, ésteres de colesterol, fosfolípidos, triacilglicéridos y otros ácidos grasos libres. Además, se ha detectado la presencia de la proteína albúmina en estas gotas.
- La expresión de diferentes tipos de filamentos intermedios como la vimentina, la desmina, la proteína acídica fibrilar glial (GFAP) o la nestina [23]. Estos marcadores sirven para identificar a esta población celular. Además, la expresión de esta gran variedad de marcadores específicos de

diferentes estirpes celulares muestra la gran diversidad funcional que tiene esta población celular.

- La expresión de las proteínas adipofilina y citoglobina [24]. La expresión de estas proteínas se ha reportado en muestras de tejido pancreático humano sano.
- Una tasa proliferativa baja [20].

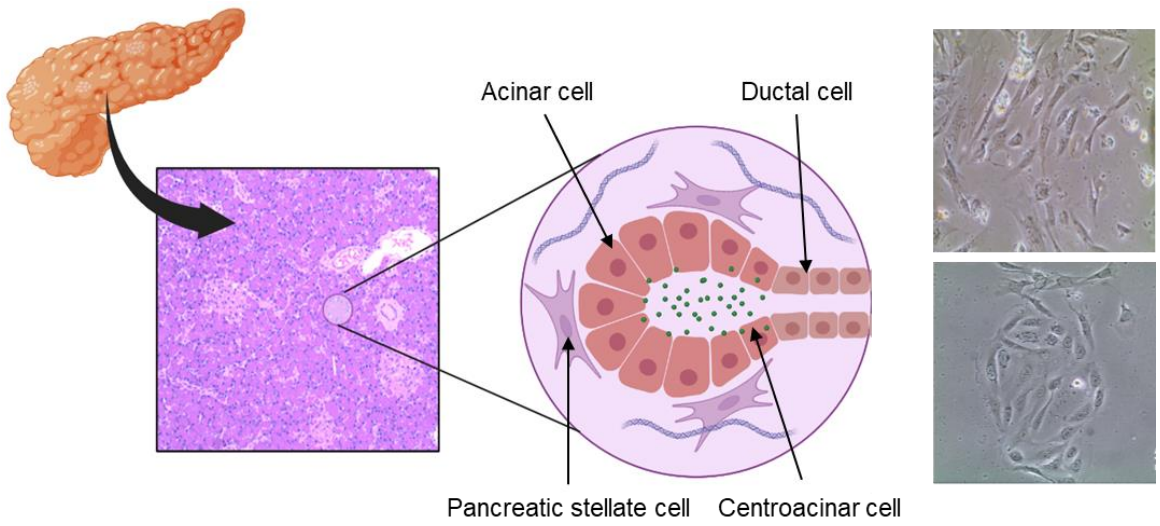

Figura 3.4. Localización de las CEP en el páncreas sano. *Las CEP se localizan en los espacios periacinares e interductales preservando la arquitectura del páncreas. Las fotografías muestran cultivos primarios de CEP obtenidas de rata y humanos. Figura elaborada con Biorender.com.*

La función principal de las CEP es el mantenimiento y la regulación de la matriz extracelular [21]. La matriz extracelular (MEC) es una red formada por proteínas fibrosas y proteoglicanos que proporciona soporte mecánico y biológico a las células, y participa en los procesos de diferenciación, migración y proliferación celular [25]. El colágeno es la proteína principal de la MEC [26]. Otros componentes fundamentales de la MEC son fibronectina, laminina y ácido hialurónico [27,28]. Las CEP son las principales células encargadas de secretar las proteínas que componen esta MEC [29]. Además, también controlan el recambio de estas proteínas, pues las CEP secretan las metaloproteinasas (MMP), enzimas encargadas de la degradación de las proteínas de la MEC, y los inhibidores de estas enzimas (TIMPs).

Se han descrito otras funciones no relacionadas con el mantenimiento de la homeostasis de la MEC para las CEP. Estas funciones están relacionadas con un papel inmunitario [30,31], como célula progenitora pluripotente [32–34] o con el proceso digestivo [35].

3.3.2. El proceso de activación de las CEP

En respuesta al daño tisular inducido durante la pancreatitis o el cáncer de páncreas, las CEP sufren un cambio fenotípico desde un estado de quiescencia hacia un estado activado [21] (Figura 3.5.). Los factores que pueden inducir la activación, denominados activadores, abarcan desde citocinas proinflamatorias, factores de crecimiento, especies reactivas de oxígeno, sustancias tóxicas exógenas como el alcohol o el tabaco, hasta estados de hipoxia o la presión mecánica [36]. Estos activadores actúan de manera paracrina sobre las CEP e inducen los siguientes cambios:

- A nivel morfológico, la activación induce un cambio de las CEP hacia un fenotipo miofibroblástico [37]. Pierden las gotas lipídicas con alto contenido en Vitamina A y expresan la proteína α-actina de musculo liso (α-sma). Esta proteína sólo se expresa en las CEP activadas y es empleada como el principal marcador de activación de estas células.
- A nivel funcional, la activación induce un incremento en la proliferación y la migración celular, en la producción y secreción de citoquinas proinflamatorias y factores de crecimiento, en la producción y depósito de proteínas de la MEC (especialmente colágeno tipo I y tipo III) [37]. Además del aumento en la producción de proteínas de MEC, durante la activación se produce un aumento en la expresión de las MMPs, lo que da lugar a una desregulación de la MEC que, consecuentemente, conduce al establecimiento del tejido fibrótico.

3.3.2.1. Activadores de las CEP

El conocimiento sobre la implicación de las CEP en la fibrosis pancreática y el desarrollo de la metodología necesaria para establecer cultivos de estas células, han permitido descubrir una gran variedad de sustancias, metabolitos y/o condiciones que inducen la activación. Estos activadores pueden catalogarse en diferentes grupos:

- Citocinas proinflamatorias y factores de crecimiento. Algunas citocinas descritas con capacidad activadora sobre las CEP son: la interleucina-1β (IL-1β), la interleucina-6 (IL-6), la interleucina-11 (IL-11), el factor de necrosis tumoral α (TNF- α), el factor de crecimiento tumoral β1 (TGF-β1), el factor de crecimiento derivado de las plaquetas (PDGF), el factor de crecimiento fibroblástico (FGF), el factor de crecimiento de tejido conectivo (CTGF), el factor de crecimiento del endotelio vascular (VEGF) o el factor de crecimiento similar a la insulina (IGF) [38–43].

- Tóxicos y toxinas exógenas. Algunos tóxicos como el etanol y sus metabolitos, acetaldehído y los ésteres etílicos de ácidos grasos, o la nicotina se han señalado como activadores de las CEP [44,45]. Dietas ricas en grasas se han relacionado con la producción de fibrosis, por los efectos activadores de las lipoproteínas de baja densidad (VLDL) sobre las CEP [46]. Toxinas exógenas como el lipopolisacárido (LPS) pueden modular la activación de estas células [47].
- Alteraciones fisicoquímicas en el páncreas. Respecto a las propiedades físicas, un aumento en la presión mecánica en el páncreas activa a las CEP [48]. Otros cambios en las condiciones del microambiente del páncreas como incremento del estado oxidativo, baja disponibilidad de oxígeno, incrementos en la concentración de glucosa [49,50].
- Otros factores biológicos. Diferentes factores biológicos han sido descritos con capacidad de inducir la activación de las CEP entre los que se encuentran: la endotelina-1 [51], el factor pigmentario derivado del epitelio [52], la galectina-1 [53], la tripsina[54] o el fibrinógeno [55].

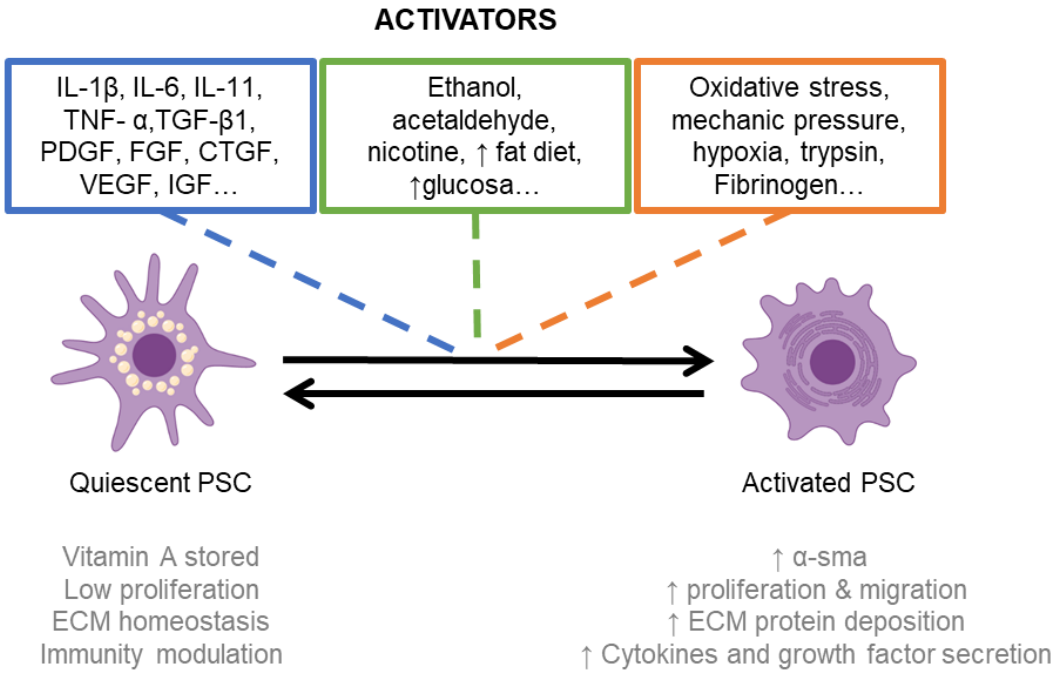

Figura 3.5. Activación de las CEP. *Ante una gran cantidad de mediadores, las CEP pueden sufrir un cambio fenotípico desde un estado de quiescencia hacia un estado de activación. En este estado activado, estas células incrementan la capacidad proliferativa y migratoria, la síntesis y secreción de proteínas de MEC y la secreción de citocinas y factores de crecimiento que contribuyen al desarrollo de un estado inflamatorio del páncreas.*

3.3.2.2. Vías de señalización intracelular como biomarcadores de la activación de las CEP.

Todos los factores que median la activación de las CEP, actúan a través de un complejo entramado de vías de señalización intracelular que inducen los cambios propios del fenotipo activado de estas células. El conocimiento de estas vías de señalización nos permite identificar biomarcadores celulares del proceso de activación de las CEP, así como determinar nuevas dianas moleculares para el control de esta población celular. Las principales vías o elementos de la señalización intracelular que intervienen en la activación de las CEP se describen a continuación.

3.3.2.2.1. Vía de señalización de las MAPKs.

Las proteínas cinasas activadas por mitógenos (MAPKs), comprenden una familia de proteínas cinasas de serina-treonina que constituyen un elemento central en la señalización intracelular que media en procesos como la proliferación, la migración, la respuesta a estrés o la muerte celular [56]. Los principales miembros de esta familia de cinasas incluyen a: cinasas reguladas por factores extracelulares (ERK-1/2 o p44/42), cinasas c-Jun N-terminal (JNK), cinasas p38 (p38) y cinasas reguladas por factores extracelulares 5 (ERK-5).

El modelo de activación canónico o estándar de estas vías de señalización de la MAPKs involucra una cascada de fosforilaciones activadoras secuenciales [57] (Figura 3.6.). Las MAPKs van a fosforilar a diferentes factores de transcripción (FT) en residuos de Ser-Thr, produciendo la activación de estos e induciendo la expresión de los genes bajo el control de estos FT. Estas MAPKs son activadas por fosforilación en residuos de Tyr y Thr por las cinasas de las MAPKs (MAP2K o MAPKK o MEK). A su vez, estas MEK son activadas por fosforilación en residuos de Ser y Thr por las cinasas de las MEK (MAP3Ks o MEKK). La activación de estas últimas cinasas MEKK está controlada por señales extracelulares acoplados a receptores de membrana con actividad cinasa o a proteínas G.

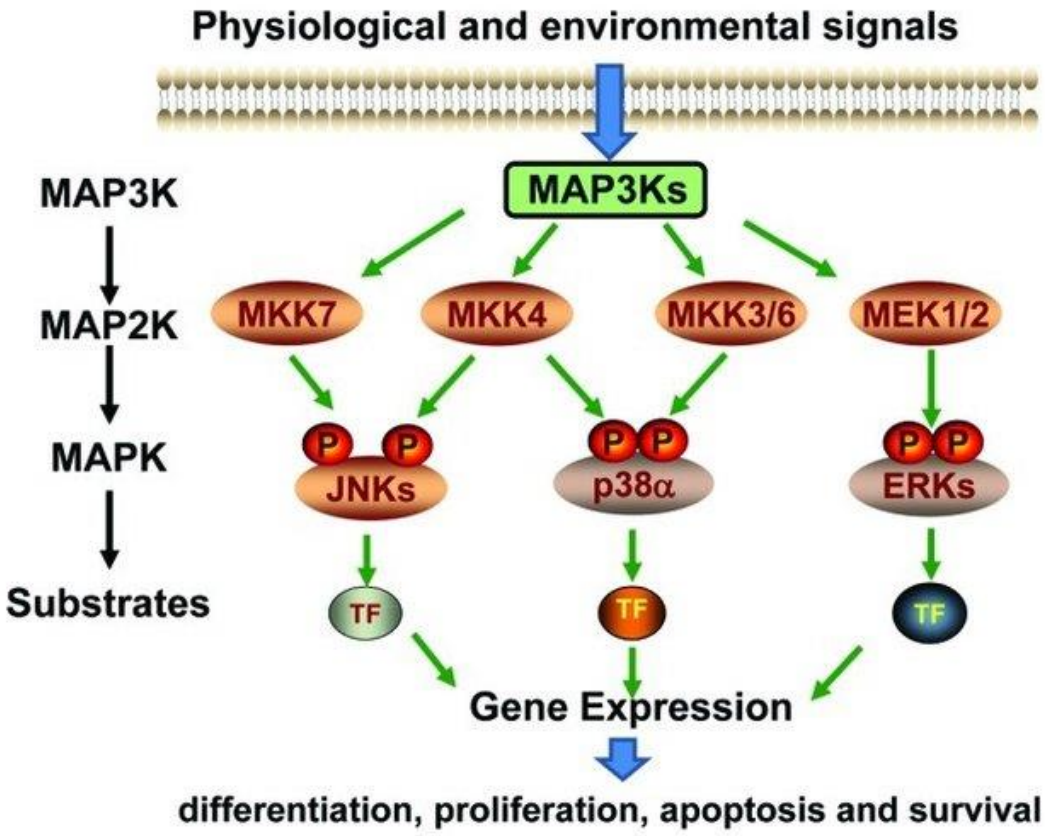

Figura 3.6. Vía de señalización de las MAPKs*. La activación de esta familia de proteínas se ejecuta como una cascada de reacciones de fosforilación que permiten la activación de factores de transcripción que modulan la expresión génica. Están involucradas en diferentes procesos celulares como la diferenciación, la proliferación o la supervivencia celular. Fuente: Assessing developmental roles of MKK4 and MKK7 in vitro*[58].

La activación de p44/42 se ha reportado tanto en las células tumorales pancreáticas como en el estroma de muestras de pacientes de CP [59]. Se han descrito multitud de activadores de las CEP que inducen la activación de p44/42 [48,60–62]. El PDGF activa la vía de Raf-1/p44/42 y la inhibición química de p44/42 con PD98059 o trapidil reduce la proliferación de las CEP [61]. El etanol y el acetaldehído también incrementan la activación de p44/42 en las CEP, sin embargo, la inhibición de estas cinasas no revierte el incremento en la expresión de α-sma inducido por estos activadores [63]. El TFG-β1 incrementa la activación de p44/42 en las CEP. La inhibición de esta cinasa revierte la expresión de α-sma y fibronectina inducida por el TFG- β1 [64]. El fibrinógeno también incrementa la activación de p44/42 en las CEP [55]. La inhibición farmacológica de p44/42 revertió parcialmente el incremento de las interleucinas 6 y 8 evocada por el fibrinógeno. Además, moléculas con capacidad de revertir la activación de las CEP hacia el estado quiescente, como la curcumina o la vitamina A, reducen la activación de estas cinasas [65,66]. Todos estos trabajos apuntan a que p44/42 tiene un claro papel “pro-activación” en las CEP.

Respecto al papel de JNK en la activación de las CEP, la inhibición de estas cinasas con el antagonista SP600125 redujo la proliferación inducida por el PDGF, la producción de colágeno inducida por el suero y la producción de la proteína quimioatrayente de monocitos 1 (MCP-1) inducida por la IL-1 y el TNF [67]. En un modelo *in vivo* de pancreatitis crónica inducida por ceruleína en ratón, el tratamiento con un extracto acuoso de *Gardenia jasminoides* redujo la desactivación de JNK y p44/42 y la deposición de tejido fibrótico en el páncreas [68]. Otros activadores de las CEP también han mostrado la capacidad de modular la activación de estas cinasas como el TFG-β1 o el etanol [63,64].

p38 es activada en respuesta a estresores y citocinas proinflamatorias [57]. En el páncreas se ha descrito la activación de esta cinasa durante la PC [69]. En relación con la función de esta cinasa en el tejido estromal, la activación de las CEP inducida por altas concentraciones de glucosa es mediada por la activación de la vía de señalización de la proteína cinasa C (PKC)/p38 [70]. La inhibición farmacológica de p38 inhibe la activación de las CEP, incluyendo la reducción de la capacidad proliferativa, de la secreción de citocinas proinflamatorias y de la expresión de diferentes marcadores de activación [71].

Hasta la fecha, no se ha explorado el papel de ERK-5 ni de ningún otro miembro de la familia de las MAPKs en la biología de las CEP.

A pesar del claro papel “pro-fibrótico” que ha mostrado la activación de los principales miembros de la familia de las MAPKs, todavía no se ha establecido ninguna herramienta terapéutica frente a la fibrosis pancreática que tenga como diana esta vía central en la activación de las CEP.

3.3.2.2.2. Vía de señalización de PI3-K/Akt

La vía de la fosfoinositol-3 cinasa (PI3-K)-proteína cinasa B (PKB o Akt) regula diferentes procesos celulares como la proliferación, el crecimiento, la muerte celular, la migración o el metabolismo [72]. La PI3-K fosforila el lípido de membrana fosfatidilinositol 4,5-bifosfato (PIP_2) para formar el fosfatidilinositol 1,4,5-trifosfato (PIP_3 o IP_3) [73]. La fosfatidilinositol-1,4,5-trifosfato 3-fosfatasa u homólogo de fosfatasa y tensina (PTEN) cataliza la reacción inversa de la PI3-K. El PIP_3 actúa como un segundo mensajero que recluta en la membrana a proteínas con dominios de homología a pleckstrina (PH) como Akt. Una vez traslocada la Akt a la membrana, esta cinasa es activada por fosforilación por otra cinasa que también es reclutada por el PIP_3, la proteína cinasa dependiente de 3-fosfoinositido (PDK1). Una vez Akt ha sido activada, esta cinasa puede activar o inhibir mediante fosforilación a una extensa red de proteínas

entre las que se incluyen (Figura 3.7.): la proteína diana de rapamicina en células de mamíferos (mTOR), la glucógeno sintasa cinasa 3-β (GSK3-β), la cinasa inhibidora de la subunidad beta de la quinasa kappa-B del factor nuclear (IKK), la proteína pro-apoptótica promotor de muerte asociado a Bcl-2 (Bad) o la caspasa 9, entre otras [72]. Estas y otras proteínas bajo control de Akt modulan distintos procesos como el ciclo celular, la apoptosis, la proliferación, el crecimiento, la síntesis de proteínas, la migración o la reparación de ADN.

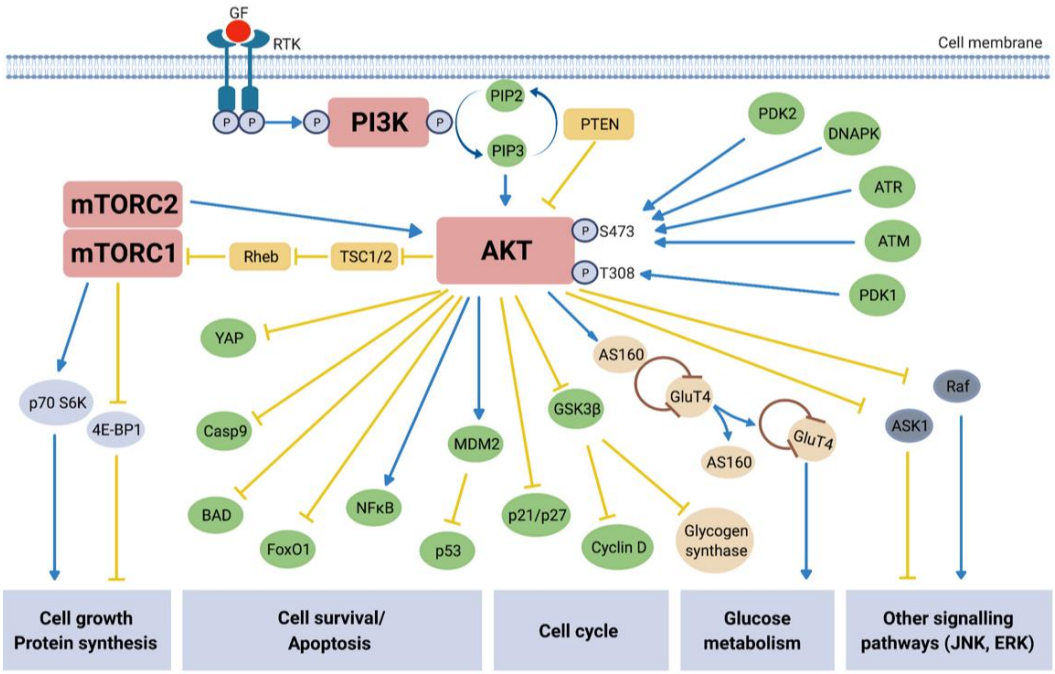

Figura 3.7. Vía de señalización de PI3K/Akt/mTOR*. Esta vía de señalización modula la activación de una gran plétora de proteínas intracelulares. Fuente: Targeting the PI3K/AKT/mTOR pathway in epithelial ovarian cancer, therapeutic treatment options for platinum-resistant ovarian cancer* [74].

Los trabajos que muestran la participación de esta ruta en la regulación de las CEP son cuantiosos. Así, la vía de PI3-K/Akt parece mediar la migración de las CEP inducida por el activador PDGF [75,76]. El tratamiento de las CEP con resveratrol reduce la activación de Akt, la proliferación y la expresión de α-sma, colágeno y fibronectina inducida por el activador TGF-β1 [77]. La molécula 2 liberadora de monóxido de carbono (CORM-2) induce la parada del ciclo celular en fase G0/G1 en las CEP modulando la vía de PI3-K/Akt/Mtor [78]. El 2,3-dehidroflavonoide ha demostrado reducir la activación de las CEP y reducir la fosforilación de PI3-K y Akt [79]. Respecto a los mecanismos que pudieran inducir la activación de Akt en el proceso de activación de las CEP, un trabajo reciente ha mostrado que la movilización de Ca^{2+} a través del canal Orai-1 media la proliferación y la secreción de TFG-β1 vía activación de Akt [80].

Contrariamente a estas observaciones, la coenzima Q, molécula descrita con capacidad inhibitoria sobre las CEP, activa la vía de señalización PI3-K/Akt/mTOR e

inhibe la autofagia [81,82]. Esta misma observación se realizó con el tratamiento con Saikosaponina D, el componente activo principal de la planta con propiedades medicinales *Bupleurum falcatum* [83].

3.3.2.2.3. Vía de señalización de NF-κB

El factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas (NF-κB) se ha descrito como el factor de transcripción máster de la respuesta inflamatoria-inmunitaria [84]. Sin embargo, esta vía de señalización también participa en el control de la proliferación celular y la apoptosis, la angiogénesis, la metástasis o el metabolismo [85]. NF-κB es una familia de factores de transcripción compuesta por cinco miembros: RelA (p65), RelB, c-Rel, p50 y p52 [86]. Estos miembros forman homodímeros o heterodímeros e interaccionan con la molécula de ADN. p65, RelB y c-Rel poseen un dominio C-terminal transactivador que permite activar la expresión de los genes dianas. p50 y p52 no contienen este dominio de interacción con el ADN, por lo que no pueden funcionar como homodímeros.

La activación de este factor de transcripción se produce en respuesta a una gran cantidad de estímulos como citocinas, factores de crecimiento, productos de microorganismos, estrés oxidativo, daños en el ADN…[85] Estos activadores pueden inducir la activación de NF-κB a través de dos rutas: la ruta canónica y la ruta alternativa. En el modelo canónico, NF-κB se encuentra secuestrado en el citoplasma por la proteína inhibidora de κB (IκB). Los estímulos arriba mencionados inducen la activación del complejo inhibidor cinasa de IκB (IKK), el cual fosforila a IκB marcándolo para su posterior ubiquitinación y degradación por el proteasoma. De esta forma, NF-κB se libera de su secuestrador y puede translocarse al núcleo donde ejerce su actividad transcripcional. El modelo alternativo, no requiere la fosforilación y degradación del IκB. En este modelo, el precursor de p52, p100, es activado por la cinasa inductora de NF-κB (NIK) lo que induce su proteólisis dando lugar a p52.

El papel de NF-κB ha sido evaluado recientemente por Nan Wu y colaboradores [87]. Estos investigadores observaron cómo la expresión y activación de este factor de transcripción se inducía en las CEP en modelos de fibrosis pancreática inducida por ceruleína. La activación de NF-κB en respuesta al activador TFG-β1 incrementaba la expresión del marcador α-sma y la producción de MCP-1. Otros hallazgos han mostrado que los efectos del TFG- β1 sobre las CEP involucran la señalización a través de NF-κB [88]. La activación de NF-κB en las CEP promueve mecanismos de inmunoevasión tumoral, reduciendo la infiltración de linfocitos T citotóxicos que destruirían a las células tumorales [89]. Diferentes activadores de las CEP han mostrado un claro efecto

activador de NF-κB [90,91], e inversamente se ha observado un efecto inhibitorio con moléculas que inducen la reversión a quiescencia [77,92–95].

3.3.2.2.4. Vía de señalización de Nrf2.

El factor 2 relacionado con el factor nuclear eritroide 2 (Nrf2) es el factor de transcripción máster que controla los elementos de la respuesta antioxidante (ARE) [96]. En condiciones en las que las células no están sometidas a un estrés oxidativo, Nrf2 forma un complejo en el citoplasma con la proteína 1 asociada a ECH similar a Kelch (KEAP1) que favorece la rápida degradación de Nrf2 por el proteasoma [97]. En condiciones de estrés oxidativo, algunos residuos de cisteína de KEAP1 sufren modificaciones que inducen un cambio conformacional de esta proteína, que ahora no impide la ubiquitinación y posterior degradación de Nrf2 por el proteasoma. Mediante este mecanismo, Nrf2 se estabiliza, acumula y transloca al núcleo donde ejerce su actividad transcripcional, induciendo la expresión de numerosas enzimas detoxificadoras de fase II (Figura 3.8.).

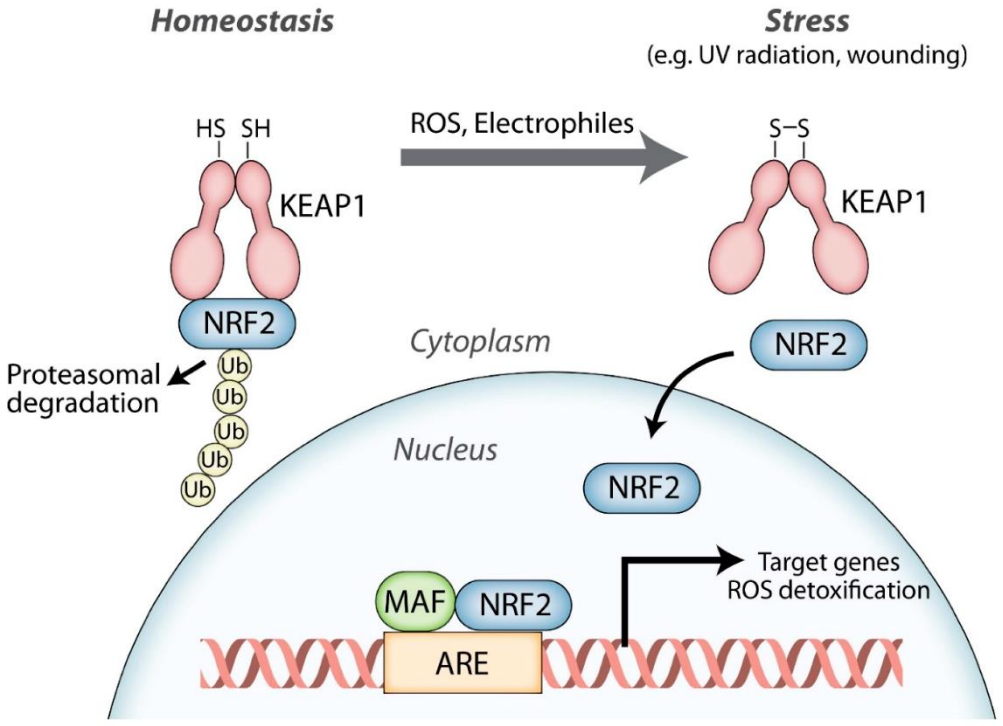

Figura 3.8. Regulación de la activación de Nrf2. *En condiciones homeostáticas, Nrf2 forma un complejo en el citoplasma con la proteína KEAP1. Esta interacción retiene a Nrf2 en el citoplasma y lo marca por ubiquitinación para su degradación por el proteasoma. Ante un estrés oxidativo, el complejo KEAP1-Nrf2 sufre cambios conformacionales que permiten la liberación del factor de transcripción. Entonces, Nrf2 puede ser activado por fosforilación y translocado al núcleo, donde se unirá a los elementos de respuesta antioxidante e inducir su expresión. Fuente: Regulation of Wound Healing by the NRF2 Transcription Factor—More Than Cytoprotection* [98].

Nrf2 se encuentra alterado en una gran variedad de patologías, que abarcan la inflamación, las enfermedades neurodegenerativas o el cáncer. En relación con la fibrosis pancreática, la actividad de Nrf2 es necesaria para mantener una elevada tasa

proliferativa y migratoria en las CEP [99]. El medio condicionado por cultivo de CEP deficientes de Nrf2 tiene una menor capacidad mitogénica sobre células tumorales pancreáticas, comparado con el medio condicionado de CEP *wild-type*. En un modelo de fibrosis pancreática inducida por ácido sulfónico 2,4,6-trinitrobenceno (TNBS), el tratamiento con L-cisteína redujo el desarrollo de tejido fibrótico [100]. Se observó un incremento en la expresión de Nrf2 y las enzimas antioxidantes hemo-oxigenasa 1 (HO-1) y N-quinona oxidorreductasa 1 (NQO-1). Estos autores proponen a Nrf2 como la diana del tratamiento con L-cisteina en este modelo de fibrosis pancreática.

3.3.2.2.5. Otras vías de señalización implicadas en la activación de las CEP

Existen otras vías de señalización cuya actividad modula la activación de las CEP que no han sido objeto de estudio de este proyecto, pero que constituyen nuevas e interesantes líneas de trabajo para el futuro. Entre estas vías destacamos la vía de JAK/STAT, la vía de Hippo, la vía de Wnt/β-catenina y la vía de Hedgehog.

Vía de señalización de JAK/STAT

La vía de señalización de la cinasa Janus (JAK), también denominada transductor de señales y activador de la transcripción (STAT), permite la recepción de un gran número de estímulos extracelulares entre los que se incluyen citocinas, hormonas o factores de crecimiento [101]. La familia de cinasas Janus incluye a 4 miembros y son proteínas tirosina cinasa asociadas a receptores. La familia de proteínas STAT la conforman 7 miembros y son factores de transcripción. La activación de esta vía comienza con la unión de una hormona, factor de crecimiento o una citocina a su receptor en la membrana plasmática [102]. Esta unión induce un cambio en la estructura del receptor en la cara interna de la membrana que permite la unión y actividad cinasa de JAK. La activación de JAK reclute a STAT que se activa y dimeriza. De esta forma, STAT se activa y se transloca al núcleo donde ejerce su actividad transcripcional.

La activación de esta vía ha sido reportada en CEP tratadas con activadores como el PDGF o la nicotina [103,104]. El tratamiento con ruxolitinib, un inhibidor de la cinasa JAK, redujo la activación de las CEP *in vitro* y la deposición de tejido fibrótico y marcadores de fibrosis en el modelo de pancreatitis crónica inducida por ceruleína [105].

Vía de señalización de Hippo

La vía de señalización de Hippo controla una gran variedad de procesos celulares como la proliferación, la diferenciación, la apoptosis, la embriogénesis o la reparación tisular, entre otros [106]. Esta vía puede ser activada por estrés mecánico, por cambios en el estado energético de la célula o por señales recibidas a través de receptores

acoplados a proteínas G[107]. Esta ruta de comunicación intracelular está compuesta por una cascada de cinasas que conducen a la activación/desactivación de los factores de transcripción *Yes-associated protein* (YAP) y su parálogo, la proteína coactivador transcripcional con motivos de unión PDZ (TAZ) [106]. Cuando la ruta se encuentra activa, se produce la fosforilación de los factores de transcripción mencionados, los cuales quedan retenidos en el citoplasma. Cuando YAP y/o TAZ se desfosforilan, estos se translocan al núcleo donde se unen a otros factores de transcripción, pues estas proteínas carecen de regiones de unión al ADN, e inducen la expresión de genes que controlan la proliferación.

Esta vía de señalización se encuentra alterada en numerosas patologías, entre las que se incluyen aquellas que afectan al páncreas [108]. En relación con la fibrosis pancreática, se ha demostrado como la proteína YAP incrementa su expresión en el tejido fibrótico y se ha correlacionado este incremento con la activación de las CEP [109,110]. Además, la inhibición de YAP suprime el desarrollo del cáncer de páncreas por mecanismos que impiden la comunicación entre estroma y tumor [111].

Vía de señalización de Wnt/β-catenina

La vía de señalización de Wnt/ β-catenina es una ruta de comunicación intracelular muy conservada, que controla procesos como la apoptosis, la proliferación, la diferenciación, la estabilidad genética o la migración [112]. Las proteínas Wnt son una familia de glicoproteínas ricas en cisteína, secretadas por las células a la MEC y que interaccionan con receptores específicos en la superficie de las células. Estos receptores pertenecen a la familia de proteínas Frizzled, que son receptores transmembranas acoplados a proteínas G. La interacción de Wnt con su receptor induce la disrupción del complejo de β-catenina, un complejo formado por las proteínas axin, la proteína *adenomatous polyposis coli* (APC), la cinasa caseina 1α, GSK3β y β-catenina. Esta última, liberado el complejo, se transloca al núcleo y se une con el factor de transcripción *T cell factor/lymphoid enhancer factor-1*.

Esta vía de comunicación parece tener diferentes funciones en la fibrogénesis del páncreas [113]. Las CEP secretan la proteína Wnt2, que actúa de manera paracrina sobre las células tumorales induciendo su proliferación [114]. El ácido retinoico, un inhibidor de la activación de las CEP, media sus acciones bloqueando la activación de esta vía [115,116]. Además, esta ruta parece estar implicada en la función de soporte energético que realizan las CEP sobre las células tumorales, modulando el aporte de glutamina para la progresión de las células tumorales[117].

Vía de señalización de Hedgehog

La vía de señalización de Hedgehog (Hh) juega un papel fundamental en el desarrollo embrionario, aunque también se encuentra alterada en procesos patológicos como la inflamación o el cáncer [118]. La familia de proteínas Hedgehog incluye tres miembros (Sonic-Hh, Indian-Hh y Desert-Hh) que actúan como ligandos extracelulares al receptor de membrana Patched[119]. Cuando este receptor se encuentra libre de ligandos, forma un complejo con la proteína Smoothened. Tras la unión del ligando, esta proteína se libera y realiza un procesamiento post-trasduccional de la proteína *glioma-associated oncogene* (GLI)1/2 (GLI). Entonces, esta proteína puede liberarse de su secuestrador citoplasmático, translocarse al núcleo e inducir la expresión de sus genes dianas.

En las CEP se ha detectado la expresión de las proteínas que componen esta ruta. Además, esta expresión se incrementó en estados patológicos en los que las CEP tienen un papel patogénico importante como la pancreatitis crónica[120]. La proteína Sonic-Hh, liberada por células tumorales, actúa de manera paracrina sobre las CEP induciendo su activación[121]. Otro interesante hallazgo ha propuesto a esta vía de comunicación celular como la responsable del dolor asociado en las patologías del CP o la PC[122]. El mecanismo propone que la activación de esta vía en las CEP induce un incremento en la producción de NFG, siendo este el responsable de la activación de fibras de dolor.

Ca^{2+} como segundo mensajero

El ion calcio (Ca^{2+}) actúa como segundo mensajero intracelular controlando numerosos procesos celulares como la expresión génica, la secreción, la contracción, la división o la muerte celular [123].

A diferencia de las células acinares pancreáticas, las CEP no movilizan Ca^{2+} cuando son estimuladas con ACh o CCK[124]. Sin embargo, estas células si responden con oscilaciones de este ion cuando son estimuladas con bradicinina, un péptido derivado de la acción proteolítica de la calicreína sobre el cininógeno[125].

En los últimos años, se está investigando en mayor profundidad el papel del Ca^{2+} en la activación de las CEP. La activación de los canales de calcio TRPC1 en respuesta a estímulos mecánico induce oscilaciones en la concentración intracelular de Ca^{2+} en las CEP, las cuales incrementan su grado de activación[126]. Se han descrito otros canales para Ca^{2+} en las CEP, con funciones relacionadas con la modulación de la migración o la respuesta de estas células a la hipoxia[126,127].

3.3.2.3. Eventos post-activación de las CEP.

La activación de las CEP o el desarrollo del tejido fibrótico tiene como objetivo controlar y reparar la estructura que se ha dañado. Se trata del mismo mecanismo o respuesta de cicatrización de las heridas[128]. Si la inflamación y el daño desaparece o se produce de forma aislada y transitoria, las CEP pueden entrar en apoptosis o regresar al estado quiescente[129]. Sin embargo, en el proceso fibrótico, las señales de activación de las CEP no se detienen tras la reparación, por diferentes razones:

- En la pancreatitis crónica, el daño repetido sobre el páncreas emite señales de daño que activan a las CEP.
- En el cáncer de páncreas, las células tumorales secretan factores paracrinos que estimulan a las CEP.
- Las propias CEP secretan factores autocrinos que amplifican su activación.

Todos estos factores condicionan la activación sostenida de las CEP y perpetúa la reacción fibrótica.

3.4. La hipoxia

El oxígeno molecular (O_2) es un reactivo necesario en muchas reacciones bioquímicas que ocurren en la inmensa mayoría de seres vivos del planeta. En la atmosfera terrestre, la concentración de este gas se sitúa en torno al 21%, condición a la que nos referimos como normoxia [130]. Sin embargo, la concentración de oxígeno en los tejidos sanos varía dependiendo de la localización del mismo [131]. En el páncreas, la concentración o porcentaje de O_2 tisular es del 6,8%. Sin embargo, de manera fisiológica o patofisiológica, la concentración de O_2 en un determinado tejido puede verse drásticamente disminuida [132]. La hipoxia se define como el estado en el que la disponibilidad o concentración de O_2 es baja (1%, o incluso inferior).

El estado de hipoxia es una condición muy frecuente en algunos cánceres, especialmente en tumores sólidos como el cáncer de páncreas [133]. De hecho, los tumores pancreáticos son uno de los tipos de tumores más hipóxicos, sufriendo un descenso de la concentración de O_2 tisular de unas 17 veces [134]. Esta condición se establece por diferentes motivos:

1) Las células que componen los tumores utilizan y agotan el O_2 presente en los tejidos.
2) Los vasos sanguíneos que se forman en el foco tumoral no son funcionales y no perfunden correctamente los tumores (angiogénesis aberrante).
3) La reacción desmoplásica o la formación del tejido estromal encapsulan el tumor e impiden la correcta difusión de este gas a través del tumor.

Bajo esta condición de hipoxia, las células presentes en los tumores sufren diferentes adaptaciones que les permiten sobrevivir y proliferar activamente en este contexto (Figura 3.9.)[134]. Conocer estas adaptaciones nos puede permitir establecer nuevas dianas terapéuticas que nos posibiliten controlar el crecimiento de las distintas poblaciones celulares del microambiente tumoral.

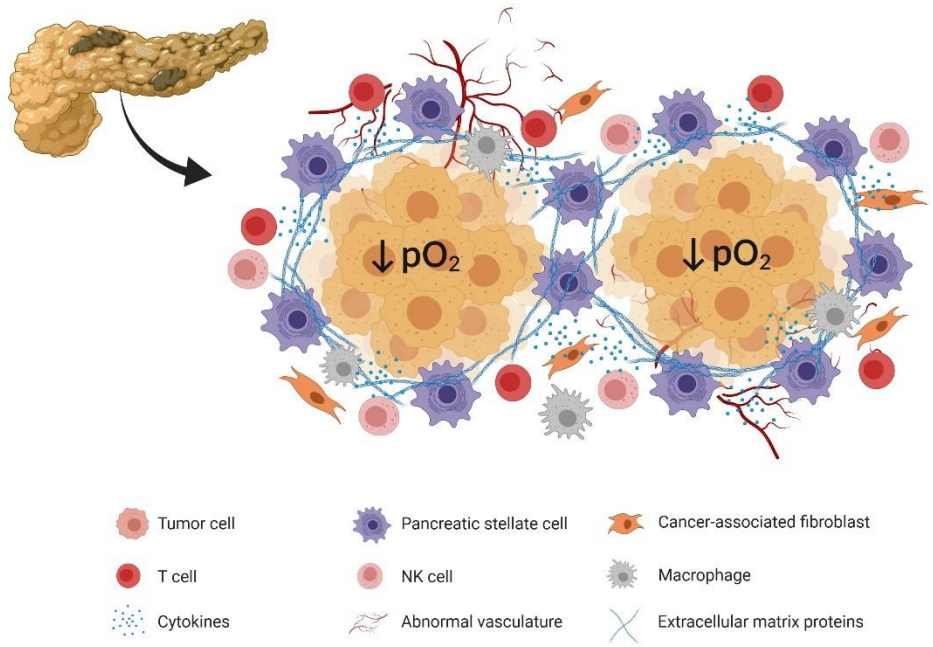

Figura 3.9. Componentes del microambiente tumoral pancreático. *Figura elaborada con Biorender.com.*

Los factores inducidos por hipoxia (HIF) son una familia de factores de transcripción que orquestan la repuesta de las células para adaptarse a la baja disponibilidad de O_2[135]. HIF forma un heterodímero constituido por una subunidad α y una subunidad β. Se han identificado 3 isoformas de la subunidad α (HIF-1α, HIF-2α y HIF-3α) y 2 subunidades β (ARNT y ARNT2) [136].

En condiciones de normoxia, los residuos de prolina de la subunidad α de HIF son hidroxilados por la acción de las enzimas prolil-4-hidroxilasas dependientes de O_2 (PHDs)[136]. Estos residuos hidroxilados actúan como sustrato para su marcaje por ubiquitinación y posterior degradación por el proteasoma. Sin embargo, bajo hipoxia la actividad de estas PHDs es suprimida. Esto permite la estabilización de HIF-α, el cual se transloca al núcleo y se une con la subunidad HIF-β. Este heterodímero constituye el factor de transcripción que localizará los elementos de respuesta a hipoxia (HRE) e inducirá la expresión de los genes dianas relacionados con la supervivencia celular, la angiogénesis, la glucolisis o la invasión y la metástasis [137].

Además de las acciones mediadas por los HIF, la hipoxia modula la fisiología celular por otras rutas de señalización y procesos celulares independientes de estos factores de transcripción [134].

En el contexto de los tumores pancreáticos, la hipoxia condiciona el fenotipo de las células tumorales[134]:

- En las células tumorales, la condición de hipoxia supone un estrés oxidativo al que las células tumorales responden activando diferentes sistemas protectores que abarcan desde un incremento en la expresión de enzimas antioxidantes hasta una disminución de la producción mitocondrial de especies oxidantes.
- La hipoxia promueve en las células tumorales pancreáticas los procesos de transformación de epitelio a mesénquima, la invasión y la migración celular.
- A nivel metabólico, las células tumorales reprograman su estado bioenergético hacia el metabolismo glucolítico. Otras vías catabólicas de obtención de energía son activadas, como la autofagia.

Estas adaptaciones de la fisiología celular a la condición de hipoxia, que realizan las células tumorales pancreáticas, han sido ampliamente estudiadas. Sin embargo, las adaptaciones que sufren otros componentes celulares del microambiente tumoral, que también se encuentran sometidos a esta misma condición, no han sido elucidados.

Todas estas adaptaciones que sufren las células a la condición de hipoxia tienen un claro efecto en cómo estas células responden a los tratamientos [138]. El contexto y la fisiología celular cambian cuando la disponibilidad de O_2 disminuye y con ello la sensibilidad a los fármacos. Esta resistencia de las células se puede producir por diferentes razones:

1) Algunas drogas o tratamientos necesitan el O_2 para ejercer su máxima citotoxicidad.
2) Las adaptaciones que sufren las células por la hipoxia permiten mecanismos de detoxificación más eficientes.
3) La hipoxia incrementa la inestabilidad genética de las poblaciones celulares del tumor y con ella aumentan la posibilidad de aparición de células resistentes al tratamiento.

3.5. Estrés oxidativo y respuesta antioxidante

El estrés oxidativo es un fenómeno causado por el desequilibrio entre la producción y la acumulación de especies oxidantes y la capacidad de los sistemas biológicos de detoxificar estos productos[139]. La ruptura de este equilibrio incrementa las especies oxidantes, las cuales pueden alterar negativamente diferentes estructuras celulares como lípidos, proteínas o ácidos nucleicos. Estos daños producidos pueden contribuir al desarrollo de procesos fisiológicos como el envejecimiento o patológicos como el cáncer o las enfermedades cardiovasculares [140–142].

3.5.1. Radicales libres y especies reactivas de oxígeno.

Los radicales libres son átomos o moléculas que poseen uno o más electrones libres o desapareados en su capa de valencia [143]. Estos electrones les confieren una gran inestabilidad química a estas especies y, por lo tanto, una gran reactividad para interaccionar con otras moléculas y sustraerle electrones para su estabilización. Esta transferencia de electrones es por definición una reacción redox, en la que una especie gana electrones y se reduce y otra los pierde y se oxida.

Los oxidantes o radicales libres tienen funciones fisiológicas fundamentales, más allá de su rol como productores del estrés oxidativo. Algunas de estas funciones incluyen la regulación del tono vascular inducida por el óxido nítrico (NO), el estallido respiratorio producido por las células fagocíticas inmunitarias o la transducción de señales acoplada a la generación de segundos mensajeros como el GMPc [144].

Las especies reactivas de oxígeno (ERO) y las especies reactivas de nitrógeno (ERN) constituyen los principales radicales libres en los sistemas biológicos [145].

Las ERO son pequeñas moléculas formadas por la reducción del oxígeno molecular, el cual participa en procesos biológicos fundamentales como la respiración celular o el metabolismo aerobio [146]. Dentro de las ERO se incluyen moléculas que son radicales libres (superóxido ($O_2^{\bullet -}$), hidroxilo ($OH^{\bullet}$), radical alcoxi ($RO^{\bullet}$), radical peroxilo ($ROO^{\bullet}$))y otras que no lo son, pero pueden provocar fácilmente reacciones de radicales libres en los organismos vivos (peróxido de hidrógeno (H_2O_2), oxígeno singlete ($^1O_2$), ozono (O_3), peróxido orgánico (ROOH), ácido hipocloroso (HOCl), ácido hipobromoso (HOBr)) [147].

En los sistemas biológicos, las ERO pueden tener un origen exógeno (la contaminación ambiental, el humo del tabaco, los alimentos, etc.) o endógeno [148]. La producción endógena de ERO puede ocurrir en diferentes estructuras celulares como las mitocondrias, el retículo endoplasmático (RE), los peroxisomas, la membrana plasmática o incluso en el espacio extracelular. Las fuentes de producción son [148]:

1) La cadena respiratoria mitocondrial. La cadena de transporte electrónico mitocondrial incluye 4 complejos (NADH-ubiquinona oxidorreductasa (I), succinato dehidrogenasa (II), ubiquinol-citocromo c oxidorreductasa (III) y citocromo c oxidasa (IV)), el coenzima Q y la proteína citocromo c. En los complejos I y III ocurre la reducción del oxígeno molecular hasta $O_2^{\bullet -}$, que posteriormente será transformado a H_2O en el complejo IV. Este proceso es uno de los principales pasos de formación de ERO en las células.
2) NADPH oxidasa. Esta enzima cataliza la oxidación del NADH o NADPH a NAD^+ o $NADP^+$ empleando O_2 y generando radical superóxido. Esta generación de superóxido es fundamental en el estallido respiratorio, en el cual una gran cantidad de ERO son liberadas al espacio extracelular o al interior del fagosoma con una función defensiva.
3) Xantina oxidasa. Esta enzima cataliza la oxidación de la hipoxantina a xantina y la xantina a ácido úrico en el proceso de catabolismo de las bases púricas. En esta reacción se generan radicales superóxidos que son transformados a peróxido de hidrógeno.
4) Mieloperoxidasas. Estas enzimas se localizan en los lisosomas de células fagocíticas y catalizan reacciones de oxidación como la cloración del peróxido de hidrógeno para formar ácido hipocloroso.
5) Ciclooxigenasas y lipooxigenasas. La metabolización del ácido araquidónico y sus derivados para formar leucotrinenos, prostaglandinas y lipoxinas implica la acción de estas enzimas. Estas enzimas catalizan la oxidación de lípidos, formando especies reactivas como radicales peroxilos, que se estabilizan generando radicales intermediarios como el $NAD^{\bullet}$ hasta formar radical superóxido.
6) Metales de transición. La oxidación del Fe^{2+} a Fe^{3+} o el Cu a Cu^{2+} por la reacción de Fenton genera radicales hidroxilos.

Como hemos descrito, la producción de ERO en los sistemas biológicos tiene implicaciones fisiológicas fundamentales. Sin embargo, una producción exacerbada y sin control puede ser perjudicial para la célula [149].

Las membranas celulares pueden ser objetivos del daño inducido por las ERO [150]. La peroxidación lipídica ocurre cuando los fosfolípidos entran en contacto con agentes oxidantes, produciéndose un lípido hidroperóxido y un radical alquilo. Esta lipoperoxidación altera la estructura de la membrana, modificando su fluidez y dañando su integridad [151]. Estas reacciones de peroxidación lipídica no afectan exclusivamente a los lípidos, pues, como consecuencia de ellas se forman especies altamente reactivas

como el hidroxinonenal o malondialdehido (MDA), que pueden reaccionar con aminoácidos o el ADN causando lesiones mutagénicas.

Las proteínas también pueden ser dianas de oxidación de las ERO [150]. Cuando los aminoácidos que componen las proteínas son oxidados pueden ocurrir diferentes escenarios. La oxidación de algunos aminoácidos puede marcar a la proteína para su degradación por el proteasoma. Además, estas reacciones de oxidación pueden modificar la química de algunos residuos de las proteínas haciendo que estas forman aductos y agregados proteicos. Otra alternativa es la ruptura de la cadena polipeptídica por efecto de la oxidación. La relación estructura-función es especialmente importante en las proteínas, por lo que cualquiera de las anteriores alteraciones tiene consecuencias para la fisiología celular.

Las ERO pueden reaccionar con los ácidos nucleicos como el ADN [150]. Algunas de las modificaciones que pueden producirse en esta molécula por la oxidación son la modificaciones de las bases nitrogenadas, la rotura de la doble hebra o uniones con otras macromoléculas como proteínas. Dada la importancia que tiene para la supervivencia de la célula mantener la integridad de la información contenida en el ADN, las células poseen sistemas de reparación de esta molécula. Sin embargo, en situaciones de estrés oxidativo, estos mecanismos pueden ser ineficientes para controlar estos daños y generar lesiones mutagénicas sobre el ADN [152].

3.5.2. Papel de las ERO en las CEP.

El estrés oxidativo se ha identificado como uno de los mecanismos de daño celular que se producen en patologías que afectan al páncreas como la pancreatitis, la diabetes o el cáncer [153–155].

En relación con la fibrosis pancreática, diferentes estudios han apuntado a las ERO como mediadoras de la activación de las CEP. La enzima NADPH oxidasa, una de las principales fuentes de generación de ERO en la célula, juega un papel activo en la activación de las CEP [156]. La inhibición farmacológica de esta enzima redujo la activación de las CEP *in vitro* y la formación de tejido fibrótico *in vivo* en un modelo de pancreatitis crónica inducida por dibutilestaño dicloruro (DBTC). En otro estudio, investigadores observaron como el H_2O_2 incrementaba la producción de colágeno en las CEP, aunque no tenía efecto sobre la proliferación o la transformación hacia fenotipo miofibroblástico [90]. La generación de cierto grado de estrés oxidativo es uno de los mecanismos por los que altas concentraciones de glucosa median la activación de las CEP [49].

Sin embargo, los datos más esclarecedores del efecto positivo que ejerce el estrés oxidativo en la activación de las CEP ha sido estudiado gracias al empleo de antioxidantes. Diferentes moléculas que han sido descritas con capacidad antioxidante como el coenzima Q, el resveratrol o el glutatión inducen la desactivación de las CEP [77,81,157].

3.5.3. Respuesta antioxidante.

La célula posee diferentes sistemas antioxidantes que le permiten detoxificar las especies oxidantes, a fin de mantener un equilibrio redox. Estos sistemas antioxidantes pueden estar mediados por la acción de enzimas antioxidantes (sistemas antioxidantes) o por moléculas con capacidad de detoxificar por ellas mismas estas moléculas.

3.5.3.1. Sistemas antioxidantes enzimáticos.

La expresión de estas enzimas se encuentra gobernada por la actividad transcripcional del factor Nrf2 y constituyen los elementos de la respuesta antioxidante (ARE) [158]. Los principales sistemas incluyen las superóxido dismutasas, la glutatión peroxidasa y glutatión reductasa, la catalasa, la hemo oxigenasa y la NADPH-quinona oxidorreductasa 1 [159].

- Superóxido dismutasa (SOD). Esta enzima cataliza la transformación del radical superóxido $O_2^{\cdot -}$ hasta H_2O_2 y O_2. Se han identificado 3 isoformas de esta proteína: SOD-1, presente en el citoplasma y dependiente de Cu y Zn; SOD-2, presente en la mitocondria y dependiente de Mn; y SOD-3, de localización extracelular y dependiente de Cu y Zn [160].
- Glutatión peroxidasa (GPX) y glutatión reductasa (GR o GSR). La GPX oxida el glutatión reducido (GSH) a glutatión oxidado (GSSG) reduciendo H_2O_2 o lípidos hidroperoxidados hasta formar H_2O o alcoholes estables, respectivamente [159]. Este sistema está acoplado a la enzima GR que restaura el GSH reduciendo el GSSG.
- Catalasa. Esta enzima cataliza la transformación del H_2O_2 a H_2O y O_2 [159]. Esta enzima contine un grupo hemo necesario para la catálisis y en las células se localiza principalmente en el peroxisoma.
- Hemo oxigenasa (HO). Esta enzima es la encargada de la degradación del grupo hemo para formar CO, biliverdina y Fe [159]. Aunque esta enzima no parece tener una función enzimática antioxidante directa, los productos producidos pueden mitigar los efectos del estrés oxidativo. Se han descrito

dos isoformas de esta enzima: HO-1, la isoforma inducida, y HO-2, la isoforma expresada de manera constitutiva.

- NADPH-quinona oxidorreductasa 1 (NQO-1). Esta enzima cataliza la reducción de dos electrones de quinonas u otros compuestos orgánicos [161]. Aunque su función biológica no ha sido completamente estudiada, se ha propuesto que esta enzima participa en la detoxificación de radicales libres y xenobióticos en la célula. Esta enzima emplea como cofactores para la catálisis el NADH o NADPH y FAD.

3.5.3.2. Sistemas antioxidantes no enzimáticos.

Dentro de este grupo de elementos de la respuesta antioxidante encontramos a moléculas que actúan como *scavengers* (amortiguadores) de radicales libres, entre los que se incluyen el sistema glutatión, o antioxidantes naturales como la melatonina.

El glutatión es un tripéptido formado por glutamato, cisteína y glicina. Es el principal antioxidante soluble presente en las células y se distribuye por los diferentes compartimentos de ésta (citosol, orgánulos y núcleo) [162]. El glutatión puede encontrase en su forma reducida (GSH) u oxidada (GSSG) (Figura 3.10.). En esta última se establece un puente disulfuro entre los grupos tioles de dos moléculas de glutatión, por lo que se conoce como disulfuro de glutatión. Y es en ese grupo funcional tiol del residuo de cisteína en el que reside su poder amortiguador de radicales libres. Este glutatión oxidado se acumula en la célula y, por ello, el ratio GSSG/GSH o consumo de glutatión es una buena medida del estrés oxidativo de las células.

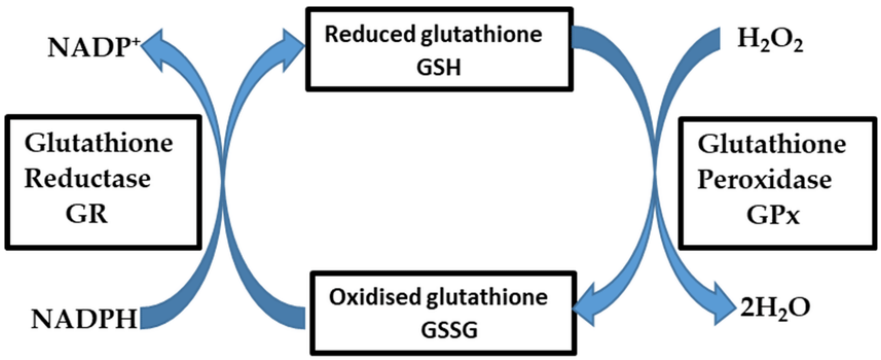

Figura 3.10. Sistema glutatión. *El sistema glutatión supone un de los principales tampones amortiguadores de radicales libres en la célula. Fuente: Differential Sensitivity of Two Endothelial Cell Lines to Hydrogen Peroxide Toxicity: Relevance for In Vitro Studies of the Blood–Brain Barrier*[163].

Otro sistema antioxidante similar al glutatión es el sistema tioredoxina. Se trata de proteínas con actividad oxidorreductasa, que poseen grupos tioles reactivos en residuos

conservados de cisteína. Sin embargo, la abundancia de este sistema es menor que la del sistema glutatión [162].

Finalmente, los antioxidantes presentes de manera natural en los sistemas biológicos por producción endógena, como la melatonina, o por la administración con la dieta, como el ácido ascórbico (u otras vitaminas), constituyen otra línea defensiva del organismo para detoxificar los radicales libres [164].

3.6. Metabolismo energético y función mitocondrial.

En condiciones de homeostasis, existe un equilibrio entre el anabolismo (síntesis de biomoléculas) y el catabolismo (degradación de biomoléculas). Sin embargo, alteraciones del estado de salud como puede ser el desarrollo de tejido fibrótico, desplazan el equilibrio hacia el anabolismo, pues la proliferación exacerbada y la sobreproducción de proteínas de MEC implica la síntesis de macromoléculas [165].

Las adaptaciones metabólicas que sufren las células tumorales para sobrevivir y proliferar de forma descontrolada fueron descritas hace más de 100 años [166]. Sin embargo, solo recientemente se ha puesto de manifiesto que estas modificaciones del metabolismo pueden ser una característica clave del desarrollo de otras patologías, entre las que se incluye la fibrosis [167].

3.6.1. Glucólisis.

La glucólisis es una vía central del metabolismo energético que transforma la glucosa en piruvato [168] (Figura 3.11.). Esta vía comienza con la entrada de la glucosa en el interior de la célula, proceso mediado por la familia de transportadores de glucosa (GLUT) [169]. Esta familia de transportadores incluye diferentes miembros cuya afinidad por la glucosa define su función y localización, dependiendo de la demanda de glucosa del tejido. El transportador GLUT-1 es el encargado del transporte basal de glucosa en la mayoría de las células.

Esta vía metabólica está constituida por diez reacciones químicas catalizadas por diez enzimas diferentes. Tres de estas diez reacciones químicas son irreversibles y son catalizadas por las enzimas hexoquinasa (HK), fosfofructoquinasa (PFK) y piruvato quinasa [170]. Estas enzimas se encuentran altamente reguladas, pues el flujo glucolítico se controla a través de su actividad.

Con suficiente O_2, el piruvato es internalizado en la mitocondria donde será usado como sustrato del ciclo de los ácidos tricarboxílicos o ciclo de Krebs (TCA) [171]. Mediante este proceso se formará energía en forma de poder reductor, que posteriormente será transformada en ATP por la fosforilación oxidativa o intermediarios

como el anabolismo de aminoácidos o lípidos. En condiciones de baja o nula disponibilidad de O_2, el piruvato presente en el citosol es reducido a lactato por la enzima lactato deshidrogenasa (LDH), generando en esta reacción poder reductor en forma de NAD^+. La metabolización de la glucosa por vía anaerobia es muy deficiente energéticamente en comparación con la vía aerobia.

Una característica de las células tumorales es el aumento de la actividad glucolítica y utilización del piruvato para la fermentación a lactato, aun en condiciones de normoxia [167]. Este efecto es conocido como efecto Warburg. La teoría más prevalente apunta a que esta alteración, que no incrementa la potencia energética de las células tumorales, les provée de intermediarios para la síntesis de macromoléculas que necesitan unas células con alta capacidad proliferativa [166].

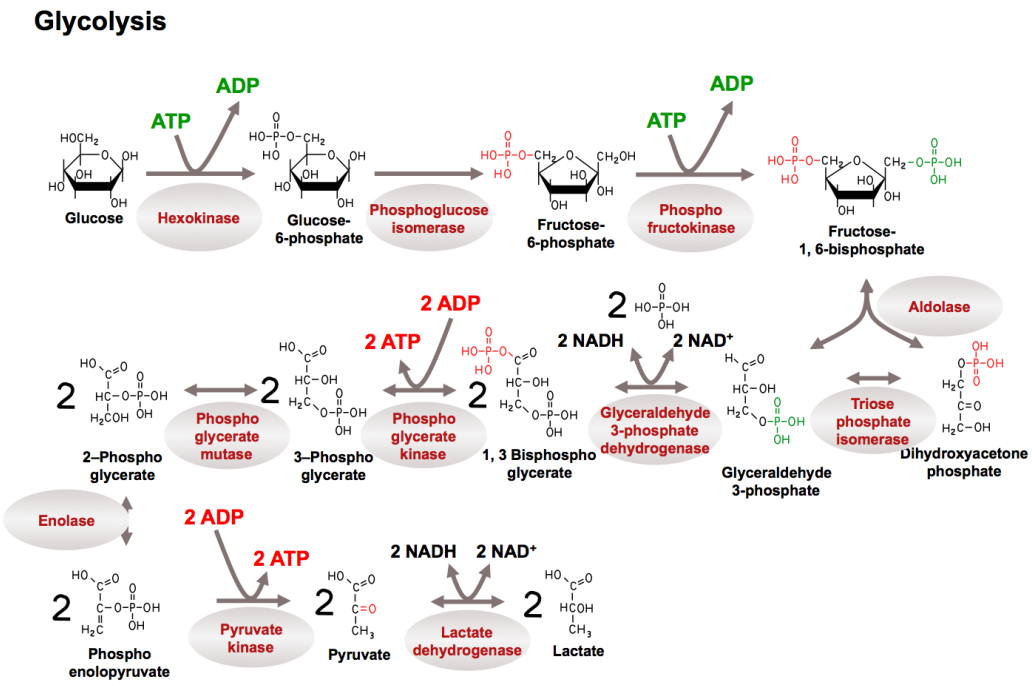

Figura 3.11. La glucolisis. *Esta vía catabólica convierte la glucosa en piruvato. Fuente:* https://quizlet.com/es/510386553/fermentacion-diagram/

En el contexto de la fibrosis, la alta proliferación de los fibroblastos y la producción de grandes cantidades de proteínas de ECM como el colágeno requiere una gran cantidad de intermediarios biosintéticos, que son generados a partir de la metabolización de la glucosa. Por ejemplo, el α-cetoglutarato es un intermediario fundamental para la síntesis de colágeno [172].

3.6.2. Función mitocondrial: el ciclo de Krebs y la fosforilación oxidativa.

La mitocondria es un orgánulo celular heredado por vía materna con una plétora de funciones que abarcan la producción de energía, la síntesis de diferentes biomoléculas

o el control del estado redox celular [173]. La cantidad de mitocondrias y su estado funcional es el resultado del balance entre los procesos de biogénesis mitocondrial (formación de nuevas mitocondrias), la fisión o fusión mitocondrial y la mitofagia (proceso de autofagia de las mitocondrias) [174].

El estado bioenergético de la célula es una función directa de la actividad mitocondrial, pues estos orgánulos son las fábricas de producción energética principales de la célula [175].

El ciclo de Krebs, un ciclo de reacciones químicas en el que intermediarios de tres átomos de carbono sufren modificaciones que generan energía en forma de poder reductor (NADH y $FADH_2$) y liberando CO_2, ocurre en el interior de la matriz mitocondrial [176]. Además, en estas reacciones se generan intermediarios para la biosíntesis de otras moléculas biológicas como aminoácidos o ácidos grasos. Las principales fuentes de moléculas de tres átomos de carbono que ciclan en este proceso proceden del piruvato generado en la glucólisis, el acetil-CoA generado en la lipolisis o algunos intermediarios generados en la oxidación de aminoácidos.

La fosforilación oxidativa ocurre en la membrana interna mitocondrial, donde se localizan los complejos encargados del transporte electrónico, la creación del gradiente de protones y la generación del ATP (Figura 3.12.) [177]. Estos complejos son:

- Complejo I o NADH-ubiquinona oxidorreductasa. La función del complejo I es transferir electrones desde el NADH presente en la matriz mitocondrial hasta la ubiquinona o coenzima Q. Este complejo transporta cuatro protones desde el interior de la matriz mitocondrial al espacio intermembrana, contribuyendo a la creación del gradiente de protones. La rotenona es un inhibidor del complejo I.
- Complejo II o succinato deshidrogenasa. Este complejo transfiere electrones desde el $FADH_2$ hasta el coenzima Q, reduciéndolo.
- Complejo III o ubiquinol-citocromo c oxidorreductasa. Este complejo recibe los electrones transportados por el coenzima Q y genera un transporte de protones con el que contribuye al gradiente o potencial de membrana. La antimicina A es un inhibidor del complejo III.
- Complejo IV o citocromo c oxidasa. Los electrones transportados por el citocromo c, liberados del complejo III, son transferidos al O_2 molecular y junto a cuatro protones generan dos moléculas de H_2O. Este complejo también contribuye a la generación del gradiente de protones.

- Complejo V o ATP-sintasa. Esta enzima acopla la cadena de transporte electrónico con la fosforilación oxidativa. El objetivo primordial de la cadena de transporte electrónico es crear un gradiente de protones entre la matriz mitocondrial y el espacio intermembranoso. La ATP sintasa es un complejo proteico que posee un poro de membrana por el que pasan los protones desde el espacio intermembranoso a la matriz. Como este transporte es a favor de gradiente, genera energía que es empleada por otras partes del complejo para catalizar la fosforilación del ADP hasta formar ATP. La oligomicina es un inhibidor del complejo V.

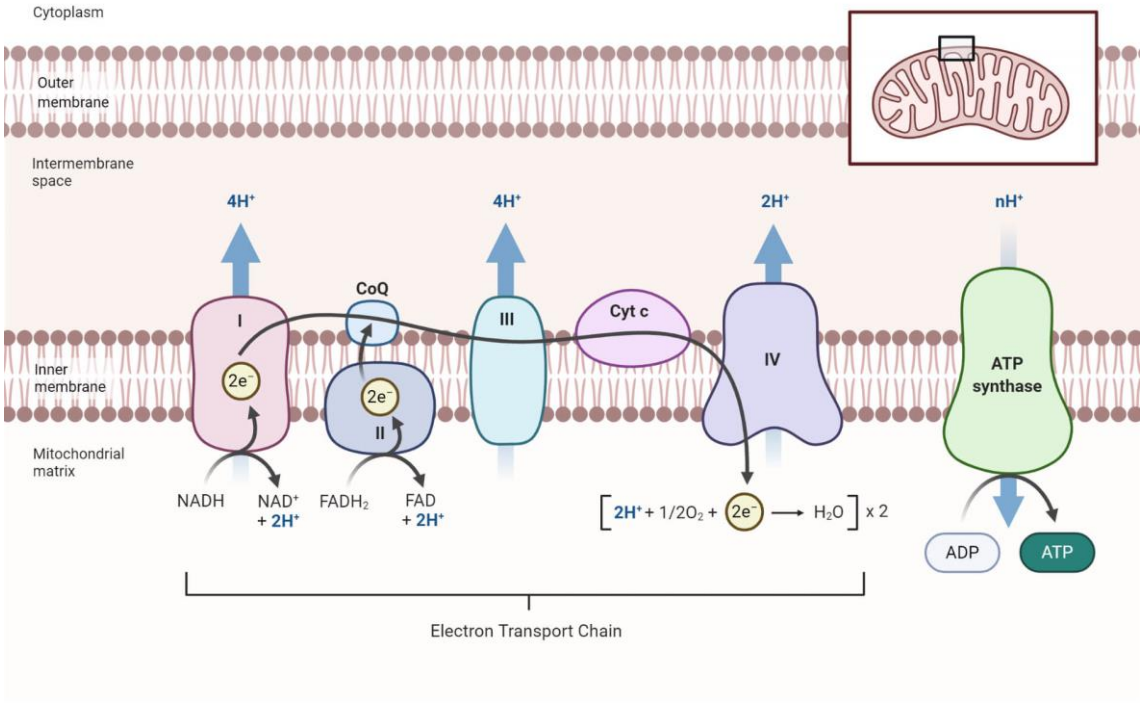

Figura 3.12. La cadena de transporte electrónico y la fosforilación oxidativa. *El transporte electrónico a través de los distintos complejos mitocondriales está acoplado a la generación de un gradiente de proteones entre la matriz mitocondrial y el espacio intermembranoso. Este gradiente es empleado posteriormente por la ATP sintasa para la generación de enlaces energéticos en el molécula de ATP. Fuente: https://microbeonline.com/oxidative-phosphorylation-mechanism-and-regulation/*

3.6.3. Adaptaciones del metabolismo en las CEP

Hasta la fecha, no existen muchos estudios sobre las adaptaciones metabólicas de las CEP activadas. Los estudios encontrados sobre estas adaptaciones metabólicas han otorgado a estas células un papel de soporte y mantenimiento de las células tumorales pancreáticas.

Un artículo publicado en la prestigiosa revista *Nature* mostró cómo las CEP secretaban aminoácidos no esenciales como la alanina, que servían de combustible

energético a las células tumorales, supliendo otras fuentes de energías escasas en el microambiente tumoral pancreático como la glucosa o la glutamina [178]. El origen de esta alanina secretada provenía de la digestión de proteína celular por el proceso de autofagia. La síntesis de glutamina es mayor en las CEP, comparada con las células tumorales [179]. Esta glutamina secretada por las CEP sirve como soporte energético para las células tumorales.

Algunas poblaciones de CEP expresan receptores para lipoproteínas de muy baja densidad (VLDL) [180]. Esta población tiene un elevado metabolismo lipídico y se ha relacionado con un aumento del fenotipo activado.

3.7. La melatonina

La melatonina (N-acetil-5-metoxitriptamina) (Figura 3.13.), también referida como indolamina, es una hormona ubicua entre casi todos los seres vivos, incluyendo bacterias, levaduras, plantas y animales [181]. Esta molécula fue descubierta por Lener y colaboradores en 1958 en extractos de glándula pineal. En los animales, esta glándula es una de las principales secretoras de esta hormona y principalmente participa en el control de los ciclos de sueño/vigilia. Sin embargo, se han descrito otras funciones fisiológicas para esta indolamina, como por ejemplo la regulación de la función reproductora o acciones en el tracto gastrointestinal [182].

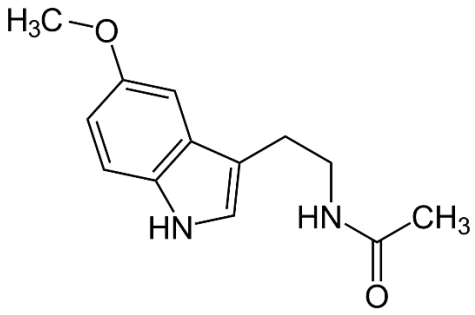

Figura 3.13. Molécula de melatonina.

3.7.1. Síntesis de la melatonina.

El aminoácido triptófano es el precursor para la síntesis de la melatonina (Figura 3.14.). Este aminoácido es transformado en serotonina y ésta es metabolizada por la acción de las enzimas serotonina-N-acetiltransferasa e hidroxindol-O-metiltransferesa hasta melatonina [182]. Una propiedad muy interesante de esta hormona, especialmente a nivel farmacológico, es su alta solubilidad tanto en agua como en lípidos. Esto permite a la melatonina poder atravesar fácilmente las membranas biológicas.

La concentración plasmática de melatonina varía con el ritmo circadiano y puede llegar hasta 0,5 nM. Sin embargo, otros tejidos extrapineales como el tracto gastrointestinal también producen esta hormona y, en esta localización, la concentración de melatonina puede llegar a alcanzar niveles locales del orden micromolar [182].

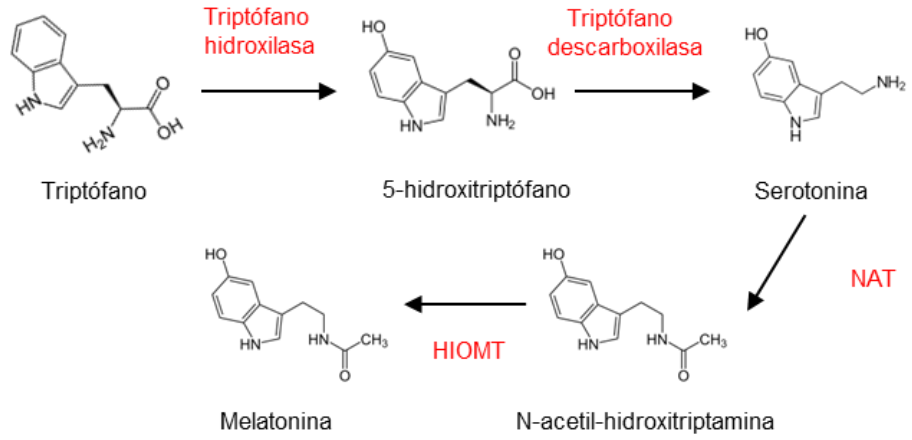

Figura 3.14. Vía de síntesis de la melatonina.

3.7.2. Mecanismos de acción.

Las acciones de la melatonina a nivel celular pueden ser dependientes o independientes de su unión a receptores específicos [182].

Entre los receptores para esta hormona, se han descrito receptores asociados a la membrana celular y receptores nucleares o citosólicos. Los receptores clásicos de membrana, MT1 y MT2, son receptores transmembranas asociados a proteínas G. La unión de la melatonina a estos receptores induce cambios en la señalización intracelular mediados por mensajeros como el AMPc, el GMPc o el ión Ca^{2+}. Otros receptores descritos para esta hormona son la proteína citoplasmática calmodulina o los receptores nucleares huérfanos RORα.

Respecto a las acciones no mediadas por receptores, estarían relacionadas con la capacidad de esta molécula para poder atravesar las membranas celulares. En el interior de la célula la melatonina, gracias a sus grupos químicos, puede actuar como una molécula con capacidad antioxidante o prooxidante [183]. Este comportamiento dual depende del contexto celular y de la concentración de dicha hormona. Como antioxidante, esta molécula puede modular el estado redox de la célula por acciones directas, actuando como una molécula que detoxifica las ERO o ERN, o indirecta, modulando la activación de los diferentes elementos de la respuesta antioxidante celular. Las acciones prooxidantes se han observado en contextos patológicos, como en el caso de las células tumorales, y se relacionan con las acciones antiproliferativas y

citotóxicas que se han observado para esta indolamina [183]. En este contexto, diferentes investigaciones han apuntado a las mitocondrias, las factorías energéticas de las células, como la dianas de la melatonina.

3.7.3. Propiedades farmacológicas de la melatonina

Tanto la comunidad científica como la sociedad han mostrado un gran interés en los últimos años sobre las múltiples propiedades farmacológicas de esta molécula. Es muy frecuente encontrar en la prensa común noticias sobre la aplicación de la melatonina para trastornos del sueño y depresivos, para enfermedades neurodegenerativas, para infecciones comunes como la gripe o, recientemente, la Covid-19, e incluso su aplicación como medida de protección frente a armas químicas. Estas noticias, encontradas en la prensa, tienen su base científica en las propiedades antioxidantes, antiinflamatorias e inmunomodulatorias, antitumorales y reguladoras del ritmo circadiano descritas para esta indolamina.

Como antioxidante, ya hemos descrito anteriormente la capacidad de esta molécula para detoxificar las ERO y ERN, así como para modular otros elementos de la respuesta antioxidante. Algunas enfermedades como las neurodegenerativas o el propio envejecimiento natural tienen como base, aunque no exclusivamente, la generación de especies oxidantes, que dañan estructuras celulares y pueden incluso producir daños a nivel del ADN [184]. La capacidad antioxidante de la melatonina para controlar dichas especies reactivas mitiga los daños producidos por éstas.

Como antiinflamatoria e inmunomoduladora, la melatonina tiene la capacidad de inhibir la activación de un factor de transcripción máster para la respuesta inflamatoria, el NF-κB [185]. Este factor de transcripción controla la expresión de diferentes citoquinas proinflamatorias como la interleucina 1β (IL-1β), la interleucina 6 (IL-6), el factor de necrosis tumoral α (TNF-α) o enzimas como la ciclooxigenasa-2, encargada de la síntesis de mediadores proinflamatorios generados, a partir del ácido araquidónico. Es por este motivo que desde un primer momento se pensó en la melatonina como posible adyuvante para el tratamiento de la Covid-19, enfermedad en la que se produce una respuesta inflamatoria excesiva y lesiva [186]. Además, algunas células del sistema inmunitario como los macrófagos o los linfocitos T y B expresan los receptores clásicos para la melatonina [187]. A través de estos receptores, la melatonina modula la proliferación y la secreción de diferentes patrones de citoquinas, orquestando diferentes perfiles de la respuesta inmunitaria.

Como antitumoral, la indolamina ha mostrado potentes acciones citotóxicas y antiproliferativas en una gran cantidad de tumores [188]. Los mecanismos moleculares

antitumorales de la melatonina son muy amplios y abarcan desde la inducción de la apoptosis, la desregulación de las rutas para la obtención de energía, la modulación de los procesos autofágicos e inducción de estrés reticular, la inhibición de la angiogénesis, la disminución de la protección del tumor frente al ataque del sistema inmunitario, hasta la inhibición de la capacidad migratoria y formación de metástasis.

Cada vez se descubren más propiedades y funciones de esta hormona. En este sentido, un efecto menos conocido es su papel como agente anti-fibrótico. Este efecto ha sido reportado en modelos de fibrosis pulmonar y renal. Sin embargo, hasta el momento de inicio de nuestras investigaciones no había estudios sobre el efecto de la melatonina en la fibrosis pancreática.

3.7.4. Melatonina y páncreas.

El sistema gastrointestinal es considerado la mayor fuente extrapineal de melatonina en el organismo [182]. En el páncreas, como parte del tracto gastrointestinal, se ha reportado la expresión de los receptores clásicos para la melatonina (MT1 y MT2) y la expresión de las enzimas que participan en su síntesis [189]. Los esfuerzos de nuestro grupo de investigación se han centrado en estudiar los efectos de la melatonina sobre la función del páncreas exocrino.

Empleando preparaciones de células acinares aisladas de ratón observamos que el tratamiento con melatonina redujo la liberación de la enzima digestiva amilasa en respuesta a una sobreestimulación con su agonista fisiológico la colecistocinina [190]. También se determinó cómo esta hormona disminuía la movilización de Ca^{2+} inducida por la colecistocinina [191]. El Ca^{2+} es un mensajero intracelular fundamental para la regulación de la secreción pancreática. Además, el tratamiento con melatonina estimuló la activación de Nrf2, el principal regulador de la respuesta antioxidante [192]. Todos estos datos indicaban que la melatonina tenía un papel "*pancreatoprotector*" frente a la sobreestimulación del páncreas por colecistocinina. Este efecto ha sido corroborado por otros grupos de investigación en modelos *in vivo* de pancreatitis aguda en rata [193]. Los investigadores, observaron que el pretratamiento con melatonina reducía la necrosis tisular, la inflamación y el daño oxidativo inducido por la pancreatitis.

Respecto a los efectos de la melatonina sobre el cáncer pancreático, nuestro grupo de investigación determinó que la melatonina disminuía significativamente la viabilidad de la línea tumoral pancreática de rata AR42J [194]. Este efecto citotóxico estaba relacionado con la alteración de la fisiología mitocondrial y la activación de la apoptosis. Este efecto ha sido también observado en diferentes líneas celulares de cáncer pancreático (MiaPaCa, SW-1990, PANC-1). En ellas, se observaba la activación de

proteínas pro-apoptóticas como Bax y diferentes caspasas y la inhibición de proteínas anti-apoptóticas de la familia de Bcl-2 [195]. Además de esta acción antiproliferativa, también se ha reportado una disminución de la migración y la invasión de las células tumorales en respuesta a la indolamina [188]. En modelos *in vivo* de cáncer pancreático, la melatonina ha mostrado un claro efecto antitumoral [196]. La combinación de esta molécula con diferentes quimioterapéuticos como el 5-fluoruracilo, el cisplatino o la capecitabina, redujo drásticamente el desarrollo de los tumores en los diferentes modelos ensayados [188]. Estos modelos han puesto de manifiesto el papel de la melatonina como una prometedora herramienta terapéutica o adyuvante en el tratamiento del cáncer pancreático. Hasta la fecha, no se han realizado ensayos clínicos para evaluar el uso farmacológico de la melatonina en humanos para el tratamiento del cáncer de páncreas. Sin embargo, sí hay ensayos clínicos en tumores de cabeza y cuello, en cáncer colorrectal, en hepatocarcinoma, en melanoma, en cáncer gástrico, en cáncer de pulmón, en cáncer de ovario, etc [197]. En estos estudios se ha mostrado que la melatonina, administrada como adyuvante generalmente, reduce los efectos adversos de la radioterapia y/o quimioterapia, mejora la calidad de vida de los pacientes y en algunos tumores incrementa la supervivencia.

4. OBJETIVOS

El objetivo principal de la propuesta presentada será evaluar las posibles acciones anti-fibróticas de la melatonina sobre la fisiología de las CEP. Además, trataremos de caracterizar las adaptaciones que las CEP sufren bajo la condición de hipoxia y estudiaremos el efecto modulador de la indolamina bajo esta condición de baja disponibilidad de O_2. Para ello, trataremos de contestar a los siguientes objetivos específicos:

1. Analizar el efecto de la melatonina y/o la hipoxia sobre la viabilidad y la proliferación en las CEP.
2. Caracterizar los efectos de la melatonina y/o la hipoxia sobre la modulación de la vía de señalización intracelular de las MAPKs de las CEP.
3. Determinar el efecto de la melatonina y/o la hipoxia sobre la activación de la vía de señalización de PI3K/Akt/mTOR en las CEP.
4. Estudiar el efecto de la melatonina y/o la hipoxia sobre el estado redox y la activación de diferentes sistemas antioxidantes celulares en las CEP.
5. Investigar el efecto de la melatonina y/o la hipoxia sobre la fisiología mitocondrial y el metabolismo glucolítico de las CEP.

4. OBJETIVES

The main objective of the presented proposal will be to evaluate the possible anti-fibrotic actions of melatonin on the physiology of PSC. In addition, we will try to characterise the adaptations that PSC undergo under the condition of hypoxia and we will study the modulating effect of the indolamine under this condition of low O_2 availability. To achieve these aims, we will try to answer the following specific objectives:

1. To analyse the effect of melatonin and/or hypoxia on viability and proliferation of PSC.
2. To characterise the effects of melatonin and/or hypoxia on the modulation of the intracellular signalling pathway of MAPKs of PSCs.
3. To determine the effect of melatonin and/or hypoxia on the activation of the PI3K/Akt/mTOR signalling pathway of PSC.
4. To study the effect of melatonin and/or hypoxia on the redox state and the activation of different cellular antioxidant systems of PSC.
5. To investigate the effect of melatonin and/or hypoxia on mitochondrial physiology and glycolytic metabolism of PSC.

5. METODOLOGÍA

5.1. Preparación de cultivos primarios de CEP de rata.

Para la preparación de los cultivos primarios de CEP (Figura 5.1.) se emplearon ratas *Wistar* neonatas no sexadas de entre dos y tres días de vida. Estos animales fueron suministrados por el Servicio de Animalario de la Universidad de Extremadura y fueron tratados según lo dispuesto en la legislación vigente. Los estudios fueron aprobados por el Comité de Experimentación animal de la Universidad de Extremadura (referencia 57/2016) y por el Comité Institucional de la Junta de Extremadura (referencia 20160915).

Los animales se sacrificaron por decapitación para conseguir una mayor exanguinación del animal y obtener menor contaminación de glóbulos rojos en los cultivos. Tras la decapitación, el cuerpo de los animales se roció con etanol al 70% para limpiar el animal y conseguir la mayor esterilidad posible para la extracción de los órganos. A partir de este paso, se trabajó en la cabina de flujo para evitar las posibles fuentes de contaminación.

El procedimiento para la extracción del páncreas es el siguiente: con el animal colocado en posición decúbito lateral derecho, se realiza un corte en la piel en la zona retrocostal (donde finalizan las costillas). Posteriormente, se abre la pared abdominal quedando expuesto el bazo. En los roedores, el páncreas es un órgano difuso, blanquecino, localizado junto al bazo. Para dañarlo lo menos posible, se extrae junto con el bazo. Posteriormente se separan con tijeras y pinzas de microcirugía. Una vez extraído el páncreas, este se coloca en medio HBSS (Hank's Balanced Salt Solution) para limpiarlo. Para cada preparación de cultivos se emplearon entre seis yocho páncreas (obtenidos de animales de la misma camada).

El siguiente paso del aislamiento es la disgregación mecánica y enzimática del páncreas. Para ello, los páncreas se mezclaron con una solución fisiológica Na-HEPES conteniendo 60 unidades/mL de colagenasa CLSPA de Worthington. La solución Na-Hepes de aislamiento tenía la siguiente composición: NaCl 130 mM, KCl 4.7 mM, $CaCl_2$ 1.3 mM, $MgCl_2$ 1 mM, KH_2PO_4 1.2 mM, glucosa 10 mM, HEPES 10 mM, inhibidor de tripsina 0.01% y albúmina bovina sérica 0.2% (pH=7.4 ajustado con NaOH). Seguidamente, se trocearon los páncreas empleando una tijera pequeña hasta

conseguir una textura de papilla. A continuación, esta suspensión de páncreas se incubó durante 45 minutos en un baño termostatizado a 37ºC y en agitación constante.

Tras la disgregación, la suspensión celular se centrifugó a 1500 rpm durante 5 minutos a RT. Para desactivar la colagenasa presente en la solución, de descartó el sobrenadante y el pellet resultante se resuspendió y se incubó en medio 199 suplementado con suero de caballo al 10% durante 5 min. A continuación, se centrifugó la suspensión celular en las mismas condiciones mencionadas anteriormente y se descartó nuevamente el sobrenadante. Finalmente, el pellet celular se resuspendió en el medio de cultivo de las CEP (medio 199 suplementado con 10% de suero fetal bovino, 4% de cuero de caballo y 0,5% de antibiótico). Para disgregar completamente la suspensión celular, se pipeteó la suspensión de células hasta conseguir una solución homogénea. Las células se sembraron en placas de Petri de 10 cm o frascos de 75 cm^2 y se dejaron crecer en un incubador a 37ºC y 5% de CO_2. Los cultivos alcanzaron la confluencia entre el cuarto y el sexto día tras la preparación. Al segundo día, los cultivos se lavaron con una solución de tampón fosfato salino (PBS: NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 2 mM; pH ajustado a 7.4) para eliminar los restos celulares de la preparación y obtener un cultivo limpio de CEP.

Al llegar al estado de confluencia, las células se despegaron mediante incubación con una solución de tripsina y se resembraron en los sustratos específicos para cada experimento. Los experimentos se realizaron con células entre los pases uno y tres, pues en pases superiores las células pierden sus propiedades.

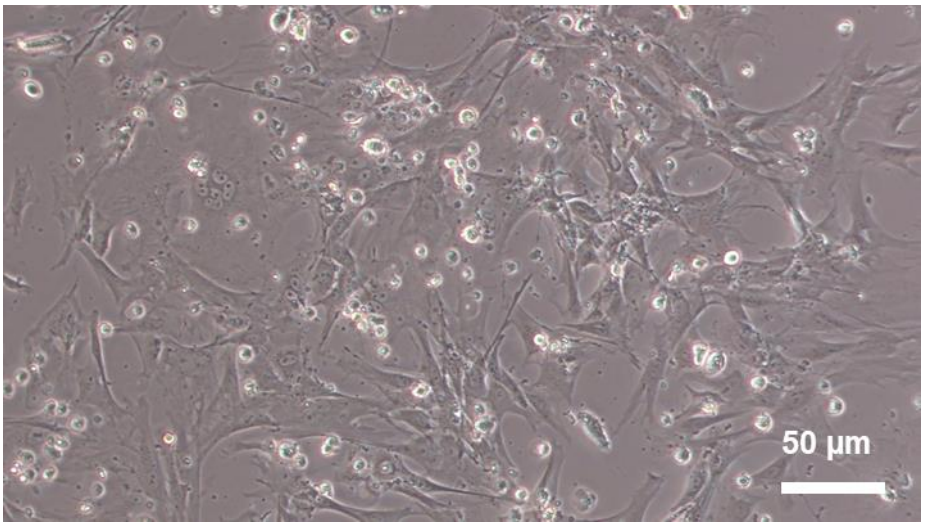

Figura 5.1. Cultivo primario de CEP de rata.

5.2. Estudio de la proliferación celular por tinción con cristal violeta.

Para la determinación de los cambios en la viabilidad celular y la proliferación se empleó la técnica de tinción con cristal violeta. Este colorante se une a las proteínas y los ácidos nucleicos de manera inespecífica y estequiométrica, permitiendo monitorizar la cantidad de células presentes en el cultivo.

Para estos estudios, las células fueron sembradas en placas de 24 pocillos a una densidad de 15.000 células por pocillo. Al día siguiente, se estimularon las células con los correspondientes estímulos y las células fueron cultivadas bajo condiciones de normoxia o hipoxia.

Finalizado el tiempo de incubación establecido para cada experimento, se retiró el medio de cultivo y las células se fijaron con una solución de p-formaldehído al 4% durante 15 min. Tras la fijación, las células se tiñeron con cristal violeta (solución preparada al 0,1%) (Sigma-Aldrich products) durante veinte minutos. Para retirar el colorante no unido a las células, se lavaron varias veces los pocillos con H_2O mili-Q y posteriormente se secaron en la estufa. De esta forma, el cristal violeta que se encuentra en la placa es aquel que se encuentra unido a los elementos celulares por los que tiene afinidad (Figura 5.2.). Para separar el colorante y mantenerlo en disolución, se añadió una solución de ácido acético al 10%. El sobrenadante coloreado se transfirió a una placa de 96 pocillos para cuantificación por lectura de absorbancia a 595 nm en un lector de placas (CLARIOstar Plus, BMG Labtech., C-Viral, Madrid, España). Los datos fueron expresados en porcentaje como la media ± error estándar de la media con respecto a la absorbancia medida en las células no tratadas.

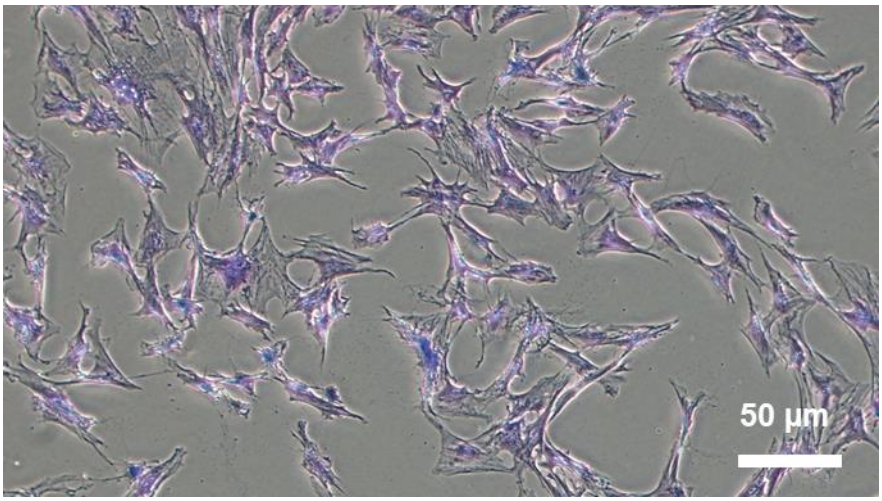

Figura 5.2. CEP teñidas con cristal violeta.

5.3. Determinación de la proliferación celular por la incorporación de BrdU.

La proliferación celular se analizó utilizando el ensayo comercial BrdU Cell Proliferation Assay Kit de Biovision. Este kit se basa en la incorporación de 5-bromo-2-deoxiuridina (BrdU), un análogo de la timidina, en la fase de síntesis del ADN previa a la división celular. La intensidad del color desarrollado es proporcional a la cantidad de BrdU incorporada en las células y puede ser usado como indicador de proliferación celular.

Las células se cultivaron en placas de 96 pocillos y se incubaron con los diferentes estímulos. Se añadió BrdU de una solución stock 10X hasta alcanzar una concentración final 1X y se dejó incubar durante una a cuatro horas a 37ºC. Tras esto se eliminó el medio con cuidado y se añadieron 100 µL de la solución de fijación, esperando 30 minutos a temperatura ambiente (21-23ºC). Seguidamente, se retiró esta solución y se añadieron 100 µL de una solución que contiene un anticuerpo específico para la detección de BrdU, dejándolo incubar durante una hora a temperatura ambiente y en agitación suave. Después se realizaron dos lavados y se añadieron 100 µL de anticuerpo secundario conjugado con HRP, incubándo ahora la preparación durante una hora a temperatura ambiente. Posteriormente, se quitó esta solución y se realizaron tres lavados. Finalmente se empleó el sustrato TMB para el desarrollo del color, que se cuantificó midiendo la absorbancia a 450 nm con el lector de placas mencionado anteriormente. Los datos muestran el cambio medio de la absorbancia expresada en porcentaje como la media ± error estándar de la media (n) con respecto a la absorbancia medida en las células control (no estimuladas), siendo n el número de experimentos independientes.

5.4. Western blot.

Previamente a la obtención de lisados proteicos, las células fueron cultivadas en placas de seis pocillos y sometidas a los correspondientes tratamientos por el tiempo determinado para cada experimento. Finalizado el tiempo de incubación, las células se colocaron en hielo y se lisaron empleando el tampón de lisis Cell Lytic suplementado con inhibidor de proteasas 1% y orto-vanadato sódico 100 mM (Sigma Chemicals Co. Madrid, España). Estos lisado fueron sonicados y centrifugados a 14.000 rpm durante quince minutos a 4ºC. Para la cuantificación del contenido en proteínas de las muestras se empleó el método de Bradford [198].

Los lisados de proteínas (15 µg/calle de electroforesis) fueron separados mediante SDS-PAGE usando geles con un porcentaje de poliacrilamida variable en función de las proteínas de interés. Posteriormente fueron transferidos a membranas de nitrocelulosa empleando el método de transferencia húmeda (110 V, 90 min). Las

membranas fueron bloqueadas mediante incubación en una solución de TBS 1X (TBS 10X: 200 mM Trizma Base + 1,37 M de NaCl, ajustado a pH 7.6 con HCl 1 N) Tween 1% + 5% de leche durante 1 hora en agitación constante. Tras ello, fueron incubadas con el anticuerpo primario específico preparado en TBT 1X + Tween 1% + 5% BSA durante toda la noche a 4ºC. Los anticuerpos empleados en este trabajo se detallan en la tabla 5.1. Una vez incubado el anticuerpo primario, se realizaron 3 lavados de 10 minutos con TBS 1X Tween 1% + 5% de leche, y posteriormente fue incubado con el correspondiente anticuerpo secundario IgG conjugado con HRP preparado en TBS 1X Tween 1% + 5% de leche. Finalmente, se realizó un lavado más con TBS 1X Tween 1% + 5% de leche y otros dos con TBS 1X + Tween 1%. Para el revelado se empleó el reactivo comercial SuperSignal™ West Pico PLUS Chemiluminescent Substrate o bien el SuperSignal™ West Femto Chemiluminescent Substrate y el lector de quimioluminiscencia G: BOX Chemi XX6 (Syngene, C-Viral, Madrid, España).

Tabla 5.1. Lista de Anticuerpos usados en este estudio.

Anticuerpo	Dilución	Casa comercial
β-Actin HRP-Conjugated	1:50000	Thermo Fisher
p-Akt (Ser473)	1:2000	Cell Signaling
p-AMPKα (Thr172)	1:1000	Cell Signaling
p-GSK-3αβ (Tyr279/Tyr216)	1:1000	Santa Cruz Biotechnology
p-mTOR (Ser2448)	1:1000	Cell Signaling
p-PTEN (Ser380)	1:1000	Cell Signaling
p-p70 S6K (Thr389)	1:1000	Cell Signaling
p-p42/44 (Thr202/Tyr204)	1:2000	Cell Signaling
p-JNK (Thr183/Tyr185)	1:1000	Cell Signaling
p-p38 (Thr180/Tyr182)	1:1000	Cell Signaling
p-Nrf2 (Ser40)	1:2000	Thermo Fisher
SOD-1	1:1000	Thermo Fisher
SOD-2	1:2000	Santa Cruz Biotechnology

Tabla 5.1. Lista de anticuerpos comerciales empleados en este estudio. En la tabla se detalla la concentración o dilución empleada del anticuerpo y la casa comercial donde se adquirió.

La cuantificación de la intensidad de las bandas se realizó empleando el software Image J (http://imagej.nih.gov/ij/). Los resultados se expresaron en porcentaje como la media ± error estándar de los valores con respecto a los en las células no tratadas.

5.5. RT-qPCR

Para la obtención de los ARN mensajeros, las células fueron cultivadas en placas de seis pocillos y sometidas a los correspondientes tratamientos por el tiempo determinado para cada experimento. Finalizado el tiempo de incubación, se procedió

con el aislamiento del ARN utilizando el kit comercial GenElute™ Mammalian Total Kit (ThermoFisher, Madrid, España). Para la RT-qPCR se empleo el kit SYBR Green RNA-to-C_T 1-Step (Applied Biosystems, Township, USA). La transcripción reversa se realizó a 48ºC durante 30 min. Las condiciones de la PCR fueron 10 min a 95ºC seguido de 40 ciclos de 15 s a 95ºC más 1 min a 55ºC. Los *primers* empleados se muestran en la tabla 5.2.

La abundancia de ARNm de cada transcrito se normalizó con respecto a la abundancia de ARNm de Gapdh obtenida en la misma muestra. Los niveles relativos de ARNm se calcularon mediante el método ΔΔCt, y se expresaron como el incremento o "*fold change"* existente entre la muestra y el control no estimulado.

Tabla 5.2. *Primers* usados en este estudio.

Gen	Primer forward	Primer Reverse
Gclc	5′-GGCACAAGGACGTGCTCAAGT-3′	5′-TGCAGAGTTTCAAGAACATCG-3′
Cat	5′-ACTTTGAGGTCACCCACGAT-3′	5′-AACGGCAATAGGGGTCCTCTT-3′
Ho-1	5′-AGCACAGGGTGACAGAAGAG-3′	5′-GAGGGACTCTGGTCTTTGTG-3′
Nqo-1	5′-GGGGACATGAACGTCATTCTCT-3′	5′-AAGACCTGGAAGCCACAGAAGC-3′
Sod-1	5′-GGGGACAATACACAAGGCTGTA-3′	5′-CAGGTCTCCAACATGCCTCT-3′
Sod-2	5′-GTGGAGAACCCAAAGGAGAG-3′	5′-GAACCTTGGACTCCCACAGA-3′
Glut-1c	5′-ATCCTTATTGCCCAGGTGTT-3′	5′-CAGAAGGGCAACAGGATACA-3′
Ldha	5′-GCAGGTGGTTGACAGTGCAT-3′	5′-ACCCGCCTAAGGTTCTTCAT-3′
Pfkp	5′-GACAAGATCCCCAAGAGCAA-3′	5′-AGCCGTCATAGATTGCGAAC-3′
Gapdh	5′-GGGTGTGAACCACGAGAAAT-3′	5′-CCTTCCACGATGCCAAAGTT-3′

Tabla 5.2. Lista de *primers* empleados en este estudio. En la tabla se indica los *primers* y sus respectivas secuencias.

5.6. Determinación de la producción de ERO.

La generación de ERO se monitorizó empleando las sondas flurescentes CM-H_2DCFDA y MitoSOX Red (Invitrogen, Barcelona, España). La sonda CM-H_2DCFDA es un derivado reducido de fluoresceína y calceina permeable a las células que permite la detección de ERO por la reactividad de su grupo clorometilo reactivo a tioles. El aducto oxidado generado en presencia de especies oxidantes forma una especie fluorescente. El MitoSOX Red permite la detección de superóxido mitocondrial.

Las CEP se despegaron del sustrato de crecimiento y se cargaron con las sondas CM-H2DCFDA (10 μM, incubación de cuarenta minutos a temperatura ambiente) o con MitoSOX Red (2,5 μM, incubación de veinte minutos a 37ºC) en medio Na-Hepes de aislamiento. A continuación, las células se incubaron con los estímulos

durante una h. Los estímulos fueron preparados empleando el medio Na-Hepes para experimentos (NaCl 130 mM, KCl 4.7 mM, $CaCl_2$ 1.3 mM, $MgCl_2$ 1 mM, KH_2PO_4 1.2 mM, glucosa 10 mM, HEPES 10 mM, pH 7,4). Para la detección de cambios en el estado redox, las células se excitaron a 530 nm y la fluorescencia emitida se detectó a 590 nm para CM-H2DCFDA, mientras que para las células cargadas con MitoSOX red se empleó excitación a 510 nm con detección a 580. Para la detección y cuantificación de la fluorescencia se utilizó un lector de placas (CLARIOstar Plus, BMG Labtech., C-Viral, Madrid, España). Los resultados expresan como el aumento medio de fluorescencia expresado en porcentaje ± error estándar de la media con respecto a los valores detectados en las células no estimuladas.

5.7. Determinación de carbonilos oxidados.

Las células fueron incubadas con los estímulos y posteriormente se lisaron para su análisis. La detección de carbonilos proteicos se realizó según los métodos descritos por Villaverde y colaboradores [199].

Para la determinación de los carbonilos oxidados se tomaron 500 µL de cada lisado celular y se trataron con una solución fría de ácido tricloroacético (TCA) al 10%. Después de la centrifugación (600 × *g* durante 5 minutos a 4 °C), se eliminaron los sobrenadantes y los pellets se incubaron secuencialmente con una solución que contenía 0,5 mL de tampón de ácido 2-(N-morfolino) etanosulfónico (MES) 250 mM pH 6.0 con 1 mM de dietilenglicol que contenía 1 mM de ácido dietilentriaminopentaacético (DTPA), una solución que contenía 0,5 mL de 50 mM de ABA en 250 mM de tampón MES de pH 6,0 y una solución que contenía 0,25 mL de 100 mM de $NaBH_3CN$ en 250 mM de tampón MES de pH 6,0. A continuación, las muestras se incubaron con una solución fría de 250 mM de tampón MES de pH 6,0 y se lavaron con agua fría. Seguidamente, las muestras se trataron con una solución fría de TCA al 50% y se centrifugaron a (1200 × g durante 10 min). Los pellets se lavaron dos veces con TCA al 10% y éter dietílico-etanol (1:1). Por último, el precipitado se trató con HCl 6 M y se mantuvo en un horno a 110 °C durante 18 horas hasta que se completó la hidrólisis. Finalizada la incubación, las muestras se secaron al vacío y el residuo generado se reconstituyó con 200 µL de agua miliQ y se filtró para el análisis por HPLC utilizando un aparato HPLC Shimadzu 'Prominence' (Shimadzu Corporation, Japón). Los eluidos se monitorizaron con longitudes de onda de excitación y emisión fijadas en 283 y 350 nm, respectivamente. Se corrieron y analizaron patrones (0,1 µL) en las mismas condiciones. Se calcularon los nmol de alisina, un producto de la oxidación de lisina, por mg de proteína. Los resultados se expresan en porcentaje como la media ± error

estándar de la media con respecto a los valores detectados en las células no estimuladas.

5.8. Determinación de peroxidación lipídica TBARS.

La determinación de la peroxidación lipídica se realizó mediante la detección de malonialdehido y otras sustancias reactivas del ácido tiobarbitúrico (TBARS).

Las células se incubaron con los estímulos y, posteriormente, se lisaron para su análisis. Se midieron el malondialdehído (MDA) y otras sustancias tiobarbitúrico-reactivas (TBARS), añadiendo 500 µL de ácido tiobarbitúrico (0,02 M) y 500 µL de ácido tricloroacético (10%) a 200 µL de una muestra de cada tratamiento. A continuación, la mezcla se incubó durante 20 min a 90 °C. Después de enfriarse, se realizó una centrifugación de 5 min a 600 × g y se midió la absorbancia del sobrenadante a 532 nm empleando un lector de placas (VariosKan Lux 3020-205, Thermo Sci., Vantaa, Finlandia). Se calcularon los mg/L de TBARS en cada muestra. Los resultados se expresan en porcentaje como la media ± error estándar de la media con respecto a los valores detectados en las células no estimuladas.

5.9. Determinación de los niveles de glutatión.

La determinación de los cambios en los niveles de glutatión reducido (GSH) y glutatión oxidado (GSSG) se llevó a cabo mediante el método de Hissin y Hilf [200].

Las células se incubaron con los diferentes estímulos ensayados. Después, las células se lavaron dos veces con PBS, se lisaron con 1 mL de una solución que contenía tampón Tris-HCl 25 mM (pH = 7,6) y Triton X-100 al 1% (p/v) y se sonicaron. A continuación, las proteínas del sobrenadante se precipitaron mediante la adición de ácido tricloroacético frío al 20%. Los viales se centrifugaron (10.000×g durante 10 min a 4 °C). En el momento de su utilización, 50 µl de cada muestra se incubaron con 1 mg/ml del reactivo fluorescente ortoftalaldehído (OPT) en un tampón básico (pH 8,0) que contenía fosfato de Na (0,1 M) y EDTA (5 mM). Se añadieron N-etilmaleimida (40 mM) y NaOH (1 M; volumen para alcanzar pH 12) a las muestras antes de la determinación de GSSG. La mezcla de reacción se incubó durante 45 minutos a 20 °C. Después, 200 µl de la mezcla de reacción fue añadida a los pocillos de una placa negra de 96 pocillos. La presencia de GSH o GSSG se determinó midiendo fluorescencia a 350 nm/420 nm (excitación/emisión) utilizando un lector de placas (Tecan Infinite M200, Grödig, Austria).

Para la cuantificación se utilizaron curvas estándar de GSH (3,20-320 µmol/ml) y GSSG (2,5-80 µmol/ml). Para la normalización se determinó la cantidad de proteínas de los lisados por el método de Bradford [198]. Se determinaron los valores de GSH y

GSSG (µmol/mg de proteínas) y se calculó la relación GSH/GSSG. Los datos se expresaron como el aumento medio de la relación GSH/GSSG expresado en porcentaje ± error estándar de la media (n) con respecto a los valores medidos en las células control (no estimuladas).

5.10. Determinación de la capacidad antioxidante total (TAC).

La capacidad antioxidante total fue determinada empleando el kit comercial Total Antioxidant Capacity (TAC) Colorimetric Assay (BioVision, Cambridge, UK). Este kit permite determinar la acción de los sistemas antioxidantes empleando una reacción colorimétrica basada en la reducción del cobre y empleando una curva patrón del antioxidante Trolox.

Las células fueron tratadas bajo las condiciones testadas y se procedió con el protocolo dado por el fabricante del kit. La absorbancia a 570 nm de la muestra se midió empleando un lector de placas (CLARIOstar Plus, BMG Labtech., C-Viral, Madrid, España). Los resultados muestran el cambio medio de absorbancia expresado en porcentaje ± error estándar de la media con respecto a los valores detectados en las células no estimuladas.

5.11. Determinación del potencial de membrana mitocondrial.

Para la determinación de cambios en el potencial de membrana mitocondrial (ψ_m) se empleó la sonda tetrametilrodamina metil éster (TMRM) (ThermoFisher, Madrid, España). El potencial de membrana mitocondrial es generado por el gradiente de protones creado entre el espacio intermembranoso y la matriz mitocondrial, y es un claro indicativo de la actividad mitocondrial.

Las células fueron despegadas del sustrato de crecimiento y cargadas con la sonda TMRM 100 nM (incubación durante 30 minutos a 37ºC). Posteriormente, se incubaron con los estímulos durante una hora y se procedió a la lectura de la fluorescencia empleando un lector de placas (CLARIOstar Plus, BMG Labtech., C-Viral, Madrid, España). La disminución de la fluorescencia derivada del TMRM indicaba una disminución del potencial de membrana mitocondrial.

5.12. Estudio de la actividad mitocondrial.

La determinación de la función mitocondrial se realizó empleando la tecnología Agilent SeaHorse XF Analysis que permite monitorizar en células vivas cambios en el consumo de oxígeno y cambios en el pH extracelular. Para determinar la alteración en

la función mitocondrial se empleó el kit Seahorse XF Cell Mito Stress Test (Figura 5.3.). Este kit nos permite determinar los siguientes parámetros:

- Respiración basal. El consumo de oxígeno necesario para suplir la demanda de energía de la célula en condiciones basales.
- Respiración ligada a la producción de ATP. Este parámetro se calcula tras la inyección de la oligomicina, un inhibidor del complejo V que desacopla el consumo de oxígeno de la producción de energía por la ATP sintasa. Permite conocer el consumo de oxígeno empleado para la producción de ATP.
- Fuga de protones. Este parámetro mide el consumo de oxígeno no ligado a la producción de ATP y se calcula restando la respiración basal a la respiración ligada a la producción de ATP.
- Respiración máxima. Este parámetro se calcula tras la adicción de FCCP, un agente desacoplante que rompe el gradiente de protones entre el espacio intermembranoso y la matriz mitocondrial. Esto emula una situación de demanda energética en la célula, la cual incrementa la oxidación de diferentes fuentes de energía para suplir esta demanda energética e incrementa, por lo tanto, el consumo energético.
- Capacidad respiratoria máxima o Spare Capacity. Este parámetro indica la capacidad máxima que posee la célula de incrementar la oxidación fosforilativa para hacer frente a un estrés energético. Este parámetro se calcula restando la respiración máxima a la respiración basal.
- Respiración no mitocondrial. La mitocondria no es la única demanda de oxígeno en la célula. Este parámetro mide el consumo de oxígeno por otras fuentes distintas a las mitocondrias. Se obtiene tras la inyección de oligomicina A y rotenona, inhibidores de los complejos mitocondrial I y III, e impide el transporte de electrones y con ello el consumo de oxígeno.

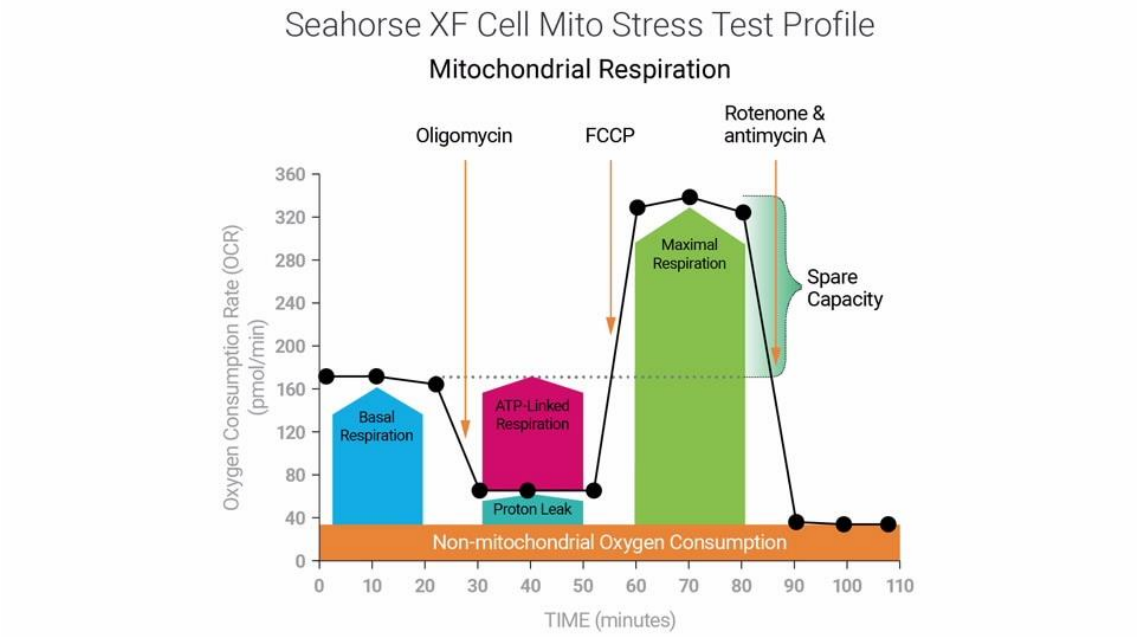

Figura 5.2. Perfil del Seahorse XF Cell Mito Stress Test. *Fuente: https://www.agilent.com/en/product/cell-analysis/real-time-cell-metabolic-analysis/xf-assay-kits-reagents-cell-assay-media/seahorse-xf-cell-mito-stress-test-kit-740885*

Las CEP se sembraron a una densidad de 30.000 células por pocillo en placas SeaHorse® de 24 pocillos. SeaHorse® de 24 pocillos. Al día siguiente, las células se incubaron en condiciones de normoxia o hipoxia, en ausencia o en presencia de melatonina, durante 24 horas. Antes de medir la tasa de consumo de oxígeno (OCR), el medio de cultivo se sustituyó por medio de cultivo DMEM sin rojo de fenol, que se complementó con 2 mM de L-glutamina, 10 mM de glucosa y 1 mM de piruvato. A continuación, las células se incubaron a 37 ºC en un incubador sin CO_2 durante 1 h. La OCR se midió en condiciones basales y en respuesta a 1 µM de oligomicina, 1,5 µM de p-(trifluoro-metoxi)fenilhidrazona de carbonilcianuro (FCCP) o 0,5 µM de rotenona más 0,5 µM de antimicina A. Los datos de OCR se expresan como valores absolutos normalizados con respecto al número de células, que se comprobó con tinción con violeta cristal.

5.13. Estudio de la actividad glucolítica.

Para la determinación de la actividad glucolítica también se empleó la tecnología Agilent SeaHorse XF Analysis. En esta ocasión, se empleó el kit Seahorse XF Glycolysis Stress Test, el cual se basa en la determinación de la ratio de acidificación extracelular (ECAR) del medio producido por el metabolismo celular (Figura 5.4.). Este kit nos permite determinar los siguientes parámetros:

- Glucolisis. Este parámetro se calcula tras la adicción de glucosa al medio.

- Capacidad glucolítica. Este parámetro permite determinar la capacidad máxima de la célula de utilizar la glucolisis como fuente de energía. Esto se calcula tras la adición de oligomicina, que inhibe el complejo V e impide la producción de energía por la fosforilación oxidativa.
- Reserva glucolítica. Este parámetro hace referencia a las reservas que posee la célula para suplir una demanda energética máxima por vía glucolítica.

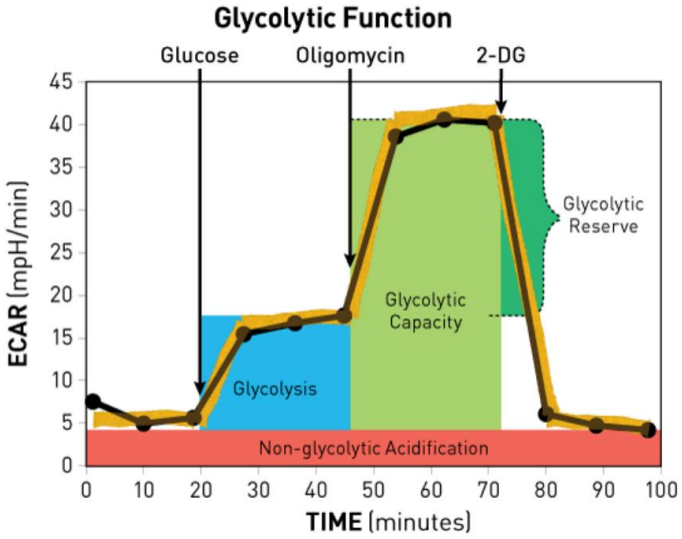

Figura 5.3. Perfil del XF Glycolysis Stress Test. *Fuente: https://www.agilent.com/cs/library/usermanuals/public/XF_Glycolysis_Stress_Test_Kit_User_Guide.pdf*

Las CEP se sembraron a una densidad de 30.000 células por pocillo en placas SeaHorse® de 24 pocillos. Al día siguiente, las células se incubaron en condiciones de normoxia o hipoxia, en ausencia o en presencia de melatonina, durante 24 horas. Antes de la medición de la tasa de acidificación extracelular, el medio de cultivo medio se sustituyó por DMEM sin rojo de fenol, que estaba suplementado con 2 mM de L-glutamina. A continuación, las células se incubaron a 37 ºC en una incubadora sin CO_2 durante una hora. El ECAR se midió en condiciones basales basales y en respuesta a 10 mM de glucosa, 1 µM de oligomicina y 100 mM de 2-Deoxiglucosa (2-DG). Los datos se expresan como valores absolutos normalizados con respecto al número de células, que se comprobó con tinción con violeta cristal.

5.14. Análisis estadístico.

Para el análisis estadístico se empleó del programa GraphPad Prism Versión 6.01. El análisis estadístico de los datos se realizó mediante un análisis de varianza de una vía (ANOVA) seguido de la prueba *post hoc* de Tukey, y sólo los valores $P < 0,05$ se consideraron estadísticamente significativos. Para las comparaciones individuales y

las estadísticas entre tratamientos individuales, se empleó la prueba *t* de Student, y sólo los valores $P < 0,05$ se consideraron estadísticamente significativos.

6. RESULTADOS

En la presente Tesis Doctoral se incluyen los siguientes artículos de investigación que contienen los resultados obtenidos y que responden a los objetivos planteados:

- Estaras M, Peña FJ, Tapia JA, Fernandez-Bermejo M, Mateos JM, Vara D, Roncero V, Blanco G, Lopez D, Salido GM, Gonzalez A. Melatonin modulates proliferation of pancreatic stellate cells through caspase-3 activation and changes in cyclin A and D expression. J Physiol Biochem. 2020 May;76(2):345-355. doi: 10.1007/s13105-020-00740-6.
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ORIGINAL ARTICLE

Melatonin modulates proliferation of pancreatic stellate cells through caspase-3 activation and changes in cyclin A and D expression

Matias Estaras[1] · Fernando J. Peña[2] · José A. Tapia[1] · Miguel Fernandez-Bermejo[3] · Jose M. Mateos[3] · Daniel Vara[3] · Vicente Roncero[4] · Gerardo Blanco[5] · Diego Lopez[5] · Gines M. Salido[1] · Antonio Gonzalez[1]

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Abstract

In this study, the effects of melatonin (1 μM–1 mM) on pancreatic stellate cells (PSC) have been examined. Cell viability and proliferation, caspase-3 activation, and the expression of cyclin A and cyclin D were analyzed. Our results show that melatonin decreased PSC viability in a time- and concentration-dependent manner. This effect was not inhibited by treatment of cells with MT1, MT2, calmodulin, or ROR-alpha inhibitors prior to melatonin addition. Activation of caspase-3 in response to melatonin was detected. The expression of cyclin A and cyclin D was decreased in cells treated with melatonin. Finally, changes in BrdU incorporation into the newly synthesized DNA of proliferating cells were also observed in the presence of melatonin. We conclude that melatonin, at pharmacological concentrations, modulates proliferation of PSC through activation of apoptosis and involving crucial regulators of the cell cycle. These actions might not require specific melatonin receptors. Our observations suggest that melatonin, at high doses, could potentially exert anti-fibrotic effects and, thus, could be taken into consideration as supportive treatment in the therapy of pancreatic diseases.

Keywords Apoptosis · Cyclin · Melatonin · Cell viability · Pancreatic stellate cells

Key points
- Melatonin, at pharmacological concentrations, modulates viability of PSC.
- Melatonin stimulates caspase-3 activation.
- Melatonin might regulate cell cycle in a cyclin-dependent manner.
- These actions might not involve specific melatonin receptors.

✉ Antonio Gonzalez
agmateos@unex.es

[1] Institute of Molecular Pathology Biomarkers, University of Extremadura, Avenida de las Ciencias s/n, E-10003 Cáceres, Spain

[2] Laboratory of Equine Reproduction and Equine Spermatology, Veterinary Teaching Hospital, University of Extremadura, Cáceres, Spain

[3] Department of Gastroenterology, San Pedro de Alcantara Hospital, Cáceres, Spain

[4] Unit of Histology and Pathological Anatomy, Veterinary Faculty, University of Extremadura, Cáceres, Spain

[5] Hepatobiliary-Pancreatic Surgery and Liver Transplant Unit, Infanta Cristina Hospital, Badajoz, Spain

Abbreviations

BrdU	5-bromo-2-deoxyuridine
FSC	Forward scatter
KN93	N-[2-[[[3-(4-Chlorophenyl)-2-propenyl] methylamino]methyl]phenyl]-N-(2-hydroxyethyl)-4-methoxybenzenesulphonamide
PSCs	Pancreatic stellate cells
SCC	Side scatter
SR1001	N-[4-Methyl-5-[[[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl]amino]sulfonyl]-2-thiazolylacetamide
Tps	Thapsigargin

Introduction

Pancreatic stellate cells (PSC) play pivotal roles in the physiology and the pathophysiology of the pancreas. PSC comprise of around 4–7% of the organ and are normally in a dormant, i.e., inactive, state. However, these cells turn into an activated state in disease. It has been highlighted that activated PSC are responsible for the desmoplastic reaction present in severe

pancreatic disorders such as chronic pancreatitis and pancreatic ductal adenocarcinoma [8].

Scientific studies are currently carried out in order to unravel their role in pancreatic cancer, which nowadays represents one of the deadliest diseases. PSC neither secrete digestive enzymes nor hormones. Additionally, they do not respond to stimuli like acetylcholine or cholecystokinin. Nevertheless, PSC release factors that may influence neighbor cells [11]. Activated PSC collaborate in tumor progression and chemoresistance. The major factor underlying this hypothesis includes the release of molecules that contribute to the growth of fibrotic tissue and to the activation of different types of cells within the pancreas [38]. Understanding how activation and regulation of growth and proliferation of PSC occurs represents a leading challenge in the therapy of pancreatic illness. This is of major interest in cancer, because the extensive fibrosis that accompanies pancreatic tumors represents a barrier for a successful action of chemo and radiotherapy.

Numerous evidences highlight a role for melatonin as regulator of pancreatic physiology. The expression of melatonin membrane-bound MT1- and MT2-type receptors has been reported in the pancreas [10]. In addition, other points of action of melatonin are located in the cytosol or in the nucleus [39]. Melatonin regulates enzyme and bicarbonate secretion [18, 35]. Additionally, melatonin exerts protective actions on the gland. Some of them involve signaling and gene expression. Other actions seem to be carried out through interactions with enzymes, ion channels, or electron transport. These actions are all related with the antioxidant effects of melatonin [13]. Moreover, melatonin has anti-inflammatory effects [17, 29]. On another side, melatonin induces antitumor effects in the pancreas [10, 19]. In addition, melatonin modulates PSC viability [7, 37].

In the present work, we continued our former studies and have further investigated the ways by which melatonin could exert its effects on PSC to control their proliferation. As a major concern of our research, we aimed at gaining further understanding of the mechanisms by which melatonin exerts its actions on the pancreas, in a search for a compound potentially useful in the therapy of the illnesses that affect the gland.

Materials and methods

Pancreatic tissues and chemicals

Pancreatic tissues, obtained from newborn Wistar rats (7 days), were used in the present study. The animals that were used in this study were provided by the animal house of the University of Extremadura (Caceres, Spain). Animals handling was approved the University Ethical Committee for Animal Research of the University of Extremadura (reference 57/2016) and by the Institutional Committee of the Junta de Extremadura (reference 20160915). Additionally, all methods and the experimental protocols were performed in accordance with the relevant guidelines and regulations of the Ethical Committee for Animal Research of the University of Extremadura and with the Institutional Committee of the Junta de Extremadura (law 32/2007 and RD 53/2013). Collagenase CLSPA was obtained from Worthington Biochemical Corporation (Labclinics, Madrid, Spain). Cell Lysis Reagent, melatonin, hydrogen peroxide (H_2O_2), Tween®-20, and thapsigargin were obtained from Sigma Chemicals Co. (Madrid, Spain). AlamarBlue® was purchased from AbD serotec (BioNova Científica, Madrid, Spain). Hank's balanced salts (HBSS), horse serum, and medium 199 were purchased from Life Technologies (Invitrogen, Barcelona, Spain). Cell Event Caspase-3/7 Green Detection Reagent (excitation, 502 nm; emission: 530 nm), ethidium homodimer (Eth-1) (excitation, 528 nm; emission, 617 nm), fetal bovine serum (FBS), Hoechst 33342 (excitation 404 nM; emission 488 nm), and polystyrene plates for cell culture were purchased from Thermo Scientific (Madrid, Spain). Penicillin/streptomycin was obtained from BioWhittaker (Lonza, Basel, Switzerland). Bradford reagent, Tris/glycine/SDS buffer (10×) and Tris/glycine buffer (10×) were from Bio-Rad (Madrid, Spain). SignalFire™ ECL Reagent was obtained from Cell Signaling Technology (C-Viral, Madrid, Spain). 5-bromo-2-deoxyuridine (BrdU) cell proliferation assay kit was purchased from BioVision (Deltaclon S.L., Madrid, Spain).

Antibodies against Cyclin A and Cyclin D were purchased from Thermo Scientific (Erembodegen, Belgium) and Abcam plc (Cambridge, UK) respectively. Anti-actin antibody was purchased from Sigma Chemicals Co. (Madrid, Spain). Secondary antibodies goat anti-rabbit and rabbit anti-goat IgG HRP conjugate were purchased from Thermo Fisher Sci. (Fisher Scientific Inc., Madrid, Spain). All other analytical grade chemicals used were obtained from Sigma Chemicals Co. (Madrid, Spain).

Preparation of pancreatic stellate cells cultures

Preparation of cultures of PSC was carried out following methods used in our laboratory [37]. Briefly, the pancreas was washed in a physiological buffer containing: 130 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 1.2 mM KH_2PO_4, 10 mM glucose, 10 mM HEPES, 0.01% trypsin inhibitor (soybean), and 0.2% bovine serum albumin (pH = 7.4 adjusted with NaOH). Next, an enzymatic digestion was carried out by incubation of the tissues for 50 min at 37 °C in buffer supplemented with 30 units/mL collagenase CLSPA from Worthington.

Thereafter, mechanical dissociation of the cells was carried out by gently pipetting the cell suspension through tips of decreasing diameter. After centrifugation at 30×*g* for 5 min at 4 °C, cells were resuspended in culture medium (medium

199 supplemented with 4% horse serum, 10% FBS, 0.1 mg/ml streptomycin, 100 IU penicillin, and 1 mM $NaHCO_3$). Finally, cells were seeded on polystyrene plates for cell culture and grown in a humidified incubator at 37 °C and 5% CO_2. With this procedure, it is possible to obtain cultures of a population of PSC as demonstrated by the expression of PSC-specific markers as desmin, α-smooth muscle actin, cytokeratin-7, vimentin, and glial fibrillary acidic protein [7, 27, 28, 37, 51]. Confluence (90–95%) was reached after 18 days of culture. Cells used in the experiments had been obtained from different preparations.

Cell viability and proliferation assays

Determination of cell viability was carried out using AlamarBlue® test as described previously [36]. Additionally, cell proliferation was analyzed by detection of BrdU incorporation into the newly synthesized DNA of proliferating cells. A commercially available kit (BrdU Cell Proliferation Assay Kit, from Biovision) was used. Proliferation of cells was determined following manufacturer's directions. Absorbance of the samples was measured employing a plate reader (CLARIOstar Plus, BMG Labtech., C-Viral, Madrid, Spain). The viability of treated cells (subjected to stimuli) was compared with that of non-stimulated cells. Data show the mean change of absorbance expressed in percentage ± S.E.M. (*n*) with respect to non-stimulated cells, where *n* is the number of independent experiments.

Cell proliferation was further studied by detection of the expression of cyclin A and cyclin D, which was carried out by Western blotting analysis.

Caspase-3 determination

Caspase-3 activation was determined by flow cytometry employing previously described methods [26].

CellEvent Caspase-3/7 Green Detection Reagent consists of a four-amino-acid peptide (DEVD) conjugated to a nucleic acid-binding dye. This cell-permeant substrate is intrinsically non-fluorescent because the DEVD peptide inhibits the ability of the dye to bind to DNA. After activation of caspase-3 in apoptotic cells, the DEVD peptide is cleaved, enabling the dye to bind to DNA and produce a bright, fluorogenic response with absorption/emission maxima of 502/530 nm. Samples were diluted in standard PBS to a final concentration 5×10^6 cells/mL. The cells were stained with 1 μL CellEvent Caspase-3/7 Green Detection Reagent (2 mM stock solution) and 1 μL of Hoechst 33342 (16.2 mM stock solution). After thorough mixing, the cell suspension was incubated at room temperature (23–25 °C) in the dark for 25 min, then cells were loaded with 1 μL ethidium homodimer (1.167 mM in DMSO) and incubated for further 5 min. Next, the samples were immediately run on the flow cytometer. The controls consisted of unstained and single-stained controls to properly set gates and compensations.

Analyses were conducted using a Cytoflex® flow cytometer (Beckman Coulter, USA) equipped with violet, blue, and red lasers. The instrument was calibrated daily using specific calibration beads provided by the manufacturer. A compensation overlap was performed before each experiment. Files were exported as FCS files and analyzed using FlowJoV 10.4.1 Software (Ashland, OR, USA). Unstained, single-stained, and Fluorescence Minus One (FMO) controls were used to determine compensations and positive and negative events, as well as to establish regions of interest, as described in previous publications [30]. Data are expressed in percentage ± S.E.M. (*n*) with respect to non-stimulated cells, where *n* is the number of independent experiments.

Western blotting analysis

Western blotting was performed using previously described methods [10]. After treatment with different stimuli, cells were washed with phosphate-buffered saline (PBS) of the following composition: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4; pH adjusted to 7.4. Next, cells were lysed and were sonicated. The protein content of each sample was determined by Bradford's method [2]. Protein lysates (12 μg/lane) were separated by SDS-PAGE and were transferred to nitrocellulose membranes. Specific primary and the corresponding IgG HRP-conjugated secondary antibody were used to detect the presence of proteins in the membranes. The intensity of the bands which appear was quantified using the software Image J (http://imagej.nih.gov/ij/). The experiments were carried out employing batches of cells obtained from different preparations. Values are expressed as the mean ± SEM of normalized values expressed as % vs. non-stimulated cells.

Statistical analysis

Statistical analysis of data was performed by one-way analysis of variance (ANOVA) followed by Tukey post hoc test, and only P values < 0.05 were considered statistically significant. For individual comparisons and statistics between individual treatments, we employed the Student's t test, and only P values < 0.05 were considered statistically significant.

Results

Effects of melatonin on cell viability

Proliferation of PSC has been related with fibrosis that takes place in the exocrine pancreas in inflammation and cancer [21, 27]. Melatonin decreases viability of pancreatic cancer cells

[10, 19]. At this point, we interested in confirming the effect of melatonin on cell viability. Therefore, PSC were incubated during 24 h, 48 h, 72 h, and 96 h in the presence of 1 mM, 100 μM, 10 μM, or 1 μM melatonin. Other cells were incubated in the absence of stimulus (non-stimulated) and were used as control to compare the effects of melatonin.

Melatonin induced a concentration-dependent effect on cell survival. Cell viability dropped after treatment of cells with 1 mM melatonin (Fig. 1a). We observed a slight, but significant, drop of cell viability beyond day 2 in the presence of 1 μM, 10 μM, or 100 μM melatonin. The drop in cell viability was stronger at 96 h of incubation (Fig. 1b and d).

Separate batches of cells were incubated during 24 h, 48 h, 72 h, and 96 h in the presence of 1 μM thapsigargin (Tps), a cell death inducer [4]. Tps induced a time-dependent decrease in cell viability compared with non-stimulated cells (Fig. 1e).

It has been shown that melatonin can bind to MT1- and MT2-type membrane receptors to induce its effects [3, 39]. However, in previous works, we showed that PSC do not exhibit classical melatonin membrane receptors MT1 and MT2 [7, 37]. To test if the effects of melatonin on cell viability are independent of the classical melatonin membrane receptors, we performed a series of experiments in which cell viability was evaluated in the presence of the combination of melatonin (1 mM–1 μM) plus a MT1-antagonist (luzindole; 10 μM) [15] or melatonin (1 mM–1 μM) plus a MT2-antagonist (K185, 5 μM) [12]. PSC were preincubated with the antagonists for 5 min prior to addition of melatonin. Thereafter, cells were incubated during 24 h, 48 h, 72 h, and 96 h. Neither luzindole nor K185 blocked the effects of melatonin on PSC viability (see Table 1).

In addition, binding of melatonin to calmodulin or to nuclear orphan receptors from the RORα/RZR family has also been proposed [3, 39]. To study if the effects of melatonin on cell viability were potentially mediated through these receptors, a series of experiments were carried out in which PSC were preincubated for 5 min in the presence of a ROR-alpha-antagonist (SR1001, 10 μM) [40] or a calmodulin-antagonist (KN-93, 1 μM) [14], prior to addition of melatonin. Thereafter, cells were incubated during 24 h, 48 h, 72 h, and 96 h. Cell viability decreased in cells incubated with KN-93 and SR1001 alone. However, none of these melatonin receptor-antagonists blocked the effect of melatonin on cell viability. The results obtained are summarized in Table 1.

Caspase-3 activation in response to melatonin

Previous work has shown that melatonin evokes caspase-3 activation to induce cell death in pancreatic cancer cells [10]. Therefore, we evaluated if melatonin induced caspase-3 activation in PSC, which might be related with the decrease of cell viability that we had observed. For this purpose, cells were incubated during 24 h with different concentrations of melatonin (1 mM–1 μM) and caspase-3 activation was determined by flow cytometry. In the presence of melatonin, caspase-3 activation was observed (melatonin 1 mM: 399.2 ± 64.6, $n = 3$, $P < 0.05$; melatonin 100 μM: 321.6 ± 21.5, n = 3, $P < 0.05$; melatonin 10 μM: 342.1 ± 33.9, $n = 3$, $P < 0.05$; melatonin 1 μM: 250.5 ± 46.8, $n = 3$, $P < 0.05$). Representative cytograms of the assay and gating strategies performed are given in Fig. 2.

Effect of melatonin on cyclin expression

In the results shown above, it can be observed that PSC survive to a certain extent following treatment with melatonin, because cell viability did not decrease completely in the presence of the indole. It might be possible that cells that survive could enter cell cycle in an effort to maintain cell population. Cyclin is a family of proteins that control the progression of cells through the cell cycle. Therefore, we were interested in studying whether melatonin exerts any effect on cyclin A, which is active in the early phases of division, and cyclin D, which regulates transition from G_1 to S phase [33, 45].

The expression of cyclin A and D was studied by western blot. PSC were incubated in the absence (non-stimulated cells) or in the presence of melatonin (1 mM–1 μM) for 1 h. Then, cell lysates were analyzed to detect cyclin A and cyclin D. In the presence of melatonin, the expression of both proteins was decreased. This was observed at all concentrations of melatonin tested, except for 1 μM melatonin in the case of cyclin D, where an increase in the expression of the protein was noted. At this concentration, the expression of cyclin A was lower compared to that found in non-stimulated cells, although the differences were not statistically significant (Fig. 3).

Effect of melatonin on cell proliferation

We next evaluated cell proliferation employing a kit based on 5-bromo-2-deoxyuridine (BrdU). BrdU incorporates into the DNA of dividing cells and can be used as indicator of cell proliferation. For this purpose, cells were incubated during 48 h in the absence (non-stimulated cells) or in the presence of melatonin (1 mM–1 μM). Thereafter, cell proliferation was analyzed. In the presence of melatonin, a decrease in BrdU content, i.e., lower cell proliferation, was observed in comparison with that detected in non-stimulated cells (Fig. 4).

Discussion

Transformed epithelia within the pancreas, along with fibroblasts, play a pivotal role in the growth and progression of carcinomas [25]. Moreover, participation of PSC in cancer growth has been documented [31]. As a major challenge,

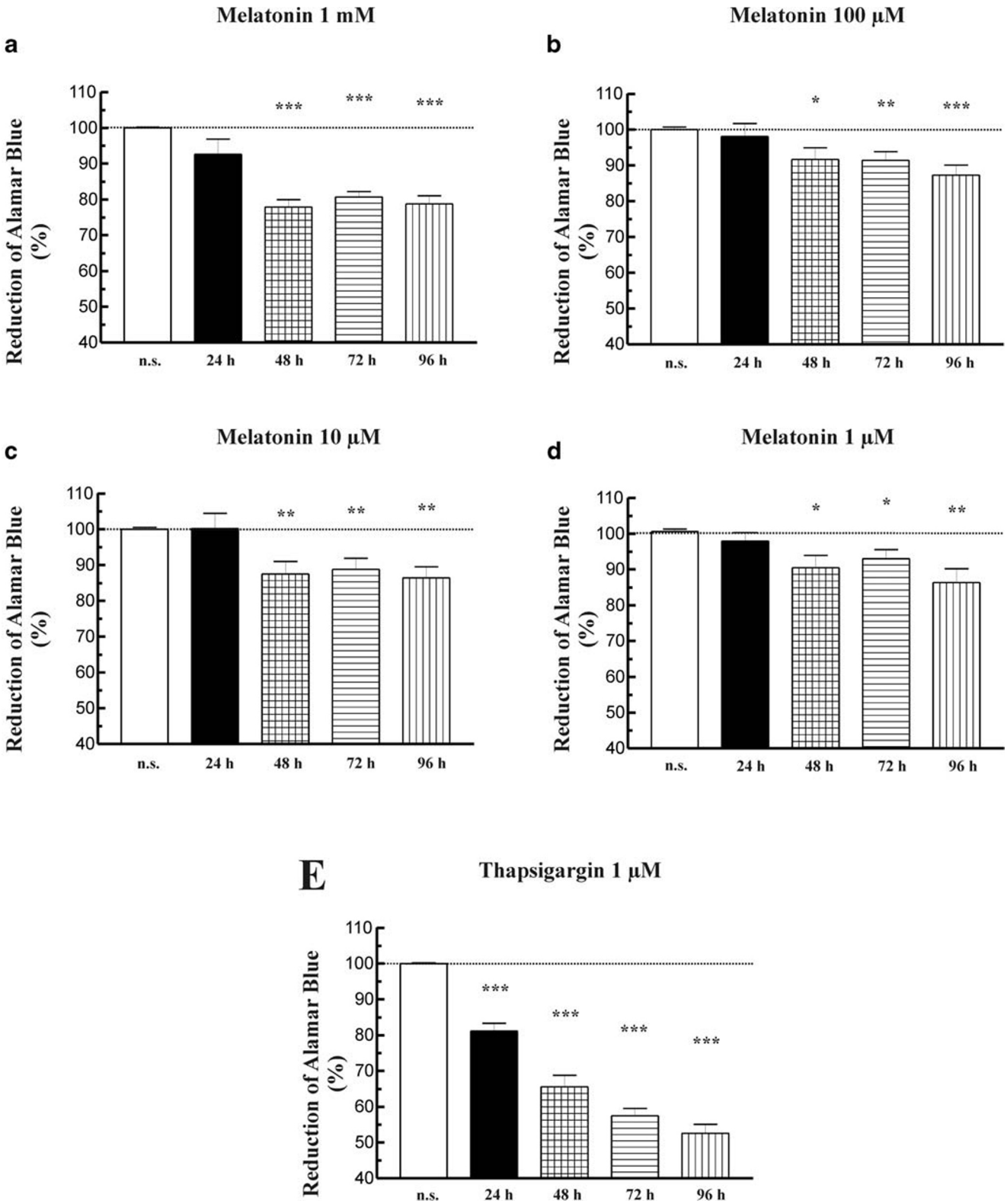


Fig. 1 Analysis of PSC viability. Cells were incubated in the presence of 1 mM (**a**), 100 μM (**b**), 10 μM (**c**), 1 μM (**d**) melatonin, or 1 μM Tps (**e**) during 24 h, 48 h, 72 h, and 96 h. Cell viability was determined and was compared with that of cells in the absence of stimulus (non-stimulated cells). A horizontal dotted line represents the viability of cells in the absence of stimulus (non-stimulated cells), which was considered 100%. Histograms are representative of three independent experiments (n.s., non-stimulated cells; $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ vs. non-stimulated cells)

Table 1 Effect of melatonin receptors inhibition on cell viability

	24 h	48 h	72 h	96 h
Mel 1 mM	92.64 ± 4.21	77.89 ± 2.03 (***)	80.66 ± 1.55 (***)	78.78 ± 2.25 (***)
Mel 100 μM	98.06 ± 3.61	91.66 ± 3.28 (*)	91.97 ± 2.69 (**)	87.30 ± 2.80 (***)
Mel 10 μM	100.2 ± 4.23	87.50 ± 3.49 (**)	88.79 ± 3.12 (**)	86.41 ± 3.06 (**)
Mel 1 μM	97.97 ± 2.29	90.48 ± 3.48 (*)	92.98 ± 5.58 (*)	86.37 ± 3.83 (**)
Luz 10 μM + Mel 1 mM	81.85 ± 5.42 (**)	81.41 ± 7.89 (**)	78.99 ± 3.85 (***)	71.99 ± 3.93 (***)
Luz 10 μM + Mel 100 μM	99.30 ± 6.35	95.89 ± 8.58	97.77 ± 1.77	83.45 ± 4.72 (**)
Luz 10 μM + Mel 10 μM	101.70 ± 3.26	91.36 ± 12.20	95.67 ± 1.66	88.34 ± 3.20 (**)
Luz 10 μM + Mel 1 μM	97.43 ± 4.49	89.54 ± 6.81 (*)	98.08 ± 2.44	91.69 ± 2.98 (*)
Luz 10 μM	94.93 ± 2.24	94.27 ± 6.97	94.63 ± 4.96	93.86 ± 3.41
K185 5 μM + Mel 1 mM	81.11 ± 3.31 (***)	84.09 ± 4.27 (***)	84.78 ± 4.62 (**)	72.70 ± 5.47 (***)
K185 5 μM + Mel 100 μM	88.13 ± 3.94 (**)	89.12 ± 6.72 (*)	95.36 ± 5.15	80.44 ± 5.95 (***)
K185 5 μM + Mel 10 μM	99.00 ± 8.50	96.21 ± 10.75	82.27 ± 4.65 (***)	92.59 ± 5.87
K185 5 μM + Mel 1 μM	103.50 ± 8.71	92.13 ± 6.08	89.91 ± 6.75 (*)	102.70 ± 8.09
K185 5 μM	105.90 ± 6.93	101.80 ± 6.39	95.96 ± 3.48	73.69 ± 9.76 (***)
SR1001 10 μM + Mel 1 mM	88.14 ± 3.06 (*)	90.63 ± 2.62 (*)	73.42 ± 4.56 (***)	79.15 ± 3.21 (***)
SR1001 10 μM + Mel 100 μM	86.61 ± 5.90 (*)	90.04 ± 3.36 (*)	79.26 ± 4.06 (***)	82.21 ± 5.88 (**)
SR1001 10 μM + Mel 10 μM	92.89 ± 8.24	98.93 ± 4.75	79.57 ± 4.49 (***)	81.89 ± 5.86 (**)
SR1001 10 μM + Mel 1 μM	95.83 ± 2.83	93.76 ± 5.83	91.52 ± 2.23 (*)	82.89 ± 3.20 (***)
SR1001 10 μM	88.99 ± 5.36 (*)	89.56 ± 3.49 (*)	89.25 ± 4.75 (*)	85.72 ± 3.16 (**)
KN93 1 μM + Mel 1 mM	95.98 ± 2.01 (*)	80.11 ± 5.61 (***)	85.85 ± 4.46 (***)	66.51 ± 2.73 (***)
KN93 1 μM + Mel 100 μM	102.20 ± 7.01	87.94 ± 6.39 (**)	92.91 ± 4.69 (*)	75.71 ± 4.55 (***)
KN93 1 μM + Mel 10 μM	112.90 ± 6.65 (*)	93.83 ± 6.26	92.20 ± 4.64 (*)	75.03 ± 3.52 (***)
KN93 1 μM + Mel 1 μM	116.10 ± 7.64 (***)	102.00 ± 4.40	94.80 ± 1.25 (*)	83.35 ± 2.54 (***)
KN93 1 μM	115.70 ± 4.79 (***)	88.16 ± 7.99 (**)	96.55 ± 2.75	88.02 ± 4.31 (***)
Tps 1 μM	81.13 ± 2.176 (***)	65.58 ± 3.18 (***)	57.48 ± 2.03 (***)	52.55 ± 2.55 (***)

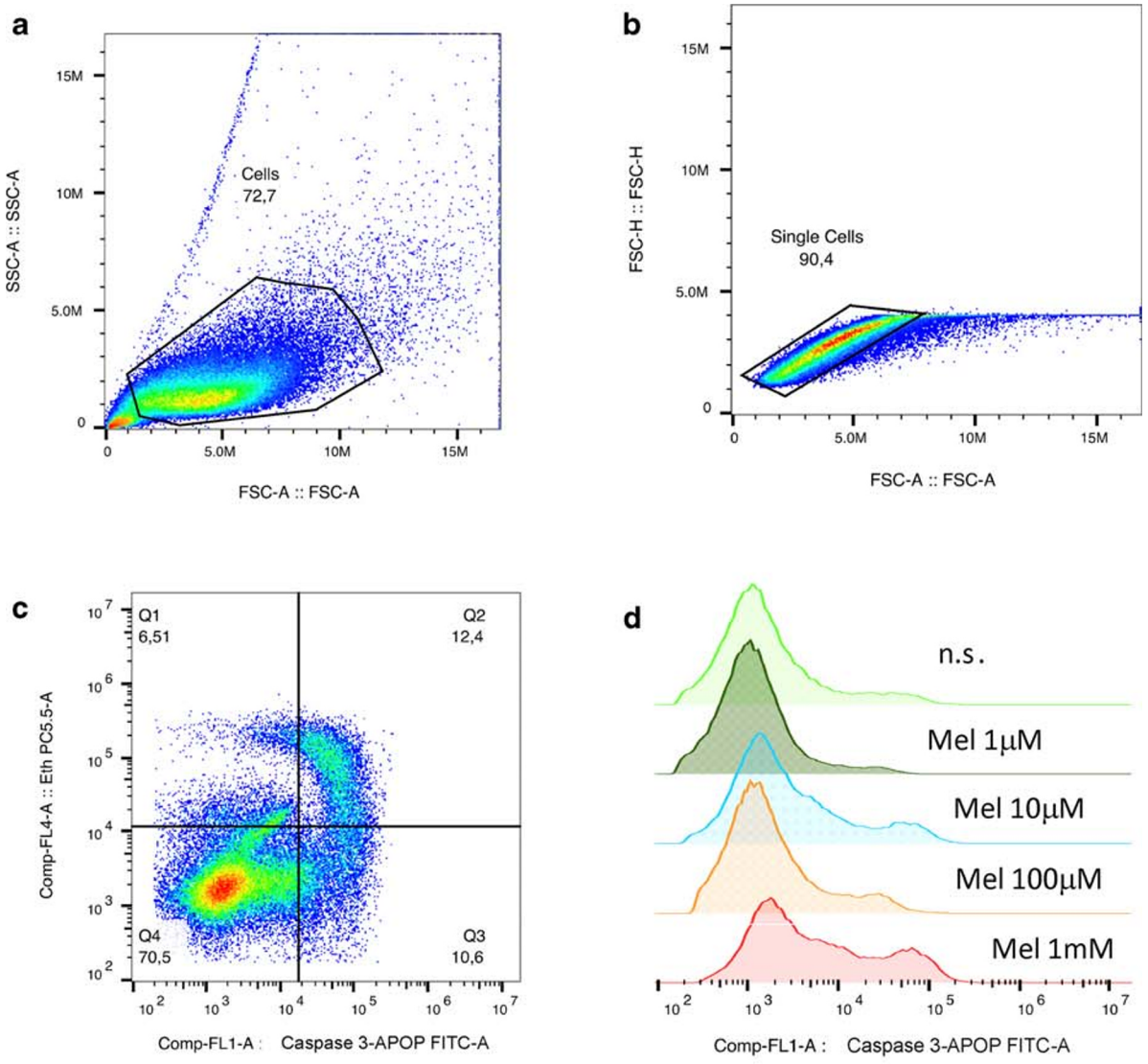
PSC were incubated during 24 h to 96 h in the presence of luzindole (10 μM), K185 (5 μM), SR1001 (10 μM), or KN93 (1 μM) alone or in combination with melatonin (1 mM to 1 μM). The antagonists were added to the cells 5 min prior to addition of melatonin. Cell viability was determined as described in "Material and methods section" and is expressed in percentage ± S.E.M. with respect to non-stimulated cells. Studies were performed in cells from three different preparations. Each stimulus was assayed in triplicate (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. non-stimulated cells)

modulation of the development of fibrotic tissue within tumors is pointed out as relevant in the treatment of cancer [27].

We have former evidences that support the capability of melatonin to regulate the viability of PSC [7, 37]. Here, we continued to investigate the effect of the indole on the physiology of PSC. Attention has been paid to the ways by which melatonin could exert its effects on PSC to control their proliferation. This is of great relevance in medicine because, in case of positive effects, melatonin could be considered of putative usefulness in the therapy of cancer and pancreatitis.

Our results show effects of pharmacological concentrations of melatonin on cell viability. The decrease in cell population might be due to activation of caspase-3-dependent apoptosis. However, treatment with melatonin did not lead to a complete cell loss. Hypothetically, those cells that survive might grow slowly due to the actions of melatonin on cyclin expression that, in turn, could regulate cell cycle. The effects of melatonin were concentration-dependent in all biomarkers studied, except for cyclin expression. The higher effect was noted with 1 mM melatonin. In our opinion, the results that we have obtained are of major interest, taking into account that PSC exhibit a pivotal role in the fibrosis that expands in pancreatic inflammation and cancer [8, 27, 38].

Fig. 2 Gating strategy and representative cytograms depicting flow cytometry detection of apoptotic cells. **a** Identification of cells was based in FSC and SCC. **b** Doublets and clumps are identified and gated out of the analysis. **c** 2D dot plot showing apoptotic cells after staining with CellEvent and Eth-1; events in Q4 are live non apoptotic cells; events in Q3 represent caspase-3 positive cells; events in Q2 are cells caspase-3 + and ETH-1 +. Events in Q1 represent necrotic cells. **d** The effect of the treatment is depicted showing increased expression of caspase-3 from top to bottom. **e** Histogram showing the values of caspase-3 activation detected for each treatment (n.s., non-stimulated cells; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. non-stimulated cells). A horizontal dotted line represents the level of caspase-3 detected in cells incubated in the absence of stimulus (non-stimulated cells), which was considered 100%. Results are representative of three different preparations (n.s., non-stimulated cells; Mel, melatonin; *$P < 0.05$, **$P < 0.01$ vs. non-stimulated cells)


a
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Q1
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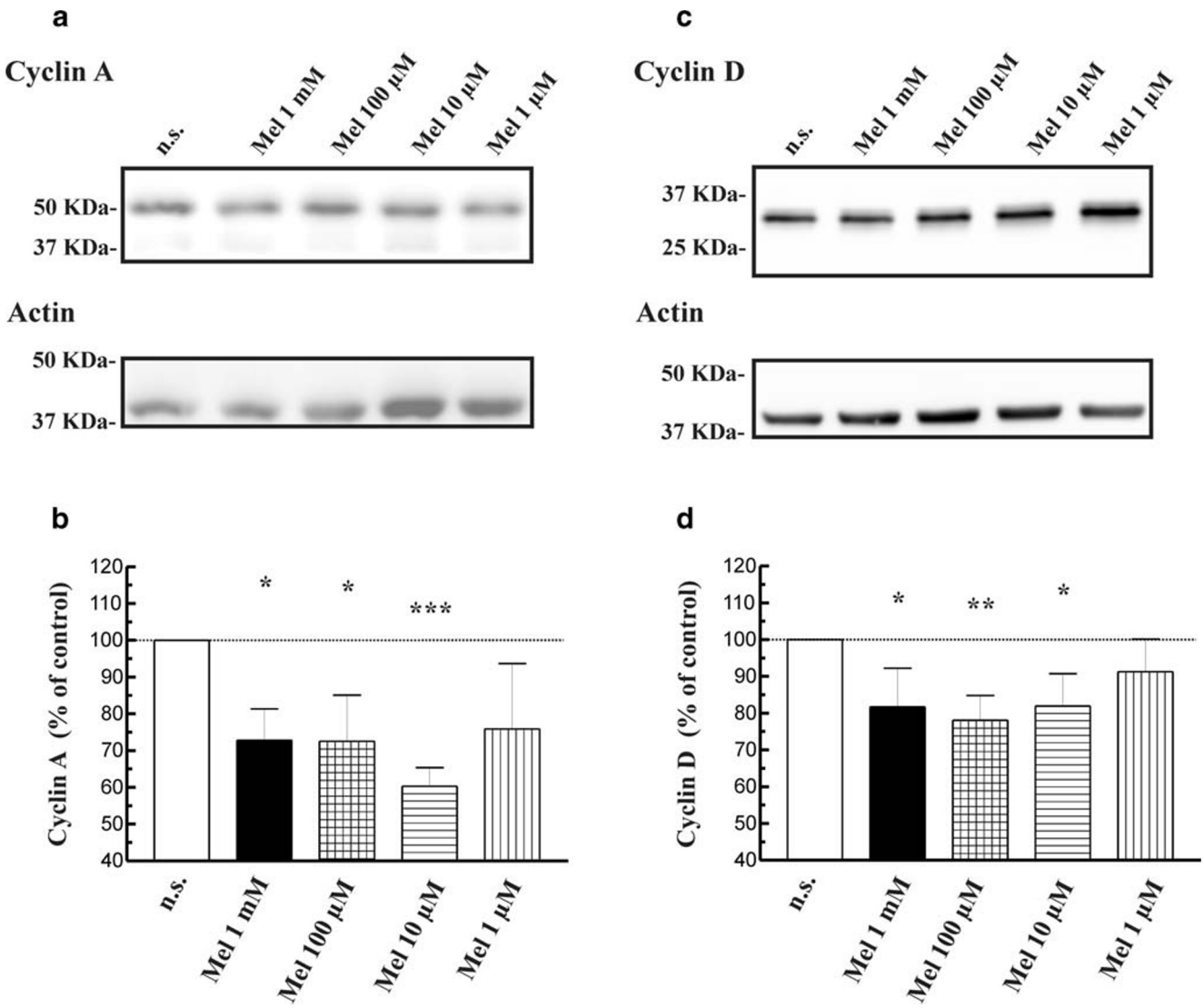

Fig. 3 Effect of melatonin on cyclin A and D expression. PSC were incubated with 1 mM–1 µM melatonin for 1 h. Then, cell lysates were analyzed to determine the level of cyclin A and cyclin D. The figure shows representative blots showing the expression of cyclin A (**a**) and D (**c**) evaluated with specific antibodies. To ensure equal loading of proteins, the levels of actin were employed as controls under the tested conditions. The graphs show the quantification of protein expression (**b** and **d**). A horizontal-dotted line represents the value observed in non-stimulated cells, which was considered 100%. Values are the mean ± SEM of normalized values expressed as % vs. non-stimulated cells. The experiments shown are representative of three others. (n.s., non-stimulated cells; Mel, melatonin; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. non-stimulated cells)

Treatment of cells with melatonin decreased cell viability. The stronger effect was noted when the cells were treated with 1 mM melatonin. These results are in accordance with previously findings of our laboratory [5, 7, 37]. The action of melatonin on cell membrane receptors might not be responsible for the decrease in cell viability, because the effect of melatonin was not abolished by pretreatment of cells with known melatonin receptor-antagonists. In fact, MT1 and MT2 receptors could not be detected in PSC [7, 37]. Melatonin also binds to calmodulin or to nuclear receptors. However, controversies exist regarding whether melatonin binds to the RORα/RZR family [39]. Our results show that neither an inhibitor of calmodulin nor of ROR-alpha receptors abolished the effect of melatonin on PSC viability. The concentrations of each inhibitor that we have used fall within the range reported by other studies that show efficacy of luzindole [15, 20, 32], K185 [12, 43], SR1001 [40, 47], and KN93 [14, 34, 48] in blocking the signals induced by melatonin. Thus, our observations rather point towards direct or receptor-independent actions of melatonin.

Melatonin may be an effective apoptosis inducer in cancer cells through regulation of a large number of molecular pathways [16, 44]. Activation of caspase-3 by melatonin has been reported in different types of tumors [6, 41, 42], including the pancreas [10]. Our results are in agreement with these previous findings and support an action of melatonin on apoptosis that might regulate proliferation of PSC.

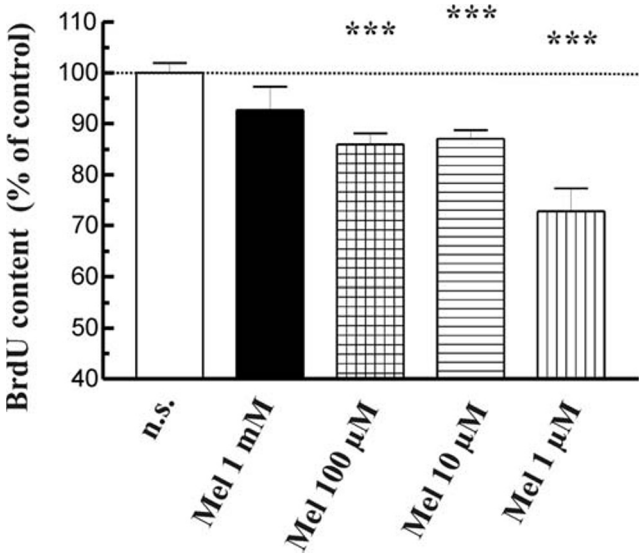

Fig. 4 Effect of melatonin on BrdU content. Cells were incubated during 48 h in the presence of different concentrations of melatonin (1 mM–1 μM) and Brdu incorporation to DNA of dividing cells was compared with that of cells incubated in the absence of melatonin (non-stimulated cells). A horizontal dotted line represents the proliferation of non-stimulated cells (which was considered 100%). Data are representative of three independent experiments (n.s., non-stimulated cells; Mel, melatonin; ***$P < 0.001$ vs. non-stimulated cells)

Our results further show an action of melatonin on the cell cycle. In the presence of melatonin, decreases in the expression of cyclin A and D were observed. Cyclin A is an essential regulator of the cell division cycle. Cyclin A is required for S, G_2, and M phase progression and increased cyclin A expression has been observed in various cancers [24, 33]. Former studies suggested that melatonin does not require changes in cyclin A to exert its antiproliferative actions [22, 23, 50]. However, a decrease in the expression of cyclin A in prostate cancer cells in response to melatonin has been documented [49]. Our observations agree with the latter study.

Another regulator protein involved in the cell cycle is cyclin D. This protein promotes the progression from G_1 to S phase. It has been found to be overexpressed in different types of cancer [45], including pancreatic cancer [46]. This fact might be associated with enhanced growth and rapid cell cycle progression of pancreatic cancer cells. Melatonin has been shown to downregulate the expression of cyclin D in tumor cells [22].

The changes induced by melatonin in the expression of pivotal regulators of cell cycle that we have observed, together with its effects on cell viability, lets us hypothesize that melatonin might be an effective modulator of PSC proliferation.

In relation with these observations, PSC exhibited a decrease in BrdU incorporation into DNA. This can be considered an additional index of changes in cell proliferation in the presence of melatonin. Interestingly, cells incubated with 1 mM melatonin showed a higher value of BrdU compared with that noted in cells incubated with 1 μM. Because a higher degree of apoptosis was observed in cells treated with 1 mM melatonin, the higher level of BrdU noted with this concentration of melatonin could be explained on the basis of a stronger response of surviving cells to divide. In the culture, these cells could tend to divide at a higher rate in comparison with cells treated with lower concentrations of melatonin, which exhibited lower values of apoptosis and of BrdU detection. However, the higher level of apoptosis observed in cells treated with 1 mM melatonin, together with the lower expression of cyclin A and D could maintain cell proliferation to a low level, resulting in diminished viability and proliferation. Conversely, in the presence of the other concentrations of melatonin used the degree of apoptosis was lower than the values noted with 1 mM melatonin. Under these circumstances, the stimulus for cell division could be smaller and, therefore, the expected values of BrdU content should be lower, as we have observed. These observations are consistent with the hypothesis that melatonin could induce cell death of highly proliferating cells and could maintain to a low level the proliferation of those cells that survive. Therefore, melatonin could be pointed out as a candidate agent with encouraging actions in the treatment of diseases in which exacerbated cell proliferation occurs, like cancer.

The concentrations of melatonin used in this study fall out of the physiological range and are rather pharmacological. However, melatonin is synthesized by many tissues where it acts then in an autocrine or paracrine manner. As a consequence, concentrations of melatonin over those found in plasma have been found in these tissues [1, 9]. In this line, local concentrations of melatonin might be acting to regulate cell physiology and to control proliferation of abnormally activated PSC. Moreover, pharmacological concentrations of melatonin might be useful in the therapy pancreatic illnesses.

In conclusion, we provide evidences which suggest that melatonin may play important actions to modulate PSC physiology. Melatonin induces caspase-3 activation and changes in the expression of cyclin A and D that might regulate cell viability and proliferation. In this cellular type melatonin might exert direct actions, independently of its receptors. Our observations therefore highlight a promising role of melatonin in the modulation of pancreatic fibrosis. These observations suggest that melatonin at high doses could be potentially taken into consideration as the supportive treatment in the therapy of pancreatic cancer.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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ORIGINAL ARTICLE

Melatonin controls cell proliferation and modulates mitochondrial physiology in pancreatic stellate cells

Matias Estaras[1] · Candido Ortiz-Placin[1] · Alba Castillejo-Rufo[1] · Miguel Fernandez-Bermejo[2] · Gerardo Blanco[3] · Jose M. Mateos[2] · Daniel Vara[2] · Pedro L. Gonzalez-Cordero[2] · Sandra Chamizo[2] · Diego Lopez[3] · Adela Rojas[3] · Isabel Jaen[3] · Noelia de Armas[3] · Gines M. Salido[1] · Juan L. Iovanna[4] · Patricia Santofimia-Castaño[4] · Antonio Gonzalez[1]

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Abstract

We have investigated the effects of melatonin on major pathways related with cellular proliferation and energetic metabolism in pancreatic stellate cells. In the presence of melatonin (1 mM, 100 μM, 10 μM, or 1 μM), decreases in the phosphorylation of c-Jun N-terminal kinase and of p44/42 and an increase in the phosphorylation of p38 were observed. Cell viability dropped in the presence of melatonin. A rise in the phosphorylation of AMP-activated protein kinase was detected in the presence of 1 mM and 100 μM melatonin. Treatment with 1 mM melatonin decreased the phosphorylation of protein kinase B, whereas 100 μM and 10 μM melatonin increased its phosphorylation. An increase in the generation of mitochondrial reactive oxygen species and a decrease of mitochondrial membrane potential were noted following melatonin treatment. Basal and maximal respiration, ATP production by oxidative phosphorylation, spare capacity, and proton leak dropped in the presence of melatonin. The expression of complex I of the mitochondrial respiratory chain was augmented in the presence of melatonin. Conversely, in the presence of 1 mM melatonin, decreases in the expression of mitofusins 1 and 2 were detected. The glycolysis and the glycolytic capacity were diminished in cells treated with 1 mM or 100 μM melatonin. Increases in the expression of phosphofructokinase-1 and lactate dehydrogenase were noted in cells incubated with 100 μM, 10 μM, or 1 μM melatonin. The expression of glucose transporter 1 was increased in cells incubated with 10 μM or 1 μM melatonin. Conversely, 1 mM melatonin decreased the expression of all three proteins. Our results suggest that melatonin, at pharmacological concentrations, might modulate mitochondrial physiology and energy metabolism in addition to major pathways involved in pancreatic stellate cell proliferation.

Keywords Cell proliferation · Fibrosis · Glycolysis · Melatonin · Mitochondria · Pancreatic stellate cells

Key Points
- Pancreatic stellate cells have been signaled as major regulators of pancreatic fibrosis.
- Melatonin modulated major pathways involved in PSC proliferation.
- Melatonin induced changes in mitochondrial physiology.
- Melatonin modulated energy metabolism.

Abbreviations

Akt	Protein kinase B
AMPK	Activated protein kinase
2-DG	2-Deoxyglucose
DMEM	Dulbecco's modified Eagle medium
ECAR	Extracellular acidification rate
ECM	Extracellular matrix
FBS	Fetal bovine serum

✉ Antonio Gonzalez
agmateos@unex.es

[1] Departamento de Fisiología, Instituto de Biomarcadores de Patologías Moleculares, Universidad de Extremadura, Avenida de Las Ciencias S/N, 10003 Cáceres, Spain

[2] Departamento de Gastroenterología, Hospital Universitario, Cáceres, Spain

[3] Unidad de Cirugía Hepatobiliopancreática Y Transplante Hepático, Hospital Universitario, Badajoz, Spain

[4] Centre de Recherche en Cancérologie de Marseille, INSERM U1068, CNRS UMR 7258, Aix-Marseille Université and Institut Paoli-Calmettes, Parc Scientifique Et Technologique de Luminy, Marseille, France

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FCCP	Carbonylcyanide p-(trifluoro-methoxy) phenylhydrazone
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N′N′-tetraacetic acid
ER	Endoplasmic reticulum
Glut-1	Glucose transporter 1
HBSS	Hanks' balanced salt solution
H_2O_2	Hydrogen peroxide
JNK	C-Jun N-terminal kinase
LDH	Lactate dehydrogenase
mTOR	Mammalian target of rapamycin and mechanistic target of rapamycin
MAPKs	Mitogen-activated protein kinases
Ψ_m	Mitochondrial membrane potential
OCR	Oxygen consumption rate
OxPhos	Mitochondrial oxidative phosphorylation
PFK	Phosphofructokinase
p44/42	Extracellular signal-regulated kinase 1/2
PSCs	Pancreatic stellate cells
mtROS	Mitochondrial reactive oxygen species
RT-qPCR	Quantitative reverse transcription-polymerase chain reaction
SERCA	Sarcoendoplasmic reticulum Ca^{2+}-ATPase
TME	Tumor microenvironment
TMRM	Tetramethylrhodamine
Tps	Thapsigargin

Introduction

Pancreatic fibrosis is a condition that can impair pancreatic function. Fibrosis is a consequence of the anomalous activation of cells forming part of the stroma of the gland, together with accumulation of components of the extracellular matrix (ECM). It usually accompanies pancreatitis and/or cancer, and there is no treatment available [1]. Pancreatic stellate cells (PSCs) have been signaled as major regulators of pancreatic fibrosis. In the normal (healthy) pancreas, PSCs remain in a quiescent (resting) state and participate in the normal regulation of the extracellular surrounding. Notably, PSCs represent approximately 4–7% of the total cell population of the pancreatic tissue under physiological conditions. Nevertheless, upon damage to the gland, PSCs turn into an activated state (considered pathological) that, if not reversed, results in an increase in the proliferation of PSCs, accompanied by migration of cells, augmented deposition of ECM, and secretion of cytokines. Additionally, other inflammatory cells can infiltrate within the tissue, which enhances the deleterious conditions that are being set within the gland. As a consequence, destruction of pancreatic (either exocrine or endocrine) cells or the development of transformed cells occurs, at the time in which the healthy tissue is replaced by massive fibrosis [2]. Therefore, the control of the growth of fibrotic tissue within the pancreas is of critical relevance in the treatment of pancreatic diseases.

Melatonin (N-acetyl-5-methoxytryptamine) is an indoleamine that is produced in the mammalian pineal gland during the night. Its production follows a circadian rhythm, with high levels released into the blood at night. Melatonin receptors are widely distributed in the body, which supports that the indoleamine is able to regulate a wide array of physiological processes [3]. Moreover, evidence exist and signal that melatonin might regulate pancreatic function [4–6].

With regard to pathologies of the pancreas, melatonin has been signaled to exert possible anti-inflammatory and anticancer effects [7]. Moreover, the effects of conventional chemotherapies in different cancers were augmented in the presence of melatonin, including pancreatic cancer [8].

Of major relevance is the fact that the development of excessive fibrosis within the pancreatic gland would need to be resolved, in order to improve the response of the tissue to chemotherapy and to diminish resistance. In this line, melatonin might represent a potential therapeutical aid. Several studies, carried out on primary cultures of human and rodent PSCs, have shown that melatonin reduced the proliferation and viability of PSCs. Several mechanisms of action have been reported for melatonin to induce its antiproliferative effects on this cellular type, which include the modulation of cell cycle and of the oxidative status of the cells and activation of apoptosis [9, 10]. Nevertheless, the exact mechanisms involved in the actions of the indolamine remain to be fully elucidated. In particular, cellular energy metabolism also undergoes adaptative changes that allow fast cellular proliferation and tumor growth [11]. However, it is not entirely known whether the indoleamine might exert regulatory effects on energy metabolism in PSCs.

In this study, we aimed at providing further insights into the signaling pathways involved in melatonin actions to modulate PSC proliferation. Specifically, we analyzed whether melatonin could induce any effects on energy metabolism in PSCs, in order to clarify its potential role to control the development of fibrosis. This work is a continuation of former studies, carried out in our laboratory, to further investigate the ways by which melatonin could exert its effects on PSCs to control their proliferation and, hence, to hypothetically diminish pancreatic fibrosis.

Materials and methods

Chemicals

Collagenase CLSPA was obtained from Worthington Biochemical Corporation (Labclinics, Madrid, Spain). Antimycin A, Cell Lysis Reagent for cell lysis and protein

solubilization, crystal violet, carbonyl cyanide m-chlorophenylhydrazone (CCCP), 2-deoxyglucose, melatonin, oligomycin, rotenone, melatonin, Tween®-20, and thapsigargin were obtained from Sigma Chemicals Co. (Madrid, Spain). Dulbecco's modified Eagle medium (DMEM), Hanks' balanced salt solution (HBSS), horse serum, hydrogen peroxide (H_2O_2), medium 199, and MitoSOX™ Red were purchased from Life Technologies (Invitrogen, Barcelona, Spain). Fetal bovine serum (FBS) was purchased from HyClone (Thermo Scientific, Erembodegen, Belgium). Penicillin/streptomycin was obtained from BioWhittaker (Lonza, Basel, Switzerland). Bradford reagent, Tris/glycine/SDS buffer (10×), and Tris/glycine buffer (10×) were from Bio-Rad (Madrid, Spain). Polystyrene plates for cell culture, SuperSignal™ West Femto Reagent, and primers for RT-qPCR were purchased from Thermo Fisher Sci. (Madrid, Spain). The list of antibodies and suppliers is given in Table 1. All other analytical grade chemicals used were obtained from Sigma Chemicals Co. (Madrid, Spain).

Preparation of cultures of pancreatic stellate cells

Primary cultures of PSCs were prepared as described previously [12]. With this procedure, cultures of activated PSCs can be prepared [9]. Pancreatic tissues were obtained from Wistar rat pups (3–5 days after birth). Animals employed in the study were supplied by the animal house of the University of Extremadura (Cáceres, Spain). Animal handling and experimental protocols were approved by the Ethical Committee for Animal Research of the University of Extremadura (reference 57/2016) and by the Institutional Committee of the Junta de Extremadura (reference 20,160,915). The experiments were carried out employing batches of cells obtained from different preparations.

Table 1 Primary antibodies used in the study

Antibody	Dilution	Supplier
B-Actin HRP-Conjugated	1:50,000	Thermo Fisher
p-Akt (Ser473)	1:2000	Cell Signaling
p-AMPKα (Thr172)	1:1000	Cell Signaling
p-mTOR (Ser2448)	1:1000	Cell Signaling
p-p42/44 (Thr202/Tyr204)	1:2000	Cell Signaling
p-p38 (Thr180/Tyr182)	1:1000	Cell Signaling
p-JNK (Thr183/Tyr185)	1:1000	Cell Signaling
Total OxPhos Rodent WB Antibody Cocktail	1:500	Abcam
Mitofusin 1	1:1000	Abcam
Mitofusin 2	1:1000	Cell Signaling
LAMP-1	1:1000	Abcam
Parkin	1:1000	Abcam
TIM-23	1:500	Abcam
LC3	1:1000	Cell Signaling

List of primary antibodies used for detection of the desired protein. Western blotting analysis was used, as described in the "Materials and methods" section. The corresponding secondary HRP-conjugated specific antibody was employed. Thermo Fisher (Madrid, Spain); Abcam plc (Cambridge, UK); Cell Signaling (C-Viral, Madrid, Spain)

Western blot analysis

Cells were detached, centrifuged, washed with a standard phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4; pH adjusted to 7.4), and sonicated in lysis buffer. For quantification of the protein content of samples, Bradford's method was employed [13]. Protein lysates (20 µg/lane) were fractionated by SDS-PAGE using 10% polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated with the specific primary and the corresponding IgG-HRP conjugated secondary antibody. The experiments were carried out employing different batches of cells, harvested on different days.

Determination of mitochondrial reactive oxygen species (mtROS) generation

Determination of mtROS generation was performed following previously described methods [10]. Briefly, PSCs were detached, resuspended in Na-HEPES solution 1 containing 130 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 1.2 mM KH_2PO_4, 10 mM glucose, 10 mM HEPES, 0.01% trypsin inhibitor (soybean), and 0.2% bovine serum albumin (pH = 7.4 adjusted with NaOH). Next, cells were loaded with the mitochondrial ROS indicator MitoSOX™ Red for 15 min at 37 °C. Next, the cells were centrifuged (30 × *g* for 5 min) and resuspended in Na-HEPES solution 2 containing 140 mM NaCl, 4.7 mM KCl, 1 mM $CaCl_2$, 1.1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose (pH adjusted to 7.4 with NaOH). Cells were then incubated with stimuli for 1 h. Generation of mitochondrial ROS was determined by measuring cellular fluorescence at 510 nm/580 nm (excitation/emission). Fluorescence was measured employing a spectrofluorimeter (CLARIOstar Plus, BMG Labtech., C-Viral, Madrid, Spain). Data show the mean increase of fluorescence expressed in percentage ± SEM (*n*) with respect to control (non-treated) cells, where *n* is the number of independent experiments.

Determination of mitochondrial membrane potential

Mitochondrial membrane potential (Ψ_m) was recorded in PSCs loaded with TMRM. Cells were incubated in the presence of 100 nM of the dye as shown previously [14]. Fluorescence was measured employing a spectrofluorimeter

(CLARIOstar Plus, BMG Labtech., C-Viral, Madrid, Spain). Results are expressed as the absolute values of fluorescence emitted at the selected excitation light, normalized to basal (pre-stimulation) fluorescence.

XF Cell Mito Stress Test (OxPhos measurement)

PSCs were seeded at a density of 30,000 cells per well in 24-well SeaHorse® plates and allowed to attach. After 24 h, cells were in the absence or in the presence of melatonin for further 24 h. Afterwards, culture medium was replaced by DMEM culture medium without phenol red (containing 2 mM L-glutamine, 10 mM glucose, and 1 mM pyruvate). Next, cells were then incubated at 37 °C in a non-CO_2 incubator for 1 h. Then, oxygen consumption rate (OCR) was measured under basal conditions or in the presence of 1 µM oligomycin, 1.5 µM carbonylcyanide p-(trifluoro-methoxy) phenylhydrazone (FCCP), or 0.5 µM rotenone plus 0.5 µM antimycin A. The OCR data show the absolute values normalized with respect to the number of cells, which was checked with crystal violet staining.

XF Glycolysis Stress Test (glycolysis experiment)

PSCs were seeded at a density of 30,000 cells per well in 24-well SeaHorse® plates and allowed to attach. The next day, cells were incubated in the absence or in the presence melatonin for 24 h. Next, the culture medium was replaced by DMEM culture medium without phenol red, containing 2 mM L-glutamine. Cells were incubated at 37 °C in a non-CO_2 incubator for 1 h. Then, extracellular acidification rate (ECAR) was measured under basal conditions and in response to 10 mM glucose, 1 µM oligomycin, and 100 mM 2-deoxyglucose (2-DG). Data show the absolute values normalized with respect to the number of cells, which was checked with crystal violet staining.

Quantitative reverse transcription-polymerase chain reaction (RT-qPCR) analysis

RT-qPCR analysis was carried out following previously described methods [12]. In brief, cells were subjected to treatments and then lysed. Lysates were subsequently used for total RNA purification and analysis of protein. The following primers were used:

Glut-1c	Forward: 5′-ATCCTTATTGCCCAGGTGTT-3′
	Reverse: 5′-CAGAAGGGCAACAGGATACA-3′
Ldha	Forward: 5′-GCAGGTGGTTGACAGTGCAT-3′
	Reverse: 5′-ACCCGCCTAAGGTTCTTCAT-3′
Pfkp	Forward: 5′-GACAAGATCCCCAAGAGCAA-3′
	Reverse: 5′-AGCCGTCATAGATTGCGAAC-3′
Gapdh	Forward: 5′-GGGTGTGAACCACGAGAAAT-3′
	Reverse: 5′-CCTTCCACGATGCCAAAGTT-3′

The relative mRNA levels were calculated and were expressed as the fold change between the sample and calibrator (*Gapdh*).

Cell viability assay

Determination of cell viability was carried out using the crystal violet test as described previously [15]. Briefly, after treatment, cells were fixed with 4% paraformaldehyde for 15 min at room temperature (23–25 °C) and washed with distilled water. Afterwards, the fixed cells were stained by incubation in the presence of 0.1% crystal violet (20 min at room temperature, 23–25 °C). After removal of the dye, the wells were washed with distilled water. The wells were then allowed to air dry. Next, 10% acetic acid was added to each well of the plate, followed by incubation for 20 min with shaking. In the end, 50 µL of each well was diluted 1:4 with milli-Q water and the absorbance of each sample was measured at 590 nm employing a plate reader (CLARIOstar Plus, BMG Labtech., C-Viral, Madrid, Spain). Data are shown as the mean change of absorbance expressed in percentage ± SEM (n) with respect to non-treated cells, where n is the number of independent experiments.

Statistical analysis

Data were checked for normal distribution applying the Shapiro–Wilk test. Statistical analysis of data was performed by one-way analysis of variance (ANOVA) followed by Tukey post hoc test, and only P values < 0.05 were considered statistically significant. For individual comparisons and statistics between individual treatments, we employed Student's t test, and only P values < 0.05 were considered statistically significant.

Results

Melatonin modulates MAPK and PI3K/Akt/mTOR signaling and reduces proliferation of PSCs

The increased proliferative rate of PSC (a classic marker of the activation process) and the increased deposition of extracellular matrix proteins are the causes of the development of the desmoplastic reaction, characteristic of pancreatic tumors [16]. Among the different pharmacological properties attributed to melatonin, antifibrotic actions have been described in different models of hepatic, renal, or pulmonary fibrosis. Our first objective was to evaluate the effect of the indolamine on PSC proliferation and viability.

In a first step, PSCs were incubated in the absence (non-treated cells) or in the presence of melatonin (the concentrations used were 1 mM, 100 µM, 10 µM, or 1 µM) for 48 h.

Separate batches of cells were incubated in the presence of 1 μM thapsigargin (Tps), a cell death inducer. After incubation of cells with the drugs, cell viability was evaluated using the crystal violet assay, which allows us to determine changes in cell number independently of metabolic activity. Viability of PSCs incubated with melatonin or with Tps was compared with that of non-treated cells, which was estimated 100% ($n=4$ experiments). In the presence of melatonin, a decrease in cell viability was noted (Fig. 1A), which was stronger in the presence of 1 mM melatonin. In the presence of 1 μM melatonin, no changes in the viability of PSC were observed. As a control of cell death, other cells were incubated in the presence of 1 μM Tps. In the presence of this, cell viability dropped, compared with the viability of non-treated cells (Fig. 1A).

In the next step, we aimed to evaluate the status of two key intracellular signaling pathways involved in the control of cell proliferation and survival: mitogen-activated protein kinases (MAPKs) and Akt/mTOR pathways.

MAPKs are considered as major regulators of signaling pathways involved in cell stress responses, cell differentiation, cell survival, and tumorigenesis. We next studied whether melatonin could induce any effect of MAPKs in PSCs. Thus, cells were challenged with melatonin (1 mM, 100 μM, 10 μM, or 1 μM melatonin) for 48 h. As a control, other batches of PSCs were incubated in the absence of melatonin. Additionally, Tps (1 μM) was tested as a positive control of cell death in separate batches of cells. Analysis of cell lysates revealed a statistically significant decrease in the phosphorylation of JNK in PSCs incubated with melatonin (1 mM, 100 μM, 10 μM, or 1 μM), compared with the level detected in non-treated cells (incubated in the absence of melatonin). In the presence of Tps (1 μM), a statistically significant drop in the phosphorylation of JNK was observed (Fig. 1C). Treatment of PSCs with melatonin evoked increases in the phosphorylation of p38. Tps also evoked an increase in the phosphorylation of JNK (Fig. 1C). When phosphorylation of p44/42 was assayed, we observed a decrease in its phosphorylation in PSCs incubated with 1 mM melatonin, whereas no statistically significant changes were detected in cells treated with the other concentrations of melatonin. In the presence of Tps (1 μM), a statistically significant decrease in the phosphorylation of p44/42 was noted (Fig. 1C).

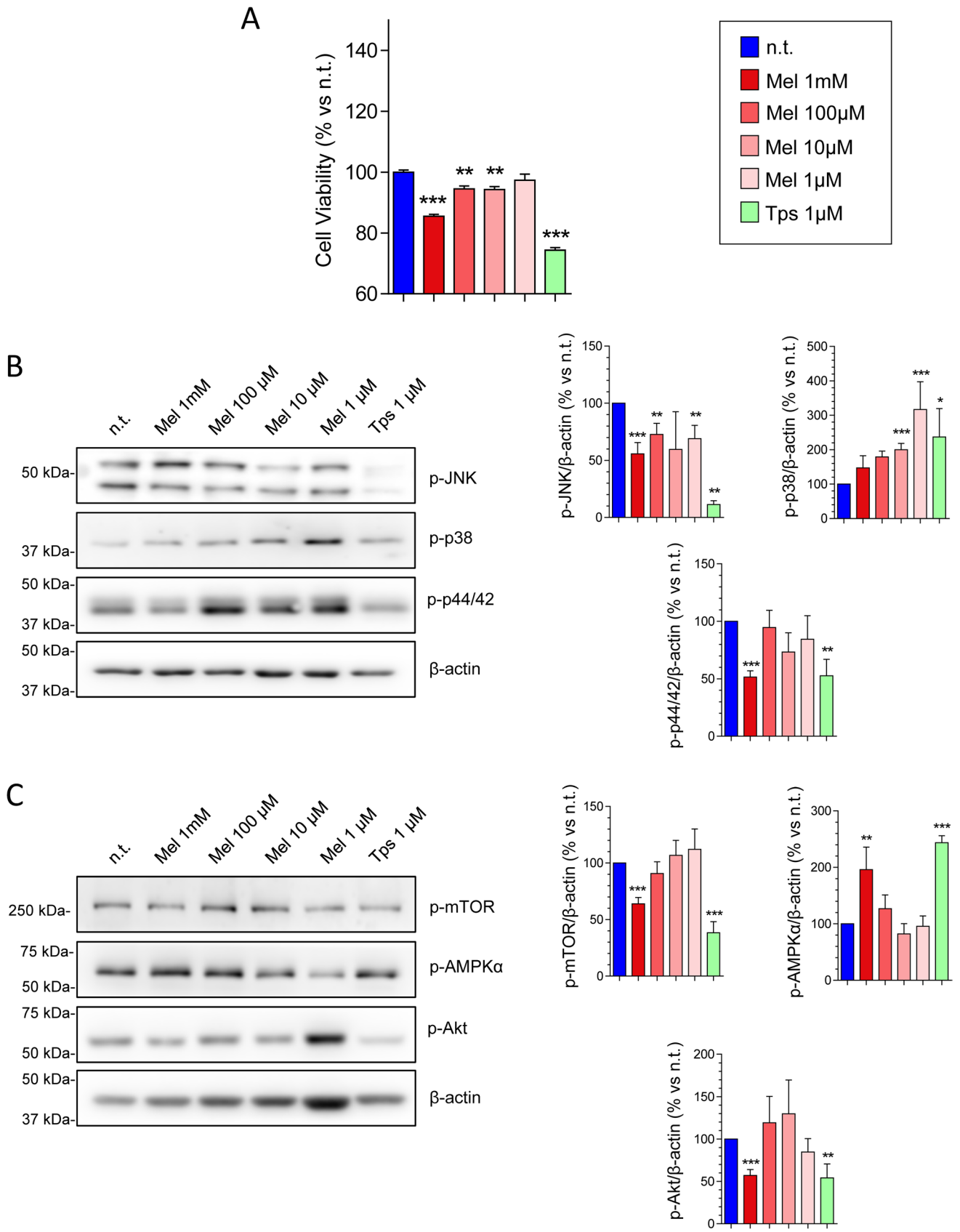
The Akt/AMPK/mTOR is a pivotal pathway with major roles in cell growth, survival, and proliferation. In addition, these proteins play major roles in the regulation of cellular metabolism. For these reasons, this group of proteins has been considered a key component in the development and progression of different types of cancer, including pancreatic cancer [17]. In this set of experiments, we incubated PSCs for 48 h in the presence of melatonin (1 mM, 100 μM, 10 μM, or 1 μM) or in its absence, and the phosphorylation state of several proteins of the Akt/AMPK/mTOR pathway was studied (Fig. 1C). In the presence of 1 mM melatonin, the phosphorylation of mTOR was significantly decreased. A slight decrease was detected in the presence of 100 μM melatonin, whereas no detectable changes in the phosphorylation of mTOR were noted in response to other concentrations of melatonin tested. In the presence of Tps (1 μM), a statistically significant drop in the phosphorylation of mTOR was noted. An increase in the phosphorylation of AMPK was detected in the presence of 1 mM and 100 μM melatonin. Nevertheless, no changes were noted in cells incubated in the presence of 10 μM or 1 μM melatonin. On its side, a statistically significant increase in the phosphorylation of AMPK was observed in cells treated with Tps (1 μM). Finally, a decrease in the phosphorylation of Akt was noted in cells incubated in the presence of 1 mM melatonin. On the contrary, the phosphorylation of Akt was increased in cells incubated in the presence of 100 μM and 10 μM melatonin, although the increase was not statistically significant. Treatment of PSCs with Tps (1 μM) diminished the phosphorylation of Akt.

Melatonin induces mtROS generation, depolarization of mitochondrial membrane potential, and OxPhos impairment

Mitochondria are the major source of energy for cellular metabolism. However, these organelles are involved in many other processes such as the maintenance of redox homeostasis, Ca^{2+} signaling, the production of biosynthetic precursors, or the regulation of apoptotic processes. The actions of melatonin on mitochondria have been studied in other cancer cell models such as pancreatic tumor cells [7] or in head and neck squamous carcinoma cells. These effects have been related with the cytotoxic or antitumor actions of melatonin [18]. At this point, our next objective was to evaluate the status of mitochondria in PSCs treated with melatonin.

In this set of experiments, we were first interested in analyzing the effect of melatonin on the generation of reactive oxygen species within the mitochondria (mtROS). For this purpose, PSCs were incubated for 1 h in the presence of melatonin (1 mM, 100 μM, 10 μM, or 1 μM). In the presence of indoleamine, a concentration-dependent increase in mtROS generation was noted, in comparison with that detected in non-treated cells (incubated in the absence of melatonin) (Fig. 2A). Separate batches of cells were challenged with hydrogen peroxide (H_2O_2, 100 μM), a known oxidant. In the presence of H_2O_2, a statistically significant increase in the oxidation of the ROS-sensitive probe was detected.

In a next step, we tested the effect of melatonin on mitochondrial membrane potential (Ψ_m). Following addition of melatonin (1 mM, 100 μM, 10 μM, or 1 μM) to PSCs for 1 h, we observed a statistically significant decrease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Cell Viability (% vs n.t.)
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◂**Fig. 1** Effect of melatonin on MAPKs and PI3K/Akt/mTOR signaling and on viability of PSC. (**A**) Histogram depicting the effect of melatonin (1 mM, 100 μM, 10 μM, 1 μM) on the viability of PSC. Cells were incubated for 48 h in the absence (n.t., non-treated) or in the presence of melatonin (Mel), and cell viability was analyzed. Separate batches of cells were incubated in the presence of 1 μM thapsigargin (Tps). Results are expressed in % as the mean ± SEM (*n*) of cell viability for each treatment vs non-treated cells (incubated in the absence of melatonin). Data are representative of three independent experiments (**, $P<0.01$; ***, $P<0.001$ vs non-treated cells). (**B**) The blots show the level of the phosphorylated state of JNK, p38, and p44/42 in PSCs incubated for 48 h in the absence (n.t., non-treated) or in the presence of melatonin (Mel; 1 mM, 100 μM, 10 μM, 1 μM). Separate batches of cells were incubated in the presence of 1 μM Tps. The levels of β-actin were employed as controls to ensure equal loading of proteins. The bars show the quantification of protein phosphorylation for each treatment. Data show the mean ± SEM of normalized values, expressed as % with respect to non-treated cells (incubated in the absence of melatonin). Four independent experiments were carried out (Mel, melatonin; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ vs non-treated cells). (**C**) The blots show the effect of melatonin (48-h incubation) on the phosphorylation state of mTOR, AMPK, and Akt, in comparison to cells incubated in its absence (n.t., non-treated cells). The levels of β-actin were employed as controls to ensure equal loading of proteins. The bars show the quantification of protein phosphorylation for each treatment. Data show the mean ± SEM of normalized values, expressed as % with respect to non-treated cells (incubated in the absence of melatonin). Separate batches of cells were incubated in the presence of 1 μM Tps. Four independent experiments were carried out (Mel, melatonin; **, $P<0.01$; and ***, $P<0.001$ vs non-treated cells)

in TMRM-derived fluorescence, compared with the value observed in non-treated cells. The effect was more noticeable in cells incubated with the higher concentrations of melatonin tested (Fig. 2B). As a control to monitor depolarization of Ψ_m, different batches of cells were challenged with the mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP; 100 nM). The compound induced a statistically significant decrease in Ψ_m, compared with that observed in non-treated cells.

Mitochondrial respiration through oxidative phosphorylation represents a major source of energy for cell function and growth, either in healthy or in pathological conditions. In this part of the research, we were interested in studying the effect of melatonin on mitochondrial respiration and its contribution to the bioenergetics status of PSCs. For this purpose, separate batches of cells were incubated in the absence (non-treated cells) or in the presence of melatonin (1 mM, 100 μM, 10 μM, or 1 μM) for 24 h. Then we analyzed several parameters related with oxidative phosphorylation. XF Cell Mito Stress revealed that melatonin decreased basal and maximal respiration, ATP production by oxidative phosphorylation, spare capacity, and proton leak. The effect was more noticeable at the concentration of 1 mM melatonin. However, no detectable changes were observed in PSCs incubated in the presence of 1 μM melatonin (Fig. 2C).

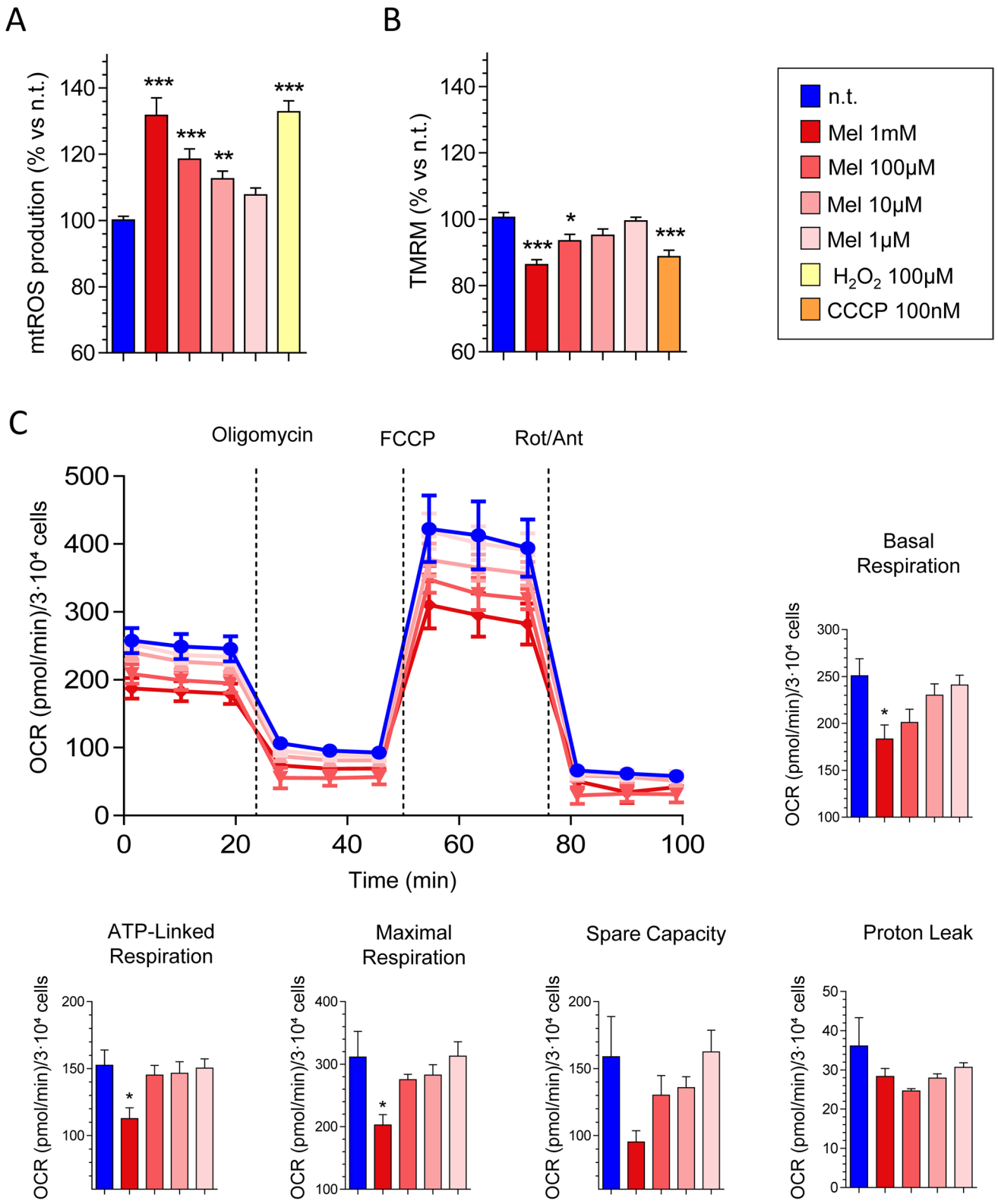
We further studied the effect of melatonin on mitochondria in PSCs. In a next step, we analyzed the effect of the compound on the expression of mitochondrial complexes I, II, III, and V of the respiratory chain. Separate batches of cells were incubated in the absence (non-treated cells) or in the presence of melatonin (1 mM, 100 μM, 10 μM, or 1 μM) for 24 h. The effect of melatonin was different depending on the complex studied, and on the concentration applied. Following melatonin treatment, statistically significant increases in the expression of mitochondrial complex I were detected, in comparison with that noted in non-treated cells. Slight changes in the expression of complexes II, III, and V, with respect to non-treated cells, were observed, although the differences were not statistically significant (Fig. 3).

Fission and fusion are pivotal processes that are involved in the health of the mitochondrial network. These regulated processes represent a quality control system that removes dysfunctional organelles and are involved in cancer [19]. In this set of experiments, we were interested in analyzing the effect of melatonin on the expression of mitofusins 1 and 2. Separate batches of cells were incubated in the absence (non-treated cells) or in the presence of melatonin (1 mM, 100 μM, 10 μM, or 1 μM) for 24 h. In PSCs incubated with 1 mM melatonin, decreases in the expression of mitofusins 1 and 2 were noted in comparison with non-treated cells, although the effect was not statistically significant. The other concentrations of melatonin tested evoked slight increases in the detection of mitofusins, although the differences were not statistically significant (Fig. 4).

Mitophagy is a pathway by which damaged mitochondria are destroyed and recycled [20]. Because melatonin modulates mitochondrial function and dynamics, we were interested in studying whether it could exert a regulatory role on the mitophagy process. For this purpose, we analyzed the changes of the expression of different proteins that participate in the mitophagy pathway: lysosomal-associated membrane protein 1 (LAMP-1), parkin, translocase of inner mitochondrial membrane 23 homolog B (TIM-23), and microtubule-associated protein 1A/1B-light chain 3 (LC3). PSCs were incubated for 24 h in the absence or in the presence of melatonin. Significant decreases in the expression of LAMP-1 were noted in the presence of 10 μM and 1 μM melatonin. However, we could not observe noticeable changes in the expression of the above-mentioned proteins in the presence of 1 mM melatonin, which was the concentration that had induced mitochondrial damage (Fig. 5).

Glycolytic activity of PSC is modulated by melatonin

As observed above, melatonin seemed to induce changes in mitochondrial activity, which might be related with energy supply to the cells. Therefore, we examined the contribution of glycolysis to cell metabolism in PSC. In



this set of experiments, separate batches of cells were incubated in the absence or in the presence of melatonin (1 mM, 100 μM, 10 μM, or 1 μM). XF Glycolysis Stress Test revealed that the glycolysis and the glycolytic capacity were diminished in PSCs treated with 1 mM or 100 μM melatonin, in comparison with those detected in non-treated cells, although the differences were not statistically significant. No detectable changes were noted in

◂**Fig. 2** Effect of melatonin on mitochondrial reactive oxygen species generation, mitochondrial membrane potential, and on oxidative phosphorylation. (**A**) Bar chart showing the effect of melatonin on mitochondrial reactive oxygen species (mtROS; Ψ_m) generation. Separate batches of cells were incubated for 1 h in the absence (n.t., non-treated) or in the presence of melatonin (Mel; 1 mM, 100 µM, 10 µM, 1 µM) or 100 µM hydrogen peroxide (H_2O_2). Values are expressed in % as the mean ± SEM of cell viability for each treatment vs non-treated cells (incubated in the absence of melatonin). Data are representative of three independent experiments (**, $P<0.01$; ***, $P<0.001$ vs non-treated cells). (**B**) Bar chart showing the effect of melatonin on Ψ_m. PSCs were incubated for 1 h in the absence (n.t.;,non-treated) or in the presence of melatonin (Mel; 1 mM, 100 µM, 10 µM, 1 µM) or the mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP; 100 nM). Values are expressed in % as the mean ± SEM of the changes in Ψ_m for each treatment vs non-treated cells (incubated in the absence of melatonin). Data are representative of four independent experiments (TMRM, tetramethylrhodamine, methyl ester; *, $P<0.05$; ***, $P<0.001$ vs non-treated cells). (**C**) Cells were incubated for 24 h in the absence (non-treated, n.t.) or in the presence of melatonin (Mel; 1 mM, 100 µM, 10 µM, 1 µM). Then, oxygen consumption rate (OCR) was measured using the XF Cell Mito Stress Test Kit. The lines in the graph show the mean of OCR ± SEM of normalized values of each measurement. The OCR was measured under basal conditions or following the addition of oligomycin, carbonylcyanide p-(trifluoro-methoxy)phenylhydrazone (CCCP), or rotenone plus antimycin A (Rot/Ant). In the histograms, the bars respectively show the quantification of basal respiration, ATP-linked respiration, maximal respiration, spare capacity, and proton leak. Data were normalized with respect to the number of cells, using the crystal violet test. Results are expressed as the mean of OCR ± SEM of normalized values. Three independent experiments were carried out (*, $P<0.05$ vs non-treated cells)

cells incubated in the presence of 10 µM or 1 µM melatonin. A decrease in the glycolytic reserve was observed at all concentrations of melatonin tested, despite the differences were not statistically significant. The effect on glycolysis was more noticeable in cells incubated with the higher concentrations of melatonin tested (1 mM and 100 µM) (Fig. 6A).

In addition, we tested the effect of melatonin (1 mM, 100 µM, 10 µM, or 1 µM) on the expression of the glucose transporter 1 (Glut-1), phosphofructokinase (PFK), and lactate dehydrogenase (LDH). Therefore, PSCs were incubated with melatonin for 24 h. The effect of melatonin varied with the concentrations tested and the proteins studied. The results are shown in Fig. 6B. In comparison with the values noted in non-treated cells, we observed increases in the expression of *PFK-1* and *LDH* in cells incubated with 100 µM, 10 µM, or 1 µM melatonin. In cells treated with 10 µM or 1 µM melatonin, the expression of *Glut-1* was increased. However, a decrease in the expression of all three proteins was detected in cells incubated in the presence of 1 mM melatonin. Additionally, a drop in the expression of *Glut-1* was noted in cells incubated in the presence of 100 µM melatonin.

Discussion

The formation of fibrotic tissue is a common pathological feature of pancreatic diseases such as inflammation and cancer. The excessive accumulation of components and cells of the extracellular matrix, at the expense of excessive secretion and/or lack of catabolism of the secreted components, impairs the balance of the normal content of the connective tissue within the gland. As a consequence, the function of the pancreas can be impaired. Moreover, the presence of growing fibrotic tissue might protect transformed cells present in the gland, which could end in the development and growth of a tumor [21]. One of the major contributors to the homeostasis of the extracellular matrix is PSC. Despite representing a low proportion of the pancreas, PSCs are considered a cell population that exhibit a critical role in health and disease because of their contribution to the preservation of the extracellular matrix and the pancreatic architecture [22]. Thus, to deal with disease, intervention might be required in order to minimize excessive deposition of extracellular components in order to prevent and/or to the successful elimination of disease [21]. Melatonin is the major product of the pineal gland and is additionally produced in other tissues and organs of the body where it could exert local effects [23]. Moreover, it can be found in vegetables and fruits and, therefore, can enter the body with diet [24]. Melatonin has exhibited antioxidant, anti-inflammatory, and anticancer effects [5]. Moreover, melatonin could be regarded as a potential antifibrotic agent, and hence as a potential therapeutical aid, provided that it has been shown to diminish the proliferation and viability of PSCs. Melatonin could induce its antiproliferative effects on PSCs through several mechanisms of action that include, among others, the cell cycle, the generation of reactive oxygen species, and apoptosis [9, 10]. Nevertheless, the exact mechanisms involved in the actions of the indolamine need to be further characterized, with particular emphasis on energy metabolism.

In this work, we have shown that, in the presence of melatonin, PSC proliferation and viability were diminished. This is in agreement with formerly reported observations [9, 10, 12]. Melatonin could involve several maneuvers to induce its effects on PSC. For example, a prooxidant effect of melatonin, which was based on the generation of ROS and a decrease in the availability of glutathione, has been observed [9, 10]. Additionally, it has been shown that melatonin induces caspase-3 activation and regulates the cell cycle through the modulation of the expression of cyclins A and D [25]. However, the molecular mechanisms involved in the antiproliferative actions of melatonin on PSCs have not been fully elucidated yet.

MAPK signaling is involved in the development of several human diseases, including neurodegenerative diseases

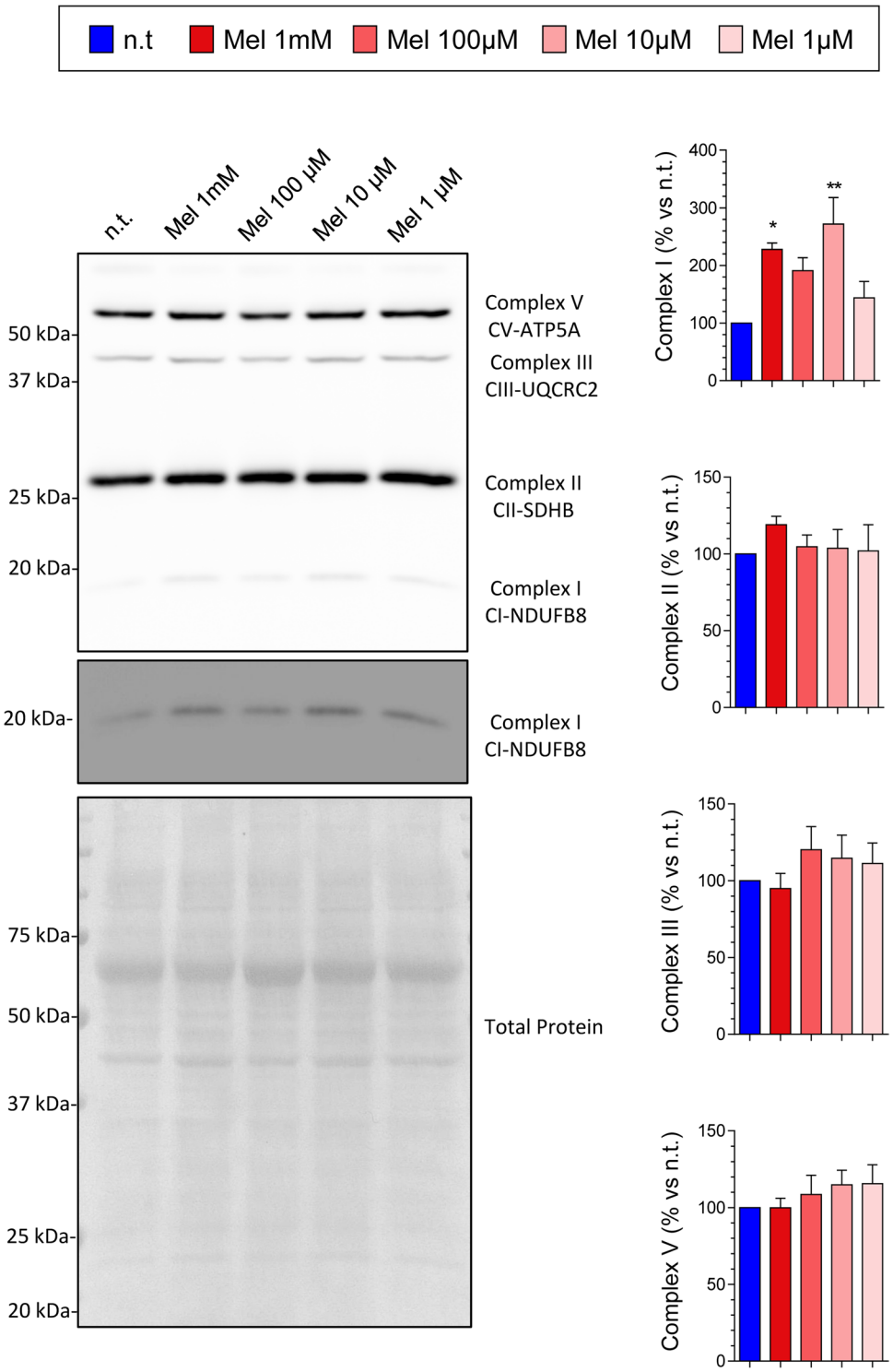


Fig. 3 Effect of melatonin on the expression of mitochondrial respiratory chain complexes in pancreatic stellate cells. Cells were incubated for 24 h in the absence (n.t., non-treated) or in the presence of melatonin (1 mM, 100 µM, 10 µM, or 1 µM). The blots show the effect of melatonin on the level of the mitochondrial respiratory chain complexes I, II, III, and V. The levels of total protein were employed as control to ensure equal loading of proteins. The bars show the quantification of protein detection for each treatment. Data show the mean ± SEM of normalized values, expressed as % with respect to non-treated cells (incubated in the absence of melatonin). Four independent experiments were carried out (Mel, melatonin; *, $P < 0.05$; **, $P < 0.01$ vs non-treated cells)

and various types of cancers, including pancreatic cancer [26]. Our results have shown that, in the presence of melatonin, the phosphorylation of JNK and of p44/42 was diminished, whereas an increase in the phosphorylation of p38 was noted. Increased phosphorylation of p38 MAPK has been related with cell death [27]. In addition, low levels of phosphorylated JNK and p44/42 have been related with diminished cell proliferation [28]. Our results are in agreement with these previous observations and suggest that melatonin might control PSC proliferation and survival through the modulation of MAPK signaling.

On its side, the Akt/AMPK/mTOR is also a major pathway that controls cell growth, survival, and proliferation. In addition, these proteins play major roles in the regulation of

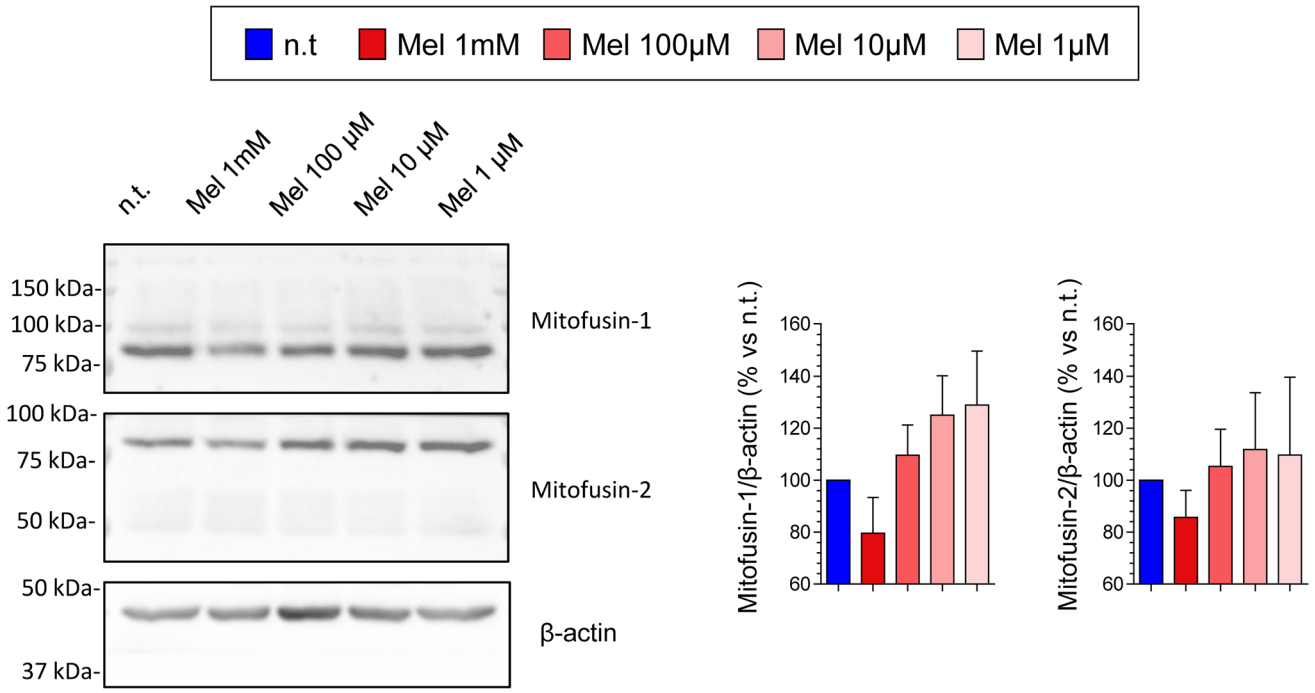


Fig. 4 Effect of melatonin on mitofusin expression in pancreatic stellate cells. Cells were incubated for 24 h in the absence (n.t., non-treated) or in the presence of melatonin (Mel; 1 mM, 100 µM, 10 µM, or 1 µM). The blots show the effect of melatonin on the level of mitofusins 1 and 2. The levels of β-actin were employed as controls to ensure equal loading of proteins. The bars show the quantification of protein detection for each treatment. Data show the mean ± SEM of normalized values, expressed as % with respect to non-treated cells (incubated in the absence of melatonin). Four independent experiments were carried out

cellular metabolism. An increase in the phosphorylation of AMPK and a decrease in the detection of phosphorylated mTOR have been shown in colorectal cancer cells. In addition, increases in the phosphorylation of Akt in the presence of low concentrations of melatonin and decreases in the phosphorylation of this protein in the presence of high concentrations of melatonin have been reported [29]. These previous findings support the results that we have obtained in relation with the effects of melatonin on Akt/AMPK/mTOR.

The mitochondrion has been described as one of the main sites of action of melatonin [30]. Furthermore, former results showed that melatonin induced changes in mitochondrial activity in pancreatic tumor cells that were accompanied by cell death [7]. It is well known that mitochondria are the major source of energy for cellular metabolism, which supports normal cell physiology in addition to abnormal growth of cancer cells [31]. In the present work, we observed an increase in mtROS generation and a decrease in Ψ_m in the presence of melatonin. These results are in agreement with previous findings of our laboratory. Furthermore, our results suggest that melatonin might regulate the oxidative status of PSCs in terms of putative actions on the mitochondrial activity, as we have previously shown [7, 9, 10]. Our results further show that, in the presence of melatonin, the basal and maximal respiration, ATP production by oxidative phosphorylation, spare capacity, and proton leak were diminished. These processes are considered critical mitochondrial parameters related with energy supply to the cell. Interestingly, an increase in the expression of mitochondrial complex I was noted in the presence of melatonin. However, mitochondrial activity was diminished upon treatment of cells with the indolamine. It has been shown that the expression of mitochondrial complex I is increased in pancreatic tumors, which is accompanied by elevated mitochondrial activity. For this reason, complex I of the mitochondrial respiratory chain has been signaled as a putative therapeutic target [32]. The increase in the expression of complex I that we have observed in cells treated with melatonin could be explained as a compensatory mechanism to enhance mitochondrial activity, which could probably be targeted by melatonin.

Additionally, decreases in the expression of mitofusins 1 and 2 were noted in cells treated with melatonin. Mitofusins are located at the outer mitochondrial membrane and are involved in the control of the plasticity of mitochondria. Fission and fusion are pivotal processes that are involved in the health of the mitochondrial network and play pivotal roles in cancer [19]. Importantly, mitofusins ensure proper energetic and metabolic cellular performance [33]. Additionally, our

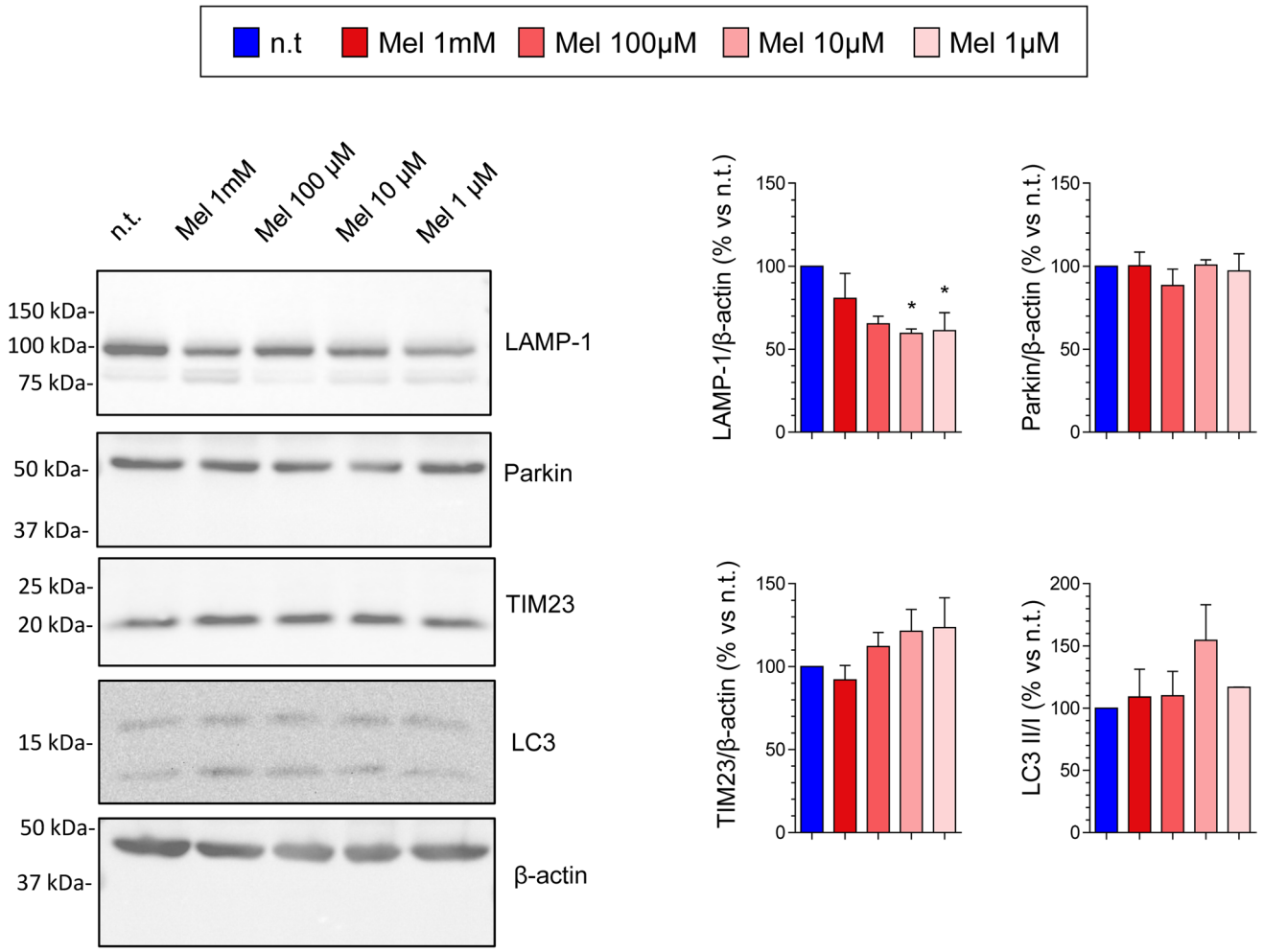


Fig. 5 Effect of melatonin on mitophagy in pancreatic stellate cells. Cells were incubated for 24 h in the absence (n.t., non-treated) or in the presence of melatonin (Mel; 1 mM, 100 µM, 10 µM, or 1 µM). The blots show the effect of melatonin on the level of LAMP-1, Parkin, TIM-23, and LC3 I/II. The levels of β-actin were employed as controls to ensure equal loading of proteins. The bars show the quantification of protein detection for each treatment. Data show the mean ± SEM of normalized values, expressed as % with respect to non-treated cells (incubated in the absence of melatonin). Four independent experiments were carried out (Mel, melatonin; *, $P < 0.05$ vs non-treated cells)

results showed no clear changes in the expression of different proteins that participate in the mitophagy pathway. Despite melatonin treatment seems to target the mitochondria, the responses directed to dispose damaged mitochondria seem not to be activated. Altogether, these results suggest that melatonin might target mitochondrial physiology in PSC, by terms of decreased energy availability for cell proliferation. The stronger effects were noted in cells treated with the highest concentration of melatonin tested. Probably, this could be an explanation for the cytostatic effect of melatonin on PSCs.

The next set of experiments showed that the glycolysis and the glycolytic capacity were diminished in PSCs treated with melatonin. Additionally, changes in the expression of Glut-1, PFK, and LDH, which are major proteins involved in the glycolytic metabolism, were observed in the presence of melatonin. In this regard, again, the stronger effects were noted in cells treated with the highest concentration of melatonin tested. A decrease in the expression of Glut-1 in the presence of melatonin has been shown in breast cancer cells [34]. On its side, PFK depicts major roles in the metabolism of cancer cells. Thus, its modulation might represent a possible target for therapeutic intervention [35]. Finally, LDH also plays a pivotal role in cancer development. Targeting of LDH decreases cell proliferation and migration. Indeed, melatonin downregulated the activity of LDH in gastric adenocarcinoma cell line SGC7901 [36]. Our results are in agreement with all these observations and support that the antiproliferative actions of melatonin on PSC might be related with the modulation of the major regulators of energy supply to the cell.

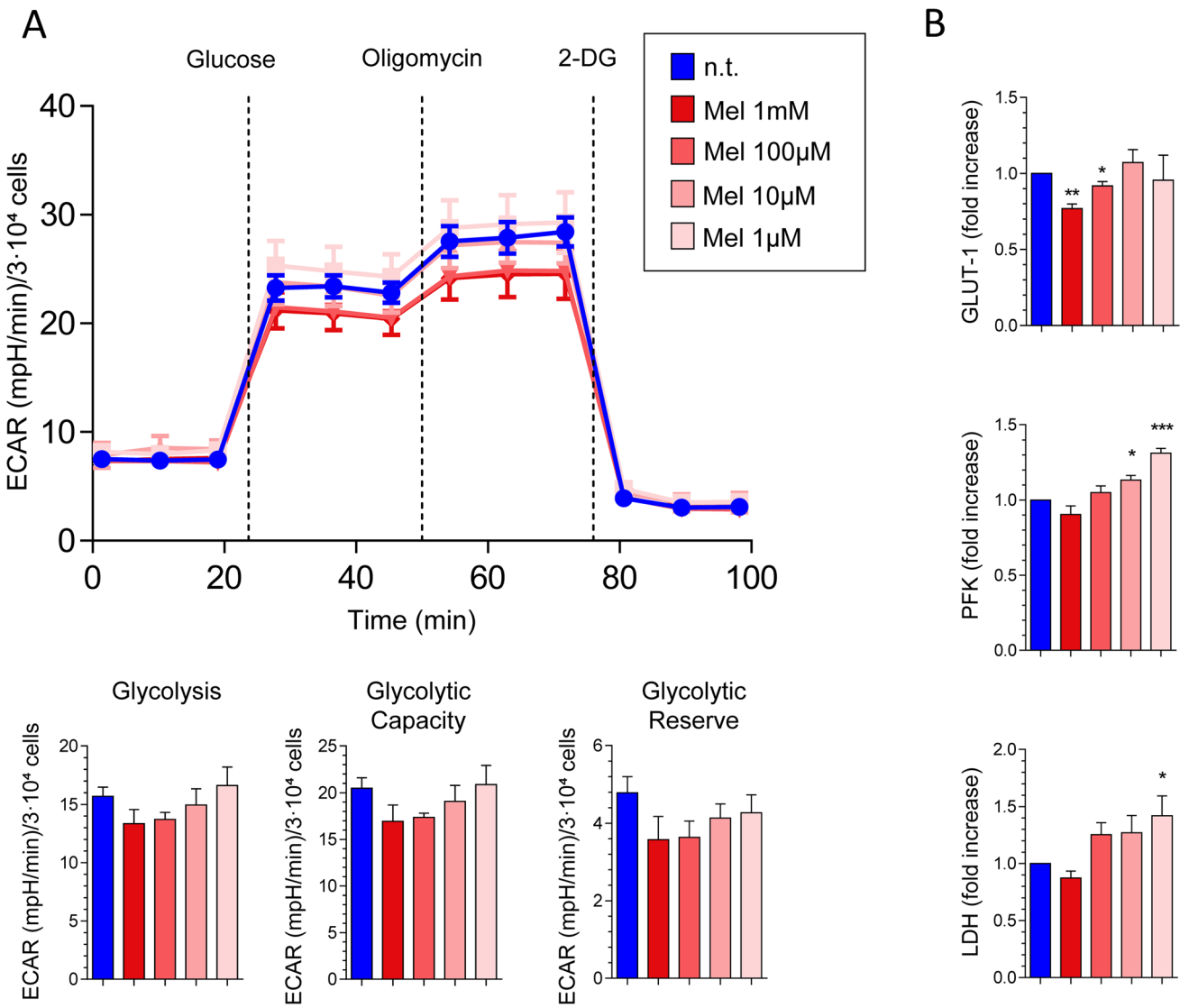


Fig. 6 Study of glycolytic metabolism in PSCs subjected to hypoxia. Effect of melatonin. (**A**) Cells were incubated for 24 h in the absence (n.t., non-treated) or in the presence of melatonin (Mel; 1 mM, 100 µM, 10 µM, or 1 µM). Then, extracellular acidification rate (ECAR) was measured using the XF Glycolysis Stress Test Kit. The line chart shows the mean of ECAR ± SEM of normalized values of each time point measurement. The ECAR was measured under basal conditions or following the addition of glucose, oligomycin, or 2-deoxyglucose (2-DG). The bar graphs show the quantification of glycolysis, glycolytic capacity, and glycolytic reserve, respectively. ECAR data were normalized with respect to the number of cells using the crystal violet test. Results are expressed as the mean of ECAR ± SEM of normalized values. Three independent experiments were carried out. (**B**) Cells were incubated with melatonin (1 mM, 100 µM, 10 µM, or 1 µM) for 48 h in the absence (n.t., non-treated) or in the presence of melatonin (1 mM, 100 µM, 10 µM, or 1 µM) and the mRNA levels of glucose transporter 1 (GLUT-1), phosphofructokinase (PFK), and lactate dehydrogenase (LDH) were analyzed by RT-qPCR. The bars show the fold increase of mRNA levels ± SEM of each mRNA relative to cells incubated in the absence of melatonin (Mel, melatonin; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ vs non-treated cells)

In conclusion, our results provide evidence for the modulation by melatonin in PSC of major pathways involved in cell proliferation, like the PI3K/Akt/mTOR and MAPKs. In addition, melatonin might decrease mitochondrial activity, which was reflected by the depolarization of Ψ_m and the generation of ROS. Interestingly, melatonin could target the complexes of the mitochondrial electron chain, which might lead to decreased energy synthesis by the organelles. Moreover, the expression of mitofusins, which are responsible for energetic and metabolic cellular performance, could be modulated by melatonin. Glycolysis could also be a point of action of melatonin. Taken together, our results provide further evidence for the mechanisms employed by melatonin to putatively modulate the physiology of PSCs, which have been signaled as major contributors to fibrosis within the pancreas.

Author contribution Matias Estaras, Patricia Santofimia-Castaño, Cándido Ortiz-Placin, and Alba Castillejo-Rufo: material preparation, data collection, and analysis.

Patricia Santofimia-Castaño, Juan L. Iovanna, and Antonio Gonzalez: designed the experimental studies, suggested discussion parts, reviewed and edited the manuscript.

Antonio Gonzalez: wrote the first draft of the manuscript.

Gines M. Salido: suggested discussion parts, reviewed and edited the manuscript.

Miguel Fernandez-Bermejo, Gerardo Blanco, Jose M. Mateos, Daniel Vara, Pedro L. Gonzalez-Cordero, Sandra Chamizo, Diego Lopez, Adela Rojas, Isabel Jaen, and Noelia de Armas suggested parts of the manuscript, reviewed and edited the manuscript.

All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Declarations

Research involving human participants and/or animals Animal handling and experimental protocols were approved by the Ethical Committee for Animal Research of the University of Extremadura (reference 57/2016) and by the Institutional Committee of the Junta de Extremadura (reference 20160915).

Conflict of interest The authors declare no competing interests.

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Melatonin modulates red-ox state and decreases viability of rat pancreatic stellate cells

Antonio Gonzalez[1*], Matias Estaras[1], Salome Martinez-Morcillo[2], Remigio Martinez[3], Alfredo García[4], Mario Estévez[5], Patricia Santofimia-Castaño[6], Jose A. Tapia[1], Noelia Moreno[1], Marcos Pérez-López[2], María P. Míguez[2], Gerardo Blanco-Fernández[7], Diego Lopez-Guerra[7], Miguel Fernandez-Bermejo[8], Jose M. Mateos[8], Daniel Vara[8], Vicente Roncero[9] & Gines M. Salido[1]

In this work we have studied the effects of pharmacological concentrations of melatonin (1 µM–1 mM) on pancreatic stellate cells (PSC). Cell viability was analyzed by AlamarBlue test. Production of reactive oxygen species (ROS) was monitored following CM-H_2DCFDA and MitoSOX Red-derived fluorescence. Total protein carbonyls and lipid peroxidation were analyzed by HPLC and spectrophotometric methods respectively. Mitochondrial membrane potential (ψ_m) was monitored by TMRM-derived fluorescence. Reduced (GSH) and oxidized (GSSG) levels of glutathione were determined by fluorescence techniques. Quantitative reverse transcription-polymerase chain reaction was employed to detect the expression of Nrf2-regulated antioxidant enzymes. Determination of SOD activity and total antioxidant capacity (TAC) were carried out by colorimetric methods, whereas expression of SOD was analyzed by Western blotting and RT-qPCR. The results show that melatonin decreased PSC viability in a concentration-dependent manner. Melatonin evoked a concentration-dependent increase in ROS production in the mitochondria and in the cytosol. Oxidation of proteins was detected in the presence of melatonin, whereas lipids oxidation was not observed. Depolarization of ψ_m was noted with 1 mM melatonin. A decrease in the GSH/GSSG ratio was observed, that depended on the concentration of melatonin used. A concentration-dependent increase in the expression of the antioxidant enzymes catalytic subunit of glutamate-cysteine ligase, catalase, NAD(P)H-quinone oxidoreductase 1 and heme oxygenase-1 was detected in cells incubated with melatonin. Finally, decreases in the expression and in the activity of superoxide dismutase were observed. We conclude that pharmacological concentrations melatonin modify the redox state of PSC, which might decrease cellular viability.

It is nowadays increasing the focus of research on the role of pancreatic stellate cells (PSC) in the physiology and the pathophysiology of the pancreas. PSC comprise of a rather small cell population of the organ. Under normal conditions PSC remain quiescent, but become activated in disease. Activated PSC are responsible for the progressive fibrosis and for the accumulation of extracellular matrix that occurs in severe pancreatic disorders such as chronic pancreatitis and pancreatic cancer[1,2]. Therefore, it is thought that activated PSC are involved in tumor progression and chemoresistance. In this regard, PSC contribute to stromal or fibrotic reaction by the release of matrix components, release signaling molecules that act on neighboring cells to modulate their proliferation and tissue growth within cancer[3]. Unraveling the mechanisms underlying growth and proliferation of PSC is of major relevance for the understanding of pancreatic diseases. In this line, it is tempting to find drugs whose anti-inflammatory, anti-fibrotic and/or anti-proliferative actions could be used in therapy.

[1]Institute of Molecular Pathology Biomarkers, University of Extremadura, Caceres, Spain. [2]Unit of Toxicology, Veterinary Faculty, University of Extremadura, Caceres, Spain. [3]Department of Animal Health, Veterinary Faculty, University of Extremadura, Caceres, Spain. [4]Department of Animal Production, CICYTEX-La Orden, Guadajira, Badajoz, Spain. [5]IPROCAR Research Institute, Food Technology, University of Extremadura, 10003, Cáceres, Spain. [6]Centre de Recherche en Cancérologie de Marseille, INSERM U1068, CNRS UMR 7258, Aix-Marseille Université and Institut Paoli-Calmettes, Parc Scientifique et Technologique de Luminy, Marseille, France. [7]Hepatobiliary-Pancreatic Surgery and Liver Transplant Unit, Infanta Cristina Hospital, Badajoz, Spain. [8]Department of Gastroenterology, San Pedro de Alcantara Hospital, Caceres, Spain. [9]Unit of Histology and Pathological Anatomy, Veterinary Faculty, University of Extremadura, Caceres, Spain. *email: agmateos@unex.es

Melatonin (N-acetyl-5-methoxytryptamine) is a compound that is produced mainly, but not exclusively, in the pineal gland. Initially, it was considered a hormone with key roles in the regulation of circadian rhythms, conveying physiological and neuroendocrine functions within the body. However, melatonin is also produced in other parts of the organism, as for example retina, Harderian gland, gastrointestinal tract, testes and lymphocytes where it can induce local effects[4]. The compound exerts its actions acting through ~~its~~ specific receptors or directly. Melatonin can bind to cellular membrane MT1- and MT2-type receptors, or can interact with intracellular proteins, as for example nuclear receptor ROR/RZR, quinone reductase 2 (termed MT3 type receptor) and calmodulin[5–8]. Beside its actions as a circadian regulator, especially of reproduction, melatonin also works as free radical scavenger, through potentiation of antioxidant defenses or via immune modulation, thereby exerting protective roles on cell physiology[8]. On the contrary, melatonin also induces cell death[8,9]. Interestingly, all these effects are cell- and context-dependent[8]. With time, widespread attention on the effects of melatonin on cellular physiology and, especially, on its ability to control cell proliferation in cancer has emerged. Melatonin induces antitumor effects in different tissues[10–13], including the pancreas[14,15]. The anticarcinogenic effects of melatonin involve different mechanisms, as for example apoptosis and cancer immunity. In addition, melatonin diminishes autophagy, metastasis and angiogenesis, leading in general to a decrease of proliferation of malignant cells[16].

As mentioned above, PSC depict an important role as components of the tumor microenvironment and have emerged as key modulators in the context of tissue injury. In this regard, we have shown that melatonin modulates proliferation of murine[17] and human PSC[18]. Our previous results showed that melatonin induced Ca^{2+} mobilization from intracellular pools and activation of key components of the mitogen-activated protein kinases (MAPKs) family. In addition, in human PSC a decrease in the GSH/GSSG ratio was observed, which could compromise cellular antioxidant defenses and induce prooxidant conditions that could diminish cell survival. Therefore, melatonin might be a compound with putative parallel effects on the cells forming part of a growing tumor, controlling their proliferation.

In the present study we aimed at identifying new actions of melatonin on the pancreas which might highlight the compound as potential candidate in therapy. We have continued our former studies to further investigate the ways by which melatonin could exert its effects on PSC to control their proliferation.

Materials and Methods

Pancreatic tissues and chemicals. Pancreatic tissues used in this study were obtained from newborn *Wistar* rats (one week). Animals employed have been purchased from the animal house of the University of Extremadura (Caceres, Spain). Animals handling, methods and experimental protocols were approved by, and were carried out according to, the University Ethical Committee (reference 57/2016) and by the Institutional Committee of the Junta de Extremadura (reference 20160915). Additionally, all methods and the experimental protocols were performed in accordance with the relevant guidelines and regulations of the Ethical Committee for Animal Research of the University of Extremadura and with the Institutional Committee of the Junta de Extremadura (law 32/2007 and RD 53/2013).

Most chemicals and reagents used for the present work were purchased from Sigma-Aldrich (Merck, Madrid, Spain) and AbD serotec (BioNova Científica, Madrid, Spain). The enzyme collagenase CLSPA for digestion of the pancreas was purchased from Worthington Biochemical Corporation (Labclinics, Madrid, Spain). The components for the preparation of culture medium and the fluorescent probes used were obtained from Invitrogen (Fisher Scientific Inc., Madrid, Spain) and from BioWhittaker (Lonza, Basel, Switzerland). Plastic materials for cell culture were purchased from Thermo Fisher Sci. (Madrid, Spain). Materials and reagents for Western blotting were purchased from Bio-Rad (Madrid, Spain) and from Cell Signaling Technology (C-Viral, Madrid, Spain). Superoxide dismutase (SOD) activity, total antioxidant capacity (TAC) kits were purchased from BioVision (Deltaclon S.L., Madrid, Spain).

The antibodies and primers used were purchased from Thermo Scientific (Fisher Scientific Inc., Madrid, Spain), Sigma-Aldrich (Merck, Madrid, Spain) and Santa Cruz Biotechnologies Inc. (Quimigen S.L., Madrid, Spain).

Pancreatic stellate cells cultures. PSC were prepared and cultured using established methods[17]. After preparation of cells suspension, small aliquots were seeded on polystyrene plates for cell culture. Culture medium consisted of medium 199, plus 4% horse serum, 10% FBS, 0.1 mg/mL streptomycin, 100 IU penicillin and 1 mM $NaHCO_3$. The cells were grown under constant temperature (37 °C) and CO_2 (5%). Confluence (90–95%) was reached after eight-ten days of culture.

Study of cell viability. Cells were treated with different stimuli for 48 h. Determination of cell viability was carried out according to previous techniques[19]. A plate reader was used to monitor absorbance (VariosKan Lux 3020–205, Thermo Sci., Vantaa, Finland). The viability of cells subjected to stimuli was compared with that of control cells (non-stimulated). Data show the change in cell viability expressed as the mean in percentage ± S.E.M. (n) with respect to non-stimulated cells (n is the number of experiments carried out).

Detection of reactive oxygen species (ROS) generation. ROS generation was monitored employing methods used in or laboratory[20]. Cells were detached and loaded with CM-H_2DCFDA (10 µM) or with MitoSOX Red (2.5 µM). Next, cells were incubated with stimuli during 1 h. For detection of changes in the red-ox state cells were excited at 530 nm and fluorescence emitted was detected at 590 nm for CM-H_2DCFDA, whereas for cells loaded with MitoSOX red excitation at 510 nm with detection at 580 were employed. A spectrofluorimeter was used to monitor fluorescence (VariosKan Lux 3020–205, Thermo Sci., Vantaa, Finland). Results show the mean increase of fluorescence expressed in percentage ± SEM (n) with respect to non-stimulated cells, where n is the number of independent experiments, as described previously[20].

Detection of protein Carbonyls (Allysine). Cells were incubated during 1 h with stimuli and, thereafter, were lysed for analysis. Detection of protein carbonyls was performed according to the methods described by Villaverde *et al.*[21]. In brief, five hundred µL of each sample were treated with cold 10% trichloroacetic acid (TCA) solution. After centrifugation (600 × *g* for 5 min at 4 °C) the supernatants were removed and the pellets were sequentially incubated with a solution containing 0.5 mL 250 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer pH 6.0 containing 1 mM diethylenetriaminepentaacetic acid (DTPA), a solution containing 0.5 mL 50 mM ABA in 250 mM MES buffer pH 6.0 and a solution containing 0.25 mL 100 mM $NaBH_3CN$ in 250 mM MES buffer pH 6.0. Next, samples were treated with a cold 50% TCA solution and centrifuged ~~at~~ (1200 × g for 10 min). The pellets were then washed twice with 10% TCA and diethyl ether-ethanol (1:1). Finally, the pellet was treated with 6 M HCl and kept in an oven at 110 °C for 18 h until completion of hydrolysis. Thereafter, the samples were dried *in vacuo* and the generated residue was reconstituted with 200 µL of milliQ water and filtered for HPLC analysis using a Shimadzu 'Prominence' HPLC apparatus (Shimadzu Corporation, Japan). The elutes were monitored with excitation and emission wavelengths set at 283 and 350 nm, respectively. Standards (0.1 µL) were run and analysed under the same conditions. The nmol of allysine per mg of protein were calculated. Results are expressed as percentage ± SEM (n) with respect to non-stimulated cells, where n is the number of independent experiments.

Analysis of thiobarbituric-reactive substances. Cells were incubated during 1 h with stimuli and, thereafter, were lysed for analysis. Malondialdehyde (MDA) and other thiobarbituric-reactive substances (TBARS) were measured, by adding 500 µL thiobarbituric acid (0.02 M) and 500 µL trichloroacetic acid (10%) to 200 µL of a sample from each treatment. Next, the mixture was incubated for 20 min at 90 °C. After cooling, a 5 min centrifugation at 600 × *g* was made and the absorbance of supernatant was measured at 532 nm employing a plate reader (VariosKan Lux 3020–205, Thermo Sci., Vantaa, Finland). The mg/L of TBARS in each sample were calculated. Results are expressed as percentage ± SEM (n) with respect to non-stimulated cells, where n is the number of independent experiments.

Determination of mitochondrial membrane potential. Changes in mitochondrial membrane potential (ψ_m) were recorded using the dye TMRM as described previously[22]. Cells were incubated during 1 h in the presence of stimuli. A decrease in TMRM fluorescence reflects depolarization of ψ_m. Fluorescence was measured employing a spectrofluorimeter (VariosKan Lux 3020–205, Thermo Sci., Vantaa, Finland). The experiments were carried out employing batches of cells obtained from different preparations. The increase of fluorescence with respect to non-stimulated cells was calculated and expressed in percentage as the mean ± SEM (n) (n is the number of experiments).

Determination of glutathione levels. The changes in the levels of reduced (GSH) and oxidized (GSSG) glutathione were determined using methods described previously[18]. Cells were incubated during 4 h with the different stimuli assayed. A spectrofluorimeter (Tecan Infinite M200, Grödig, Austria) was employed to detect GSH or GSSG at 350 nm/420 nm (excitation/emission) respectively. For quantification, standard curves of GSH and GSSG were used. Normalization was carried out based on the total protein concentration in each sample[23]. A standard curve was prepared using bovine serum albumin. The experiments were carried out employing batches of cells obtained from different preparations.

Data are shown as the mean increase in GSH/GSSG ratio expressed in percentage ± SEM (n) with respect to non-stimulated cells, where n is the number of independent experiments.

Quantitative reverse transcription-polymerase chain reaction (RT-qPCR) analysis. This procedure was carried out as previously described[24]. PSC in culture were incubated during 4 h with different stimuli and lysed. Total RNA samples were purified using a commercially available kit (Sigma, Madrid, Spain). The Power SYBR Green RNA-to-C_T 1-Step kit (Applied Biosystems, Township, USA) was used. Reverse transcription was performed for 30 min at 48 °C, and PCR conditions were 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C plus 1 min at 55 °C using the following primers:

Gclc:5′-GGCACAAGGACGTGCTCAAGT-3′ and 5′-TGCAGAGTTTCAAGAACATCG-3′
Cat:5′-ACTTTGAGGTCACCCACGAT-3′ and 5′-AACGGCAATAGGGGTCCTCTT-3′
Ho-1:5′-AGCACAGGGTGACAGAAGAG-3′ and 5′-GAGGGACTCTGGTCTTTGTG-3′
Nqo-1:5′-GGGGACATGAACGTCATTCTCT-3′ and 5′-AAGACCTGGAAGCCACAGAAGC-3′
Gapdh:5′-GGGTGTGAACCACGAGAAAT-3′ and 5′-CCTTCCACGATGCCAAAGTT-3′
SOD1: 5′-GGGGACAATACACAAGGCTGTA-3′ and 5′-CAGGTCTCCAACATGCCTCT-3′
SOD2: 5′-GTGGAGAACCCAAAGGAGAG-3′ and 5′-GAACCTTGGACTCCCACAGA-3′

The mRNA abundance of each transcript was normalized to the *Gapdh* mRNA abundance obtained in the same sample. The relative mRNA levels were calculated using the ΔΔCt method, and were expressed as the fold change between sample and calibrator. The experiments were carried out employing batches of cells obtained from different preparations.

Determination of SOD activity. This procedure was carried out using a commercially available kit from BioVision. Stimuli were added to the cells and were incubated during 1 h. Thereafter SOD activity was determined following the manufacturer's directions. The sensitive SOD assay kit utilizes WST-1 that produces a water-soluble formazan dye upon reduction with superoxide anion.

The activity of SOD can be determined by a colorimetric method. Absorbance at 450 nm of the samples was measured employing a spectrofluorimeter (VariosKan Lux 3020–205, Thermo Sci., Vantaa, Finland). The experiments were carried out employing batches of cells obtained from different preparations. Results show the mean

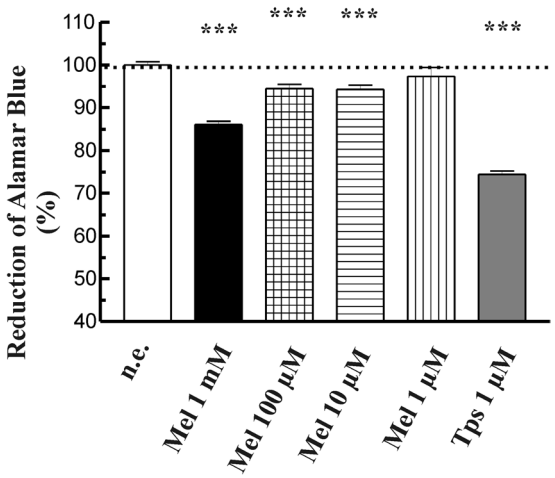

Figure 1. Analysis of PSC viability. Cell viability was analyzed studying AlamarBlue reduction by viable cells. Cells were incubated during 48 h in the presence of melatonin (Mel; 1 mM, 100 µM, 10 µM or 1 µM) or thapsigarging (Tps, 1 µM) and viability was compared with that of cells in the absence of stimulus (control). In the graph, a dotted line represents the viability of control cells (non-treated cells). Histograms are representative of three independent experiments (n.e.,non-stimulated cells; Mel, melatonin; Tps, thapsigargin; $***P < 0.001$ *vs* non-stimulated cells).

change of absorbance expressed in percentage ± SEM (n) with respect to non-stimulated cells, where n is the number of independent experiments.

Determination of total antioxidant capacity. Total antioxidant capacity (TAC) was determined using a commercially available kit from BioVision, following manufacturer's directions. Absorbance at 570 nm of the sample was measured employing a plate reader (CLARIOstar Plus, BMG Labtech., C-Viral, Madrid, Spain). Results show the mean change of absorbance expressed in percentage ± SEM (n) with respect to non-stimulated cells, where n is the number of independent experiments.

Western blotting analysis. Western blotting was performed using previously described methods[14]. Cells in culture were incubated in the presence of different stimuli during 1 h and lysed. Bradford's method was used for quantification of the protein content of lysates[23]. Protein lysates (12 µg/lane) of each sample were separated by SDS-PAGE, using 10% polyacrylamide gels, and were transferred to nitrocellulose membranes. Specific primary and the corresponding IgG-HRP conjugated secondary antibody were used for detection of proteins. Quantification of the intensity of the bands which appear was performed using the software *ImageJ* (http://imagej.nih.gov/ij/). The experiments were carried out employing batches of cells obtained from different preparations. Values are expressed as the mean ± SEM of normalized values expressed as % *vs* control (non-stimulated) cells.

Statistical analysis. Statistical analysis of data was performed by one-way analysis of variance (ANOVA) followed by Tukey *post hoc* test, and only P values < 0.05 were considered statistically significant. For individual comparisons and statistics between individual treatments we employed the Student's t test, and only P values <0.05 were considered statistically significant.

Results

Effects of melatonin on cell viability. It has been suggested that melatonin modulates cell viability of different cellular types[9,14,25,26], including PSC[17,18]. At this point it was of interest to corroborate the effect of melatonin on cell viability. Thus, PSC were incubated in the absence (non-treated cells) or in the presence of 1 mM, 100 µM, 10 µM or 1 µM melatonin, and cell viability was evaluated after 48 h of culture. The viability of cells that had been incubated in the presence of melatonin was compared with that of non-treated cells.

Cell viability dropped in the presence of 10 µM to 1 mM of melatonin (Fig. 1). A maximal effect was noted with 1 mM melatonin. Separate batches of cells were treated with 1 µM thapsigargin (Tps), which served as control for cell death[27]. In the presence of Tps a strong decrease in cell viability was observed.

Effect of melatonin on cellular oxidative state. It has been suggested the melatonin may exert a pro-oxidant action that could underlie its antiproliferative actions[28]. To study this possibility we analyzed the effect of melatonin on ROS production. For this purpose PSC were loaded with the ROS-sensitive fluorescent dyes CM-H_2DCFDA or MitoSOX Red. Thereafter, cells were incubated during 1 h with melatonin (1 mM, 100 µM, 10 µM or 1 µM). The compound evoked a concentration-dependent increase in ROS production both in the cytosol and in the mitochondria. Hydrogen peroxide (100 µM) was used as a control of oxidation. For this purpose the oxidant was added to the cells, which were then incubated during 1 h. In the presence of hydrogen peroxide a statistically significant increase in dye-derived fluorescence was observed, reflecting an increase in oxidation (Fig. 2A,B).

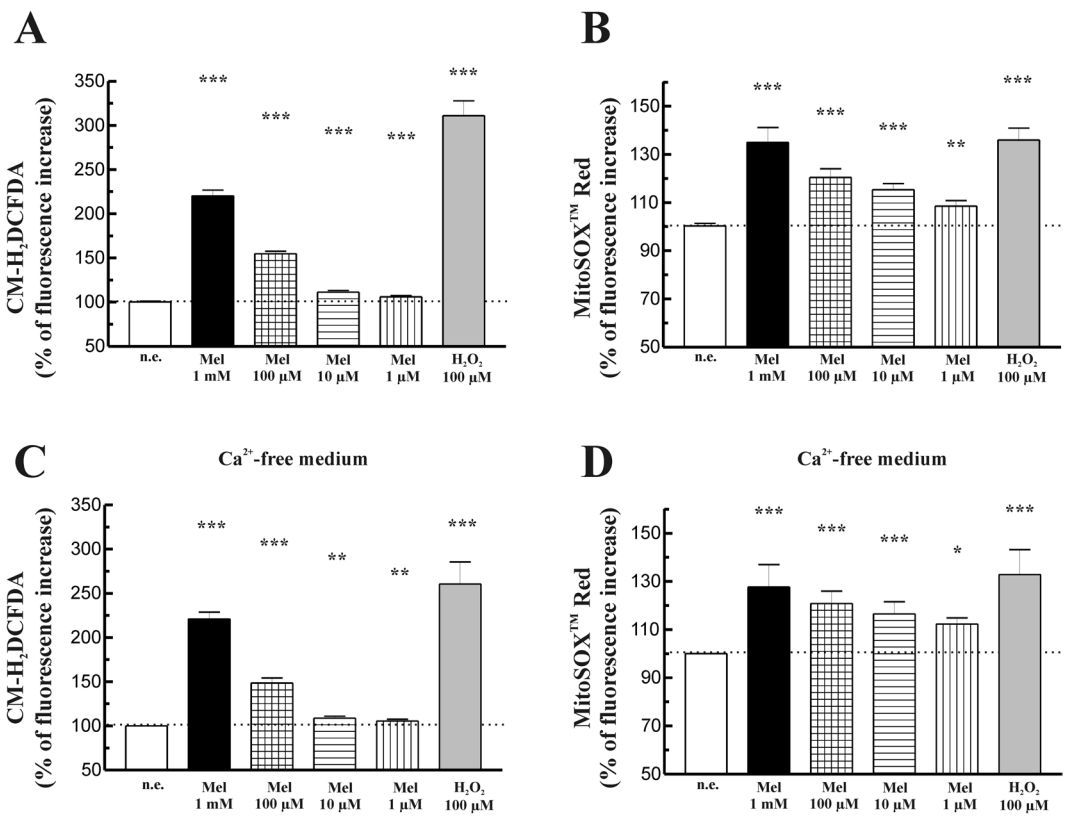

Figure 2. Generation of ROS in response to melatonin. (**A**) Cells were loaded with the red-ox-sensitive dye CM-H_2DCFDA and were challenged with different concentrations of melatonin (1 mM, 100 µM, 10 µM or 1 µM). As a control, cells were incubated in the presence of 100 µM hydrogen peroxide (H_2O_2). (**B**) Cells were loaded with the mitochondrial superoxide indicator MitoSOX Red and were incubated in the presence of melatonin (1 mM, 100 µM, 10 µM or 1 µM). Separated batches of cells were incubated with 100 µM hydrogen peroxide (H_2O_2). (**C** and **D**) Cells, loaded with either of the mentioned dyes, were challenged with melatonin in the absence of Ca^{2+} in the extracellular medium (medium containing 0.5 mM EGTA). The bars show the mean increase of dye-derived fluorescence expressed in percentage ± SEM with respect to control (non-stimulated) cells. A horizontal dotted line represents the value observed in non-stimulated cells. Results are representative of six independent experiments (n.e., non-stimulated cells; Mel, melatonin; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ *vs* non-stimulated cells).

Increases of cellular calcium (Ca^{2+}) have been related with ROS generation and with pancreatic disease[22,29]. In a former work we have shown that melatonin induces mobilization of Ca^{2+} in PSC[17]. In order to check whether ROS generation in response to melatonin was dependent on Ca^{2+}, we performed a series of experiments in which PSC were challenged in the absence of extracellular Ca^{2+} (medium containing 0.5 mM EGTA). Under these conditions ROS production evoked by melatonin did not differ from that observed in the presence of Ca^{2+} (Fig. 2C,D).

In order to investigate whether the increase in ROS production was accompanied by lipid and/or protein oxidation, the effect of melatonin on protein carbonyl levels and on TBARS were assayed. For this purpose, cells were incubated during 1 h in the presence of melatonin (1 mM, 100 µM, 10 µM or 1 µM). H_2O_2 (100 µM) was used as control. We observed a concentration-dependent increase in the total protein carbonyls content in cells treated with melatonin in comparison with that noted in non-stimulated cells. A maximal effect was observed in response to 1 mM melatonin (Fig. 3A). However, no statistically significant changes were detected in the levels of TBARS (Fig. 3B). Treatment of cells with H_2O_2 (100 µM) induced statistically significant increases in both total protein carbonyls and TBARS (Fig. 3A,B).

Effect of melatonin on mitochondrial membrane potential. It has been suggested that oxidative stress and changes in ψ_m are closely related[30]. In order to analyze whether melatonin induces changes in ψ_m, we performed a series of experiments in which PSC were loaded with the mitochondria-specific voltage-sensitive dye TMRM. The cells were then incubated during 1 h in the presence of melatonin (1 mM, 100 µM, 10 µM or 1 µM). We could only observe a statistically significant decrease in ψ_m in cells treated with 1 mM melatonin. No detectable changes in ψ_m were noted in response to the other concentrations of melatonin employed. As a control, different batches of cells were incubated in the presence of the mitochondrial uncoupler CCCP[22,31]. In the presence of CCCP (100 nM) a statistically significant decrease in ψ_m was detected (Fig. 4).

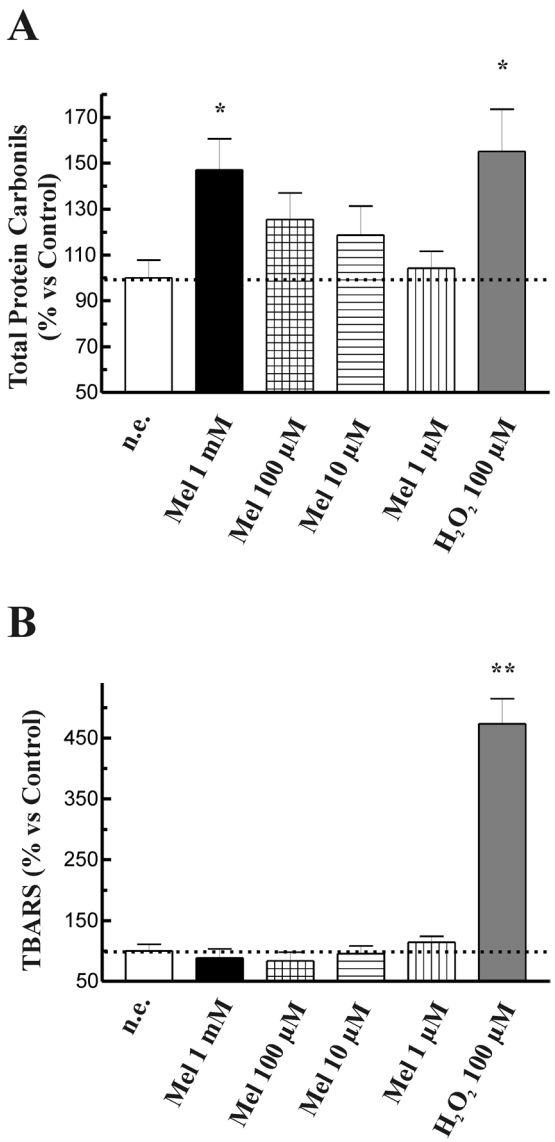

Figure 3. Effect of melatonin on protein and lipid oxidation. PSC were incubated during 1 h in the presence of melatonin (1 mM, 100 µM, 10 µM or 1 µM), and the effect on total protein carbonyls (**A**) or TBARS (**B**) were assayed. 100 µM H_2O_2 was used as control of oxidation. The bars show the mean change expressed in percentage ± SEM with respect to control (non-stimulated) cells. A horizontal dotted line represents the value observed in non-stimulated cells. Results are representative of six independent experiments (n.e., non-stimulated cells; Mel, melatonin; H_2O_2, hydrogen peroxide; $^*P < 0.05$; $^{**}P < 0.01$ *vs* non-stimulated cells).

Effect of melatonin on glutathione levels. Glutathione represents a major antioxidant defense against oxidative stress[32]. Because we had observed ROS production in the presence of the melatonin, it was of interest to test its effect on the glutathione system in PSC. Therefore, cells were incubated during 4 h in the presence of melatonin (1 mM, 100 µM, 10 µM or 1 µM) and the levels of GSH and GSSG were analyzed. We observed a concentration-dependent decrease in GSH/GSSG ratio in cells treated with melatonin in comparison with that noted in non-stimulated cells. A maximal effect was observed in response to 1 mM or 100 µM melatonin. A slight decrease in GSH/GSSG ratio was observed in response to 10 µM melatonin, which was not statistically significant. Whereas we did not detect changes in GSH/GSSG ratio in cells treated with 1 µM melatonin (Fig. 5A).

Effect of melatonin on Nrf2-dependent antioxidant enzymes. Nrf2 is a transcription factor that enhances the expression of a multitude of antioxidant and phase II enzymes, which regulate redox homeostasis[33]. The results shown above indicate that melatonin induces changes in the redox status of PSC. Therefore, we decided to study whether melatonin could stimulate the transcriptional activation of certain antioxidant enzymes through the activation of Nrf2. For this purpose PSC were incubated during 4 h in the presence of melatonin (1 mM, 100 µM, 10 µM or 1 µM) and RT-qPCR of the relative mRNA abundance was performed. Melatonin evoked statistically significant increases in the expression of GCLc, CAT, NQO1 and HO-1 (Fig. 5B–D). As a control, cells were incubated in the presence of H_2O_2 (100 µM), a known Nrf2 activator[34]. The oxidant increased the expression of all four antioxidant enzymes studied.

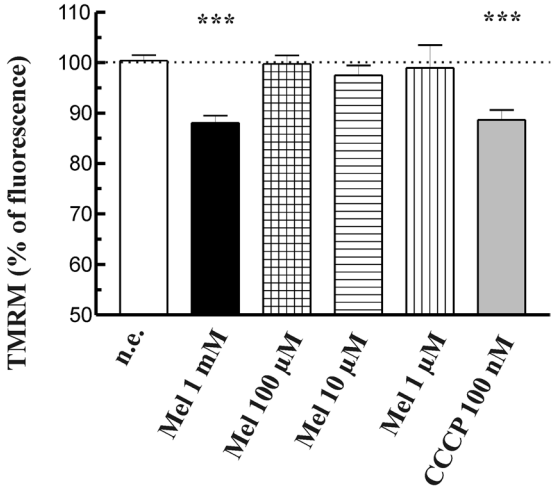

Figure 4. Effect of melatonin on mitochondrial membrane potential. PSC were loaded with the mitochondria-specific voltage-sensitive dye TMRM. The cells were then incubated during 1 h in the presence of melatonin (1 mM, 100 µM, 10 µM or 1 µM). As a control, different batches of cells were incubated in the presence of the mitochondrial uncoupler CCCP (100 nM). The bars show the changes in ψ_m of treated and non-stimulated (control) cells, and are presented as the mean increase of fluorescence expressed in percentage ± SEM with respect to non-stimulated cells. A horizontal dotted line represents the value observed in non-stimulated cells (n.e., non-stimulated cells; Mel, melatonin; ***$P < 0.001$ *vs* non-stimulated cells; n = four independent experiments).

Effect of melatonin on superoxide dismutase. Superoxide dismutases (SOD) catalyze the dismutation of superoxide anion ($O2^-$) to H_2O_2, which is then catalyzed to innocuous O_2 and H_2O by glutathione peroxidase and catalase. Thus, SOD is involved in the defense system against ROS[35]. Several classes of SOD have been identified: Cu/Zn SOD (SOD1), which is localized in cytosol, and MnSOD (SOD2), which is localized in mitochondria[36,37]. We were intereseted in analyzing whether melatonin exerted any affect on SOD. Thus, PSC were incubated during 1 h with the compound (1 mM, 100 µM, 10 µM or 1 µM) and SOD activity was then analyzed. In the presence of melatonin a concentration-dependent decrease in SOD activity was observed (Fig. 6).

We further analyzed the effect of melatonin on SOD and decided to study the protein levels of the enzyme by Western blotting. The results show that PSC that had been incubated with melatonin exhibited lower levels of both SOD1 and SOD2, compared with non-treated cells. The stronger decrease of protein expression was noted for SOD1 (Fig. 7A–D).

Additional studies were carried out to confirm the effect of melatonin on SOD expression. PSC were incubated during 1 h in the presence of melatonin (1 mM, 100 µM, 10 µM or 1 µM) and RT-qPCR of the relative mRNA abundance of SOD1 and SOD2 were performed. In cells treated with melatonin, statistically significant decreases in the mRNA of both proteins were observed (Fig. 7E–F).

Effect of melatonin on the total antioxidant capacity. We additionally evaluated the TAC of PSC. As shown in Fig. 8, the TAC of cells incubated in the presence of melatonin was decreased in comparison with that noted in non-stimulated cells (incubated in the absence of melatonin). The effect did not depend on the concentration of melatonin used. Incubation of PSC with the oxidant H_2O_2 (100 µM) evoked a statistically significant decrease in TAC compared with non-stimulated cells. These results confirm that melatonin induces changes in the oxidative state of PSC.

Discussion

It is well known that tumors undergo adaptive responses that lead to resistance and accelerated repopulation. This allows them to overcome doses of radiation and chemotherapy. Resistance can occur following different adaptive responses, which are due to the nature of the tumor cells or to the release of factors by immune cells as well as to participation of other cell types present in the tumor microenvironment[9]. In this line a major contributing factor is the characteristic extensive stromal or fibrotic reaction found in tumors[2].

In some cancer cells, melatonin itself induces apoptosis[10,13,14] or aids sensitizing cancer cells to therapy[38–41]. In addition, previous results of our laboratory showed that melatonin modulates viability of PSC. This is of relevance because PSC have been pointed out as major players in stromal formation within tumors[17,18]. Therefore melatonin is emerging as a potential tool in the treatment of cancer.

In this study, we provide further evidences that support a potential role for melatonin in the regulation of PSC proliferation by setting-up a prooxidant environment within the cells, which decreases their viability. The oxidative conditions that we have observed might be based on ROS production together with a decrease in TAC of the cells. The latter might have a basis on a reduction of glutathione levels and a decrease in SOD activity. As a whole, the results that we have obtained can be considered relevant bearing in mind that PSC play major roles in fibrosis developed in pancreatic diseases.

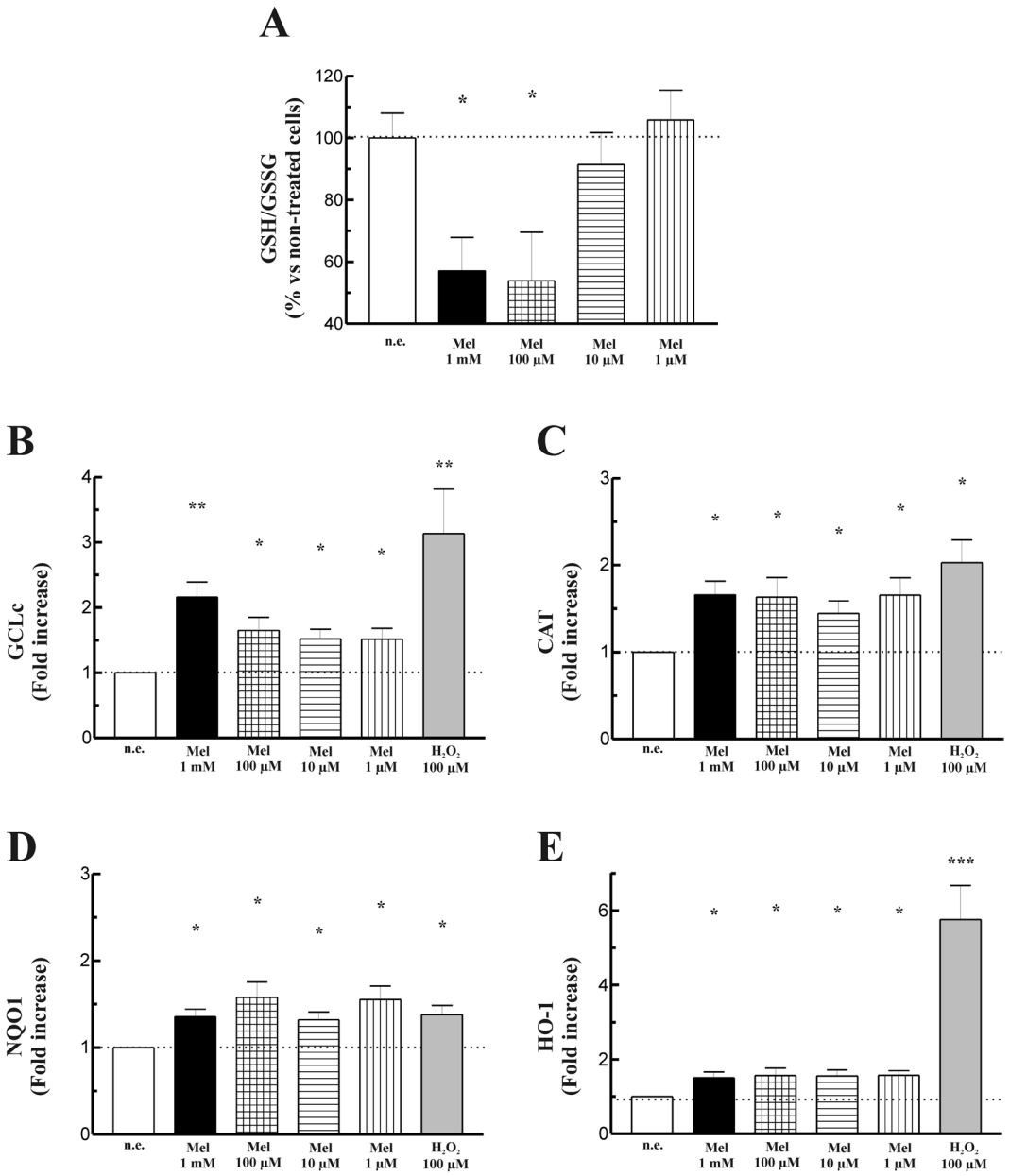

Figure 5. Effect of melatonin on glutathione. (**A**) PSC were incubated during four h in the presence of melatonin (1 mM, 100 µM, 10 µM or 1 µM), and the effect on glutathione was analyzed. The bars show the mean increase in GSH/GSSG ratio expressed in percentage ± SEM with respect to non-stimulated cells. (**B**-**D**) RT-qPCR analysis of Nrf2-target genes glutamate cysteine ligase-catalytic subunit (GClc), catalase (CAT), NAD(P)H quinone oxidoreductase 1 (NQO1) and heme-oxygenase-1 (HO-1) reveals statistically significant increases in the levels of Nrf2-dependent antioxidant enzymes in cells incubated in the presence of melatonin. Incubation of cells with H_2O_2 (100 µM) also evoked an increase in the expression of all four antioxidant enzymes. *Gapdh* mRNA was used for normalization. Data are expressed as the mean ± S.E.M. of the change relative to non-stimulated cells. A horizontal dotted line represents the value observed in non-stimulated cells. Three different cellular preparations were used (n.e., non-stimulated cells; Mel, melatonin; $*P < 0.05$; $**P < 0.01$).

The drop in PSC viability that we have observed confirms previous studies of our laboratory[17,18]. Interestingly, a decrease in the proliferation of this cellular type would be a helpful maneuver that could help in diminishing the fibrosis present in the pancreas under pathological conditions, especially in tumors.

Maintenance of adequate cellular red-ox equilibrium is critical for cell function and viability[42]. Conversely to the protective role of melatonin against oxidative stress[43] the compound can also exhibit prooxidant effects, which have been related with a cytotoxic effect[28]. The analysis of the results that we have obtained showed that melatonin induced ROS production. The generation of ROS could be detected in both the cytosol and the mitochondria, but the contribution of Ca^{2+} was negligible. Participation of mitochondria in ROS generation has been demonstrated[44,45]. These results are in agreement with previous findings of our laboratory, which showed that ROS

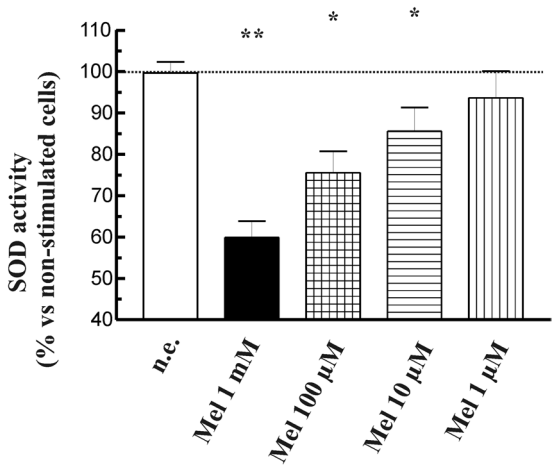

Figure 6. Effect of melatonin on SOD activity. PSC were incubated during 1 h in the presence of melatonin (1 mM, 100 µM, 10 µM or 1 µM). The bars show the mean change of SOD activity expressed in percentage ± SEM with respect to control (non-stimulated) cells. A horizontal dotted line represents the value observed in non-stimulated cells. Results are representative of five independent experiments (n.e., non-stimulated cells; Mel, melatonin; $*P < 0.05$; $**P < 0.01$ *vs* non-stimulated cells).

production was increased in PSC treated with melatonin[17,18], and confirm the hypothesis of putative prooxidant actions of melatonin in this cellular type. The present research was conducted in order to further investigate other possible points of action of melatonin to exert its prooxidant effects that could explain its actions on PSC viability.

Our results additionally show that melatonin treatment might be accompanied by oxidation of certain cellular structures. This could be reflected by the increase in the oxidation of cellular proteins that we have noted; however, we could not detect changes in the oxidation of lipids (TBARS). From these observations we could assume that melatonin might differentially affect lipids and proteins within the cell. Besides, it could be possible that certain proteins are more prone to oxidation that lipids upon melatonin treatment. Therefore, melatonin effects on protein redox state could lead to the modulation of metabolic pathways regulated by such proteins, which are activated/inactivated due to changes in their oxidative state.

In addition, impairment of mitochondria leads to ROS generation[22,46]. Our results also show that ψ_m decreased in the presence of melatonin. At this point we could hypothesize that melatonin might affect mitochondrial physiology in PSC. In fact, different studies have suggested that melatonin alters mitochondrial physiology which is related with cell death[9,14]. Moreover, a few studies using cultured cells found that melatonin stimulated ROS generation at pharmacological concentrations (micro-molar to milli-molar range) in several tumor and non-tumor cells; thus, melatonin functioned as a conditional pro-oxidant[47].

Additional evidences for a disruption by melatonin of the redox balance in PSC derives from the experiments directed to analyze its effect on glutathione. The glutathione system is a major tool used in the defense against damage caused by ROS. A defeat of antioxidant systems, like a decrease in the GSH content, can lead cells to fault in the control ROS production and, therefore, can induce cell damage and death[48]. Our results show that, in the presence of melatonin the ratio GSH/GSSG decreased. This action depended on the concentration of melatonin used. Higher effects were found at 100 µM and 1 mM of the indole, whereas no detectable changes were noted in cells treated with 1 µM melatonin. The decline in GSH/GSSG ratio that we have noted points towards an increase in oxidized glutathione. This observation might reflect a pro-oxidant action of melatonin. In other words, the decrease in the availability of reduced glutathione could be related with the increase in ROS generation evoked by melatonin. These results are in agreement with previous observations of our laboratory, obtained in human PSC, in which we showed that melatonin evoked concentration-dependent changes in glutathione oxidation[18]. Interestingly, it could be feasible that melatonin might exert the same effects in human cells as those noted in murine cells, thus providing putative beneficial actions of the compound on human health as expected from the results obtained in studies carried out on animal cells.

In another set of experiments we have detected an increase in the expression of the Nrf2-regulated antioxidant enzymes GClc, CAT, HO-1 and NQO1. Specifically, GCLc is involved in glutathione synthesis[49]. Nrf2 is required for systemic protection against redox-mediated injury. Under oxidative conditions the Keap1-ARE (antioxidant response element) pathway is activated via the upregulation of Nrf2[50]. Melatonin activates this pathway to induce protective antioxidant actions[24,51]. In our study, the prooxidant conditions evoked by melatonin might activate the Nrf2-regulated pathway in an attempt to counteract the pro-oxidative state that we have observed.

SOD is another enzyme with pivotal role in cellular antioxidant defence[52]. Our results show that SOD activity is decreased in the presence of melatonin. This effect could be explained by a diminished expression of both SOD1 and SOD2, whith a higher effect on SOD1. Our results further suggest that melatonin regulates SOD at the translation level. To our knowledge, this is the first time to show that melatonin decreases the expression of SOD. Findings of other researchers show that melatonin either increases SOD expression[53,54] or does not induces changes in the levels of these proteins[55].

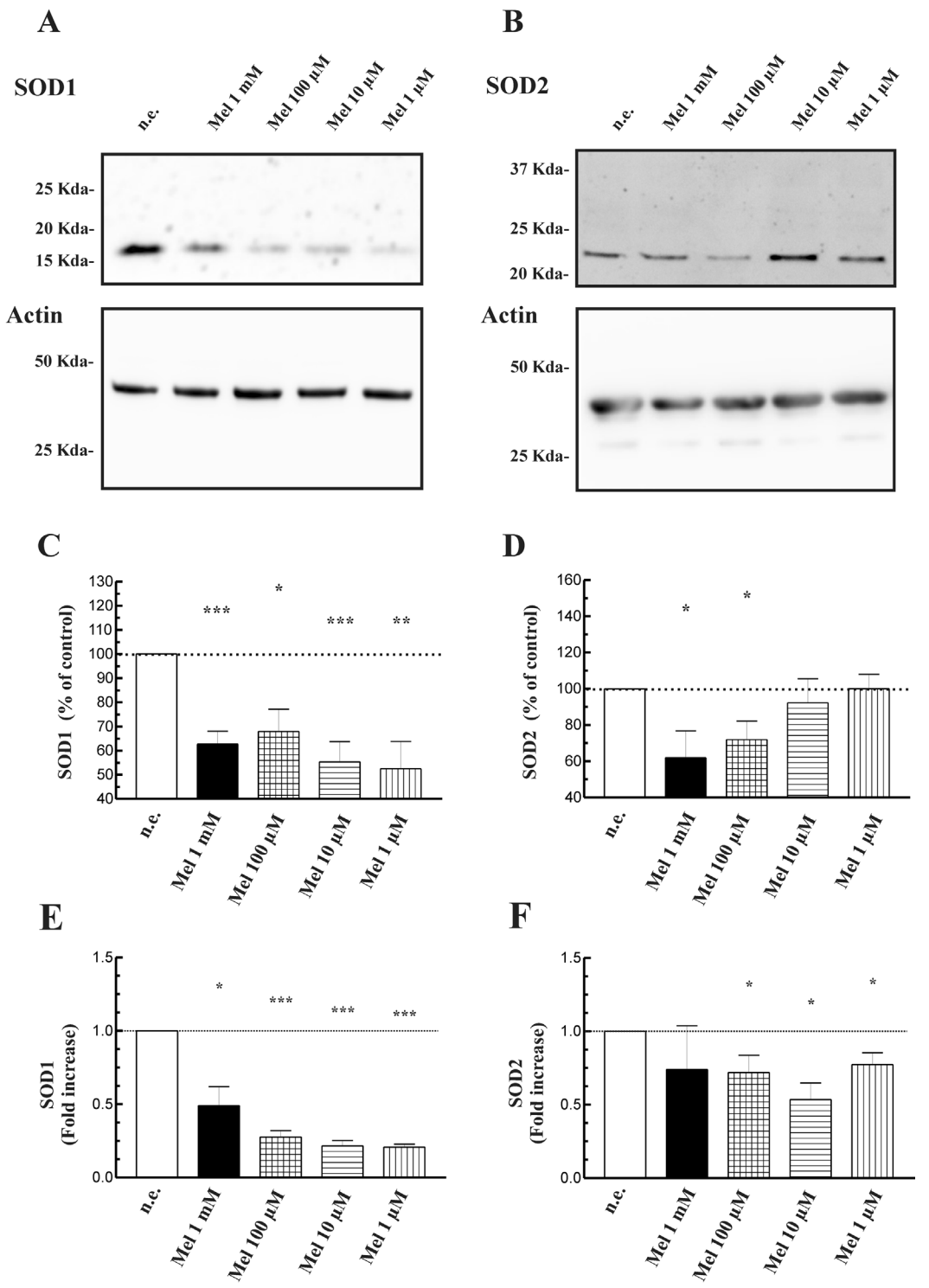

Figure 7. Expression of SOD in PSC treated with melatonin. PSCs were incubated during 1 h in the absence (Control) or in the presence of the desired concentration of melatonin (1 mM, 100 µM, 10 µM or 1 µM). The figure shows representative blots showing the effect of melatonin on the level of the antioxidant enzymes SOD1 (**A**) and SOD2 (**B**), evaluated with specific antibodies. The levels of actin were employed as controls to ensure equal loading of proteins. (**C** and **D**) The graphs show the quantification of protein expression. A horizontal dotted line represents the value observed in non-stimulated cells. Values are the mean ± S.E.M. of normalized values expressed as % of phosphorylation in control (non-stimulated) cells. (**E** and **F**) RT-qPCR analysis was performed to detect mRNA levels of SOD1 and SOD2 respectively. The bars show the mean ± S.E.M. of the change in mRNA levels of each protein relative to non-stimulated cells. *Gapdh* mRNA was used for normalization. A horizontal dotted line represents the value observed in non-stimulated cells. Three different cellular preparations were used (n.e., non-stimulated cells; Mel, melatonin; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ *vs* non-stimulated cells). The experiments shown are representative of three different preparations.

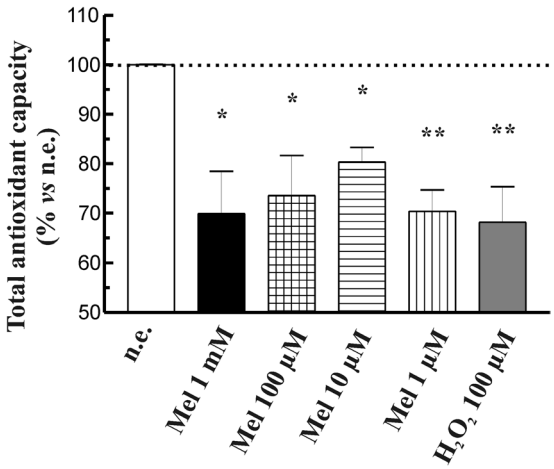

Figure 8. Effect of melatonin on total antioxidant capacity. PSC were incubated with melatonin (1 mM, 100 µM, 10 µM or 1 µM) and then TAC was determined. H_2O_2 (100 µM) was used as a control of oxidation. Values show the mean ± S.E.M. of normalized values expressed as % with respect to non-stimulated cells. A horizontal dotted line represents the value observed in non-stimulated cells. Data are representative of three independent experiments (n.e., non-stimulated cells; Mel, melatonin; H_2O_2, hydrogen peroxide; $^*P < 0.05$; and $^{**}P < 0.01$ *vs* non-stimulated cells).

Interestinlgy, melatonin exerts prooxidant effects[28] (Sanchez-Sanchez *et al*., 2011). A SOD activity under a certain level could lead to diminished antioxidant pretection of the cell that, if is not counteracted by other antioxidant defenses, might lead to prooxidant conditions that could compromise cell function and viability. As a consequence, and taking also into account the effects on glutathione that we have mentioned above, the TAC of the cells should be expected to decrease, as we have observed. Therefore, our results point out that melatonin modulates pivotal points of the cellular antioxidant machinery and leads to prooxidant conditions that could drive the mechanisms involved in PSC viability and/or proliferation. In fact, we have shown previously that melatonin induced changes in the phosphorylation state of members of the mitogen-activated protein kinases family, which are involved in cell proliferation and survival. This resulted in a decrease in cell viability[17].

The concentrations of melatonin that we have employed are not physiological and fairly fall within the pharmacological range[56]. However, pharmacological concentrations of melatonin have been used in a plethora of studies directed to the study of disease[57–59], including studies carried out our laboratory[14,17,24,60,61].

In conclusion, we present evidences that stand out melatonin as a compound with the ability to regulate PSC physiology. Despite the protective role that melatonin exerts in a wide variety of cellular types, here we show that the compound induces pro-oxidative conditions that might have consequences on cell viability. It is noteworthy to bear in mind that the actions of melatonin on cellular physiology might be cell- and context-dependent. Contribution of stellate cells to survival and development of transformed epithelia within the pancreas has been documented[62,63]. Thus, strategies directed to controlling the growth of fibrotic tissue within tumors might be challenging in the treatment of cancer[2]. In this line, our results suggest a probable mechanism by which melatonin modulates fibrosis within the pancreas. Therefore, melatonin could be considered a hopeful aid in the therapy of pancreatic cancer.

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Author contributions

Antonio Gonzalez: designed the study and wrote the manuscript. Matias Estaras, Salome Martinez-Morcillo, Remigio Martinez, Alfredo García, Mario Estévez, Jose A. Tapia, Noelia Moreno: acquisition and data analysis. Patricia Santofimia-Castaño: designed the primers for PCR. Marcos Pérez-López, María P. Míguez and Vicente Roncero: interpreted and discussed results. Gerardo Blanco, Diego Lopez, Miguel Fernandez-Bermejo, Jose M. Mateos, Daniel Vara: reviewed and corrected the manuscript. Gines M. Salido: suggested discussion parts and corrected the manuscript. All authors have approved and agreed with the submitted version.

Competing interests

The authors declare that there are no competing interest (there are no financial and non-financial interests that could directly undermine, or be perceived to undermine, the objectivity, integrity and value of this publication).

Additional information

Correspondence and requests for materials should be addressed to A.G.

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Research article

Pancreatic stellate cells exhibit adaptation to oxidative stress evoked by hypoxia

Matias Estaras*, Salome Martinez-Morcillo†, Alfredo García‡, Remigio Martinez§, Mario Estevez‖, Marcos Perez-Lopez†, Maria P. Miguez†, Miguel Fernandez-Bermejo#, Jose M. Mateos#, Daniel Vara#, Gerardo Blanco, Diego Lopez**, Vicente Roncero††, Gines M. Salido* and Antonio Gonzalez*[1]**

*Institute of Molecular Pathology Biomarkers, University of Extremadura, Caceres, Spain, †Unit of Toxicology, Veterinary Faculty, University of Extremadura, Caceres, Spain, ‡Department of Animal Production, Cicytex-La Orden, Badajoz, Spain, §Department of Animal Health, Veterinary Faculty, University of Extremadura, Caceres, Spain, ‖IPROCAR Research Institute, Food Technology, University of Extremadura, Caceres 10003, Spain, #Department of Gastroenterology, San Pedro de Alcantara Hospital, Caceres, Spain, **Hepatobiliary-Pancreatic Surgery and Liver Transplant Unit, Infanta Cristina Hospital, Badajoz, Spain, and ††Unit of Histology and Pathological Anatomy, Veterinary Faculty, University of Extremadura, Caceres, Spain

Background information. Pancreatic stellate cells play a key role in the fibrosis that develops in diseases such as pancreatic cancer. In the growing tumour, a hypoxia condition develops under which cancer cells are able to proliferate. The growth of fibrotic tissue contributes to hypoxia. In this study, the effect of hypoxia (1% O_2) on pancreatic stellate cells physiology was investigated. Changes in intracellular free-Ca^{2+} concentration, mitochondrial free-Ca^{2+} concentration and mitochondrial membrane potential were studied by fluorescence techniques. The status of enzymes responsible for the cellular oxidative state was analyzed by quantitative reverse transcription-polymerase chain reaction, high-performance liquid chromatography, spectrophotometric and fluorimetric methods and by Western blotting analysis. Cell viability and proliferation were studied by crystal violet test, 5-bromo-2-deoxyuridine cell proliferation test and Western blotting analysis. Finally, cell migration was studied employing the wound healing assay.

Results. Hypoxia induced an increase in intracellular and mitochondrial free-Ca^{2+} concentration, whereas mitochondrial membrane potential was decreased. An increase in mitochondrial reactive oxygen species production was observed. Additionally, an increase in the oxidation of proteins and lipids was detected. Moreover, cellular total antioxidant capacity was decreased. Increases in the expression of superoxide dismutase 1 and 2 were observed and superoxide dismutase activity was augmented. Hypoxia evoked a decrease in the oxidized/reduced glutathione ratio. An increase in the phosphorylation of nuclear factor erythroid 2–related factor and in expression of the antioxidant enzymes catalytic subunit of glutamate-cysteine ligase, catalase, NAD(P)H-quinone oxidoreductase 1 and heme oxygenase-1 were detected. The expression of cyclin A was decreased, whereas expression of cyclin D and the content of 5-bromo-2-deoxyuridine were increased. This was accompanied by an increase in cell viability. The phosphorylation state of c-Jun NH_2-terminal kinase was increased, whereas that of p44/42 and p38 was decreased. Finally, cells subjected to hypoxia maintained migration ability.

Conclusions and Significance. Hypoxia creates pro-oxidant conditions in pancreatic stellate cells to which cells adapt and leads to increased viability and proliferation.

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

In the pancreas, pancreatic stellate cells (PSC) represent a rather small cell population in comparison with the secretory tissue. PSC appear forming strings around large lobules or individually at the periphery of the acini (Gryshchenko et al., 2016). Under normal conditions, PSC remain quiescent but, in case of pancreatic injury, the activation of PSC occurs. Activated PSC play a key role in the fibrogenesis process that develops within the gland under certain diseases, such as inflammation or cancer. Under these circumstances, PSC actively participate in the development of fibrosis. This process is based on the secretion by PSC, and on the accumulation, of extracellular matrix components (Sherman, 2018).

The available studies suggest that PSC play an important role in the development and progression of pancreatic cancer. This is due to the specific interactions that are established between cancerous cells and PSC. This symbiosis favours cancer growth and proliferation (Xiao et al., 2019; Yu et al., 2019). Moreover, it has been suggested that activated PSC are involved in tumour resistance against chemo- and radiotherapy (Moir et al., 2015). Therefore, understanding the mechanisms by which activated PSC grow and proliferate is of critical relevance for the understanding of pancreatic diseases.

Pancreatic adenocarcinoma is characterized by hypoxia. This low availability of oxygen is a condition derived from the rapid growth and accumulation of cells within the tumour. Inside the malignant growing tissue, cells undergo an adaptation to hypoxia and are able to grow with a resultant expansion of the tumour (Gupta et al., 2019). Activated PSC that are included in the tumour mass will also be subjected to hypoxia. Like tumour cells, PSC must undergo adaptation to this low oxygen availability. Moreover, interactions between cancer and PSC under hypoxia perpetuate the fibrosis cycle (Erkan et al., 2009; Masamune et al., 2008).

Although adaptation of pancreatic cancer cells to low oxygen availability is well documented (Erkan et al., 2016), less is known about how PSC adapt to and resist hypoxia. It has been shown that PSC subjected to hypoxia exhibit increased influx of calcium (Ca^{2+}) through TRPC6 channels, which are involved in their proliferation (Nielsen et al., 2017). Moreover, cellular stress evoked by hypoxia may rapidly induce transactivation of PSC and induce their proliferation (Rebours et al., 2013). However, additional studies are required to clarify the ways by which PSC adapt to hypoxia and the intracellular pathways involved in their survival under this stressing condition. In the present study, our goal was to shed more light on the mechanisms that may underlie PSC survival and proliferation under hypoxia.

Results

Induction of hypoxia

PSC in culture were subjected to a low O_2 (1%) atmosphere. This percentage of O_2 has been successfully used to induce hypoxia in this cell type by others (Sarcar et al., 2019). Following incubation of PSC under hypoxia during 4 h, an increase in the expression of hypoxia-inducible factor (HIF)-1α and HIF-2 α was detected (supplementary Figure 1). Thereafter, we used 1 % of O_2 during our experiments.

Effect of hypoxia on intracellular free-Ca^{2+} concentration ($[Ca^{2+}]_i$), mitochondrial free-Ca^{2+} concentration ($[Ca^{2+}]_m$) and mitochondrial membrane potential (Ψ_m)

Because Ca^{2+} is a major regulator of the physiology of pancreas (Li et al., 2014; Gryshchenko et al., 2018), we were interested in analyzing the effect of hypoxia on this biomarker. For this purpose, PSC loaded with the Ca^{2+} indicator fura-2 were subjected to hypoxia. As it can be seen in Figure 1A, hypoxia induced an increase in $[Ca^{2+}]_i$ that remained elevated. The pattern of Ca^{2+} mobilization consisted of a progressive increase of $[Ca^{2+}]_i$ towards a plateau. In occasions, transient increases in $[Ca^{2+}]_i$ were observed. In order to investigate the source for Ca^{2+} mobilization, cells were challenged in the absence of Ca^{2+}

[1]To whom correspondence should be addressed (email: agmateos@unex.es)

Key words: Calcium, Glutathione, Hypoxia, Pancreatic stellate cells, Reactive oxygen species.

Abbreviations: BrdU, 5-bromo-2-deoxyuridine; *CAT*, catalase; CM-H_2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N′N′-tetraacetic acid; ER, endoplasmic reticulum; GSH, reduced glutathione; GSSG, oxidized glutathione; HIF, hypoxia-inducible factor; HPLC, high-performance liquid chromatography; JNK, c-Jun NH_2-terminal kinase; MAPKs, mitogen-activated protein kinases; MDA, malondialdehyde; MES, 2-(N-morpholino) ethanesulfonic acid; *NQO1*, NAD(P)H quinone oxidoreductase 1; Nrf2, nuclear factor erythroid 2-related factor; PSC, pancreatic stellate cells; ROS, reactive oxygen species; SOD, superoxide dismutase; TAC, total antioxidant capacity; TBARS, thiobarbituric-reactive substances; TMRM, tetramethyl rhodamine; Tps, thapsigargin

Figure 1 | Time-courses of changes in $[Ca^{2+}]_i$ observed in PSC in response to hypoxia. (**A**) Effect of hypoxia (1% O_2) on $[Ca^{2+}]_i$ in cells loaded with the Ca^{2+}-indicator fura-2 in the presence of Ca^{2+} (1 mM) in the extracellular medium. (**B**) Cells were challenged with hypoxia in the absence of extracellular Ca^{2+} (medium containing 0.5 mM EGTA). (**C**) The bars show the area under the curve (in arbitrary units of fluorescence) for Ca^{2+} mobilization in cells subjected to hypoxia in the presence or in the absence of Ca^{2+} in the extracellular medium. (**D**) The graph shows the effect of addition of thapsigargin (Tps, 1 μM) to PSC subjected to hypoxia in a Ca^{2+}-free medium (the inset shows the response evoked by Tps alone in a Ca^{2+}-free medium). (**E**) Cells were incubated under hypoxia in a Ca^{2+}-free medium and thereafter Ca^{2+} (1 mM) was added to the extracellular solution. The horizontal bars indicate the time during which hypoxia, Ca^{2+}-free medium, thapsigargin and/or Ca^{2+}-containing medium were applied to the cells. The traces show the typical response of one cell taken from 28 to 36 cells analyzed in four to five different experiments (e.g., extracellular Ca^{2+}).

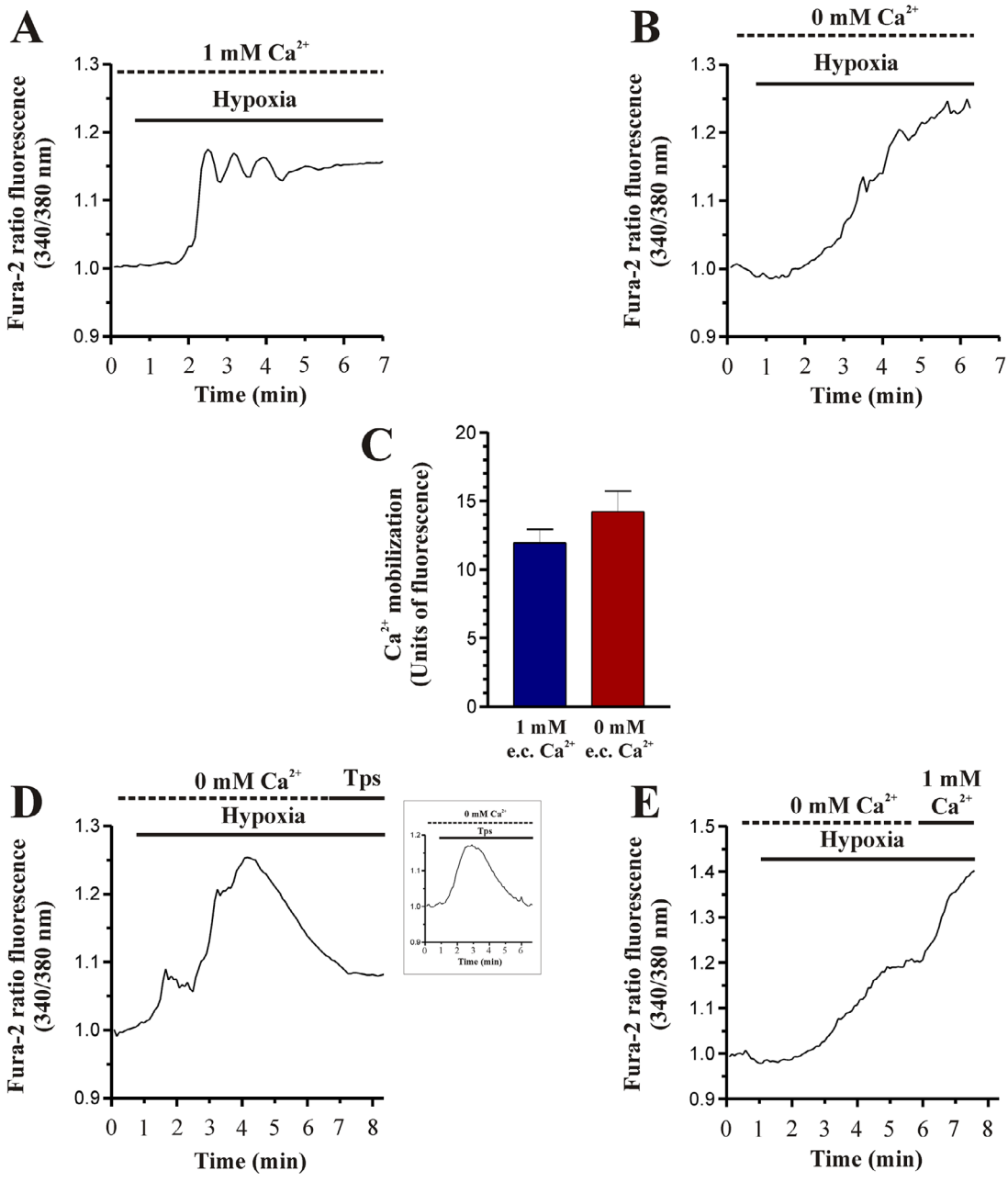
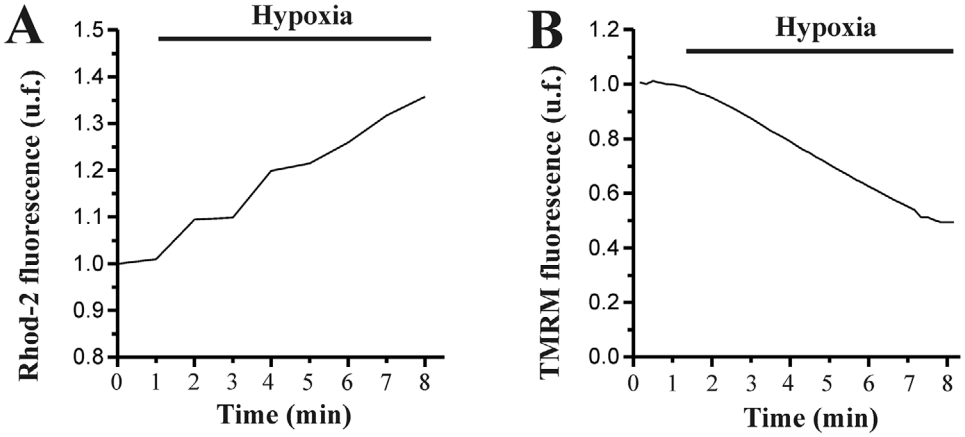


Figure 2 | Time-courses of changes in $[Ca^{2+}]_m$ and Ψ_m observed in PSC in response to hypoxia. (**A**) Effect of hypoxia in cells loaded with the mitochondrial Ca^{2+} indicator rhod-2. (**B**) Cells were loaded TMRM to detect changes in Ψ_m. The horizontal bar indicates the time during which hypoxia was applied to the cells. The traces show the typical response of one cell taken from three different preparations. The experiments were carried out in the presence of Ca^{2+} (1 mM) in the extracellular medium.



in the extracellular medium (containing 0.5 mM ethylene glycol-bis(2-aminoethylether)-N,N,N′N′-tetraacetic acid [EGTA]). In the absence of external Ca^{2+}, an increase in $[Ca^{2+}]_i$ was observed in cells subjected to hypoxia (Figure 1B). The total Ca^{2+} mobilization, measured in arbitrary units of fluorescence (u.f.), that was observed in cells subjected to hypoxia in the presence of Ca^{2+} in the extracellular medium (11.94 ± 0.99 u.f., $n = 28$ cells) did not differ significantly from that noted in cells that were challenged in the absence of extracellular Ca^{2+} (14.22 ± 1.50 u.f., $n = 36$ cells; Figure 1C). These results suggest that Ca^{2+} is mobilized from an intracellular source when cells are subjected to hypoxia.

To further investigate the source for Ca^{2+} mobilization, separate sets of experiments were performed in which cells were subjected to hypoxia in the absence of extracellular Ca^{2+} and then were additionally incubated with thapsigargin (Tps, 1 μM), a sarcoendoplasmic reticulum Ca^{2+}-ATPase inhibitor. The inhibitor failed to further increase $[Ca^{2+}]_i$ (Figure 1D). In other set of experiments, cells were incubated under hypoxia in the absence of extracellular Ca^{2+} and then Ca^{2+} (1 mM) was added to the extracellular solution. Upon inclusion of Ca^{2+} in the extracellular medium, an increase in $[Ca^{2+}]_i$ was noted, reflecting Ca^{2+} entry from the extracellular space (Figure 1E).

These observations show that the intracellular Ca^{2+} stores were depleted following treatment of cells with hypoxia and involve the endoplasmic reticulum (ER). Moreover, the results show that because intracellular Ca^{2+} stores were depleted, Ca^{2+} entry from the extracellular space was activated.

We were further interested in investigating the effect of hypoxia on $[Ca^{2+}]_m$. Thus, cells loaded with the mitochondrial Ca^{2+} indicator rhod-2 were challenged with hypoxia. As a result, we observed an increase rhod-2-derived fluorescence compared with the value observed at the beginning of the experiment, indicating that $[Ca^{2+}]_m$ was changed by the treatment (Figure 2A; 1.35 ± 0.10 u.f. vs. 1.00 ± 0.05 u.f., $n = 19$ cells; $P < 0.01$).

The effect of hypoxia on Ψ_m also was investigated. When cells were subjected to hypoxia, a decrease in tetramethyl rhodamine (TMRM)-derived fluorescence was noted indicating a decrease in Ψ_m compared with the value observed at the beginning of the experiment (Figure 2B; 0.51 ± 0.02 u.f. vs. 1.00 ± 0.01 u.f., $n = 34$ cells; $P < 0.001$).

These observations suggest that hypoxia induces changes in mitochondrial parameters that might influence the activity of the organelle.

Effect of hypoxia on the oxidative state of PSC

In the next set of experiments, we evaluated the effect of hypoxia on reactive oxygen species (ROS) production. PSC were loaded with 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl

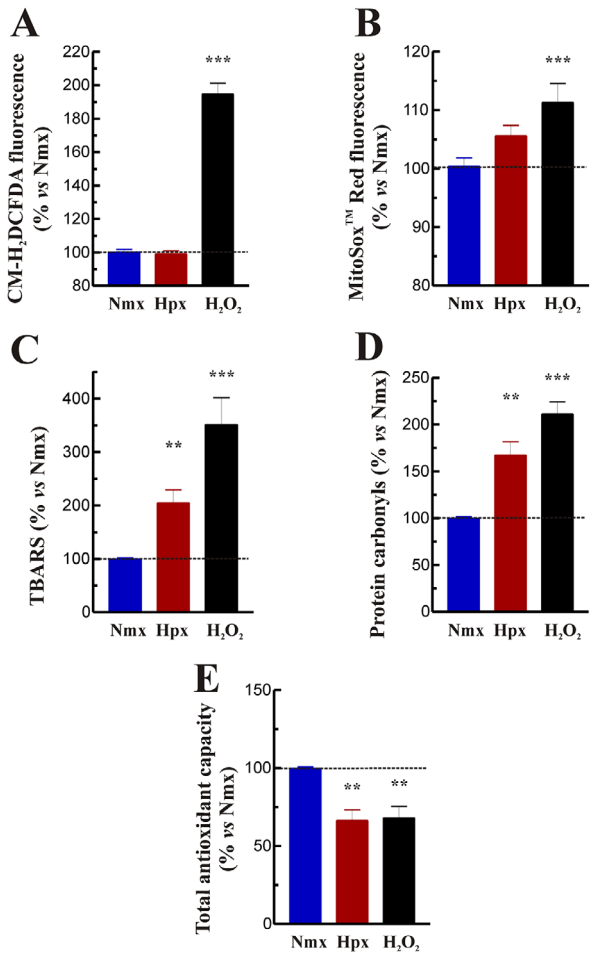
ester (CM-H_2DCFDA) to follow cytosolic ROS production, or with MitoSOX^{TM} Red, to detect production of ROS in the mitochondria (Gonzalez and Salido, 2016). Once loaded, cells were subjected to hypoxia during 1 h in the presence of Ca^{2+} (1 mM) in the extracellular medium. The generation of ROS was compared with that detected in non-treated cells (incubated in normoxia). We could not observe increases in ROS production in the cytosol (in %, 99.04 ± 1.88 vs. 100.10 ± 0.58, $n = 4$; Figure 3A). However, a slight increase in mitochondrial ROS generation was observed (in %, 105.60 ± 1.88 vs. 100.40 ± 1.44, $n = 4$; Figure 3B). As a control of oxidation, cells were challenged with hydrogen peroxide (H_2O_2, 100 μM) that evoked a statistically significant increase in oxidation of the ROS-sensitive probes in comparison with cells incubated in normoxia (in %, 194.57 ± 6.48, $n = 4$; $P < 0.001$, and 111.30 ± 3.25, $n = 4$, $P < 0.01$, respectively, for cytosol and mitochondria vs. cells incubated in normoxia; Figure 3A and B).

Thereafter, we studied the effect of hypoxia on lipid peroxidation and protein oxidation. The experiments were performed in the presence of Ca^{2+} (1 mM) in the extracellular medium. As shown in Figure 3C and D, PSC subjected to hypoxia exhibited statistically significant increases in thiobarbituric-reactive substances (TBARS; in %, 204.70 ± 24.54, $n = 3$, $P < 0.01$) and protein oxidation (in %, 167.20 ± 14.13, $n = 3$, $P < 0.01$) in comparison with non-treated cells (incubated in normoxia; 100.00 ± 1.10, $n = 3$). Again, H_2O_2 (100 μM) evoked statistically significant increases in these parameters (in %, 351.20 ± 50.27, and 211.10 ± 13.07, for TBARS and carbonyls, respectively, $n = 3$, $P < 0.001$).

We additionally evaluated the total antioxidant capacity (TAC) of PSC subjected to hypoxia, in the presence of Ca^{2+} (1 mM) in the extracellular medium. As shown in Figure 3E, the TAC of cells incubated in hypoxia was decreased (in %, 66.33 ± 6.79, $n = 3$, $P < 0.01$) in comparison with that observed in cells incubated in normoxia (in %, 100.00 ± 0.83, $n = 3$, $P < 0.01$). Incubation of PSC with the oxidant H_2O_2 (100 μM) evoked a statistically significant decrease in TAC (in %, 68.15 ± 7.28, $n = 3$, $P < 0.01$) compared with non-treated cells.

These results suggest that hypoxia induces changes in the oxidative state of PSC.

Figure 3 | Effect of hypoxia on the oxidative state of PSC. (**A** and **B**) The generation of ROS was evaluated in PSC loaded with the ROS-sensitive dyes CM-H_2DCFDA (which detects cytosolic ROS production) or MitoSox^{TM} Red (which detects generation of ROS within mitochondria). (**C** and **D**) The levels of TBARS and protein carbonyls were studied in cells subjected normoxic conditions or to hypoxia. (**E**) PSC were incubated under normoxia or hypoxia and then TAC was determined. Results report the changes in the parameters analyzed of cells subjected to hypoxia compared with that of non-treated cells (cells in normoxia). H_2O_2 (100 μM) was used as a control of oxidation. The experiments were carried out in the presence of Ca^{2+} (1 mM) in the extracellular medium. A horizontal dashed line represents the value observed in non-treated cells. Data are representative of three to four separate experiments (Nmx, normoxia; Hpx, hypoxia; H_2O_2, hydrogen peroxide; $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. cells incubated in normoxia).



Effect of hypoxia on superoxide dismutase

Superoxide dismutase (SOD) is a family of antioxidant enzymes that provide defence against free radicals (Balamurugan et al., 2018). SOD can be found in the cytosol (Cu/Zn SOD), which is termed SOD1, or in the mitochondria (MnSOD), which is termed SOD2 (Milani et al., 2011; Zou et al., 2017). We were interested in analyzing whether SOD was affected by hypoxia. The experiments were performed in the presence of Ca^{2+} (1 mM) in the extracellular medium.

Following incubation of PSC under hypoxia for 1 h, an increase in the activity of SOD was detected, which was statistically significant compared with that noted in non-treated cells (in %, 114.10 ± 2.55 vs. 100.00 ± 3.22, $n = 4$, $P < 0.01$; Figure 4A).

We next studied the protein levels of the enzyme by Western blotting. Antibodies against SOD1 and SOD2 were purchased from Thermo Scientific (Fisher Scientific Inc., Madrid, Spain) and from Santa Cruz Biotechnologies Inc. (Quimigen S.L., Madrid, Spain), respectively. Secondary HRP-conjugate antibodies were purchased from Thermo Fisher (Madrid, Spain). Separate batches of cells were incubated during 4 h under hypoxia or in normoxic conditions (nontreated cells). The results show that hypoxia induced an increase in the expression of SOD1 (in %, 136.90 ± 12.20 vs. 100.00 ± 0.01, $n = 4$; $P < 0.05$) and SOD2 (in %, 208.40 ± 21.00 vs. 100.00 ± 0.01, $n = 4$; $P < 0.01$), in comparison with non-treated cells (Figure 4B and C).

Effect of hypoxia on glutathione levels and on nuclear factor erythroid 2–related factor related antioxidant enzymes

Glutathione is a tripeptide that plays a key role among the mechanisms of the cellular defence against oxidative stress (Sies, 1999). Because we had observed changes in the oxidative state under hypoxia, which involved ROS generation, we were interested in studying the effect of hypoxia on glutathione. The experiments were performed in the presence of Ca^{2+} (1 mM) in the extracellular medium.

Following incubation of PSC during 4 h under hypoxia, a decrease in the ratio of reduced/oxidized glutathione (GSH/GSSG) was detected (in %, 75.82 ± 15.91, $n = 4$; $P < 0.05$) in comparison with the values noted in cells incubated in normoxia (100.00 ± 7.70, $n = 4$; Figure 5A). This observation could reflect a consumption of GSH under a pro-oxidative condition created by hypoxia.

The transcription factor Nrf2 (nuclear factor erythroid 2-related factor) is closely related with glutathione synthesis. Nrf2 controls the expression of an array of antioxidant and phase II enzymes that play key roles in the regulation of redox status within the cells (Tian et al., 2018). Now, we decided to study the effect of hypoxia on the transcriptional activation of Nrf2-regulated antioxidant enzymes. In a first instance, we evaluated phosphorylation of the transcription factor. Anti phospho-Nrf2 antibody and the secondary HRP-conjugate antibody were purchased from Thermo Fisher. Cells subjected to hypoxia (4 h) exhibited a statistically significant increase in the phosphorylation of Nrf2 (387.80 ± 108.40, $n = 4$, $P < 0.05$; Figure 5B and C) in comparison with the level detected in non-treated cells (incubated in normoxia). Moreover, incubation of PSC under hypoxia (4 h) evoked a statistically significant increase in the expression of the catalytic subunit of glutamate-cysteine ligase (*GCLc*, 1.89 ± 0.20, $n = 3$, $P < 0.01$), *catalase* (1.57 ± 0.21, $n = 3$, $P < 0.01$), NAD(P)H quinone oxidoreductase 1 (*NQO1*, 1.52 ± 0.20, $n = 3$, $P < 0.05$) and heme oxygenase-1 (*HO1*, 3.48 ± 0.41, $n = 3$, $P < 0.001$), which are antioxidant enzymes regulated by Nrf2 (values are expressed as fold increase with respect to cells incubated in normoxia; Figure 5D).

Effect of hypoxia on cellular proliferation and viability

Bearing in mind that the oxidative status of PSC was affected under hypoxia, we were interested in evaluating cell viability and proliferation in these conditions.

In a first instance, we tested the effect of hypoxia on key regulators of cell cycle. Cells were subjected to normoxia or hypoxia during 4 h in the presence of Ca^{2+} in the extracellular medium (1 mM) and the expression of cyclin A and cyclin D was studied. The expression of both proteins varied inversely under hypoxia; the expression of cyclin A decreased (in %, 63.90 ± 9.54, $n = 4$; $P < 0.01$), whereas cyclin D expression was increased (in %, 150.80 ± 23.94, $n = 4$; $P < 0.01$) with respect to cells incubated in normoxia (in %, 100.00 ± 0.01, $n = 4$; Figure 6A to D).

Figure 4 | Effect of hypoxia on SOD. (**A**) PSC were incubated during 1 h under hypoxia. Bars show the mean change of SOD activity expressed in percentage ± SEM with respect to non-treated cells (cells in normoxia). (**B** and **C**) Western blotting with specific antibodies was carried out to detect the expression of SOD1 and SOD2. The levels of actin were employed as controls to ensure equal loading of proteins. The arrows show the molecular weight corresponding to the analyzed band in each blot. (**D** and **E**) The graphs show the quantification of protein expression. The experiments were carried out in the presence of Ca^{2+} (1 mM) in the extracellular medium. A horizontal dashed line represents the value observed in non-treated cells. Values are the mean ± SEM of normalized values expressed as percent with respect to non-treated cells (incubated in normoxia). Results are representative of four separate experiments (Nmx, normoxia; Hpx, hypoxia; $^{*}P < 0.05$; $^{***}P < 0.001$ vs. cells incubated in normoxia).

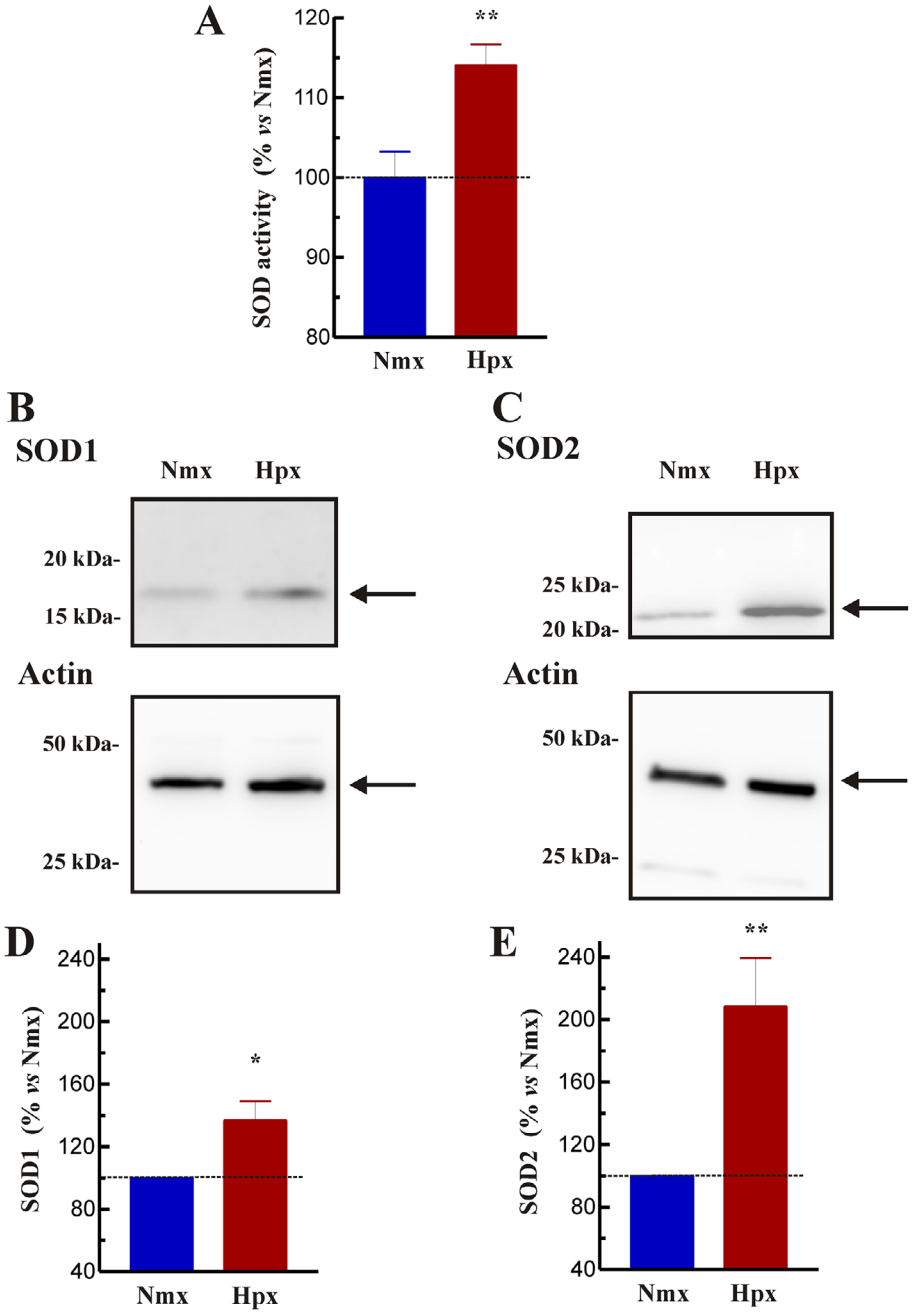


Figure 5 | Effect of hypoxia on glutathione and on Nrf2-regulated enzymes. (**A**) PSC were incubated during 4 h under normoxia or hypoxia and the levels of GSH and GSSG were determined and compared with those found in cells incubated in normal conditions. The bars show the mean increase in GSH/GSSG ratio expressed in percentage ± SEM with respect to non-treated cells (incubated in normoxia). (**B**) Western blotting analysis with a specific antibody was carried out to detect the phosphorylation of Nrf2 in cells subjected to normoxia or hypoxia during 4 h. The levels of actin were employed as controls to ensure equal loading of proteins. The arrows show the molecular weight corresponding to the analyzed band in each blot. (**C**) The graphs show the quantification of protein phosphorylation. The experiments were carried out in the presence of Ca^{2+} (1 mM) in the extracellular medium. A horizontal dashed line represents the value observed in non-treated cells (cells in normoxia). Values are the mean ± SEM. of normalized values expressed as percent with respect to non-treated cells. (**D**) Quantitative reverse transcription-polymerase chain reaction (RT-qPCR) analysis revealed statistically significant increases in the levels of Nrf2-dependent antioxidant enzymes cysteine ligase-catalytic subunit (*GCIc*), catalase (*CAT*), NAD(P)H quinone oxidoreductase 1 (*NQO1*) and heme-oxygenase-1 (*HO-1*) in cells incubated in hypoxia. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) mRNA was used for normalization. A horizontal dashed line represents the value observed in non-treated cells (cells in normoxia). Data are expressed as the mean ± SEM of the change relative to non-treated cells (incubated in normoxia). Three to four different cellular preparations were used (Nmx, normoxia; Hpx, hypoxia; **P* < 0.05; ***P* < 0.01; ****P* < 0.001 vs. cells incubated in normoxia).

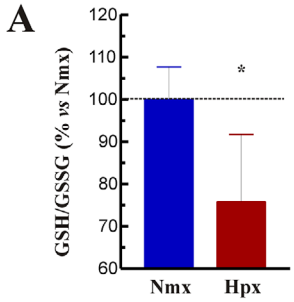
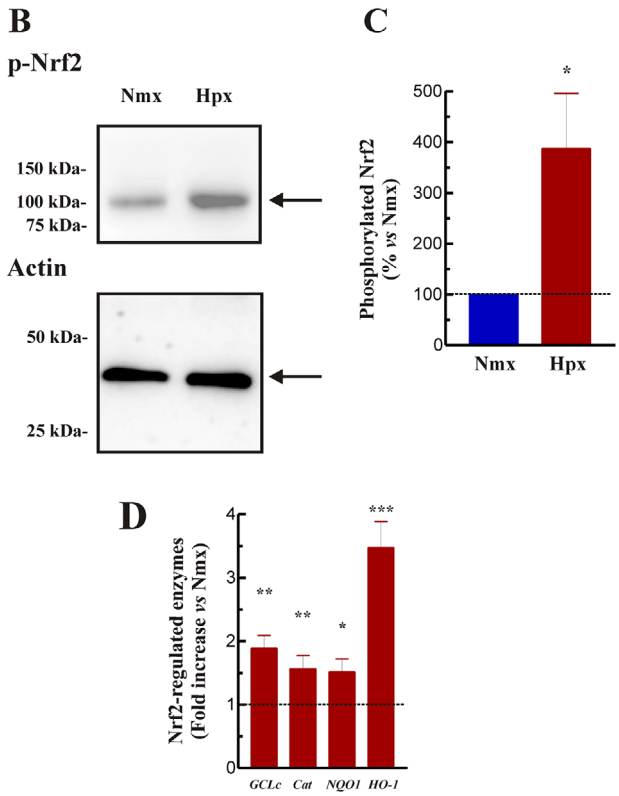


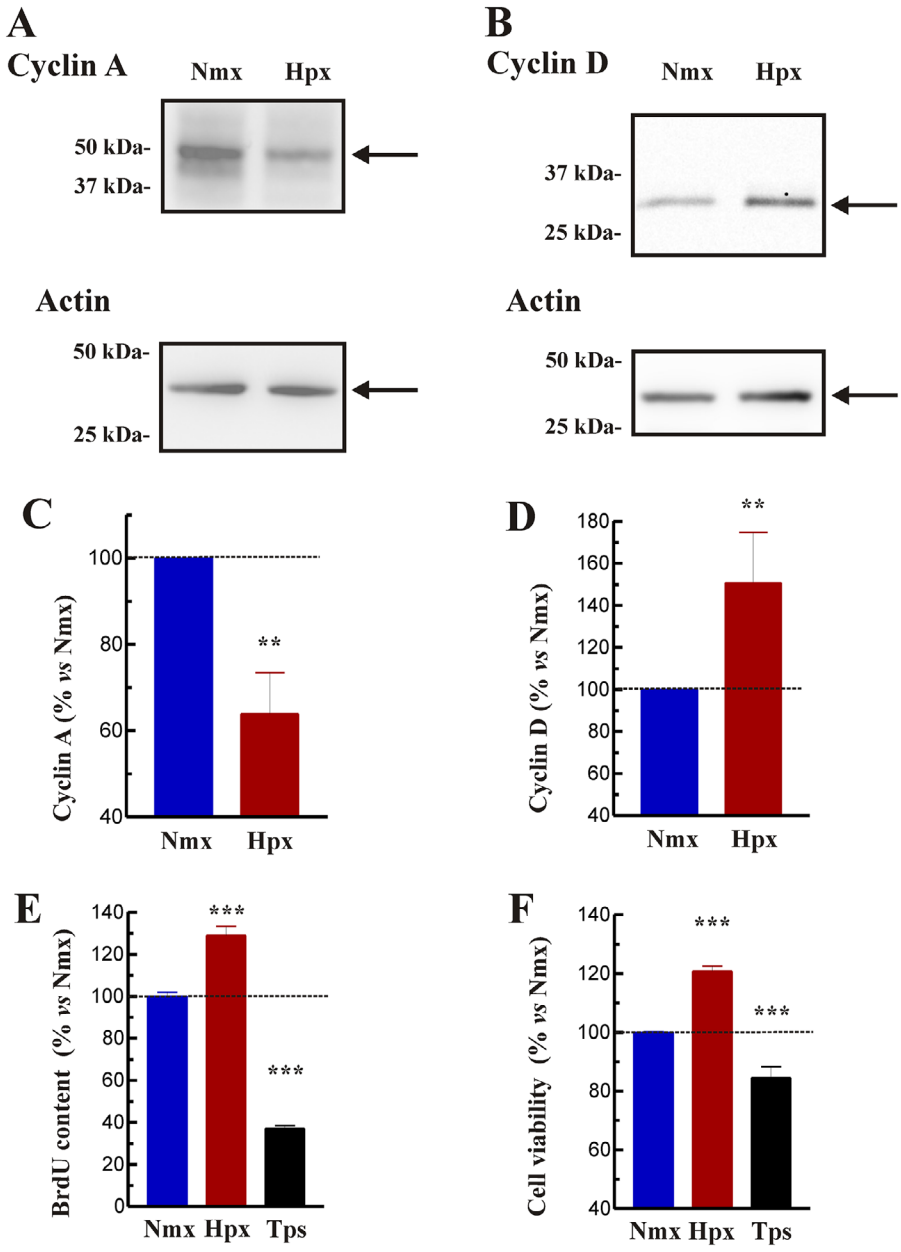
Figure 5 | *Continued*



We next evaluated cell proliferation by 5-bromo-2-deoxyuridine (BrdU) assay kit. BrdU incorporates into the newly synthesized DNA and thus serves as an indicator of cell proliferation. In this set of experiments, cells were incubated during 48 h in normoxia or hypoxia in the presence of Ca^{2+} in the extracellular medium (1 mM) and proliferation was analyzed and compared. Cells that had been incubated in hypoxia exhibited higher BrdU content, that is, higher proliferation, compared with those incubated in normoxia (129.00 ± 4.22 vs. 100.00 ± 0.01, $n = 4$; $P < 0.001$). Separate batches of cells were treated with 1 μM Tps, which served as control for cell death (Wu et al., 2019). Treatment of cells with Tps induced a decrease in BrdU content (in %, 37.18 ± 1.26, $n = 4$; $P < 0.001$; Figure 6E).

In another set of experiments, PSC were incubated during 48 h in hypoxic conditions, in the presence of Ca^{2+} in the extracellular medium (1 mM), and viability was estimated. Cell viability increased when PSC were incubated in hypoxia, compared with the values obtained in cells incubated in normoxia (in %, 120.80 ± 1.72, $P < 0.001$ vs. 100.00 ± 0.01, $n = 3$; $P < 0.001$; Figure 6F). Separate batches of cells were treated with 1 μM Tps. In the presence of Tps,

Figure 6 | Effect of hypoxia on cell viability and proliferation. (**A** and **B**) Western blotting analysis with specific antibodies was carried out to detect the expression of cyclin A or cyclin D in cells subjected to normoxia or hypoxia during 4 h. The arrows show the molecular weight corresponding to the analyzed band in each blot. (**C** and **D**) The levels of actin were employed as controls to ensure equal loading of proteins. The graphs show the quantification of protein phosphorylation. Values are the mean ± SEM of normalized values expressed as percent with respect to non-treated cells (incubated in normoxia). (**E**) Cells were incubated during 48 h under hypoxia and BrdU incorporation to DNA of dividing cells was compared with that of cells incubated in normoxia. Thapsigarging (Tps, 1 μM) was used as control of cell death. (**F**) Cells were incubated during 48 h under hypoxia and viability was compared with that of cells incubated in normoxia. Tps (1 μM) was used as control of cell death. In the graphs, a dashed line represents the viability of non-treated cells (incubated in normoxia). The experiments were carried out in the presence of Ca^{2+} (1 mM) in the extracellular medium. Data are representative of three to four separate experiments (Nmx, normoxia; Hpx, hypoxia; Tps, thapsigargin; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. cells incubated in normoxia).



a decrease in cell viability was observed (in %, 84.54 ± 3.77, $n = 3$; $P < 0.001$; Figure 6F).

Altogether, these observations suggest that viability and proliferation of PSC are increased under hypoxia.

Effect of hypoxia on mitogen-activated protein kinases activation

In view of the results obtained above it was now of interest to evaluate the effect of hypoxia on mitogen-activated protein kinases (MAPKs) activation. MAPKs pathway plays an important role in stress response, cell differentiation, cell survival, and tumourigenesis (Capolongo et al., 2019).

In order to study whether MAPKs were involved in the responses of PSC to hypoxia, cells were incubated during 4 h under normoxia or hypoxia. The experiments were carried out in the presence of Ca^{2+} (1 mM) in the extracellular medium. Primary antibodies against the phosphorylated forms of c-Jun NH_2-terminal kinase (JNK), p44/42, or p38 MAPK were obtained from Cell Signaling Technology (C-Viral, Madrid, Spain). Secondary HRP-conjugate antibodies were purchased from Thermo Fisher. Analysis of cell lysates revealed an increase in the phosphorylation of JNK in cells subjected to hypoxia (in %, 120.10 ± 7.23 vs. 100.00 ± 0.01, $n = 5$; $P < 0.05$), whereas the phosphorylated state of p44/42 and p38 was lower compared with non-treated cells (incubated in normoxia) (in %, 67.20 ± 9.48, $n = 4$ and 62.40 ± 10.25, $n = 4$, respectively, vs. non-treated cells; $P < 0.01$) (Figure 7).

In order to evaluate the involvement of JNK in the increase of viability that we observed in cells incubated under hypoxia, we carried out a series of experiments in which PSC were incubated for 48 h under hypoxia and in the presence of the JNK inhibitor SP600125 (10 μM; Tocris, Biogen Científica, Madrid, Spain). Separate batches of cells were incubated in hypoxia but in the absence of the SP600125. Inhibition of JNK significantly diminished viability of cells subjected to hypoxia compared with that of cells incubated in the absence of the inhibitor (in %, 85.69 ± 2.76 vs. 100.00 ± 0.16, $n = 3$; $P < 0.01$; Figure 8). Again, Tps (1 μM) decreased cell viability, compared with that noted in cells incubated in its absence (in %, 80.19 ± 4.65 vs. 100.00 ± 0.16, $n = 3$; $P < 0.001$; Figure 8).

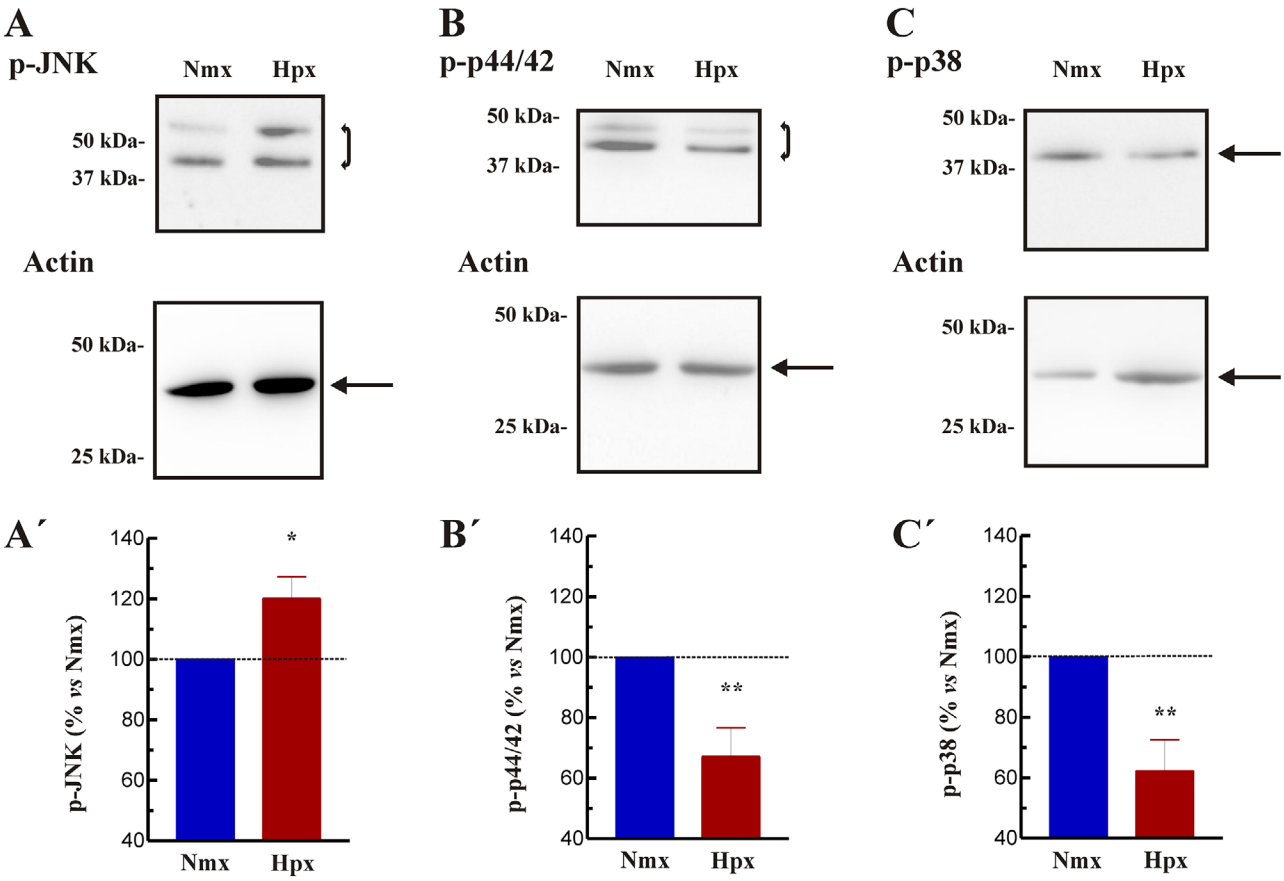
Effect of hypoxia on matrix metalloproteinases expression

As mentioned before in this manuscript, PSC participate actively in the development of fibrosis. This is a process in which secretion and accumulation of extracellular matrix components occurs (Sherman, 2018). Matrix metalloproteinases (MMPs) constitute a family of proteins that convey enzymatic activity and are involved in angiogenesis, invasiveness, and metastasis, which are major steps in cancer progression. The levels of one or several members of this family of proteins are increased in pancreatitis and tumours (Hadler-Olsen et al., 2013). In our study, we decided to evaluate the effect of hypoxia on the expression of two members of this family of proteins: MMP-2 and MMP-3. Increases in the expression of MMP-2 have been related with higher proliferation rate and migration ability of PSC (Tang et al., 2018). MMP-3 is secreted by PSC and contributes to invasion of cancer cells (Ikenaga et al., 2010). PSC were incubated during 4 h under hypoxia and in the presence of extracellular Ca^{2+} (1 mM). For comparisons, separate batches of cells were incubated in normoxic conditions. Western blot analysis employing specific antibodies for these proteins were carried out in samples of PSC subjected to the mentioned treatments (antibodies were purchased from Abcam plc, Cambridge, UK). We detected statistically significant increases in the expression of both MMP-2 (in %, 163.40 ± 24.89, $n = 4$, $P < 0.05$) and MMP-3 (in %, 336.80 ± 73.20, $n = 4$, $P < 0.01$) compared with cells incubated in normoxia (in %, 100.00 ± 0.01, $n = 4$; Figure 9).

Effect of hypoxia on cell migration

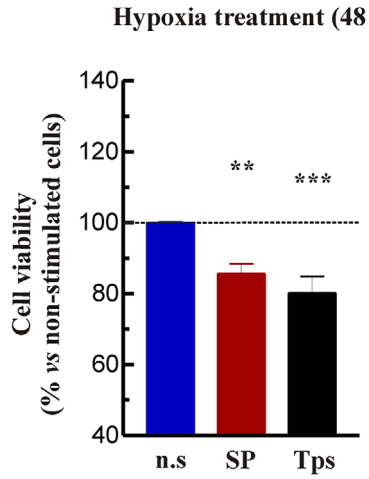
We finally analyzed whether PSC maintained the ability of migration under hypoxia. For this purpose, PSC were grown until confluence and then a single wound was created. Thereafter, cells were incubated under normoxia, hypoxia or Tps (1 μM). Images were taken to evaluate migration and protrusion of cells into the wound area at day 0 (just after wound) at 12, 24, 36 and at 48 h of culture. The experiments were carried out in the presence of Ca^{2+} (1 mM) in the extracellular medium. Cells, either in normoxia or subjected to hypoxia, could be observed growing within the wound area at 12 h of culture (Figure 10). Protrusion of cells into the wound was faster in cells incubated under hypoxia, compared with that observed

Figure 7 | Analysis of JNK, p44/42 and p38 MAPK phosphorylation in response to hypoxia. Cells were incubated during 4 h in normoxia or in hypoxia. Then, cell lysates were processed for Western blotting analysis with phosphospecific antibodies. (**A–C**) Representative blots showing the phosphorylation state of JNK, p44/42 and p38, respectively. To ensure equal loading of proteins, the levels of actin were employed as controls. The arrows show the molecular weight corresponding to the analyzed band in each blot. (**A′–C′**) The graphs show the quantification of protein phosphorylation. The experiments were carried out in the presence of Ca^{2+} (1 mM) in the extracellular medium. A horizontal dashed line represents the value observed in non-treated cells (cells in normoxia). Values show the mean ± SEM of normalized values expressed as percentage of phosphorylation in non-treated cells (incubated in normoxia). Data are representative of four to five separate experiments (Nmx, normoxia; Hpx, hypoxia; *$P < 0.05$; **$P < 0.01$ vs. cells incubated in normoxia).



in cells incubated in normoxia. At 36 h of incubation under hypoxia, areas could be observed containing cells that were growing and connecting both sides of the wound. Nevertheless, this could not be observed in cells grown under normoxia. After 48 h in culture, the wound was mostly covered by cells growing in the hypoxia treatment, whereas areas free of cells in the wound could still be observed in the normoxia treatment (areas marked by arrows). In the case of cells treated with Tps, the wound was not covered by cells to the extent noted in the experiments performed in the absence of Tps; moreover, dying cells could be observed in the supernatant, especially at 48 h of incubation. The number of cells growing was higher under hypoxia (26.06 ± 1.73; 49.42 ± 2.00; 58.85 ± 2.18 and 71.47 ± 2.41 for 12, 24, 36 and 48 h, respectively; $P < 0.001$) in comparison with that noted in normoxia (2.86 ± 0.30; 8.59 ± 0.76; 15.59 ± 0.92 and 32.67 ± 1.67 for 12, 24, 36 and 48 h, respectively) along the incubation time (Figure 11). On the contrary, the wound could be observed majorly free of cells in the case of those that had been

Figure 8 | Effect of JNK-inhibition on the viability of PSC subjected to hypoxia. Cells were incubated during 48 h under hypoxia in the presence of the JNK-inhibitor SP600125 (10 μM) or in the presence of thapsigargin (1 μM) and viability was compared with that of cells incubated in the absence of drugs. The experiments were carried out in the presence of Ca^{2+} (1 mM) in the extracellular medium. A horizontal dashed line represents the viability of cells incubated in hypoxia and in the absence of drugs. Data are representative of three separate experiments (n.s., non-stimulated; Nmx, normoxia; Hpx, hypoxia; SP, SP600125; Tps, thapsigargin; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. cells incubated in the absence of drugs).



incubated in the presence of Tps (3.167 ± 0.66; 7.86 ± 1.34; 7.15 ± 1.28 and 9.57 ± 1.55 for 12, 24, 36 and 48 h, respectively). These observations suggest that proliferation and migration of PSC is increased under hypoxia.

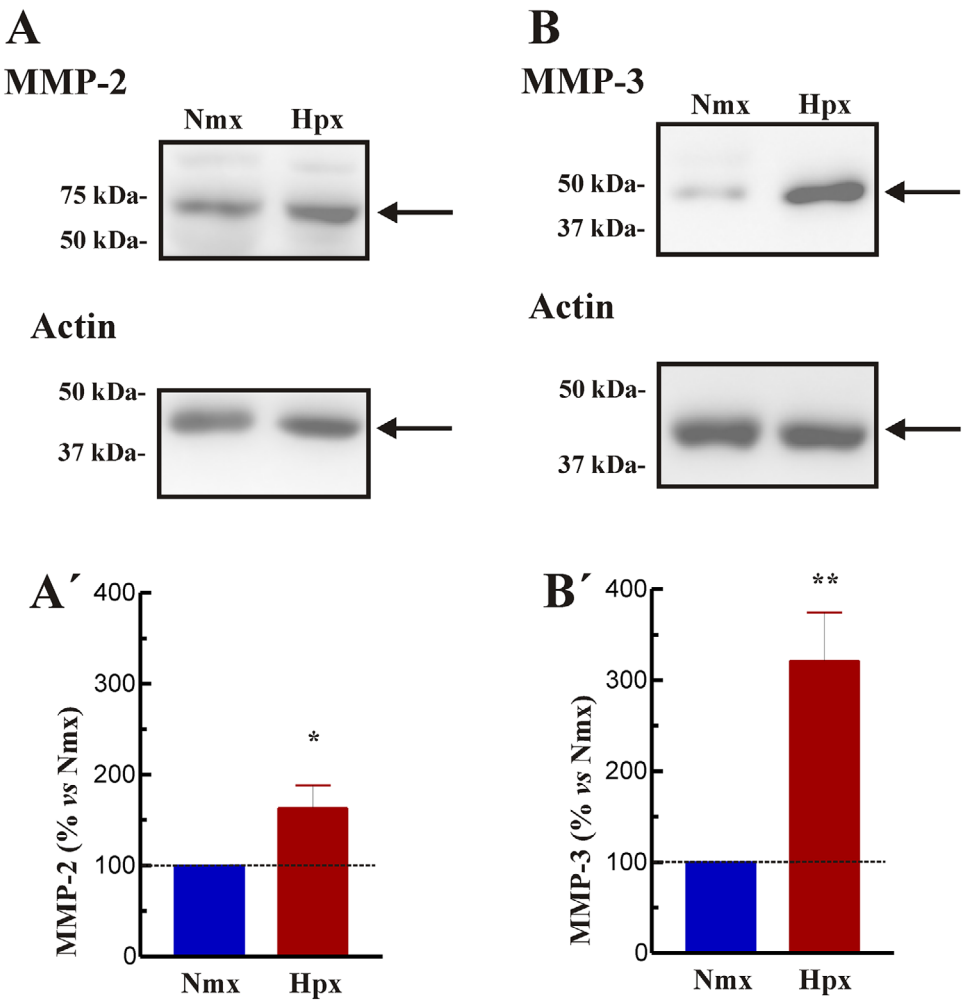
Discussion

PSC are actively involved in the growth and progression of pancreatic cancer. This is due to specific interactions that are established among them and cancer cells (Xiao et al., 2019; Yu et al., 2019). PSC contribute decisively to the progressive fibrosis that accompanies the disease (Mahadevan et al., 2007; Pothula et al., 2016). Fibrotic tissue growing within the tumour has been pointed out as a major target in the treatment of cancer (McCarroll et al., 2014). Another condition that develops in the growing cancerous tissue is hypoxia. This condition is caused by the rapid and uncontrolled proliferation of cells within the malignant mass of tissue. All cells included in the mass will be subjected to a low availability of O_2 and must undergo adaptation. This allows cells to survive and to proliferate, determining cancer growth. It is well known that cancer cells adapt to hypoxic conditions present in tumours (Erkan et al., 2016). However, little is known about the responses of PSC to low O_2 availability.

Our results show that hypoxia induced an increase in $[Ca^{2+}]_i$ in PSC. Former works showed that PSC exhibit Ca^{2+} signals when stimulated with certain agonists (Gryshchenko et al., 2016; Estaras et al., 2019b). This observation points out that Ca^{2+} may be a major signalling molecule in this cell type. Moreover, contribution of Ca^{2+} to proliferation of PSC under hypoxia has been suggested (Nielsen et al., 2017). In their work, Nielsen et al. showed that PSC expressed TRPC6 channels that allowed Ca^{2+} entry from the extracellular space, which could then contribute to the regulation of PSC function. In our study, we show that hypoxia induces the release of Ca^{2+} from intracellular stores. Because the increase in $[Ca^{2+}]_i$ was also observed in the absence of Ca^{2+} in the extracellular medium, Ca^{2+} mobilization must derive from an intracellular source, most probably the endoplasmic reticulum. In this line, the additional incubation of cells subjected to hypoxia with the SERCA inhibitor thapsigargin evoked a rather small Ca^{2+} response, which was smaller than that induced by the inhibitor when it is applied alone (Martinez-Morcillo et al., 2019). This fact reflects that the intracellular Ca^{2+} stores had been depleted by the treatment of cells under hypoxia and, moreover, suggests that the source for Ca^{2+} mobilization under hypoxia is the ER. However, we cannot exclude the contribution of extracellular Ca^{2+} to hypoxia-induced changes in $[Ca^{2+}]_i$. Therefore, Ca^{2+} signals (which can be the result of Ca^{2+} release from intracellular stores, together with the contribution of Ca^{2+} entry from the extracellular space) represent a major mechanism that might be involved in the responses of PSC to hypoxia.

Cells also exhibited an increase in $[Ca^{2+}]_m$, whereas Ψ_m was decreased. Former studies have revealed that mitochondria undergo changes in their physiology that follow changes in $[Ca^{2+}]_i$ (Gonzalez et al., 2003). The fact that hypoxia evoked changes in Ca^{2+} and in mitochondrial parameters suggests that PSC might undergo a shift in

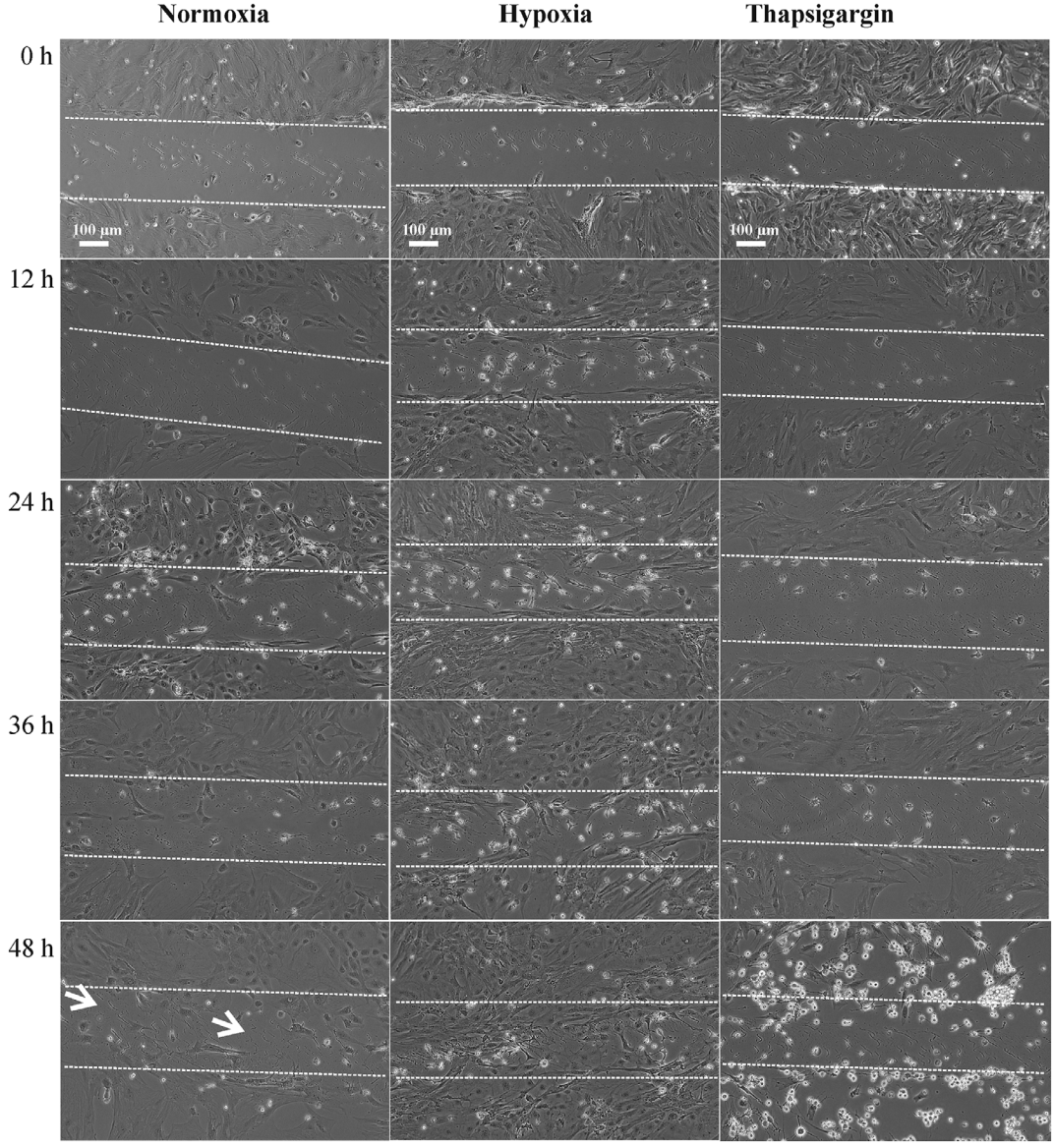
Figure 9 | Effect of hypoxia on MMP-2 and MMP-3 expression. Cells were incubated in normoxia or in hypoxia during 4 h. Then, cell lysates were processed for Western blotting analysis with antibodies. (**A** and **B**) Representative blots showing the expression of MMP-2 and MMP-3, respectively. To ensure equal loading of proteins, the levels of actin were employed as controls. The arrows show the molecular weight corresponding to the analyzed band in each blot. (**A′** and **B′**) The graphs show the quantification of protein expression. The experiments were carried out in the presence of Ca^{2+} (1 mM) in the extracellular medium. A horizontal dashed line represents the value observed in cells in normoxia. Values show the mean ± SEM of normalized values expressed as percentage of phosphorylation in incubated in normoxia. Data are representative of three to four separate experiments (Nmx, normoxia; Hpx, hypoxia; $*P < 0.05$; $**P < 0.01$ vs. cells incubated in normoxia).



their physiology in order to adapt to the low O_2 availability.

Development of pro-oxidative conditions inside the tumour mass has been reported, to which cancer cells adapt to survive (Policastro et al., 2013). When PSC were subjected to hypoxia a pro-oxidant status was observed. This was suggested by the increase in the oxidation of proteins and lipids and the slight increase in the generation of ROS within the mitochondria that we detected. However, no increase in cytosolic ROS production was noted. A decrease in the GSH/GSSG ratio was also observed. Moreover, the decrease in the availability of GSH also is an index of a pro-oxidative environment that could be created by hypoxia. Furthermore, a significant decrease of TAC was observed under hypoxic conditions. This could be a consequence of GSH consumption. Therefore, our results show that PSC

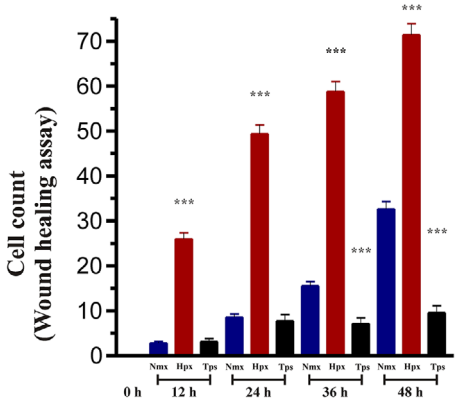
Figure 10 | Migration study of cells subjected to hypoxia. A single wound was created and cells were then incubated under normoxia, hypoxia or Tps (1 μM). Images were taken at 0, 12, 36, 24 and 48 h of culture. Migration and protrusion of cells into the wound area were monitored. The experiments were carried out in the presence of Ca^{2+} (1 mM) in the extracellular medium. The horizontal dashed lines mark the edges of the wound. The arrows indicate some areas free of cells. The images are representative of three different preparations (the horizontal bar indicates 100 μm).



undergo a pro-oxidant condition under hypoxia. As a consequence, cells might react to exhibit an increase in the antioxidant response in order to survive.

In this line, SOD activity was augmented. This could be explained by the increase in the expression of SOD1 and SOD2 that we have observed. Although SOD1 is present in the cytosol, SOD2 has a mitochondrial localization (Milani et al., 2011; Zou et al., 2017). Because of the low availability of O_2, mitochondrial respiration might be affected. This could induce the increase in SOD2 expression that we have noted. However, this effect might not be effective enough and could explain the mild increase in ROS generation that we have observed. Indeed, it has been

Figure 11 | Cell count in the wound healing assay. Cells that appeared into the wound area (considered as index of migration and protrusion) at different time periods (0, 12, 24, 36 and at 48 h of culture) were counted and is shown by the bars in the graphic. The experiments were carried out in the presence of Ca^{2+} (1 mM) in the extracellular medium. Data are representative of three different preparations (Nmx, normoxia; Hpx, hypoxia; Tps, thapsigargin; ***$P < 0.001$ vs. cells incubated in normoxia).



hypothesized that the major site of intracellular superoxide generation during ischemia-reperfusion is the cytoplasm (Sasaki et al., 2011). Therefore, it could be possible that the antioxidant defences activated during hypoxia are specially directed towards the cytosol to diminish oxidative stress to the compartment. This could explain the increase in the expression of SOD1 that we have observed. We also detected an increase in the phosphorylation of Nrf2 and in the expression of *GCLc*, *CAT*, *NQ01* and *HO-1*, which are antioxidant enzymes regulated by this transcription factor.

Altogether, it seems feasible that PSC exhibit an activation of major antioxidant mechanisms of defence to counteract the pro-oxidative status that they undergo when subjected to hypoxia. These observations could explain the absence of detection of significant ROS production in our experimental conditions.

Proliferation of PSC subjected to hypoxia was increased. Our results show that PSC exhibited an increase in BrdU incorporation into DNA, an index of cell proliferation. Additional evidence for an increase of PSC proliferation under hypoxia are shown by the increase in the expression of cyclin D that we observed. This protein is a key regulator of the cell cycle and division (Roskoski, 2016; Thakur et al., 2018). Another major regulator of progression through the cell cycle is cyclin A (Wang et al., 2018). Conversely to the changes observed in cyclin D, we observed a decrease in the expression of cyclin A. Mateo et al. (2010) suggested that cyclin A must be degraded at prometaphase in order to allow mitosis progression. Accordingly, it has been shown that the S phase arrest is accompanied by the upregulation of cyclin A (Xu et al., 2017). Therefore, PSC subjected to hypoxia exhibit changes in the expression of regulators of the cell cycle that allow them progress through the cell cycle and divide. In relation with this observation, we detected an increase of cell viability compared with cells incubated in normoxia.

MMPs consist of a multigene family of extracellular matrix (ECM) remodelling endopeptidases implicated in pathological processes, such as carcinogenesis. In this regard, their activity plays a pivotal role in tumour growth and the multistep processes of invasion and metastasis (Gialeli et al., 2011; Hadler-Olsen et al., 2013). Moreover, changes in the expression of these MMPs are involved in the remodelling of extracellular matrix during fibrosis (Popov et al., 2006).

Our results show that PSC subjected to hypoxia exhibit an increase in the expression of MMP-2 and MMP-3. Release of molecules by PSC subjected to hypoxia, such as cytokines or lactate, which can signal to neighbouring cells, was shown by Nielsen et al. (2017). Moreover, PSC migration capability depends on the release of signalling factors by PSC. Altogether, accumulation of substances released by activated PSC can lead to further activation of PSC and other surrounding cells. Provided that these proteins play a critical role in fibrosis and in many phases of cancer progression, our results show evidence in favour of activation of PSC by hypoxia and of secretion of components that can influence in the remodelling of the extracellular matrix. The latter could be involved in the progression of abnormal tissue within the pancreas under hypoxia.

We also noted changes in the phosphorylation state of c-Jun N-terminal kinase (JNK), which was increased, and of p44/42 and p38, which were decreased. Parallel to other MAPKs, the JNK pathway is important for proliferation. Phosphorylation of JNK contributes to proliferation of lung adenocarcinoma cells, potentiates hepatocellular carcinoma and

colorectal cancer progression and metastases (Xiao et al., 2016; Chan et al., 2017; Karatug Kacar et al., 2019; Cao et al., 2020). Furthermore, activation of JNK was related with increased proliferation and growth of human pancreatic cancer (Choi et al., 2018). Our observations suggest that JNK signalling is majorly involved in the adaptation and proliferation of PSC under hypoxia. Finally, PSC subjected to hypoxia maintained migration ability. All these results are in agreement with former observations which suggest that, when stressed, PSC change to an activated status that leads to an increase in their proliferation (Rebours et al., 2013; Ferdek and Jakubowska, 2017).

Finally, migration of activated PSC towards the surroundings is a major occurrence in the development of fibrosis. Our results show that PSC maintain their ability to migrate under hypoxia. Our observations are in agreement with those shown by Nielsen et al. (2017) who showed augmented migration of PSC subjected to hypoxia. This might be supported by the increase in proliferation, the changes in cyclin expression and the differential activation of MAPKs that we have observed.

In summary, here we present evidence that support changes in the physiology of PSC under hypoxic conditions. Cells exhibit an increase in the antioxidant mechanisms of defence against the pro-oxidative conditions induced by hypoxia. Far from dying, under hypoxia PSC react with an increase in cell proliferation and migration capabilities. Major regulators of the cell cycle and proliferation are involved. Altogether, our observations point towards the typical features occurring in pancreatic cancer microenvironment. Bearing in mind the close interrelationship existing between activated PSC and cancer cells, our observations shed light on the mechanisms by which PSC adapt to hypoxic conditions and that could account for how this cell type might hypothetically contribute to the progression of pancreatic cancer.

Materials and methods

Preparation of cultures of PSC

Cultures of PSC were prepared following previously used methods. With this procedure, cultures of activated PSC can be prepared (Estaras et al., 2019b; Santofimia-Castaño et al., 2015). Pancreatic tissues were obtained from *Wistar* rats pups (3–5 days after birth). Animals employed in the study were supplied by the animal house of the University of Extremadura (Caceres, Spain). Animals handling and experimental protocols were approved by the Ethical Committee for Animal Research of the University of Extremadura (reference 57/2016) and by the Institutional Committee of the Junta de Extremadura (reference 20160915).

Briefly, the pancreas was subjected to enzymatic digestion with a physiological buffer containing: 130 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 1.2 mM KH_2PO_4, 10 mM glucose, 10 mM HEPES, 0.01% trypsin inhibitor (soybean) and 0.2% bovine serum albumin (pH = 7.4 adjusted with NaOH) that was supplemented with 30 units/m/ collagenase CLSPA from Worthington (Labclinics, Madrid, Spain). After centrifugation (30 × *g* for 5 min at 4 °C) to remove the supernatant with the enzyme, culture medium was added to the pellet. Culture medium consisted of medium 199 supplemented with 4% horse serum, 10% FBS (Invitrogen, Fisher Scientific), a mixture of antibiotics (0.1 mg/ml streptomycin, 100 IU penicillin) (BioWhittaker, Lonza, Basel, Switzerland) and 1 mM $NaHCO_3$. Next, mechanical dissociation of the cells was carried out by gently pipetting the cell suspension through tips of decreasing diameter. After centrifugation, cells were resuspended in culture medium. Finally, cells were seeded on different substrates depending on the studies to be carried out (round glass coverslips, 100 mm diameter Petri dishes, or multiwell polystyrene plates) (Thermo, Fisher Scientific) and grown in a humidified incubator at 37°C and 5% CO_2. The experiments were carried out employing batches of cells obtained from different preparations.

Induction of hypoxia

Cells, growing on the preferred substrate, were incubated in a low O_2 (1%) atmosphere employing an incubator chamber (Okolab; Izasa Scientific, Madrid, Spain). Temperature (37 °C), humidity (95%) and air atmosphere (content of $O_2/CO_2/N_2$) were controlled using dedicated software. For fluorescence imaging, this chamber was placed on the stage of an epifluorescence inverted microscope (Nikon Ts2; Izasa Scientific).

Determination of changes in $[Ca^{2+}]_i$

Cells used in these studies had been seeded on independent glass coverslips. For monitoring of changes of $[Ca^{2+}]_i$, PSC were loaded with fura-2 (Invitrogen, Fisher Scientific) employing previous methods (Santofimia-Castaño et al., 2015). Loading was carried out by incubation of cells in the presence of 4 μM of fura-2 acetoxymethyl ester. Some tests were performed in the absence of extracellular Ca^{2+}. In this case, the medium was prepared with no Ca^{2+} added and contained 0.5 mM of the Ca^{2+} chelator EGTA (Sigma Chemicals, Madrid, Spain). Results are shown as the absolute values of fluorescence emitted at the selected excitation light, normalized to basal (pre-stimulation) fluorescence. For comparisons, the total Ca^{2+} mobilization was estimated as the integral of the rise in $[Ca^{2+}]_i$ over the basal values for 4 min after induction of hypoxia.

Determination of changes in $[Ca^{2+}]_m$ and Ψ_m

Changes in $[Ca^{2+}]_m$ were followed in rhod-2-loaded cells (Invitrogen, Fisher Scientific). Cells were loaded by incubation with 8 μM of this dye following previously described methods (González et al., 2000).

Ψ_m was recorded in PSC loaded with TMRM (Invitrogen, Fisher Scientific). Cells were loaded by incubation in the presence

of 100 nM of the dye as shown previously (Santofimia-Castaño et al., 2014).

Coverslips with dye-loaded cells were mounted into an experimental perfusion chamber and subjected to hypoxia using the above-mentioned incubation chamber, which was placed on the stage of an epifluorescence inverted microscope (Nikon Ts2; Izasa Scientific). The microscope was equipped with led light for each fluorophore. Fluorescence images were captured employing a digital camera and analyzed with *image-J* software (http://imagej.nih.gov/ij/).

Results are expressed as the absolute values of fluorescence emitted at the selected excitation light, normalized to basal (prestimulation) fluorescence.

Determination of ROS

ROS generation was monitored using previously described methods (Estaras et al., 2019a). For this purpose, cells were detached and loaded with the fluorescent probes CM-H_2DCFDA (10 μM), for cytosolic ROS detection, or with MitoSOX^{TM} Red (2.5 μM) for mitochondrial-ROS detection (Gonzalez and Salido, 2016) (dyes were purchased from Invitrogen, Fisher Scientific). For detection of changes in the redox state, cells were excited at 530 nm and fluorescence emitted was detected at 590 nm for CM-H_2DCFDA, whereas for cells loaded with MitoSOX^{TM} red excitation at 510 nm with detection at 580 were employed. A spectrofluorimeter was used to monitor fluorescence (VariosKan Lux 3020–205, Thermo Sci., Vantaa, Finland).

Results show the mean increase of fluorescence expressed in percentage ± SEM (*n*) with respect to non-treated cells, where *n* is the number of independent experiments.

Determination of protein carbonyls (allysine)

The methods described by Villaverde et al. (2014) were used for detection of protein carbonyls. Briefly, cells were incubated during 1 h with stimuli and, thereafter, were lysed for analysis. Five hundred microlitre of each sample was treated with cold 10% trichloroacetic acid (TCA) solution. Each sample was vortexed and centrifuged (600 × *g* for 5 min at 4°C) and the supernatants were removed. Next, the pellets were incubated in the following solutions: 0.5 ml 250 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer pH 6.0 containing 1 mM diethylenetriaminepentaacetic acid (DTPA), 0.5 ml 50 mM ABA in 250 mM MES buffer pH 6.0 and 0.25 ml 100 mM $NaBH_3CN$ in 250 mM MES buffer pH 6.0. Samples were incubated at 37°C for 90 min and then a cold 50% TCA solution was added. Samples were next centrifuged at 1200 × *g* for 10 min. The pellet was treated with 6 M HCl and kept in an oven at 110°C for 18 h until completion of hydrolysis. Next, the samples were dried in an evaporator. The residue obtained was reconstituted with 200 μl of milliQ water and filtered for high-performance liquid chromatography (HPLC) analysis (Shimadzu 'Prominence' HPLC apparatus, Shimadzu Corporation, Japan). Reagents were purchased from Sigma Chemicals. The namomole of allysine per milligram of protein was calculated. Results are expressed as percentage ± SEM (*n*) with respect to non-stimulated cells, where *n* is the number of independent experiments.

Analysis of TBARS

Malondialdehyde (MDA) and other TBARS were measured. In brief, cells were incubated during 4 h under normoxia or hypoxia and were then lysed for analysis. Thiobarbituric acid (0.02 M; 500 μl) and trichloroacetic acid (10%; 500 μl) were added to 200 μl of each sample. Samples were next incubated during 20 min at 90°C and then centrifuged (5 min, 600 × *g*). The absorbance of supernatant was measured at 532 nm. The major reagents needed were purchased from Sigma Chemicals. The milligram of TBARS per litre of sample was calculated. Results are expressed as percentage ± SEM (*n*) with respect to non-treated cells, where *n* is the number of independent experiments.

Determination of glutathione levels

The changes in the levels of reduced (GSH) and oxidized (GSSG) glutathione were determined using methods described previously (Estaras et al., 2019b). Cells were incubated during 4 h with the different stimuli assayed. A spectrofluorimeter (Tecan Infinite M200, Grödig, Austria) was employed to detect GSH or GSSG at 350 nm/420 nm (excitation/emission), respectively. For quantification, standard curves of GSH and GSSG were used. Normalization was carried out based on the total protein concentration in each sample (Bradford, 1976). A standard curve was prepared using bovine serum albumin. The major reagents needed were purchased from Sigma Chemicals, whereas Bradford's reagent was obtained from Bio-Rad (Madrid, Spain).The experiments were carried out employing batches of cells obtained from different preparations. Data show the mean increase in GSH/GSSG ratio expressed in percentage ± SEM (*n*) with respect to non-treated cells, where *n* is the number of independent experiments.

Determination of SOD activity

SOD activity was determined using a commercially available kit from BioVision (Deltaclon S.L., Madrid, Spain). Stimuli were added to the cells and were incubated during 1 h. Thereafter SOD activity was determined following the manufacturer's directions. The sensitive SOD assay kit utilizes WST-1 that produces a water-soluble formazan dye upon reduction with superoxide anion. Absorbance at 450 nm of the samples was measured employing a plate reader (VariosKan Lux 3020–205, Thermo Sci., Vantaa, Finland). Results show the mean change of absorbance expressed in percentage ± SEM (*n*) with respect to non-treated cells, where *n* is the number of independent experiments.

Determination of TAC

TAC was determined using a commercially available kit from BioVision (Deltaclon S.L., Madrid, Spain), following manufacturer's directions. Cells were treated with the different stimuli during 1 h. Next, absorbance of each sample was measured at 570 nm employing a plate reader (CLARIOstar Plus, BMG Labtech., C-Viral, Madrid, Spain). Results show the mean change of absorbance expressed in percentage ± SEM (*n*) with respect to non-treated cells, where *n* is the number of independent experiments.

Western blotting analysis

Determination of protein expression and/or phosphorylation was carried out by Western blotting. After treatment, cells were

lysed and processed as described previously (Gonzalez et al., 2011). In brief, protein lysates (12 μg/lane) of each sample were separated by SDS-PAGE, using 10% polyacrylamide gels, and were transferred to nitrocellulose membranes. Specific primary and the corresponding IgG-HRP-conjugated secondary antibodies were used for detection of proteins. The major reagents were obtained from Bio-Rad and from Sigma Chemicals. Chemiluminescent solutions kit SignalFire^{TM} ECL was obtained from Cell Signaling Technology. The software Image J (http://imagej.nih.gov/ij/) was used for quantification of the intensity of the bands. Values are expressed as the mean ± SEM of normalized values expressed as percentage versus non-treated cells.

Quantitative reverse transcription-polymerase chain reaction analysis

Cells were subjected to treatments and then lysed. Lysates were subsequently used for total RNA purification and analysis of protein expression as described previously (Santofimia-Castaño et al., 2015). Total RNA samples were purified using a commercially available kit (Sigma). The Power SYBR Green RNA-to-C_T^{TM} 1-Step kit (Applied Biosystems, Township, USA) was used. Reverse transcription was performed for 30 min at 48°C, and PCR conditions were 10 min at 95°C followed by 40 cycles of 15 s at 95°C plus 1 min at 55°C. The following primers were used:

Gclc: 5′-GGCACAAGGACGTGCTCAAGT-3′ and 5′-TGCAGAGTTTCAAGAACATCG-3′
Cat: 5′-ACTTTGAGGTCACCCACGAT-3′ and 5′-AACGGCAATAGGGGTCCTCTT-3′
Ho-1: 5′-AGCACAGGGTGACAGAAGAG-3′ and 5′-GAGGGACTCTGGTCTTTGTG-3′
Nqo-1: 5′-GGGGACATGAACGTCATTCTCT-3′ and 5′-AAGACCTGGAAGCCACAGAAGC-3′
Gapdh: 5′-GGGTGTGAACCACGAGAAAT-3′ and 5′-CCTTCCACGATGCCAAAGTT-3′

The primers were purchased from Thermo Fisher Scientific. The relative mRNA levels were calculated and were expressed as the fold change between sample and calibrator.

Cell viability and proliferation assays

Determination of cell viability was carried out using crystal violet test. After treatment, the plate with growing cells was rapidly placed on ice and cells were then washed with cold standard PBS. PBS was removed, 4% paraformaldehyde was added to each well and the plate was then incubated during 15 min at room temperature (23–25 °C). The wells were washed with distilled water. Afterwards, the fixed cells were stained by incubation in the presence of 0.1% crystal violet during 20 min at room temperature (23–25°C). The dye was removed by aspiration and the wells were washed three times with distilled water. Water was removed and the wells were allowed to air dry. Thereafter, 10% acetic acid was added to each well of the plate, followed by incubation during 20 min with shaking. Next, the content of each well was pipetted up and down. Finally, 50 μl of each well was diluted 1:4 with milli-Q water and the absorbance of each sample was measured at 590 nm employing a plate reader (VariosKan Lux 3020–205; Thermo Sci., Vantaa, Finland). The major reagents needed, together with crystal violet, were purchased from Sigma Chemicals.

Additionally, cell proliferation was analyzed by detection of BrdU incorporation into the newly synthesized DNA of proliferating cells. A commercially available kit (BrdU Cell Proliferation Assay Kit, from Biovision; Deltaclon S.L., Madrid, Spain) was used. Proliferation of cells was determined following manufacturer's directions. Absorbance of the samples was measured at 650 and 450 nm employing a plate reader (VariosKan Lux 3020–205, Thermo Sci., Vantaa, Finland).

The viability of cells subjected to hypoxia was compared with that of control cells (non-treated). Data are shown as the mean change of absorbance expressed in percentage ± SEM (n) with respect to non-treated cells, where n is the number of independent experiments.

Cell proliferation was further studied by detection of the expression of cyclin A and cyclin D1, which are key regulators of the cell cycle (Roskoski, 2016; Thakur et al., 2018). Western blotting analysis was used for this purpose. Antibody against cyclin D was purchased from Abcam plc (Cambridge, UK), whereas antibody against cyclin A and the secondary HRP-conjugate antibodies were obtained from Thermo Scientific (Madrid, Spain).

Cell migration assay

Cell migration was studied employing a wound healing assay. Cells were seeded (20,000 cells per well) in six well culture plates. When cells had reached confluence, the culture medium was replaced by serum free medium and the cells were incubated for 24 h. This manoeuvre was used to synchronize the cells. Next, a single horizontal wound was created. This was carried out by scratching the surface of each well with a sterile 200 μl pipette tip. The medium was aspirated to eliminate the debris and was replaced by complete medium. The cells were then subjected to hypoxia (1% O_2) during 48 h. Acquisition of images of the wound area was performed at 0, 12, 24, 36 and 48 h using an inverted microscope (Nikon Ts2; Izasa Scientific). Migration and protrusion of cells into the wound area were studied by manually counting the cells found within the wound at each time period.

Statistical analysis

Statistical analysis of data was performed by one-way analysis of variance followed by Tukey's post hoc test, and only P values < 0.05 were considered statistically significant. For individual comparisons and statistics between individual treatments, we employed the Student's t test, and only P values < 0.05 were considered statistically significant.

Author contribution

Antonio Gonzalez designed the study and wrote the manuscript. Matias Estaras, Salome Martinez-Morcillo, Remigio Martinez, Alfredo Garcia, Marcos Perez-Lopez and Mario Estevez performed acquisition and data analysis. Maria P. Miguez and Vicente Roncero interpreted and discussed results. Gerardo Blanco, Diego Lopez, Miguel Fernandez-Bermejo,

Jose M. Mateos, Daniel Vara and Gines M. Salido suggested discussion parts, reviewed and corrected the manuscript. All authors have approved and agreed with the submitted version.

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Conflict of interest statement

The authors have declared no conflict of interest.

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Melatonin Induces Apoptosis and Modulates Cyclin Expression and MAPK Phosphorylation in Pancreatic Stellate Cells Subjected to Hypoxia

Matias Estaras [1], Manuel R. Gonzalez-Portillo [1], Miguel Fernandez-Bermejo [2], Jose M. Mateos [2], Daniel Vara [2], Gerardo Blanco-Fernandez [3], Diego Lopez-Guerra [3], Vicente Roncero [4], Gines M. Salido [1] and Antonio González [1,*]

[1] Institute of Molecular Pathology Biomarkers, University of Extremadura, 10003 Caceres, Spain; meh@unex.es (M.E.); ramonglezpor@unex.es (M.R.G.-P.); gsalido@unex.es (G.M.S.)
[2] Department of Gastroenterology, San Pedro de Alcantara Hospital, 10003 Caceres, Spain; mfbermejo@unex.es (M.F.-B.); josemaria.mateos@salud-juntaex.es (J.M.M.); daniel.vara@salud-juntaex.es (D.V.)
[3] Hepatobiliary-Pancreatic Surgery and Liver Transplant Unit, University Hospital, 06080 Badajoz, Spain; gerardoblanco@unex.es (G.B.-F.); diego.lopezg@salud-juntaex.es (D.L.-G.)
[4] Unit of Histology and Pathological Anatomy, Veterinary Faculty, University of Extremadura, 10003 Caceres, Spain; roncero@unex.es
* Correspondence: agmateos@unex.es

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Abstract: In certain diseases of the pancreas, pancreatic stellate cells form an important part of fibrosis and are critical for the development of cancer cells. A hypoxic condition develops within the tumor, to which pancreatic stellate cells adapt and are able to proliferate. The consequence is the growth of the tumor. Melatonin, the product of the pineal gland, is gaining attention as an agent with therapeutic potential against pancreatic cancers. Its actions on tumor cells lead, in general, to a reduction in cell viability and proliferation. However, its effects on pancreatic stellate cells subjected to hypoxia are less known. In this study, we evaluated the actions of pharmacological concentrations of melatonin (1 mM–1 μM) on pancreatic stellate cells subjected to hypoxia. The results show that melatonin induced a decrease in cell viability at the highest concentrations tested. Similarly, the incorporation of BrdU into DNA was diminished by melatonin. The expression of cyclins A and D also was decreased in the presence of melatonin. Upon treatment of cells with melatonin, increases in the expression of major markers of ER stress, namely BIP, phospho-eIF2α and ATF-4, were detected. Modulation of apoptosis was noticed as an increase in caspase-3 activation. In addition, changes in the phosphorylated state of p44/42, p38 and JNK MAPKs were detected in cells treated with melatonin. A slight decrease in the content of α-smooth muscle actin was detected in cells treated with melatonin. Finally, treatment of cells with melatonin decreased the expression of matrix metalloproteinases 2, 3, 9 and 13. Our observations suggest that melatonin, at pharmacological concentrations, diminishes the proliferation of pancreatic stellate cells subjected to hypoxia through modulation of cell cycle, apoptosis and the activation of crucial MAPKs. Cellular responses might involve certain ER stress regulator proteins. In view of the results, melatonin could be taken into consideration as a potential therapeutic agent for pancreatic fibrosis.

Keywords: apoptosis; fibrosis; hypoxia; mitogen-activated protein kinases; melatonin; pancreatic stellate cells

1. Introduction

Pancreatic cancer and pancreatitis are diseases that affect the pancreas and are characterized by the development of a fibrotic tissue that overgrows within the gland and progressively occupies most of the abnormal mass [1]. Activated pancreatic stellate cells (PSCs) are majorly involved in the process of fibrosis. Their increase in number, together with the release of signaling factors towards surrounding cells, creates an environment favorable for the growth of malignant cells. Under these circumstances, the growing tissue can evolve towards pancreatic cancer [2]. Moreover, fibrosis acts as a barrier against antitumor agents, thus emerging as an element of resistance in cancer therapy [3].

Due to the fast proliferation and accumulation of malignant cells within the tumor, the oxygen supply to the cells is compromised, and therefore, a condition of hypoxia develops [4]. Conversely to what would be expected from the low oxygen availability, the cells contained in the abnormal mass exhibit adaptation and survive. As a consequence, the tumor increases in size and can expand [5]. In relation to these observations, we have recently shown that PSCs proliferate under hypoxia. Similar to tumor cells, PSCs undergo certain changes that allow them to survive under a low oxygen supply [6]. Interestingly, the maneuvers developed by PSCs under low oxygen availability could contribute to fibrosis in the tumor and aid the growth of malignant cells [7].

Accumulation of metabolites that can enter signaling cascades and contribute to inflammation has been observed in hypoxia [8]. Additionally, tumor–stroma interactions are of key importance for inflammation within the gland and for the development of pancreatic ductal adenocarcinoma (PDAC), which exhibits a significant inflammatory response [9]. Therefore, it seems feasible that controlling the inflammatory response and the release of cytokines by cells forming the tumor mass might facilitate the treatments and restrain tumor growth. In this line, Porcelli et al. showed that activation of the TGF-β signaling in cancer-associated fibroblasts promoted tumor invasion [10]. The authors suggested that counteracting the high level of circulating proinflammatory/immunosuppressive cytokines might serve as a strategy for the treatment of PDAC. Thus, at present, it is widely accepted that a major challenge in the treatment of pancreatic cancer is the modulation of fibrosis development within the tumor.

Melatonin, the major product of the pineal gland, exhibits pleiotropic effects on cell physiology [11]. With time, research has given numerous pieces of evidence that signal its potential as a therapeutic agent. With respect to cancer disease, it has been shown that melatonin exerts antitumor action in brain cancer [12], breast cancer [13], colon cancer [14], lung cancer [15], liver cancer [16] and pancreatic cancer [17]. Interestingly, melatonin modulates the viability and proliferation of PSCs [18–21]; thus, its potential antifibrotic action should be explored.

Former research of our laboratory has revealed that the oxidative state of PSCs can be modulated by melatonin, which could influence their proliferative state. In addition, we have observed that PSCs exhibit adaptation to hypoxia and increase their proliferation (findings mentioned above). Because PSCs are majorly involved in the process of fibrosis that develops in pancreatic cancer and contribute to creating conditions that favor the growth of cancer cells, including hypoxic conditions, in the present work we have investigated the effects of melatonin on PSCs cultured under hypoxia. Bearing in mind the antitumoral actions of melatonin and the effects that we have observed on PSCs, we aimed to shed more light on the ways by which melatonin exerts its antiproliferative actions on PSCs. Clarification of the mechanisms involved in the actions of melatonin to modulate PSC physiology might help in the understanding of the ways by which pancreatic fibrosis could be resolved or diminished by melatonin. In this sense, putative actions of melatonin to decrease PSC proliferation could ease the treatment of pancreatic cancer. Thus, our major objective was to investigate whether melatonin might control the development of fibrotic tissue and might help in the therapy of cancer.

2. Results

2.1. Effect of Melatonin on Cell Viability and Proliferation

We have previously shown that PSCs proliferate under hypoxia [6]. Moreover, we have shown that melatonin decreases the viability of pancreatic cancer cells [22] and also of PSCs incubated under normoxia [19–21]. To study whether melatonin also decreases the viability of PSCs subjected to hypoxia, different preparations of PSCs were incubated under hypoxia for 48 h in the absence (nontreated cells) or in the presence of melatonin (1 mM, 100 μM, 10 μM or 1 μM). Separate batches of cells were exposed to thapsigargin (Tps; 1 μM), which served as control for cell death [23]. In the presence of melatonin, a statistically significant decrease in cell viability was observed at the higher concentrations used (1 mM and 100 μM). Treatment of cells with Tps, used as control for cell death, evoked a statistically significant decrease in cell viability (Figure 1A).

In the next step, we evaluated cell proliferation employing a kit based on 5-bromo-2-deoxyuridine (BrdU). BrdU incorporation into the DNA of dividing cells is an indicator of cell proliferation. In this set of experiments, cells were incubated for 48 h under hypoxia, in the absence (nontreated cells) or in the presence of melatonin (1 mM, 100 μM, 10 μM or 1 μM). Treatment of cells with melatonin induced a statistically significant decrease in BrdU content at the concentration of 1 mM. In the presence of the other concentrations of melatonin, slight decreases in BrdU content were observed, which were not statistically significant in comparison with that noted in nontreated cells (Figure 1B). Incubation of cells with Tps (1 μM) evoked a statistically significant decrease in BrdU content (Figure 1B).

Cyclins are a family of proteins with pivotal roles in the control of the cell cycle. In this part of the study, we were interested in analyzing whether melatonin exerts any effect on these proteins. Cyclin A is active in the early phases of division, and cyclin D regulates the transition from G1 to S phase [24,25].

The expression of cyclins was studied by Western blot. For this purpose, cells were incubated with melatonin (1 mM, 100 μM, 10 μM or 1 μM) for 4 h under hypoxia. Then, cell lysates were analyzed to determine the levels of cyclin A and cyclin D. The effects of melatonin on each cyclin are shown in Figure 1C–E. In general, melatonin induced a decrease in the expression of cyclins A and D. However, in the case of cyclin A, melatonin only decreased the level of protein at the concentrations of 1 mM and 100 μM. In the presence of Tps (1 μM), the detection of cyclins A and D was decreased (Figure 1C–E).

2.2. Effect of Melatonin on Endoplasmic Reticulum Stress

ER stress is a condition that develops in cancer and inflammation [26]. It has also been observed in viral infections and in metabolic, neurodegenerative and cardiovascular diseases [27]. BiP/GRP78 is an endoplasmic reticulum (ER) chaperone that plays a key role in the regulation of ER responses to stress. BiP exhibits antiapoptotic properties and has the ability to control the activation of transmembrane ER stress sensors such as IRE1, PERK and ATF6 [28].

In a first step, we incubated PSCs under hypoxia for 4 h in the absence of melatonin, and the levels of BiP, phosphorylated eIF2α and ATF-4 were studied by Western blotting. Under these conditions, we did not observe increases in the expression of the mentioned proteins. On the contrary, the detection of such proteins was decreased in comparison with the levels noted in cells incubated under normoxia (Supplementary Figure S1).

Next, we investigated whether ER stress is involved in the responses of PSCs to melatonin treatment under hypoxia. For this purpose, cells were incubated under hypoxia and in the presence of melatonin (1 mM, 100 μM, 10 μM or 1 μM) for 4 h. Under these conditions, we detected an increase in the level of BiP (Figure 2A,B), in the phosphorylation of eukaryotic initiation factor 2 (eIF2α; Figure 2A,C) and in the level of ATF-4 (Figure 2A,D) in comparison with the levels noted in cells incubated in hypoxia but in the absence of melatonin. Treatment of cells with Tps (1 μM), an ER stress inducer [29] (Figure 4A,B), induced increases in the levels of the three proteins studied (Figure 2A–D).

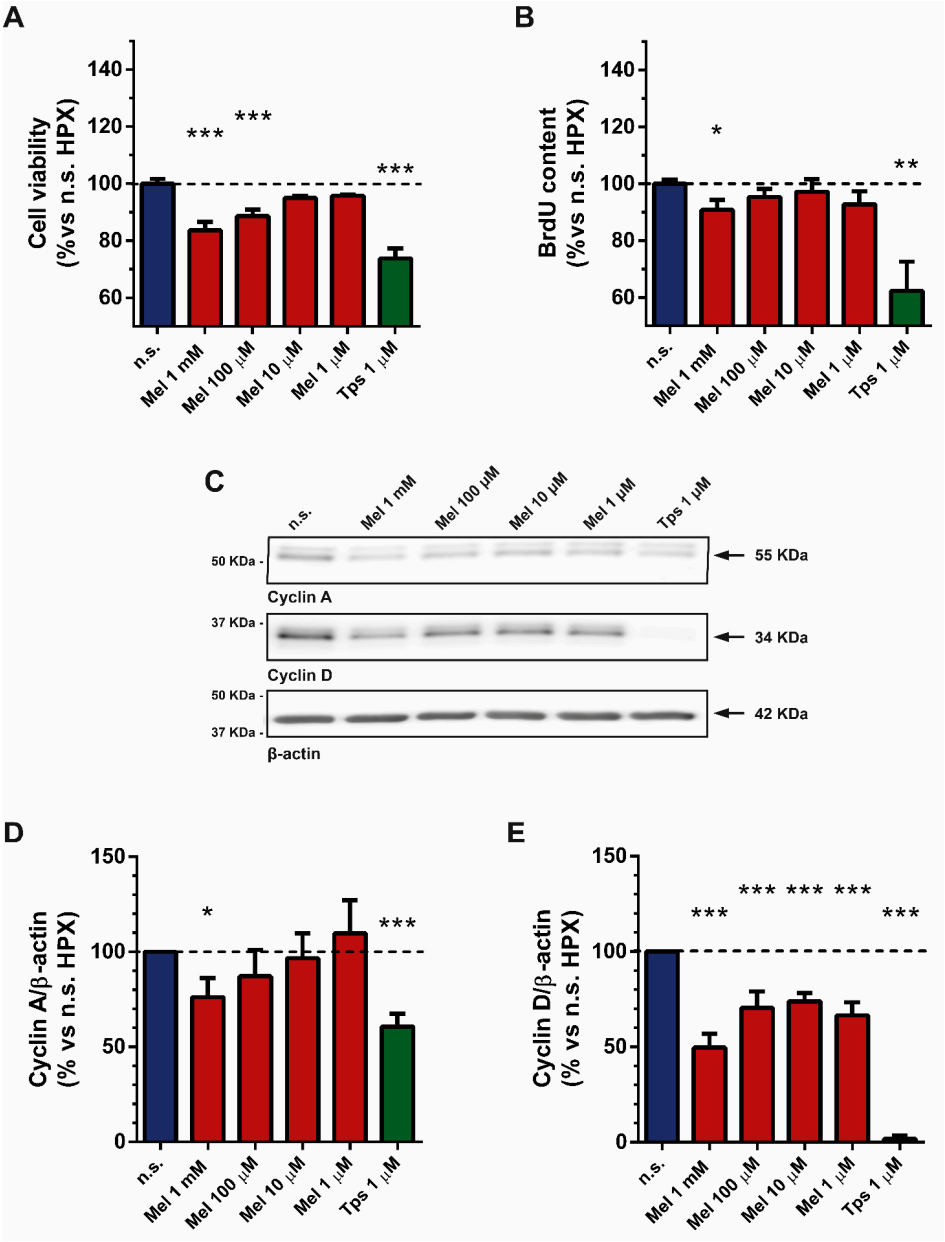

Figure 1. Effect of melatonin on cell viability and proliferation. (**A**) The bars show the effect of melatonin (1 mM, 100 μM, 10 μM or 1 μM) on the viability of PSCs incubated under hypoxia (in %: 86.77 ± 1.15; 94.16 ± 1.41; 97.38 ± 1.39; 98.39 ± 2.11; respectively for each concentration of melatonin vs. nontreated cells under hypoxia, which was considered 100%). Tps (1 μM) was used as control for cell death (in %: 63.67 ± 2.21). (**B**) The bars show the effect of melatonin on BrdU incorporation to DNA of dividing cells (in %: 90.88 ± 3.50; 95.36 ± 2.84; 97.19 ± 4.42; 92.81 ± 4.52; 62.38 ± 10.25; respectively for 1 mM, 100 μM, 10 μM or 1 μM melatonin and 1 μM Tps). (**C**) The figure shows representative blots of the expression of cyclin A and cyclin D, which were evaluated by Western blotting with specific antibodies. The band corresponding to each protein is marked by an arrow. The molecular weight of each specific protein is given on the right side of each blot. To ensure equal loading of proteins, the levels of β-actin were employed as controls under the tested conditions. (**D**,**E**) The bars show the quantification of protein levels for cyclin A (76.13 ± 10.01; 87.20 ± 13.91; 96.71 ± 13.01; 109.7 ± 17.56; 60.68 ± 6.79; respectively for 1 mM, 100 μM, 10 μM or 1 μM melatonin and 1 μM Tps) and cyclin D (49.69 ± 7.24; 70.57 ± 8.50; 73.90 ± 4.23; 66.61 ± 6.83; 1.79 ± 1.80; respectively for 1 mM, 100 μM, 10 μM or 1 μM melatonin and 1 μM Tps). Values show the mean ± SEM of normalized values expressed as % vs. nontreated cells (incubated under hypoxia and in the absence of melatonin or Tps), which was considered 100%. In the graphs, a horizontal dashed line represents the value achieved in nontreated cells. Data are representative of three to four independent experiments (n.s., nontreated cells; HPX, hypoxia; Mel, melatonin; Tps, thapsigargin; *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ vs. nontreated cells).

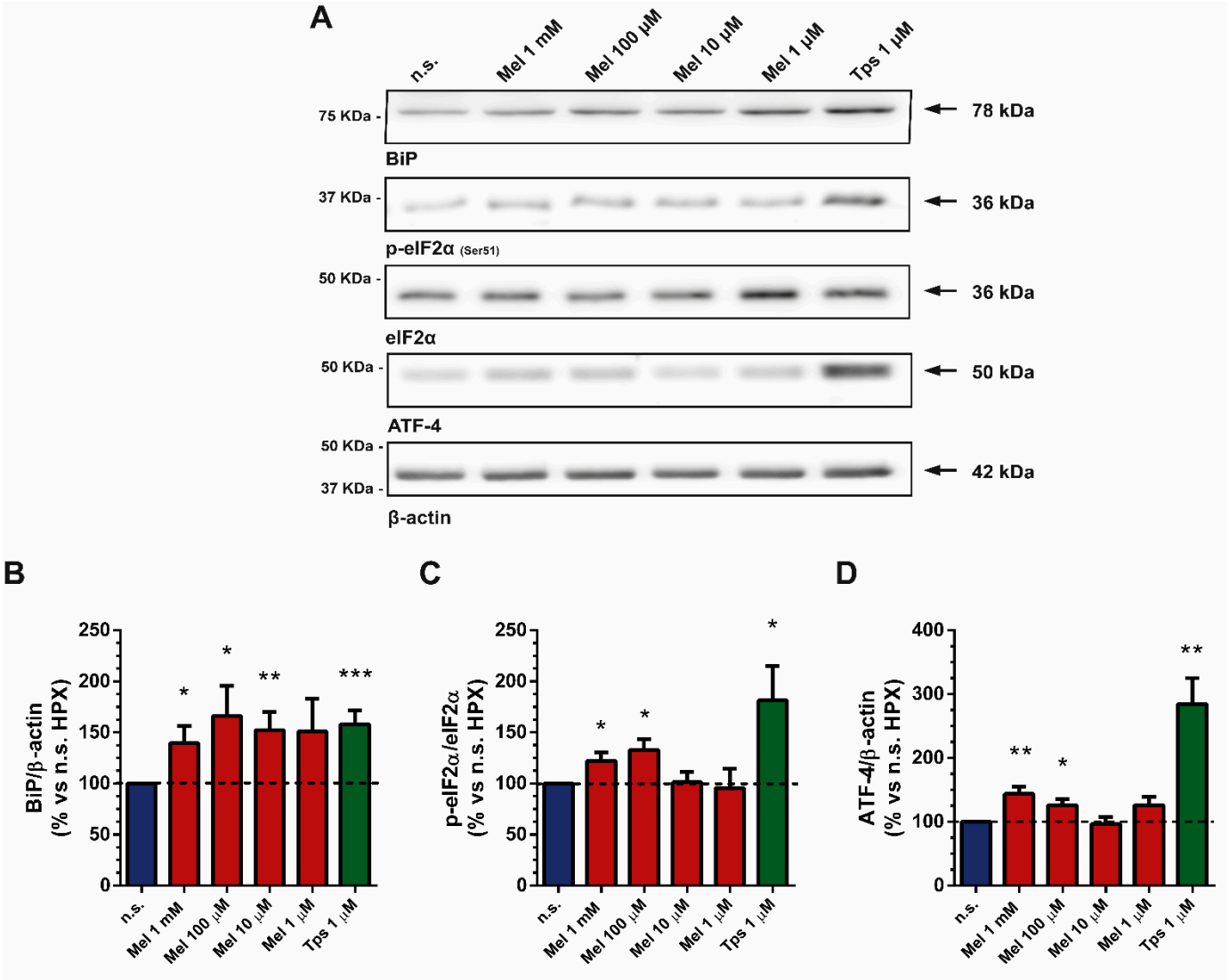

Figure 2. Effect of melatonin on ER stress markers in cells subjected to hypoxia. After treatment, cell lysates were processed for Western blotting analysis with specific antibodies. (**A**) Representative blots showing the effect of melatonin (1 mM, 100 μM, 10 μM or 1 μM) on the detection of the ER chaperone protein BiP/GRP78, the phosphorylation status of eIF2α and the level of ATF-4. The band corresponding to each protein is marked by an arrow. The molecular weight of each specific protein is given on the right side of each blot. To ensure equal loading of proteins, the levels of β-actin were employed as controls under the tested conditions for BIP and ATF-4, whereas the total expression level of eIF2α was used as control for p-eIF2α. (**B**–**D**) The bars show the quantification of protein levels for BIP (139.8 ± 16.64; 166.0 ± 29.82; 152.20 ± 17.94; 150.90 ± 32.34; 158.20 ± 13.37; respectively for 1 mM, 100 μM, 10 μM or 1 μM melatonin and 1 μM Tps), p-eIF2α (122.1 ± 7.90; 133.00 ± 10.57; 101.20 ± 10.30; 95.44 ± 19.29; 181.40 ± 33.74; respectively for 1 mM, 100 μM, 10 μM or 1 μM melatonin and 1 μM Tps) and ATF-4 (143.80 ± 10.93; 125.80 ± 9.83; 96.60 ± 11.03; 125.70 ± 13.38; 284.40 ± 40.88; respectively for 1 mM, 100 μM, 10 μM or 1 μM melatonin and 1 μM Tps). Values show the mean ± SEM of normalized values expressed as % vs. nontreated cells (incubated under hypoxia and in the absence of melatonin or Tps), which was considered 100%. In the graphs, a horizontal dashed line represents the value achieved in nontreated cells. Data are representative of four independent experiments (n.s., nontreated cells; HPX, hypoxia; Mel, melatonin; Tps, thapsigargin; *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ vs. nontreated cells).

2.3. Effect of Melatonin on Apoptosis

The decrease in cell viability that we have observed could be due to activation of apoptosis. Caspase-3 activation is a marker of apoptosis [22,30]. Therefore, we next evaluated the activation of caspase-3 in PSCs subjected to hypoxia and treated with melatonin for

24 h (1 mM, 100 µM, 10 µM or 1 µM). In the presence of melatonin, caspase-3 activation was observed (Figure 3). An increase in the level of activated caspase-3 was also noted in cells treated with the inducer of apoptosis Tps [31] (Figure 3).

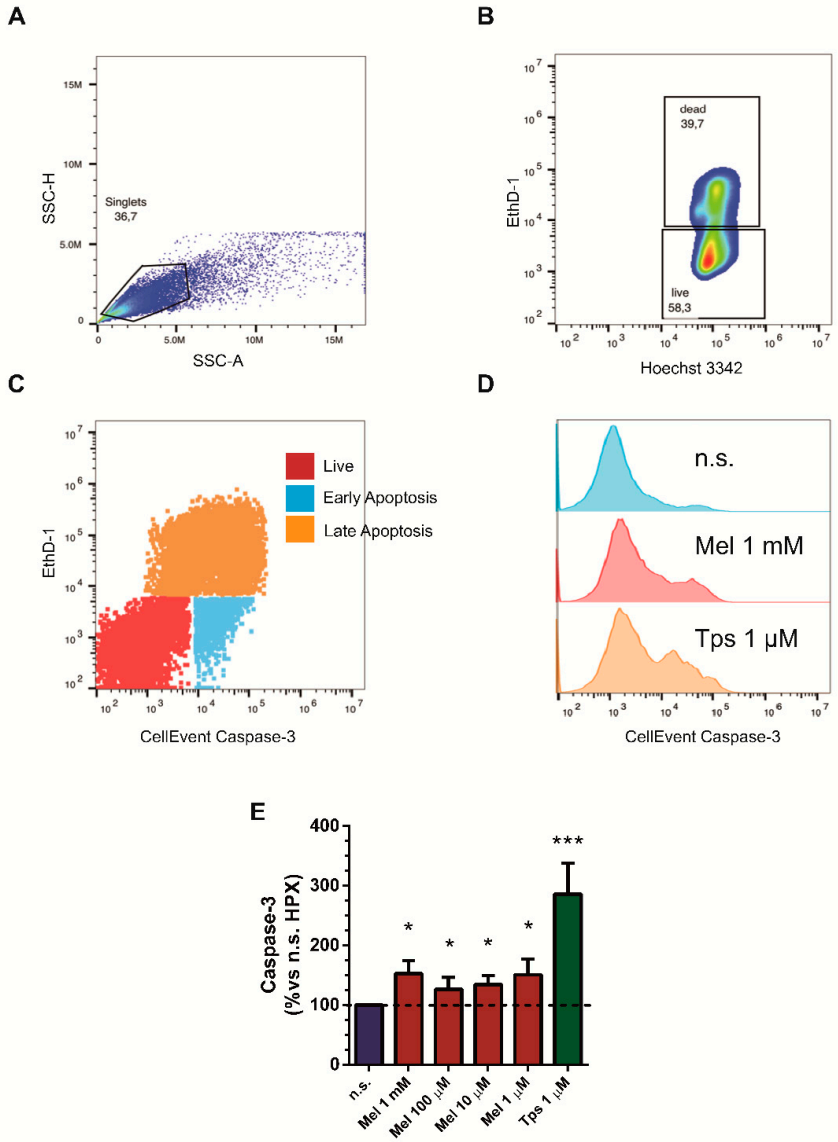

Figure 3. Study of caspase-3 activation in PSCs subjected to melatonin treatment under hypoxia. Cells were incubated for 24 h under hypoxia in the absence (nontreated) or in the presence of different concentrations of melatonin (1 mM, 100 µM, 10 µM or 1 µM) or with thapsigargin (Tps; 1 µM). (**A**) Doublets and clumps are identified and gated out to restrict the analysis to single cells. (**B**) 2D plot showing live and dead cells after staining with Eth-1 (dead) and Hoechst 3342 (live). (**C**) 2D dot plot showing apoptotic cells after staining with CellEvent and Eth-1. (**D**) The effect of the treatment is depicted, showing increased expression of caspase-3 from top to bottom. (**E**) The bars show the quantification of caspase-3 activation (152.80 ± 21.80; 126.40 ± 20.00; 134.40 ± 14.90; 150.70 ± 26.40; 285.90 ± 52.00; respectively for 1 mM, 100 µM, 10 µM or 1 µM melatonin and 1 µM Tps) in comparison with that detected in cells incubated in the absence of melatonin (nontreated cells), which was considered 100%. Tps (1 µM) was used as control. In the graph, a horizontal dashed line represents the level noted in nontreated cells (incubated under hypoxia and in the absence of melatonin or Tps), which was considered 100%. Results are representative of three different preparations (n.s., nontreated cells; HPX, hypoxia; Mel, melatonin; Tps, thapsigargin; *, $p < 0.05$; ***, $p < 0.001$ vs. nontreated cells).

2.4. Effect of Melatonin on MAPK Activation

The MAPK pathway plays a pivotal role in cellular signaling. This family of proteins transduces extracellular stimuli into phosphorylation events that control different cellular responses, which include inflammation, stress response, differentiation, survival and tumorigenesis [32].

Taking into account our results, we next evaluated the effect of melatonin treatment on MAPK activation in PSCs subjected to hypoxia. For this purpose, cells were incubated for 4 h under hypoxia and in the absence (nontreated cells) or in the presence of melatonin (1 mM, 100 μM, 10 μM or 1 μM).

Analysis of cell lysates revealed a statistically significant decrease in the phosphorylated state of JNK in cells treated with 1 mM melatonin. Conversely, an increase in phosphorylation was noted in samples from cells treated with the other concentrations of melatonin, although the differences were not statistically significant compared with the level detected in nontreated cells (Figure 4A,B). Melatonin treatment induced statistically significant increases in the phosphorylation of p38 and of p44/42 at all concentrations tested, in comparison with the values noted in nontreated cells (Figure 4A,C,D).

We next evaluated the involvement of p44/42 and p38 in the modulation of cell viability in cells treated with melatonin under hypoxia. Thus, PSCs were incubated for 5 min in the presence of U0126 (10 μM) and SB203580 (10 μM), which are specific inhibitors of p44/42 and p38, respectively. Thereafter, cells were incubated for an additional 48 h under hypoxia and in the presence of melatonin (1 mM–1 μM). Separate batches of cells were incubated in hypoxia and in the presence of the MAPK inhibitors. Cell viability was compared with that observed in nontreated cells (incubated in the absence of melatonin and without MAPK inhibitors). Inhibition of p44/42 and p38 significantly diminished viability of cells in comparison with nonstimulated cells. Moreover, treatment with the inhibitors decreased cell viability with respect to that noted in cells incubated in the presence of the respective concentration of melatonin (Figure 4E).

2.5. Effect of Melatonin on α-sma Expression

Alpha-smooth muscle actin (α-sma) is a specific marker for activated PSCs. Moreover, it has been signaled to exhibit an important role in fibrogenesis [33]. In this set of experiments, we studied the expression of α-sma in cells that had been incubated with melatonin (1 mM–1 μM) for 4 h under hypoxia. The results revealed a decrease in the content of α-sma in cells that had been treated with melatonin in comparison with cells incubated in its absence. A similar effect was noted in cells treated with 1 μM Tps (Figure 5).

2.6. Effect of Melatonin on Matrix Metalloproteinase Expression

A major contributor to the development of fibrosis is the secretion and accumulation of extracellular matrix components [34]. Matrix metalloproteinases (MMPs) are a family of proteins involved in processes such as angiogenesis, invasiveness and metastasis, which are considered important signs in cancer progression. The levels of members of this family of proteins are increased in pancreatitis and tumors [35]. Moreover, high levels of MMPs have been related to increased proliferation and migration of PSCs [36]. Because we had observed that melatonin induced changes in different biomarkers that are related to cell proliferation and that melatonin induced a decrease in cell viability, we decided to evaluate the effect of melatonin on the expression of MMP-2, MMP-3, MMP-9 and MMP-13.

PSCs were incubated for 4 h under hypoxia and in the presence of melatonin (1 mM, 100 μM, 10 μM or 1 μM). Separate batches of cells were incubated in the absence of melatonin but under hypoxia (nontreated cells). In general, treatment of cells with melatonin decreased the expression of MMPs, in comparison with the levels detected in nontreated cells (Figure 6). Tps (1 μM) also decreased the expression of MMPs (Figure 6).

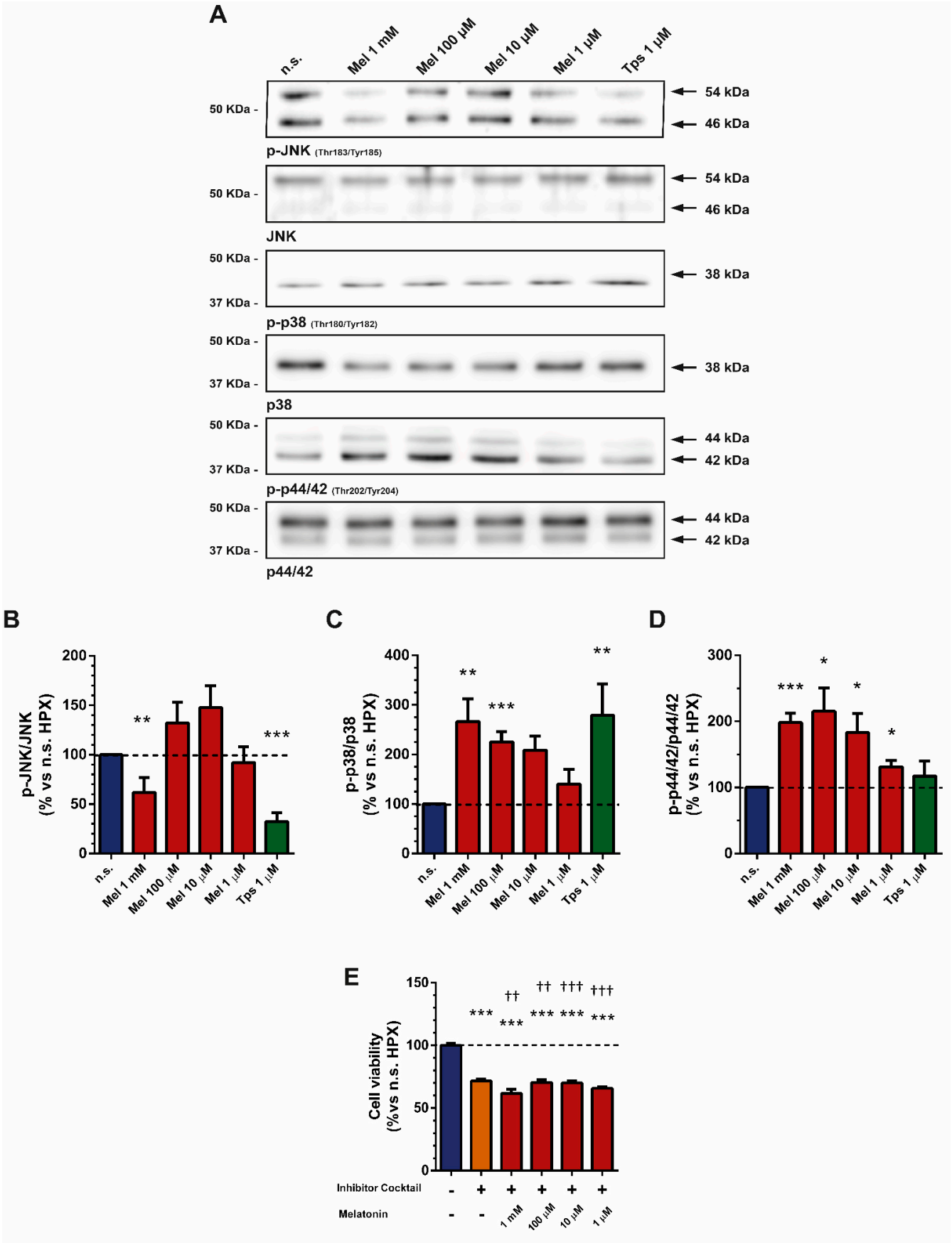

Figure 4. Analysis of JNK, p38 and p44/42 MAPK activation in response to melatonin under hypoxia. After treatment,

cell lysates were processed for Western blotting analysis with specific antibodies against the phosphorylated form of each protein. (**A**) Representative blots showing the phosphorylation states of JNK, p38 and p44/42. The band corresponding to each protein is marked by an arrow. The molecular weight of each specific protein is given on the right side of each blot. (**B**–**D**) The bars show the quantification of protein phosphorylation for p-JNK (61.99 ± 15.09; 132.2 ± 21.21; 147.7 ± 22.09; 92.04 ± 16.27; 32.18 ± 9.016; respectively for 1 mM, 100 μM, 10 μM or 1 μM melatonin and 1 μM Tps), p-p38 (266.3 ± 45.96; 225.40 ± 20.73; 208.9 ± 28.02; 140.4 ± 29.78; 279.30 ± 62.77; respectively for 1 mM, 100 μM, 10 μM or 1 μM melatonin and 1 μM Tps) and p-p44/42 (198.70 ± 14.43; 215.30 ± 35.56; 183.1 ± 28.91; 131.00 ± 9.67; 117.1 ± 23.03; respectively for 1 mM, 100 μM, 10 μM or 1 μM melatonin and 1 μM Tps). A horizontal dashed line represents the value observed in nontreated cells (incubated under hypoxia and in the absence of melatonin or Tps), which was considered 100%. Values show the mean ± SEM of normalized values expressed as % of phosphorylation vs. nontreated cells. Data are representative of four to five independent experiments (n.s., nontreated cells; HPX, hypoxia; Mel, melatonin; *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ vs. nontreated cells). (**E**) The bars show the viability of cells incubated for 48 h under hypoxia and in the presence of different concentrations of melatonin (1 mM, 100 μM, 10 μM or 1 μM) in combination with a cocktail of specific inhibitors of p44/42 (U0126; 10 μM) and p38 (SB203580; 10 μM) (in %: 71.57 ± 1.52; 61.73 ± 3.36; 70.50 ± 2.07; 70.09 ± 1.59; 65.82 ± 1.14; respectively for inhibitor cocktail alone and inhibitor cocktail plus 1 mM, 100 μM, 10 μM or 1 μM melatonin vs. nontreated cells, which was considered 100%). The inhibitors were added to the cells 5 min prior to addition of the respective concentration of melatonin. Viability was compared with that of cells incubated in the absence of melatonin (nontreated cells; *) and with the respective concentration of melatonin (†). Tps (1 μM) was used as control for cell death. In the graph, a horizontal dashed line represents the mean value of viability of nontreated cells (incubated under hypoxia and in the absence of drugs), which was considered 100%. Data are representative of three independent experiments (n.s., nontreated cells; Inhib. cocktail, cocktail of p44/42 and p38 inhibitors; Mel, melatonin; Tps, thapsigargin; ***, $p < 0.001$ vs. nonstimulated cells; ††, $p < 0.01$; †††, $p < 0.001$ vs. the respective concentration of melatonin).

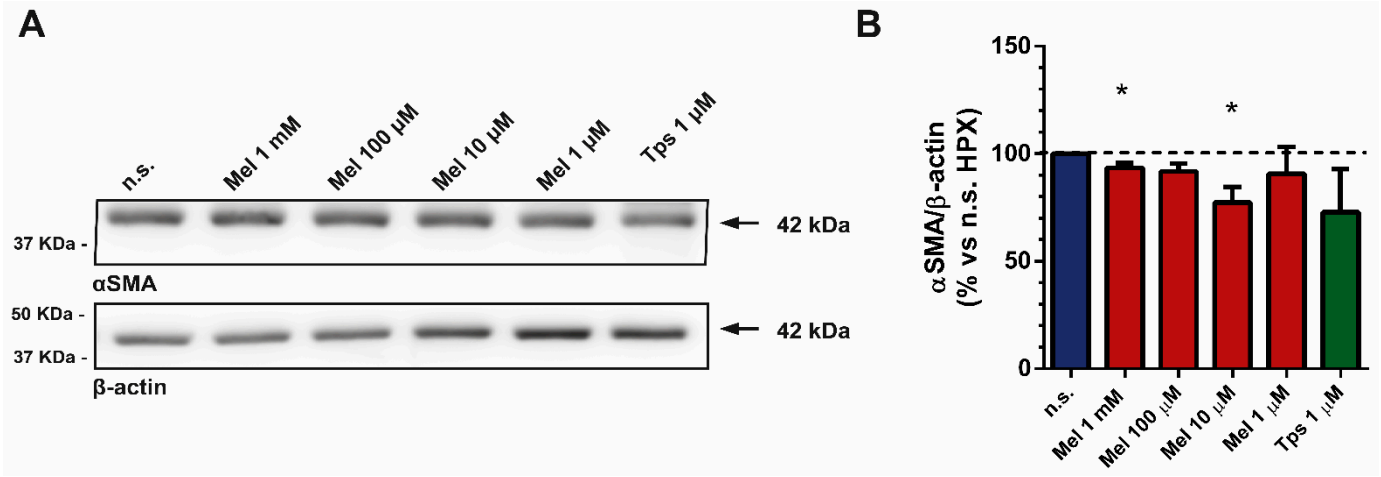

Figure 5. Analysis of α-smooth muscle actin expression in response to melatonin under hypoxia. After treatment, cell lysates were processed for Western blotting analysis with a specific antibody. (**A**) Representative blot showing the expression of α-sma. To ensure equal loading of proteins, the levels of β-actin were employed as controls under the tested conditions. The band corresponding to each protein is marked by an arrow. The molecular weight of each specific protein is given on the right side of each blot. (**B**) The bars show the quantification of α-sma expression (93.42 ± 2.30; 91.86 ± 3.52; 77.37 ± 7.16; 90.71 ± 12.49; 72.87 ± 20.01; respectively for 1 mM, 100 μM, 10 μM or 1 μM melatonin and 1 μM Tps). A horizontal dashed line represents the value observed in nontreated cells (incubated under hypoxia and in the absence of drugs), which was considered 100%. Values show the mean ± SEM of normalized values expressed as % vs. nontreated cells. Data are representative of three separate experiments (n.s., nontreated cells; HPX, hypoxia; Mel, melatonin; Tps, thapsigargin; *, $p < 0.05$ vs. nontreated cells).

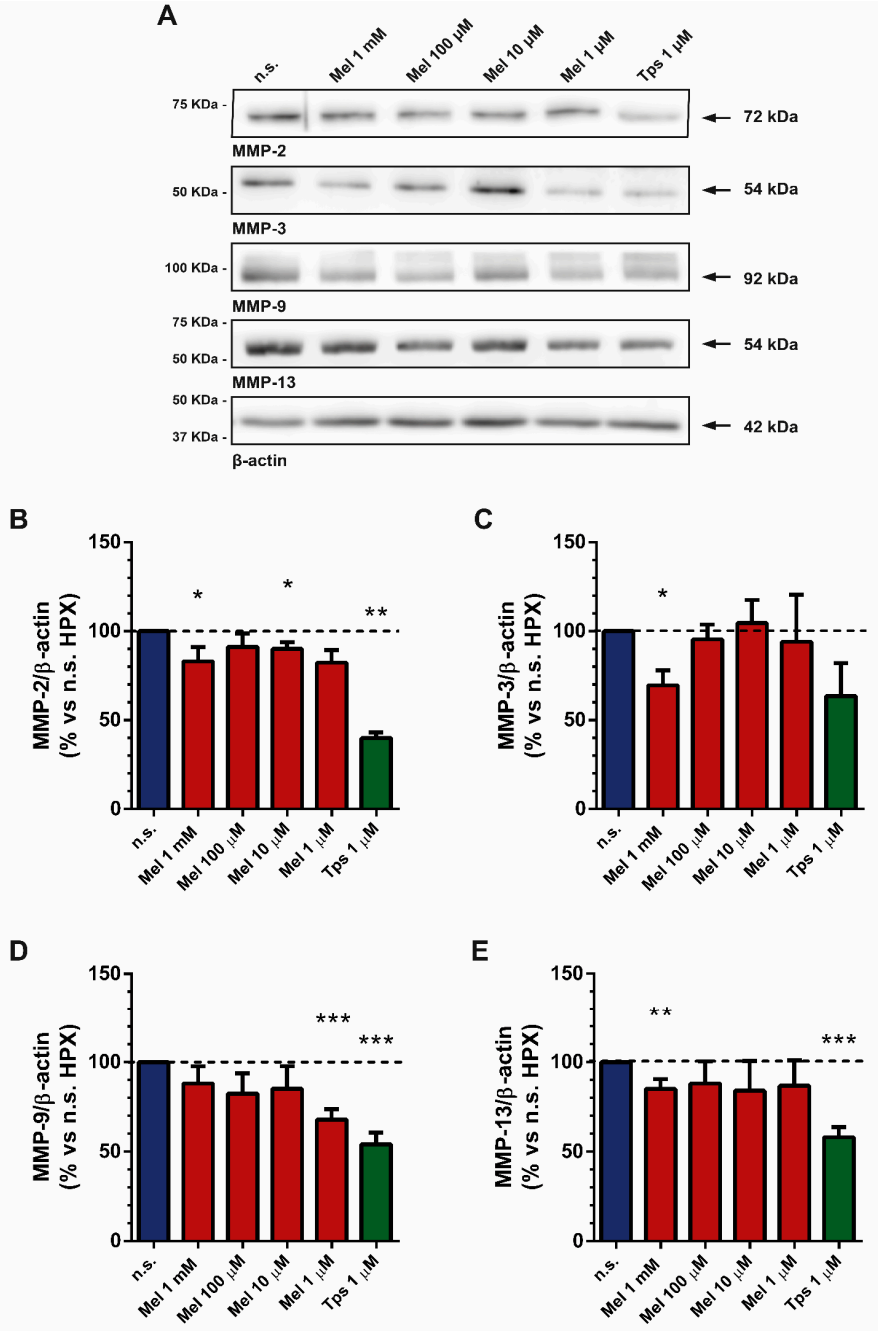

Figure 6. Analysis of MMP expression in response to melatonin under hypoxia. After treatment, cell lysates were processed for Western blotting analysis with specific antibodies. (**A**) Representative blots showing the expression of MMP-2, MMP-3, MMP-9 and MMP-13. To ensure equal loading of proteins, the levels of β-actin were employed as controls. The band corresponding to each protein is marked by an arrow. The molecular weight of each specific protein is given on the right side of each blot. (**B**) The bars show the quantification of protein expression for MMP-2 (83.12 ± 7.94; 91.20 ± 7.53; 90.11 ± 3.64; 82.29 ± 7.08; 39.98 ± 3.12; respectively for 1 mM, 100 µM, 10 µM or 1 µM melatonin and 1 µM Tps), MMP-3 (69.55 ± 8.51; 95.46 ± 8.22; 104.60 ± 12.99; 94.12 ± 26.45; 63.51 ± 18.61; respectively for 1 mM, 100 µM, 10 µM or 1 µM melatonin and 1 µM Tps), MMP-9 (88.03 ± 9.71; 82.43 ± 11.33; 85.19 ± 12.62; 67.94 ± 5.69; 54.12 ± 6.56; respectively for 1 mM, 100 µM, 10 µM or 1 µM melatonin and 1 µM Tps) and MMP-13 (85.08 ± 5.43; 88.03 ± 12.44; 84.03 ± 16.66; 86.81 ± 14.15; 58.09 ± 5.64; respectively for 1 mM, 100 µM, 10 µM or 1 µM melatonin and 1 µM Tps). A horizontal dashed line represents the value observed in nontreated cells (incubated under hypoxia and in the absence of drugs), which was considered 100%. Values show the mean ± SEM of normalized values expressed as % vs. nontreated cells. Data are representative of three to four separate experiments (n.s., nontreated cells; HPX, hypoxia; Mel, melatonin; Tps, thapsigargin; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs. nontreated cells).

3. Discussion

PSCs are resident cells of the pancreas. Normally inactive, under certain conditions, these cells undergo an activated state, and then a profibrogenic profile is set [37]. Particularly, PSCs have been pointed out as key contributors to the growth of pancreatic cancer, because of their contribution to stroma formation [38,39].

Remarkably, a special condition of hypoxia develops within abnormal tissues that exhibit a rapid development, as cancer tissues, due to the uncontrolled proliferation of cells contained in the mass [4]. In conjunction with cancer cells, PSCs will also be subjected to the low availability of O2 existing within the growing tumor. In order to survive, all types of cells forming part of the mass will exhibit adaptation to the low availability of O2 [40]. In this line, we have recently shown that PSCs adapt to hypoxia and exhibit increased proliferation capability with respect to PSCs grown in normoxia [6]. Under pathological conditions, such as inflammation and pancreatic cancer, PSCs contribute decisively to the development of the fibrotic reaction within the tissue. Therefore, maneuvers to reduce the amount of fibrotic tissue are considered a key tool in the treatment of these diseases [35,41,42]. In fact, it is now well accepted that the microenvironment contributes to the normalization of tumor cells. Hence, it might be feasible that modulation of stromal cells, rather than their removal, could be effective for cancer treatment [43].

Melatonin has been proposed to exhibit potential as a therapeutic agent in inflammation and cancer. With respect to cancer disease, it has been shown that melatonin exerts actions against a variety of tumors (for references, see introduction). As a part of the effects of melatonin, a decrease in the viability of cancer cells has been highlighted [44–46]. Its anticancer effects include the pancreas [22,47]. In addition, studies on PSCs have been conducted that show a decrease in cell viability in response to melatonin [18,20,21]. Moreover, PSCs exhibit adaptation to hypoxia and increases in their proliferation and migration ability [6]. However, the ways by which melatonin exerts its antiproliferative actions on PSCs are not completely understood, with special interest to hypoxia, and need further study.

Here we have shown that PSCs subjected to hypoxia exhibited a decrease in their viability after treatment with melatonin. These observations are in agreement with those reported formerly. In addition, our results have also shown that indoleamine decreases the expression of cyclins, which are pivotal proteins for the control of the cell cycle [24,25]. A clearer effect was exerted on cyclin D. A decrease in the expression of cyclin A was only noted with 1 mM and 100 μM melatonin. With regard to cyclin D, we have recently shown that PSCs subjected to hypoxia displayed an increase in its expression. This could be related to the increased proliferation that these cells exhibited [6]. Each of these cyclins plays a key role in the control of a certain step within the cell cycle. Interestingly, melatonin could regulate cell cycle via modulation of cyclin expression. In other words, those cells that survive upon melatonin treatment could exhibit a slowing down of the cell cycle, which might bring PSCs to a low proliferation rate. In relation to this, our results have shown that melatonin treatment diminished BrdU incorporation into DNA, which is an index of reduced cell proliferation. Therefore, our results suggest that melatonin could counteract the increased proliferation of PSCs that has been detected under hypoxia [6].

The unfolded protein response (UPR) is a condition that develops in cells subjected to stress, which promotes cell survival and adaptation to environmental conditions. The sensors PERK, IRE1 and ATF6 are pivotal to the UPR, together with their downstream transcription factors. When activated, the consequent UPR will trigger adaptation or apoptosis depending on the level of ER stress [48]. These proteins aim to restore homeostasis, but they can also induce cell death [49]. Melatonin has been shown to stimulate ER stress in different tumor cells, including the pancreas [50–52]. Our results have shown that PSCs subjected to hypoxia did not exhibit ER stress. However, upon treatment with melatonin, ER stress was observed. Our results are therefore in agreement with those previous observations.

Previous results of our laboratory showed that melatonin induced apoptosis in PSCs treated under normoxic conditions through caspase-3 activation [19]. This protein forms part of a regulated pathway that controls cell death, and its activation in cancer cells determines a decrease in cell viability [53]. Activation of caspase-3 by melatonin in pancreatic cancer cells has also been shown [22]. In the present work, we have shown that melatonin also induced the activation of caspase-3 under hypoxia. Moreover, the connection of ER stress with apoptosis has been signaled [54]. Thus, the induction of ER stress together with the activation of apoptosis that we have detected could represent two major pathways that might be recruited by melatonin in order to modulate the proliferation of PSCs. Interestingly, the cells that survive might enter the cell cycle in order to proliferate. However, melatonin might modulate the cell cycle to prevent the exacerbation of cell proliferation. This might be the reason why we observed a decrease in cell viability in the presence of melatonin, which was not as dramatic as that exerted by Tps; i.e., there is a remaining population of PSCs that survive and that could proliferate at a low rate, because the loss of cells was not complete following treatment with melatonin.

Another effect that we have observed is the activation of key components of the MAPK pathway in cells treated with melatonin. In a recent work, we showed that incubation of PSCs under hypoxia induced decreases in the phosphorylated state of p44/42 and p38, whereas an increase in the phosphorylation of JNK was noted [6]. In the present work, we have detected an increase in the phosphorylation of p38 protein. Activation of this MAPK is involved in cell death [55]. Moreover, it has been shown that melatonin-induced cell death involves phosphorylation of p38 [56]. Our results agree with these observations and suggest that the increase in p38 phosphorylation detected after treatment with melatonin could be related to the decrease in cell viability and proliferation that we have observed.

We also detected a decrease in the phosphorylation of JNK. This was only detected with the highest concentration of melatonin that we tested. Increases in its phosphorylation were observed at the other concentrations tested, but the differences were not statistically significant with respect to nontreated cells. Interestingly, 1 mM melatonin was the concentration that exerted the stronger effect on cell viability, cyclin expression and caspase-3 activation. It has been suggested that the JNK pathway plays a pivotal role in cell proliferation. JNK is phosphorylated in lung adenocarcinoma cells, hepatocellular carcinoma, colorectal cancer and pancreatic cancer, where it contributes to the progression of malignant cells and metastases [57–61]. Moreover, JNK phosphorylation is increased in PSCs subjected to hypoxia, and its inhibition is related to a decrease in cell viability [6].

In addition, we observed an increase in the phosphorylation of p44/42 in PSCs treated with melatonin. It is well known that this protein exerts a protective role [62]. From our point of view, the response that we have observed could be regarded as a counterpart by which, upon treatment with melatonin, PSC viability does not drop to an extent similar to that caused by Tps. In fact, increased phosphorylation of p44/42 by melatonin has been shown in HepG2 cells, a tumor cell line [56], where viability dropped upon melatonin treatment.

Our study suggests a parallel modulation of p44/42, p38 and JNK by melatonin, with probable competitive eventual consequences. In other words, handling of MAPK equilibrium could be an additional mechanism by which melatonin controls the proliferation of PSCs. Evidence in favor of this assumption is derived from the experiments in which cell viability dropped in cells treated with the inhibitors of p44/42 and p38.

It has been shown that activation of PSCs subjected to hypoxia occurs. A major marker of PSC activation is the expression of α-sma [63]. Interestingly, melatonin decreases the expression of this protein in myofibroblasts [64], endothelial cells [65], liver [66] or lung [67]. These observations support an antifibrogenic action of melatonin in these tissues. Our results have shown that melatonin induced a slight decrease in α-sma content. One possible explanation for the lack of a strong effect of melatonin on α-sma expression could be that melatonin does not completely reverse the activated state of PSCs subjected to hypoxia.

The development of fibrosis as a consequence of hypoxia is documented in pancreatic cancer [68]. MMPs comprise a multigene family of endopeptidases that are involved in remodeling of extracellular matrix during fibrosis. Moreover, MMPs are implicated in pathological processes, including cancer [69]. The activity of these proteins plays a pivotal role in tumor growth, invasion and metastasis [70,71]. Recent findings of our group showed that PSCs subjected to hypoxia exhibited an increase in the expression of MMP-2 [6]. Now we have shown that melatonin induced decreases in the expression of MMP-2, MMP-3, MMP-9 and MMP-13 in PSCs subjected to hypoxia. It has been suggested that, under hypoxia, PSCs release molecules that can act on neighboring cells to modulate their physiology. This might be related to the growth and proliferation of malignancy [72]. In addition, the migration capability of PSCs depends on the release of signaling factors by themselves. Accumulation of substances released by activated PSCs towards the extracellular medium can stimulate the activation of additional PSCs and other cells in the neighborhood. Because these proteins play a pivotal role in fibrosis and in cancer progression, our results show evidence in favor of a potential antifibrotic action of melatonin. A decrease in the secretion of components that could influence the remodeling of the extracellular matrix might be the basis of a putative action of melatonin to reduce the progression of abnormal tissue within the pancreas under hypoxia.

Taking into account the slight drop in cellular proliferation that was noted in the presence of melatonin under hypoxia, which might be related to the changes observed in the other biomarkers that we have studied, we could argue that melatonin might induce a putative lower activated state of PSCs. However, we cannot assure that cells reached complete quiescence. In a previous study by Estaras et al. [6], we showed that proliferation of PSCs was increased by 29% and that the expression of cyclin D was increased by 50% in cells subjected to hypoxia only, in comparison with the values detected in cells incubated in normoxia. Furthermore, the phosphorylation of JNK was increased in hypoxia by 20%, whereas the phosphorylation of p38 and of p44/42 dropped by 38% and 33%, respectively, with respect to the levels detected in cells incubated in normoxia. Additionally, in PSCs incubated under hypoxia, the expression of MMP-2, MMP-3, MMP-9 and MMP-13 was increased by 63%, 236%, 18% and 98.5%, respectively, in comparison with the values achieved in cells incubated in normoxia. All these previous observations are interesting, because our present results show effects of melatonin that are contrary to those observed in PSCs subjected to hypoxia only [6] and, hence, might support potential proquiescent effects of melatonin.

Altogether, our observations agree with the antiproliferative effects of melatonin that have been shown in pancreatic tumor cells [22] and in activated PSCs [19–21], the latter being cultured under normoxic conditions. Moreover, our results suggest that these effects also occur in hypoxia, a condition under which PSCs proliferate actively. The concentrations of melatonin that we have used fall within a range over that found in blood and, therefore, could be considered pharmacological and not physiological. However, melatonin is synthesized in various tissues, including the gastrointestinal system, where it could work in an autocrine or paracrine manner. This means that, in these tissues, melatonin could reach concentrations that are higher than those found in blood. Accordingly, the concentrations of melatonin found in blood cannot strictly define the local concentrations of melatonin that are considered physiological, because the latter have not been defined to date [73–76]. Concentrations of melatonin in the range of those employed in the present study have been used previously in healthy cells. In pancreatic acinar cells, melatonin stimulated the synthesis of antioxidant enzymes through Nrf2. This was considered a protective action of melatonin on healthy cells [77]. Additionally, melatonin protected pancreatic acinar cells against overstimulation with the secretagogue cholecystokinin, avoiding accumulation of Ca^{2+} in the cytosol and modulating amylase release [78,79]. Thus, pharmacological concentrations of melatonin might be useful in the therapy of pancreatic illnesses. Hypothetically, our results might have translational value to the clinic.

However, in vivo studies need to be conducted to ascertain whether melatonin exerts the effects that have been observed in vitro.

The hypoxic response and the circadian clock exhibit reciprocal regulation. It has been suggested that hypoxia is gated by the circadian clock in vivo. In addition, hypoxia conversely regulates the clock genes by slowing the circadian cycle and smoothening the amplitude of oscillations. Hypoxia-inducible factor 1 is the key factor in charge of this regulation [80]. Moreover, metabolic adaptation to hypoxia elevates acid production within the tumor microenvironment. The acid produced during the cellular metabolic response to hypoxia suppresses the circadian clock through diminished translation of clock constituents. Suppression of the molecular clock's oscillation affects the circadian transcriptome involving silencing of rapamycin complex 1 (mTORC1) signaling [81]. Another study revealed that O_2 use and management of its byproducts (ROS) exhibit circadian variation due to changes in activity and metabolism during the day vs. the night. Thus, the metabolic products generated under hypoxia are among the most important physiological regulators of cellular differentiation/dedifferentiation. It is well known that blood flow is disrupted in tumors. As a consequence, transport of O_2 or endocrine circadian regulators to the cells acquires key importance for circadian disruption and hypoxia in tumors. This is the reason why it has been suggested that circadian rhythms and hypoxia are involved in tumor growth and metastasis [82].

Finally, immune-based therapeutic strategies could be used to enhance PDAC cytotoxicity in order to restore immunity, which is disturbed in PDAC. In this line, the combination of melatonin with well-known immune-targeting agents hitting the microenvironment might reinforce the actions of treatments against fibrosis in PDAC [83]. In addition to immune modulation, other intracellular pathways could be used to modulate fibrosis. In this line, challenging the PI3K/mTOR pathway using inhibitors has been proposed to be a valuable tool for the treatment of cancer [84]. Modulation of the PI3K/mTOR pathway might enhance the therapeutic efficacy of melatonin by adding a significant antifibrosis effect. This could represent a line for future research.

4. Materials and Methods

4.1. Chemicals

Collagenase was obtained from Worthington Biochemical Corporation (Labclinics, Madrid, Spain). Cell Lytic for cell lysis and protein solubilization, crystal violet, protease inhibitor cocktail (Complete, EDTA-free), thapsigargin and Tween-20 were purchased from Sigma Chemicals Co. (Madrid, Spain). Fetal bovine serum, Hank's balanced salts (HBSS), horse serum, medium 199 and SuperSignal West Femto were obtained from Fisher Scientific Inc. (Madrid, Spain). Polystyrene plates for cell culture were obtained from Thermo Fisher Sci. (Madrid, Spain). Penicillin/streptomycin was purchased from BioWhittaker (Lonza, Basel, Switzerland). Acrylamide, Bradford´s reagent, Tris/glycine/SDS buffer (10×) and Tris/glycine buffer (10×) were from Bio-Rad (Madrid, Spain). 5-Bromo-2-deoxyuridine (BrdU) cell proliferation assay kit was purchased from BioVision (Deltaclon S.L., Madrid, Spain). SB203580 and U0126 were obtained from Tocris (Biogen Científica, Madrid, Spain). Species-specific HRP-conjugated secondary antibodies were purchased from Thermo Fisher Sci. (Madrid, Spain). All other analytical-grade chemicals used were obtained from Sigma Chemicals Co. (Madrid, Spain).

4.2. Culture of Pancreatic Stellate Cells

PSCs were prepared and cultured using methods described previously. With the procedure employed, an enriched culture of activated PSCs with no contamination of other cell types was obtained [21]. The pancreas was obtained from Wistar rat pups (4–5 days after birth). Briefly, the pancreas was subjected to enzymatic digestion with a physiological buffer containing 130 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 1.2 mM KH_2PO_4, 10 mM glucose, 10 mM HEPES, 0.01% trypsin inhibitor (soybean) and 0.2% bovine serum albumin (pH = 7.4 adjusted with NaOH) that was supplemented with

30 units/mL collagenase from Worthington. After centrifugation (30× *g* for 5 min at 4 °C) to remove the supernatant with the enzyme, culture medium was added to the pellet. Culture medium consisted of medium 199 supplemented with 4% horse serum, 10% FBS, a mixture of antibiotics (0.1 mg/mL streptomycin, 100 IU penicillin) and 1 mM $NaHCO_3$. Next, mechanical dissociation of the cells was carried out by gently pipetting the cell suspension through tips of decreasing diameter. After centrifugation, cells were resuspended in culture medium. Finally, cells were seeded on different substrates depending on the studies to be carried out (round glass coverslips, 100 mm diameter Petri dishes, or multiwell polystyrene plates) and grown in a humidified incubator at 37 °C and 5% CO_2. The experiments were carried out employing batches of cells obtained from different preparations. The number of passages of the cells was kept to a minimum (at most one passage was performed). The animals were supplied by the animal house of the University of Extremadura (Caceres, Spain). Handling of animals and the experimental protocols used were approved and performed according to the guidelines of the Ethical Committee for Animal Research of the University of Extremadura (identification code 44/2016; 14 July 2016) and the General Directorate of Agriculture and Livestock-Junta de Extremadura (identification code 20160810; 10 August 2016). The mentioned guidelines comply with EU bioethical law.

4.3. Induction of Hypoxia

Hypoxia was induced by incubation of cells in a low-O_2 (1%) atmosphere [6]. An incubator chamber (Okolab; Izasa Scientific, Madrid, Spain) was employed. Temperature (37 °C), humidity (90%) and air atmosphere (content of 1% O_2/5% CO_2/94% N_2) were electronically controlled.

4.4. Determination of Cell Viability and Proliferation

The crystal violet test was used to study the effect of treatments on cell viability [6]. Briefly, after treatment of cells with drugs, cells were washed with cold standard PBS and fixed with 4% paraformaldehyde (15 min at room temperature, 23–25 °C). Next, the cells were stained by incubation in the presence of 0.1% crystal violet (20 min at room temperature, 23–25 °C). This incubation was followed by washing with distilled water. Thereafter, water was removed, the wells were allowed to air dry and then 10% acetic acid was added to each well of the plate. Finally, the absorbance of each sample was measured at 590 nm employing a plate reader (CLARIOstar Plus, BMG Labtech., C-Viral, Madrid, Spain).

Cell proliferation was further analyzed by detection of BrdU incorporation into the DNA of growing cells as shown previously [19]. For this purpose, a commercially available kit (BrdU Cell Proliferation Assay Kit, from Biovision) was used. The protocol used was that suggested by the manufacturer. Absorbance of the samples was measured at 650 and 450 nm employing a plate reader (CLARIOstar Plus, BMG Labtech., C-Viral, Madrid, Spain).

Cells were subjected to drugs, and cellular viability or proliferation was compared with that of nontreated cells (subjected to hypoxia, but incubated in the absence of drugs). Data are shown as the mean change of absorbance expressed in percentage ± SEM (n) with respect to cells subjected to hypoxia in the absence of drugs (nontreated cells; n is the number of independent experiments).

Cell proliferation was further studied by detection of the expression of cyclin A and cyclin D and by detection of phosphorylation of MAPKs (p44/42, p38 and JNK). These determinations were carried out by Western blotting analysis, employing specific antibodies against respective proteins. Values are expressed as the mean ± SEM of normalized values expressed as percentage vs. the level of the respective protein found in cells subjected to hypoxia in the absence of drugs (nontreated cells; n is the number of independent experiments).

4.5. Determination of Apoptosis

Induction of apoptosis was detected by determination of capase-3 activation. Caspase-3 activation was determined employing previously described methods [19]. The cell-permeant substrate CellEvent Caspase-3/7 Green was used. This detection reagent consists of a four amino acid peptide (DEVD) conjugated to a nucleic acid binding dye. This cell-permeant substrate is intrinsically nonfluorescent because the DEVD peptide inhibits the ability of the dye to bind to DNA. After activation of caspase-3 in apoptotic cells, the DEVD peptide is cleaved, enabling the dye to bind to DNA and produce a bright, fluorogenic response with absorption/emission maxima of 502/530 nm. Samples were diluted in standard PBS to a final concentration 5×10^6 cells /mL. The cells were stained with 1µL CellEvent Caspase-3/7 Green Detection Reagent (2 mM stock solution) and 1 µL of Hoechst 33342 (16.2 mM stock solution). After thorough mixing, the cell suspension was incubated at room temperature (23–25 °C) in the dark for 25 min; then, cells were loaded with 1 µL ethidium homodimer (1.167 mM in DMSO) and incubated for a further 5 min. Next, the samples were immediately run on the flow cytometer. The controls consisted of unstained and single-stained controls to properly set gates and compensations. Quantification of the fluorogenic response was performed by flow cytometry (Cytoflex flow cytometer; Beckman Coulter, Brea, CA, USA), with absorption/emission maxima of 502/530 nm. Unstained, single-stained, and fluorescence minus one (FMO) controls were used to determine compensations and positive and negative events, as well as to establish regions of interest. FlowJoV 10.4.1 Software (Ashland, OR, USA) was used for the analysis. Data are expressed in percentage of increase ± SEM (n) with respect to cells subjected to hypoxia in the absence of drugs (nontreated cells; n is the number of independent experiments).

4.6. Determination of Endoplasmic Reticulum Stress

Activation of endoplasmic reticulum (ER) stress leads to the expression of certain proteins that can be detected by Western blotting. Several markers of ER stress were detected: BiP/GRP78, phospho-eIF2α and ATF-4. Specific antibodies against the respective protein were used. Data are expressed in percentage ± SEM (n) with respect to cells subjected to hypoxia in the absence of drugs (nontreated cells; n is the number of independent experiments).

4.7. Western Blot Analysis

Western blotting analysis was employed for the determination of protein expression and/or phosphorylation, as described previously [22]. In brief, protein lysates (12 µg/lane) of each sample were separated by SDS-PAGE, using 10% polyacrylamide gels, and were transferred to nitrocellulose membranes. After blocking, the membranes were incubated overnight with the desired specific primary antibody. The list of primary antibodies that were used can be found in Table 1. After washing, the membranes were incubated for 1 h with the corresponding species-specific HRP-conjugated secondary antibody. Western blot was revealed and the bands were detected using Syngene GBOX Chemi-XX9 Gel Documentation System (C-Viral, Madrid, Spain). The software Image J (http://imagej.nih.gov/ij/; accessed on 4 December 2020) was used for quantification of the intensity of the bands [85]. Values are expressed as the mean ± SEM of normalized values expressed as percentage vs. nontreated cells.

4.8. Statistical Analysis

Normality of data was analyzed using Shapiro–Wilk test. Statistical analysis was performed by Mann–Whitney U test, and only p values < 0.05 were considered statistically significant. The software employed was GraphPad Prism (version 6.01). For individual comparisons and statistics between individual treatments, we employed the Student's t-test, and only p values < 0.05 were considered statistically significant.

Table 1. Primary antibodies used in the study.

Antibody	Dilution	Supplier
ATF-4	1:2000	Abcam
β-Actin HRP-conjugated	1:50000	Thermo Fisher
BiP	1:2000	Cell Signaling
Cyclin A	1:2000	Thermo Fisher
Cyclin D	1:10000	Abcam
MMP-2	1:2000	Abcam
MMP-3	1:2000	Abcam
MMP-9	1:2000	Abcam
MMP-13	1:2000	Abcam
p-eIF2α	1:2000	Abcam
eIF2α	1:1000	Cell Signaling
p-JNK	1:1000	Cell Signaling
JNK	1:1000	Cell Signaling
p-p38	1:1000	Cell Signaling
p38	1:1000	Cell Signaling
p-p44/42	1:2000	Cell Signaling
p44/42	1:2000	Cell Signaling
α-sma	1:1000	Thermo Fisher

The primary antibodies listed were specific for each protein. The detection of the desired protein was carried out by Western blotting analysis, as described in Section 4. Thermo Fisher (Madrid, Spain); Abcam plc (Cambridge, UK); Cell Signaling (C-Viral, Madrid, Spain).

5. Conclusions

In summary, melatonin decreased the viability and proliferation of PSCs, which we have recently shown to be stimulated under hypoxia. Proliferating PSCs might contribute to the growth of malignant cells and, thus, could lead to the growth of abnormal tissue within a damaged pancreas. The effects that we have reported could be based on a proapoptotic action of melatonin. Moreover, a certain level of ER stress and major members of the MAPK family could be involved in the antiproliferative actions of melatonin. In addition, the cell cycle could be repressed, leading to a lower cellular proliferation. Finally, a decrease in the release of MMPs could contribute to the actions of melatonin. A summary of our findings can be seen in Figure 7. Because PSCs play a pivotal role in the fibrotic reaction that takes place in inflammation or cancer in the pancreas, where hypoxia exists, the conditions created by melatonin might slow down the proliferation of PSCs. This could control the fibrotic processes that can evolve under hypoxia and that could contribute to the survival and development of transformed epithelia within the pancreas. Our findings, therefore, support probable antiproliferative mechanisms by which melatonin could modulate fibrosis within the pancreas. Melatonin could thus serve as an aid in the strategies directed to control the development of fibrotic tissue within tumors and might help in the therapy of cancer.

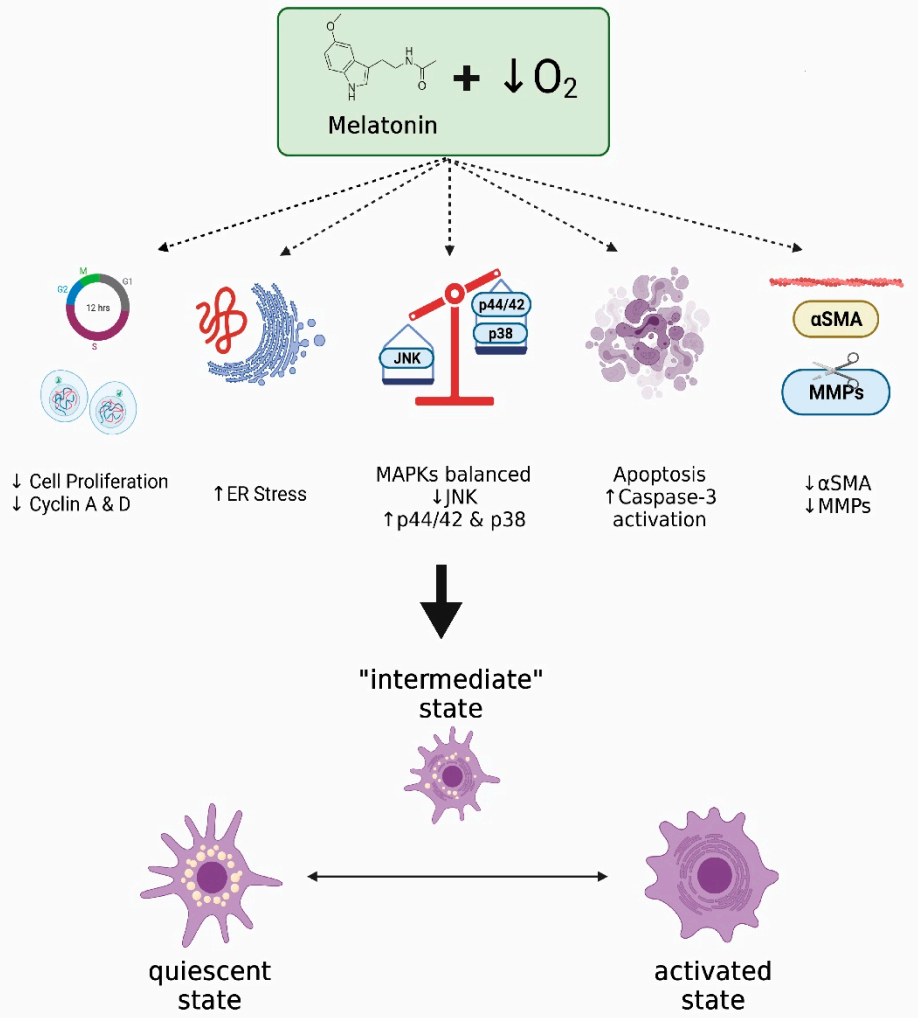

Figure 7. Summary of the effects of melatonin on PSCs subjected to hypoxia. PSCs exhibit increased proliferation under hypoxia. Thus, proliferating PSCs might contribute to the growth of abnormal tissue within a damaged pancreas, allowing the growth of malignant cells included in the mass. Melatonin may exert a certain level of ER stress and a proapoptotic action that could lead to a certain degree of cell death. In addition, melatonin could influence the cell cycle, thereby slowing down the proliferation of surviving cells. Major members of the MAPK family could be involved. Additionally, a decrease in the release of MMPs and a decrease in the expression of α-sma were also observed in cells treated with melatonin. Melatonin might therefore control the viability and proliferation of PSCs under hypoxia, leading the cells to a hypothetic "intermediate state". Hence, melatonin might decrease the fibrotic reaction that could lead to impairment of the pancreatic function and that could contribute to survival and development of transformed epithelia within the pancreas (α-sma, α smooth muscle actin; ER, endoplasmic reticulum; JNK, c-Jun N-terminal kinase; MMP, matrix metalloproteinase). Figure created with BioRender software (BioRender.com).

6. Strengths and Limitations

Our results strengthen the hypothesis for a therapeutical use of melatonin in different diseases, including those that affect the pancreas, particularly inflammation and cancer. We provide evidence that sheds light on the effects of melatonin on the physiology of major cells that participate in the fibrotic processes that affect the gland. Moreover, our findings support former results obtained in other cell types of the pancreas, with which we have worked in the past: acinar cells, which were protected in the presence of melatonin, and tumor cells, which exhibited apoptosis in the presence of melatonin. The limitations of our study refer to the fact that we performed an in vitro study. In vivo studies need to be

carried out in order to confirm the results obtained in vitro. Additionally, the involvement of other intracellular pathways and/or adaptations of metabolism in the cellular responses to melatonin needs to be further explored.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms22115555/s1, Figure S1: Detection of ER stress markers in PSCs subjected to normoxia or to hypoxia only. Supplementary material: original, uncropped and unadjusted images for blots.

Author Contributions: A.G.—Designed the study and wrote the manuscript. M.E. (Matias Estaras), M.R.G.-P.—Acquisition and data analysis, interpreted and discussed results, reviewed and edited the manuscript. V.R.—Interpreted and discussed results, reviewed and edited the manuscript. M.F.-B., J.M.M., D.V., G.B.-F., D.L.-G.—Suggested parts of the manuscript, reviewed and edited the manuscript. G.M.S.—Suggested discussion parts, reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

Data Availability Statement: Data are available from the corresponding author upon reasonable request.

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Conflicts of Interest: The authors declare that there is no conflict of interest.

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Melatonin Modulates the Antioxidant Defenses and the Expression of Proinflammatory Mediators in Pancreatic Stellate Cells Subjected to Hypoxia

Matias Estaras [1], Manuel R. Gonzalez-Portillo [1], Remigio Martinez [2], Alfredo Garcia [3], Mario Estevez [4], Miguel Fernandez-Bermejo [5], Jose M. Mateos [5], Daniel Vara [5], Gerardo Blanco-Fernández [6], Diego Lopez-Guerra [6], Vicente Roncero [7], Gines M. Salido [1] and Antonio Gonzalez [1,*]

[1] Institute of Molecular Pathology Biomarkers, University of Extremadura, 10003 Caceres, Spain; meh@unex.es (M.E.); ramonglezpor@unex.es (M.R.G.-P.); gsalido@unex.es (G.M.S.)
[2] Department of Animal Health, Veterinary Faculty, University of Extremadura, 10003 Caceres, Spain; remimar@unex.es
[3] Department of Animal Production, CICYTEX-La Orden, 06187 Badajoz, Spain; alfredo.garcia@juntaex.es
[4] IPROCAR Research Institute, Food Technology, University of Extremadura, 10003 Cáceres, Spain; mariovet@unex.es
[5] Department of Gastroenterology, San Pedro de Alcantara Hospital, 10003 Caceres, Spain; mfbermejo@unex.es (M.F.-B.); josemaria.mateos@salud-juntaex.es (J.M.M.); daniel.vara@salud-juntaex.es (D.V.)
[6] Hepatobiliary-Pancreatic Surgery and Liver Transplant Unit, Infanta Cristina Hospital, 06080 Badajoz, Spain; gerardoblanco@unex.es (G.B.-F.); diego.lopezg@salud-juntaex.es (D.L.-G.)
[7] Unit of Histology and Pathological Anatomy, Veterinary Faculty, University of Extremadura, 10003 Caceres, Spain; roncero@unex.es
* Correspondence: agmateos@unex.es

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Abstract: Pancreatic stellate cells (PSC) play a major role in the formation of fibrotic tissue in pancreatic tumors. On its side, melatonin is a putative therapeutic agent for pancreatic cancer and inflammation. In this work, the actions of melatonin on PSC subjected to hypoxia were evaluated. Reactive oxygen species (ROS) generation reduced (GSH) and oxidized (GSSG) levels of glutathione, and protein and lipid oxidation were analyzed. The phosphorylation of nuclear factor erythroid 2-related factor (Nrf2), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), and the regulatory protein nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-alpha (IκBα) was studied. The expression of Nrf2-regulated antioxidant enzymes, superoxide dismutase (SOD) enzymes, cyclooxygenase 2 (COX-2), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were also studied. Total antioxidant capacity (TAC) was assayed. Finally, cell viability was studied. Under hypoxia and in the presence of melatonin generation of ROS was observed. No increases in the oxidation of proteins or lipids were detected. The phosphorylation of Nrf2 and the expression of the antioxidant enzymes catalytic subunit of glutamate-cysteine ligase, catalase, NAD(P)H-quinone oxidoreductase 1, heme oxygenase-1, SOD1, and of SOD2 were augmented. The TAC was increased. Protein kinase C was involved in the effects of melatonin. Melatonin decreased the GSH/GSSG ratio at the highest concentration tested. Cell viability dropped in the presence of melatonin. Finally, melatonin diminished the phosphorylation of NF-kB and the expression of COX-2, IL-6, and TNF-α. Our results indicate that melatonin, at pharmacological concentrations, modulates the red-ox state, viability, and the expression of proinflammatory mediators in PSC subjected to hypoxia.

Keywords: cell viability; glutathione; hypoxia; inflammation; melatonin; pancreatic stellate cells; reactive oxygen species

1. Introduction

Activated pancreatic stellate cells (PSC) play a major role in the fibrotic process that develops in the pancreas in inflammation and cancer [1]. Fibrosis acts as a barrier against antitumor agents and, thereby, represents a factor of resistance in cancer [2]. In the three-dimensional matrix that is built up by the fibrotic components, cells establish specific interactions and cooperate between each other. This leads to tumor growth in parallel with resistance against chemo- and radiotherapy [3,4].

Hypoxia is a condition that is present in the vast majority of tumors and is a consequence of the fast proliferation and accumulation of cells within the growing tissue [5]. The cells comprising the mass exhibit adaptation to the low oxygen availability and set up different mechanisms that will help them survive. These changes allow the growth of the tumor [6].

In a recent work, we have shown that PSCs exhibited adaptation to hypoxia and were able to proliferate [7]. This is a major change that takes place in PSCs, which might contribute to the perpetuation of the fibrosis cycle inside a malignant tissue and could also contribute to inflammation [8]. Therefore, modulation of the growth of fibrotic tissue within tumors might be of outstanding importance in the treatment of inflammation and cancer.

Melatonin is the product of the pineal gland and is subjected to a rhythmic production and secretion during the dark phase of the day [9]. Melatonin depicts antioxidant properties on the exocrine pancreas [10]. Mainly, its mechanisms of action involve detoxification of free radicals by direct electron donation and by modulation of antioxidant defenses [11]. To cite some examples, melatonin reverted glutathione peroxidase activity in cerulein-induced acute pancreatitis [12], increased the levels of superoxide dismutase (SOD) activity and of reduced glutathione (GSH) in plasma and macrophages of animals infected with *Trypanosoma cruzi* [13], increased glutathione reductase content in liver, kidney, heart, and testis tissues [14], and induced the activation of the nuclear factor erythroid 2-related factor (Nrf2) and the antioxidant-responsive element, leading to related antioxidant enzymes, in pancreatic acinar cells [15]. In addition, melatonin exhibits a wide range of anticancer activities as for example in lung cancer [16], liver cancer [17], colorectal cancer [18], or pancreatic cancer [19]. Effects of melatonin against inflammation in the pancreas have also been shown [20]. With regard to PSC, we have shown previously that melatonin decreases the viability of this cell type under normoxia [21,22].

The major actions of melatonin are carried out through the activation of membrane-bound receptors [23,24]. However, direct actions of melatonin have also been proposed [22]. Interestingly, PSCs do not exhibit such membrane-located receptors for melatonin [21,22]. Therefore, the means by which melatonin exerts its actions on PSC physiology is intriguing. As mentioned above, PSCs are able to proliferate under hypoxia. To some extent, PSCs exhibited antioxidant responses that might underlie the mechanisms by which cells adapt to the low availability of oxygen (O_2) [7]. Hence, bearing in mind the antiproliferative actions of melatonin and the contribution of PSC to inflammation and cancer growth, the study of the mechanisms of action of melatonin on PSC physiology needs further consideration.

In the present study, we have continued our previous work and have investigated the effects of melatonin on PSC subjected to hypoxia. We were interested in clarifying the mechanisms by which the indoleamine could modulate PSC physiology in order to demonstrate its therapeutic potential in the treatment of pancreatic inflammation and cancer.

2. Materials and Methods

2.1. Chemicals

Collagenase was purchased from Worthington Biochemical Corporation (Labclinics, Madrid, Spain). Cell Lytic for cell lysis and protein solubilization, crystal violet, hydrogen

peroxide (H_2O_2), N-ethylmaleimide, oxidized glutathione, O-phthalaldehyde, protease inhibitor cocktail (Complete, EDTA-free), reduced glutathione, thapsigargin, and Tween®-20 were obtained from Sigma Chemicals Co. (Madrid, Spain). CM-H_2DCFDA (5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester), fetal bovine serum (FBS), Hank's balanced salts (HBSS), horse serum, and medium 199 were obtained from Invitrogen (Fisher Scientific Inc., Madrid, Spain). Polystyrene plates for cell culture and primers for RT-qPCR were purchased from Thermo Fisher Sci. (Madrid, Spain). Penicillin/streptomycin was purchased from BioWhittaker (Lonza, Basel, Switzerland). Bradford's reagent, Tris/glycine/SDS buffer (10×), and Tris/glycine buffer (10×) were from Bio-Rad (Madrid, Spain). SignalFire™ ECL Reagent was obtained from Cell Signaling Technology (C-Viral, Madrid, Spain). Total antioxidant capacity (TAC) assay kit was obtained from BioVision (Deltaclon S.L., Madrid, Spain). Ro-31-8220 was purchased from Calbiochem (Sigma Chemicals Co., Madrid, Spain). The primary antibodies used in the study are listed in Table 1. The corresponding HRP-conjugated species-specific secondary antibody was employed. All other analytical grade chemicals used were obtained from Sigma Chemicals Co. (Madrid, Spain).

Table 1. Primary antibodies used in the study.

Antibody	Dilution	Supplier
B-Actin HRP-Conjugated	1:50,000	Thermo Fisher
COX-2	1:2000	Cell Signaling
p-Nrf2 (Ser40)	1:2000	Thermo Fisher
p-p65 NF-κB (Ser536)	1:1000	Cell Signaling
p-IκB α (Ser32)	1:500	Santa Cruz Biotechnology
SOD-1	1:1000	Thermo Fisher
SOD-2	1:2000	Santa Cruz Biotechnology

The primary antibodies listed were specific for each protein. The detection of the desired protein was carried out by Western blotting analysis, as described in the Methods section. Thermo Fisher (Madrid, Spain); Santa Cruz Biotechnology (Quimigen S.L., Madrid, Spain); Cell Signaling (C-Viral, Madrid, Spain).

2.2. Culture of Pancreatic Stellate Cells.

To prepare the cultures of PSC, we employed methods described previously [22]. The pancreas was obtained from Wistar rat pups (3–5 days after birth). Animals were supplied by the animal house of the University of Extremadura (Caceres, Spain). A suspension of pancreatic cells was prepared by enzymatic digestion of the pancreas during 50 min at 37 °C with a Na-Hepes buffer (130 mM NaCl, 4.7 mM KCl, 1.3mM $CaCl_2$, 1 mM $MgCl_2$, 1.2 mM KH_2PO_4, 10 mM glucose, 10 mM HEPES, 0.01% trypsin inhibitor (soybean), and 0.2% bovine serum albumin -pH 7.4 adjusted with NaOH-) supplemented with collagenase (30 Units/mL). Next, the tissue was subjected to gentle pipetting through tips of decreasing diameter to obtain a cell suspension. Thereafter, the suspension was centrifuged at 30× *g* for 5 min. at 4 °C, the supernatant was discarded, and the pellet was resuspended in culture medium, which was comprised of: Medium 199 supplemented with 4% horse serum, 10% FBS, a mixture of antibiotics (0.1 mg/mL streptomycin, 100 IU penicillin), and 1 mM $NaHCO_3$. Small aliquots of cell suspension were then seeded on polystyrene plates for cell culture. The cells were grown in a humidified incubator with controlled temperature (37 °C) and CO_2 (5%). With this procedure, an enriched culture of activated PSC with no contamination of other cell types is obtained [21,22]. Purity of the cultures was checked by determination of the expression of α-smooth muscle actin and of collagen type 1 (Supplementary Figure S1), which are specific markers for activated PSC [25,26]. Confluence (90–95%) was reached after eight-ten days of culture. Different cell preparations were used in the studies.

2.3. Induction of Hypoxia

For induction of hypoxia, cells were incubated in a low O_2 atmosphere employing an incubator chamber (Okolab; Izasa Scientific, Madrid, Spain) and following previous protocols [7]. Temperature (37 °C), humidity (90%), and air atmosphere (content of 1% O_2/5% CO_2/94% N_2) were electronically controlled. The pH of the medium (7.30–7.35) did not change upon addition of drugs nor during the incubation periods.

2.4. Experimental Conditions for Melatonin Treatment

In the experimental procedures employed, separate batches of cells were incubated under hypoxia and in the presence of varying concentrations of melatonin (1000 μM, 100 μM, 10 μM, or 1 μM), or H_2O_2 (100 μM) or thapsigargin (Tps; 1 μM). For comparisons, separate batches of cells were incubated under hypoxia and in the absence of drugs (non-treated cells). Unless specifically stated, the incubation period lasted 4 h either in the absence or in the presence of drugs. In the experiments in which the PKC inhibitor Ro-31-8220 (5 μM) was used, cells were preincubated during 5 min in the presence of the inhibitor, prior to addition of the corresponding concentration of melatonin.

2.5. Determination of Reactive Oxygen Species Generation

Production of cytosolic reactive oxygen species (ROS) was detected following the manufacturer's directions and using methods described previously [27]. Briefly, cells were detached and loaded with the fluorescent probe CM-H_2DCFDA (10 μM). Cells were subjected to hypoxia and incubated with the indicated compounds for 1 h. Redox state of cells was monitored by measuring cellular fluorescence at 530 nm/590 nm (excitation/emission). Detection of fluorescence was performed using a plate reader (CLARIOstar Plus, BMG Labtech., C-Viral, Madrid, Spain). The experiments were carried out employing separate batches of cells, obtained from different preparations. Results are expressed as the mean increase of fluorescence in percentage ± SEM (n) with respect to cells that were subjected to hypoxia, but treated with no drug (non-treated cells) (n is the number of independent experiments).

2.6. Determination of Protein Carbonyls (Allysine)

After treatment, cells were washed with standard phosphate saline solution and were lysed for analysis. Protein carbonyls were determined following the methods described by Villaverde et al. [28], with slight modifications [29]. Five hundred μL of each sample were dispensed in 2 mL microtubes and treated with cold 10% trichloroacetic acid (TCA) solution. Each microtube was vortexed and then subjected to centrifugation at 600× g for 5 min at 4 °C. The supernatants were removed, and the pellets were incubated with the following freshly prepared solutions: 0.5 mL 250 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer pH 6.0 containing 1 mM diethylenetriaminepentaacetic acid (DTPA), 0.5 mL 50 mM ABA in 250 mM MES buffer pH 6.0, and 0.25 mL 100 mM NaBH3CN in 250 mM MES buffer pH 6.0. The tubes were vortexed and then incubated in water bath at 37 °C for 90 min. The samples were stirred every 15 min. After derivatization, samples were treated with a cold 50% TCA solution and centrifuged at 1200× g for 10 min. The pellets were then washed twice with 10% TCA and diethyl ether-ethanol (1:1). Finally, the pellet was treated with 6 M HCl and kept in an oven at 110 °C for 18 h until completion of hydrolysis. The hydrolysates were dried in vacuo in a centrifugal evaporator. The generated residue was reconstituted with 200 μL of milliQ water and then filtered through hydrophilic polypropylene GH Polypro (GHP) syringe filters (0.45 μm pore size, Pall Corporation, USA) for HPLC analysis.

The content of carbonyls was determined employing a Shimadzu "Prominence" HPLC apparatus (Shimadzu Corporation, Japan), equipped with a quaternary solvent delivery system (LC-20AD), a DGU-20AS on-line degasser, a SIL-20A auto-sampler, a RF-10A XL fluorescence detector, and a CBM-20A system controller. An aliquot (1 μL) from

the reconstituted protein hydrolysates was injected and analyzed in the above mentioned HPLC equipment. AAS- ABA was eluted in a Cosmosil 5C18-AR-II RP-HPLC column (5 μm, 150 × 4.6 mm) equipped with a guard column (10 × 4.6 mm) packed with the same material. The flow rate was kept at 1 mL/min and the temperature of the column was maintained constant at 30 °C. The eluate was monitored with excitation and emission wavelengths set at 283 and 350 nm, respectively. Standards (0.1 μL) were run and analyzed under the same conditions. Identification of both derivatized semialdehydes in the FLD chromatograms was carried out by comparing their retention times with those from the standard compounds. The peak corresponding to allysine-ABA was manually integrated from FLD chromatograms and the resulting areas plotted against an ABA standard curve with known concentrations that ranged from 0.1 to 0.5 mM. The nmol of allysine per mg of protein were calculated. Results are expressed as percentage of change of allysine content ± SEM (*n*) with respect to non-treated cells under hypoxia (n is the number of independent experiments).

2.7. Analysis of Thiobarbituric-Reactive Substances

After treatment, cells were lysed for analysis. Malondialdehyde (MDA) and other thiobarbituric-reactive substances (TBARS) were measured in 200 μL samples of each treatment, by adding 500 μL thiobarbituric acid (0.02 M) and 500 μL trichloroacetic acid (10%), and following incubation during 20 min at 90 °C. After cooling, a 5 min centrifugation at 600× *g* was made, and the absorbance of supernatant was measured at 532 nm using a microplate reader. The mg of TBARS per L of sample were calculated. Results are expressed as percentage of TBARS ± SEM (n) with respect to non-treated cells under to hypoxia (n is the number of independent experiments).

2.8. Determination of Glutathione Levels

The changes in the levels of reduced (GSH) and oxidized (GSSG) glutathione were determined using methods described previously [21]. Cells were incubated with the different stimuli assayed. A spectrofluorimeter (Tecan Infinite M200, Grödig, Austria) was employed to measure the fluorescence of each sample at 350 nm/420 nm (excitation/emission), respectively. For quantification, standard curves of GSH and GSSG were used. Normalization was carried out based on the total protein concentration in each sample (Bradford, 1976). A standard curve was prepared using bovine serum albumin. The experiments were carried out employing batches of cells obtained from different preparations. Data show the mean increase in GSH/GSSG ratio expressed in percentage ± SEM (n) with respect to non-treated cells under hypoxia (n is the number of independent experiments).

2.9. Determination of Total Antioxidant Capacity

Total antioxidant capacity (TAC) was determined using a commercially available kit, following manufacturer's directions, as described previously [29]. In brief, a working solution containing Cu^{2+}, provided with the assay kit, was added to all standard and sample wells. Samples were then incubated during 1.5 h in the dark. Trolox standard curve was used for calibration, as described in the assay kit's datasheet. A plate reader (CLARIOstar Plus, BMG Labtech., C-Viral, Madrid, Spain) was used to measure the absorbance (570 nm) of each sample. Results show the mean change of absorbance expressed in percentage ± SEM (n) with respect to non-treated cells incubated under hypoxia (n is the number of independent experiments).

2.10. Western Blotting Analysis

Western blotting analysis was employed for the determination of protein expression and/or phosphorylation, as described previously [30]. Bradford's method was used for quantification of the protein content of lysates [31]. Protein lysates (15 μg/lane) of each sample were separated by SDS-PAGE, using 10% polyacrylamide gels, and were

transferred to nitrocellulose membranes. Specific primary antibody (Table 1) and the corresponding IgG-HRP conjugated secondary antibody were used for detection of proteins. The software Image J (http://imagej.nih.gov/ij/ accessed on 4 December 2020) was used for quantification of the intensity of the bands. Values are expressed as the mean ± SEM of normalized values expressed as % vs. cells subjected to hypoxia in the absence of melatonin (non-treated cells).

2.11. Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-qPCR) Analysis

RT-qPCR was used to detect the expression of antioxidant enzymes. Cells were incubated under hypoxia with or without melatonin and were then lysed. Lysates were thereafter used for total RNA purification and analysis of protein expression as described previously [15]. Total RNA samples were purified using a commercially available kit (Sigma, Madrid, Spain). The Power SYBR Green RNA-to-CTTM 1-Step kit (Applied Biosystems, Township, USA) was used. Reverse transcription was performed for 30 min at 48 °C, and PCR conditions were 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C plus 1 min at 55 °C using the primers listed in Table 1. The mRNA abundance of each transcript was normalized to the *Gapdh* mRNA abundance obtained in the same sample. The relative mRNA levels were calculated using the ΔΔCt method, and were expressed as the fold change between sample and calibrator. The experiments were carried out employing batches of cells obtained from different preparations. The primers used are listed in Table 2.

Table 2. List of primers used in the study.

Primer	Forward	Reverse
Cat	5′-ACTTTGAGGTCACCCACGAT-3′	5′-AACGGCAATAGGGGTCCTCTT-3′
Gapdh	5′-GGGTGTGAACCACGAGAAAT-3′	5′-CCTTCCACGATGCCAAAGTT-3′
Gclc	5′-GGCACAAGGACGTGCTCAAGT-3′	5′-TGCAGAGTTTCAAGAACATCG-3′
IL-6	5′-GTTTGGAAGCATCCATCATTT-3′	5′-TGGAAATGAGAAAAGAGTTGTG-3′
Ho-1	5′-AGCACAGGGTGACAGAAGAG-3′	5′-GAGGGACTCTGGTCTTTGTG-3′
Nqo-1	5′-GGGGACATGAACGTCATTCTCT-3′	5′-AAGACCTGGAAGCCACAGAAGC-3′
Sod-1	5′-GGGGACAATACACAAGGCTGTA-3′	5′-CAGGTCTCCAACATGCCTCT-3′
Sod-2	5′-GTGGAGAACCCAAAGGAGAG-3′	5′-GAACCTTGGACTCCCACAGA-3′
TNF-α	5′-CCACCAGTTGGTTGTCTTTG-3′	5′-TAGCCCACGTCGTAGCAAAC-3′

RT-qPCR was performed for 30 min at 48 °C. PCR conditions were 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C plus 1 min at 55 °C using the primers listed above (purchased from Thermo Fisher; Madrid, Spain). The abundance of *Gapdh* mRNA in each sample was used for normalization (n = 3 independent experiments).

2.12. Determination of Cell Viability

Cell viability was studied using crystal violet test, as described previously [7]. Briefly, cells were subjected to different treatments under hypoxia and fixed with 4% paraformaldehyde. After washing, cells were stained with crystal violet (0.1%). Following several washing steps with distilled water, the wells were allowed to air dry and then acetic acid (10%) was added to each well of the plate to dissolve the precipitate. Finally, the absorbance (590 nm) of each sample was measured. A plate reader (CLARIOstar Plus, BMG Labtech., C-Viral, Madrid, Spain) was used. The absorbance was related with the viability of cells subjected to each treatment.

The viability of cells subjected to drugs was compared with that of non-treated cells. Data show the mean change of absorbance expressed in percentage ± S.E.M. (n) with

respect to non-treated cells incubated under hypoxia (n is the number of independent experiments).

2.13. Statistical Analysis

Data were analyzed for statistics using one-way analysis of variance (ANOVA) followed by Tukey post hoc test, and only p values < 0.05 were considered statistically significant. For comparisons and statistics between individual treatments, we employed the Student's t test and only p values < 0.05 were considered statistically significant.

3. Results

3.1. Effect of Melatonin on the Oxidative State of PSC

We were interested in testing whether melatonin evokes ROS production in PSC subjected to hypoxia. In the presence of melatonin, cells exhibited a concentration-dependent change in cytosolic ROS generation. The effect was stronger at the higher concentration of melatonin used (Figure 1A). For comparisons, separate batches of cells were incubated in the presence of hydrogen peroxide (H_2O_2, 100 μM) alone, which was used as control of oxidation.

We next evaluated whether treatment with melatonin was accompanied by lipid and/or protein oxidation. Thus, the effects of melatonin on protein carbonyl levels and on TBARS were assayed. In the presence of melatonin, we did not detect increases in the total protein carbonyls content (Figure 1B) nor in the levels of TBARS (Figure 1C). Conversely to what we would have expected, the values detected were lower compared with those detected in non-treated cells, which had been incubated in the absence of melatonin. On its side, treatment of cells with H_2O_2 (100 μM) induced statistically significant increases in both total protein carbonyls and TBARS (Figure 1B,C), reflecting an increase in oxidation.

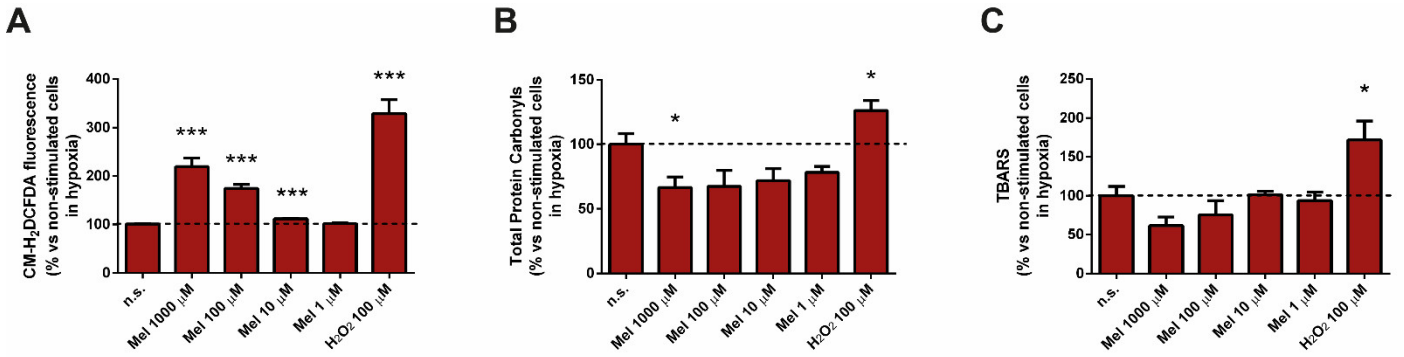

Figure 1. Effect of melatonin on the oxidative state of pancreatic stellate cells (PSC). (**A**) The cytosolic generation of reactive oxygen species (ROS) was evaluated in PSC loaded with the ROS-sensitive dye CM-H_2DCFDA. (**B**) The effect of melatonin on the levels of protein carbonyls was studied. (**C**) The effect of melatonin on lipid-peroxidation (TBARS) was assayed. H_2O_2 (100 μM) was used as a control of oxidation. A horizontal dashed line represents the value observed in cells incubated under hypoxia and in the absence of melatonin (non-treated). Data are representative of three to five independent experiments (n.s., non-treated cells; Mel, melatonin; H_2O_2, hydrogen peroxide; *, $p < 0.05$; and ***, $p < 0.001$ vs. non-treated cells in hypoxia).

3.2. Effect of Melatonin on Nuclear Factor Erythroid 2-Related Factor and Related Antioxidant Enzymes

Next, we analyzed the phosphorylated state of Nrf2 in cells treated with melatonin. In the presence of melatonin, the phosphorylation of Nrf2 was increased in comparison with that detected in its absence (non-stimulated cells; Figure 2A,B).

We next studied the effect of melatonin on the transcriptional activation of Nrf2-related antioxidant enzymes. Treatment of cells with melatonin evoked concentration-

dependent increases in the expression of catalytic subunit of glutamate-cysteine ligase (GCLc), catalase (CAT), NAD(P)H quinone oxidoreductase 1 (NQO1), and heme oxygenase-1(HO1), which are antioxidant enzymes regulated by Nrf2 (Figure 2C–F).

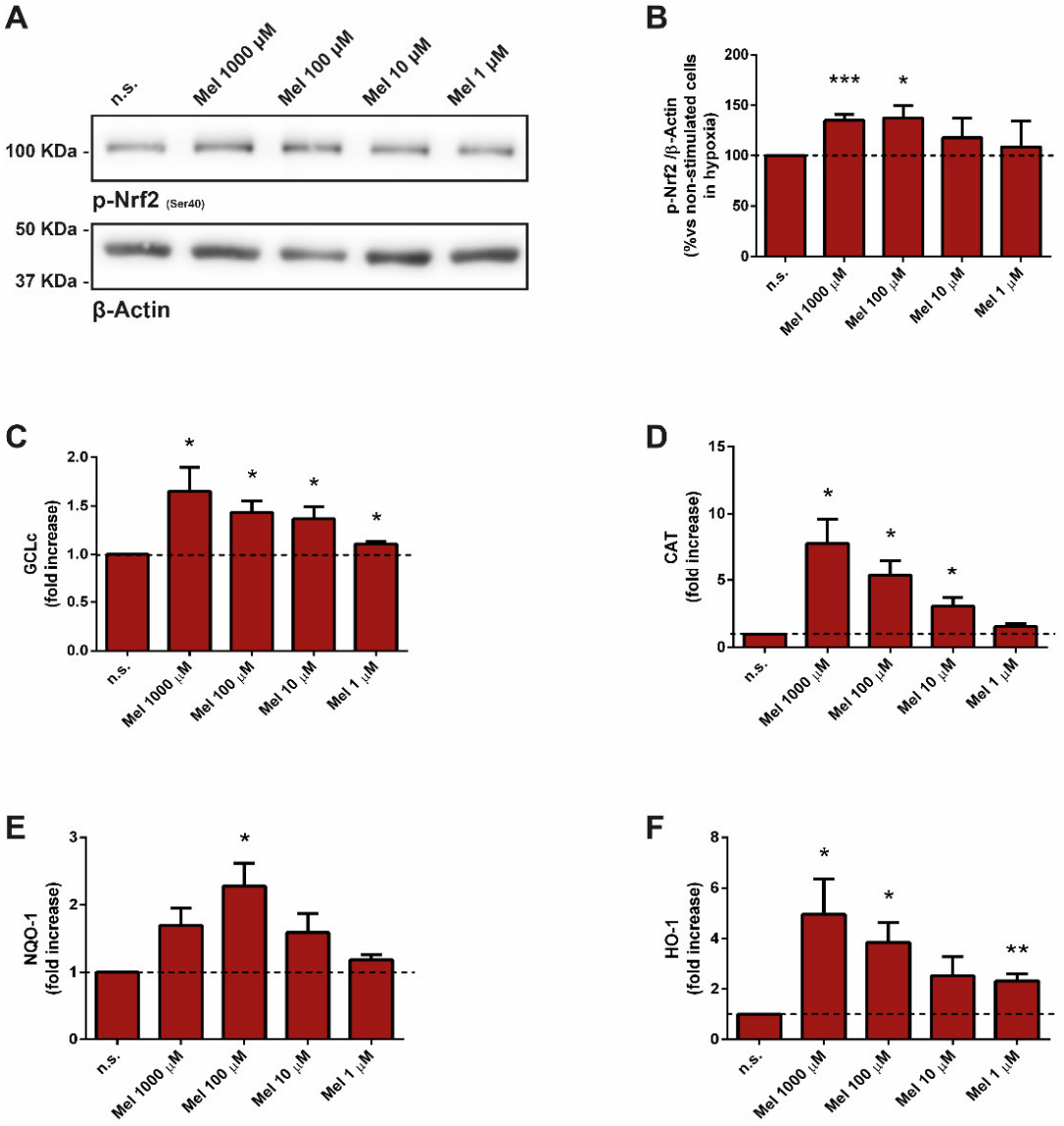

Figure 2. Effect of melatonin on Nrf2-phosphorylation and on Nrf2-regulated enzymes. PSC were incubated with melatonin (1000 μM, 100 μM, 10 μM, or 1 μM) during 4 h under hypoxia. (**A**) Blots showing the effect of melatonin on the phosphorylation of Nrf2. Western blotting analysis was carried out using a specific antibody against the protein. The levels of β-actin were employed as controls to ensure equal loading of proteins. (**B**) The bars show the quantification of protein phosphorylation. A horizontal dashed line represents the value observed in non-treated cells (n.s., incubated under hypoxia but in the absence of melatonin). Results are the mean ± S.E.M. of normalized values, expressed as % with respect to non-treated cells. Four independent experiments were carried out. (**C**–**F**) RT-qPCR analysis was used to study the effect of melatonin on the levels of various Nrf2-dependent antioxidant enzymes: Cysteine ligase-catalytic subunit (**A**; *GClc*), catalase (**B**; *CAT*), NAD(P)H quinone oxidoreductase 1 (**C**; *NQO1*), and heme-oxygenase-1 (**D**; *HO-1*). *Gapdh* mRNA was used for normalization. The bars show the fold increase of mRNA levels ± S.E.M. of each protein relative to non-treated cells (n.s., incubated under hypoxia but in the absence of melatonin). Three different preparations were used (n.s., non-stimulated cells; Mel, melatonin; *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ vs. non-treated cells in hypoxia).

3.3. Effect of Melatonin on Superoxide Dismutase

First, we tested the effect of melatonin on the protein levels of SOD1 and SOD2, which were analyzed by Western blotting. The expression levels SOD1 and SOD2 were increased in cells incubated in the presence of melatonin, compared with cells that had been incubated in the absence of melatonin (non-treated cells; Figure 3A–C).

In an additional step, we studied the relative mRNA abundance of SOD1 and SOD2 by RT-qPCR. Treatment of cells with melatonin increased the mRNA levels of SOD2 at all four concentrations tested, compared with that noted in cells incubated in its absence (non-treated cells). However, we only detected an increase in the levels of SOD1-mRNA in cells incubated with 1000 μM melatonin with respect to non-treated cells (Figure 3D,E).

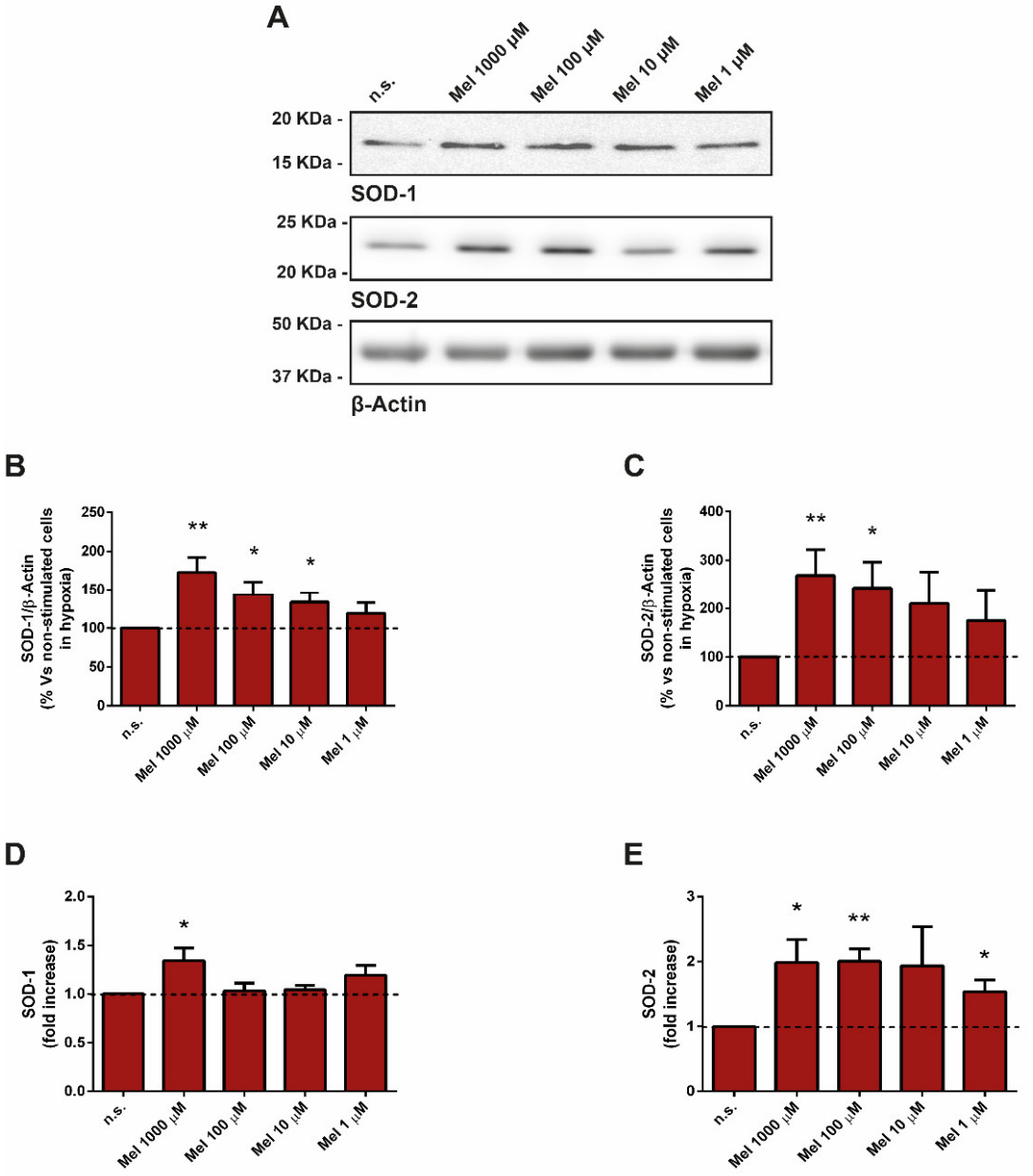

Figure 3. Effect of melatonin on superoxide dismutase (SOD). PSCs were incubated with melatonin (1000 μM, 100 μM, 10 μM, or 1 μM) during 4 h under hypoxia. (**A**) Determination of the expression of SOD1 and SOD2 was carried out by Western blotting, employing specific antibodies. The blots show the effect of melatonin on the expression of these proteins. The levels of β-actin were used as controls to ensure equal loading of proteins. (**B**,**C**) The graphs show the quantification of protein expression. A horizontal dashed line represents the value observed in cells incubated in the absence of melatonin (non-treated). Values are the mean ± S.E.M. of normalized values expressed as % with respect to non-treated cells

(incubated under hypoxia and in the absence of melatonin). (**D**,**E**) Detection of mRNA levels of SOD1 and SOD2 was carried out by RT-qPCR analysis. The bars show the fold increase of mRNA levels ± S.E.M. of each protein relative to non-treated cells (incubated under hypoxia and in the absence of melatonin). *Gapdh* mRNA was used for normalization. A horizontal dashed line represents the value observed in non-treated cells. Results are representative of three to four independent experiments (n.s., non-treated; Mel, melatonin; *, $p < 0.05$; and **, $p < 0.01$ vs. non-treated cells in hypoxia).

3.4. Effect of Melatonin on Glutathione and on Total Antioxidant Capacity (TAC)

Having evaluated the effect of melatonin on the above-mentioned antioxidant defenses, it was of interest to analyze the TAC of PSC after treatment with melatonin under hypoxia. The TAC was increased in cells incubated in the presence melatonin, in comparison with that noted in cells incubated in its absence (non-treated cells). The highest values were achieved with 1000 μM and 100 μM melatonin (Figure 4A).

The next part of the experiments was directed to study the effect of melatonin on glutathione. We detected a decrease in the ratio of GSH/GSSG in cells treated with 1000 μM melatonin. On the contrary, GSH/GSSG was increased in cells treated with 10 μM or 1 μM melatonin, in comparison with cells incubated in its absence (non-treated cells; Figure 4B).

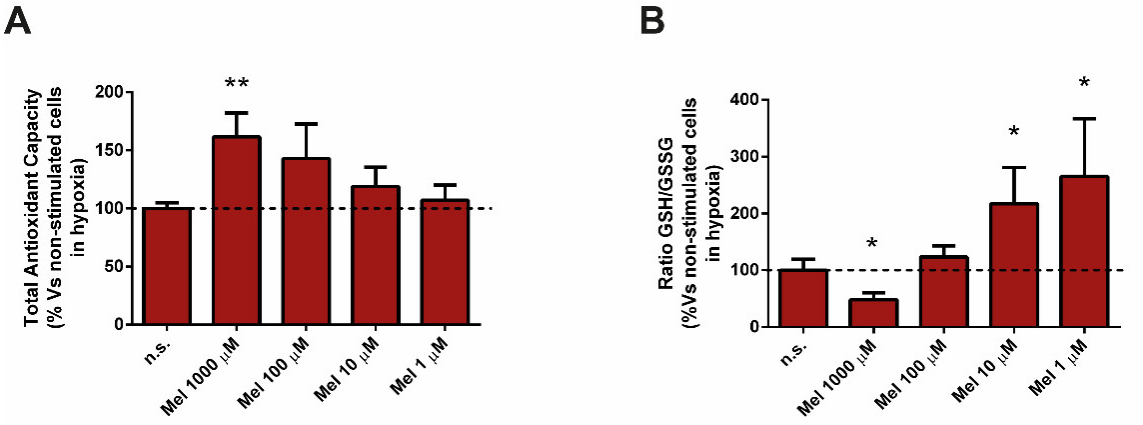

Figure 4. Effect of melatonin on glutathione and on total antioxidant capacity. PSC were incubated with melatonin (1000 μM, 100 μM, 10 μM, or 1 μM) under hypoxia during 4 h. (**A**) Total antioxidant capacity (TAC) of the cells was determined. (**B**) The ratio of GSH/GSSG was studied. Values are the mean ± S.E.M. of normalized values expressed as % with respect to cells incubated under hypoxia, but in the absence of melatonin (non-treated cells). A horizontal dashed line represents the value observed in non-treated cells. Data are representative of five independent experiments (n.s., non-treated cells; Mel, melatonin; H_2O_2, hydrogen peroxide; *, $p < 0.05$; and **, $p < 0.01$ vs. non-treated cells in hypoxia).

3.5. Involvement of Protein Kinase C in Melatonin-Induced Changes in Nrf2-Related Antioxidant Enzymes and in SOD

At this stage, we were interested in analyzing the dependency on PKC of melatonin-evoked changes in the antioxidant enzymes that we had observed. Thus, cells were pre-incubated in the presence of the PKC inhibitor Ro-31-8220 (5 μM), prior to addition of melatonin. Upon addition of melatonin, and in the presence of Ro-31-8220, cells were further incubated during 4 h under hypoxia. The inhibition of PKC led to a decrease in the phosphorylation of Nrf2 and in the detection of SOD1 and SOD2, i.e., PKC inhibition abolished the increases induced by melatonin that we had noted when cells were incubated with melatonin alone (Figure 5).

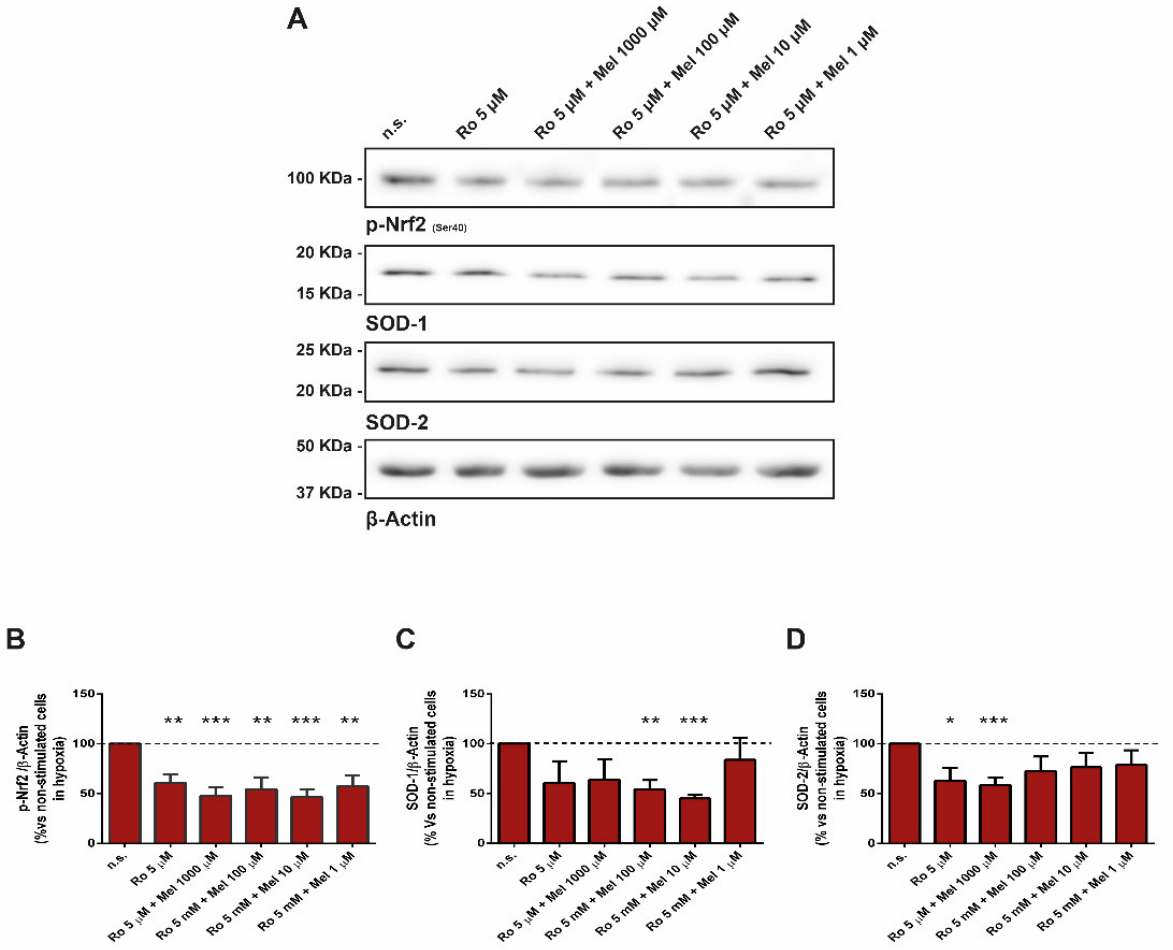

Figure 5. Effect of PKC inhibition on melatonin-evoked changes in Nrf2 and SOD. PSCs were incubated during 5 min in the presence of the PKC inhibitor Ro-31-8220 (5 μM) under hypoxia. Thereafter, melatonin (1000 μM, 100 μM, 10 μM, or 1 μM) was added to the incubation medium and cells were incubated for further 4 h under hypoxia. (**A**) Western blotting analysis was carried out to detect the phosphorylation of Nrf2 and the expression of SOD1 and SOD2 in cells treated with the PKC inhibitor. The levels of β-actin were employed as controls to ensure equal loading of proteins. The blots show the effect of melatonin on protein expression. (**B**–**D**) The graphs show the quantification of Nrf2 phosphorylation and of SOD1 and SOD2 expression. A horizontal dashed line represents the value observed in non-treated cells (incubated in the absence of melatonin). Values are the mean ± S.E.M. of normalized values expressed as % with respect to non-treated cells. Results are representative of four independent experiments (n.s., non-treated cells; Mel, melatonin; Ro, Ro-31-8220; *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ vs. non-treated cells in hypoxia).

3.6. Effect of Melatonin on Cell Viability

Bearing in mind the increase in ROS production that we had detected in the presence of the high (micro to millimolar) concentrations of melatonin, we were interested in evaluating the effect of the indolamine on the viability of PSC subjected to hypoxia. For this purpose, PSC were incubated under hypoxia during 48 h in the absence (non-treated cells) or in the presence of melatonin. Separate batches of cells were incubated in the presence of Tps (1 μM) alone, which served as control for cell death. A decrease of cell viability was noted with the higher concentrations of melatonin tested (1000 μM and 100 μM). The drop in cell viability (around 14%) was stronger in cells incubated with 1000 μM melatonin. A drop of approximately 5% in cell viability was noted in cells incubated with 100 μM melatonin. Nevertheless, the effect on cell viability was negligible in the presence of 10 μM or 1 μM melatonin. As expected, incubation of PSC in the presence of Tps alone induced a decrease of cell viability, which dropped by 35% (Figure 6).

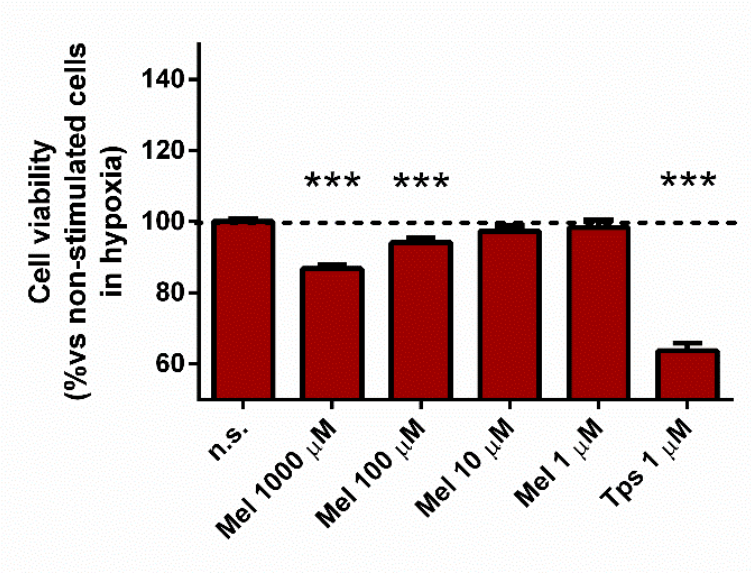

Figure 6. Effect of melatonin on cell viability. Cells were incubated during 48 h under hypoxia and in the presence of different concentrations of melatonin (1000 μM, 100 μM, 10 μM, or 1 μM). Viability was compared with that of cells incubated in the absence of melatonin (non-treated cells). Tps (1 μM) was used as control of cell death. In the graphs, a dashed line represents the viability of non-treated cells. Data are representative of five independent experiments (n.s., non-treated cells; Mel, melatonin; Tps, thapsigargin; ***, $p < 0.001$ vs. non-treated cells).

3.7. Effect of Melatonin on Pivotal Members of Inflammation

To investigate whether melatonin modulates major regulators of the intracellular inflammation pathways in PSC, we carried out the next set of experiments. Western blotting was used to detect the phosphorylation of NF-kB and of the regulatory protein nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-alpha (IκBα). The expression of cyclooxygenase-2 (COX-2) also was analyzed by Western blotting. In the presence of melatonin, a decrease in the phosphorylation of NF-kB was observed, compared with non-treated cells (incubated under hypoxia and in the absence of melatonin). The effect was stronger at the concentration of 1000 μM melatonin (Figure 7A,B). Accordingly, a decrease in the phosphorylation of IκBα was detected (Figure 7A,C).

In a separate set of experiments, we assayed the effect of melatonin on COX-2 expression. In the presence of melatonin, a decrease in the detection of COX-2 was noted, compared with non-treated cells (incubated in the absence of melatonin but under hypoxia). Similar to what had been observed with NF-kB and IκBα, the stronger effect was achieved with the concentration of 1000 μM melatonin (Figure 7A,D).

In the next set of experiments, we evaluated the effect of melatonin on the expression of two major pro-inflammatory interleukins, interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). For this purpose, PSC were incubated during 24 h under hypoxia and in the presence of melatonin. Separate batches of cells were incubated in the absence of melatonin and under hypoxia (non-treated cells). The mRNA levels of IL-6 and TNF-α were studied by RT-qPCR. The analysis of the samples revealed a decrease in the mRNA levels of IL-6 in cells treated with 1000 μM melatonin, in comparison with non-treated cells (Figure 7E). With respect to TNF-α, a decrease in the mRNA levels was noted in cells incubated with 1000 μM and 100 μM melatonin, in comparison with non-treated cells (Figure 7F).

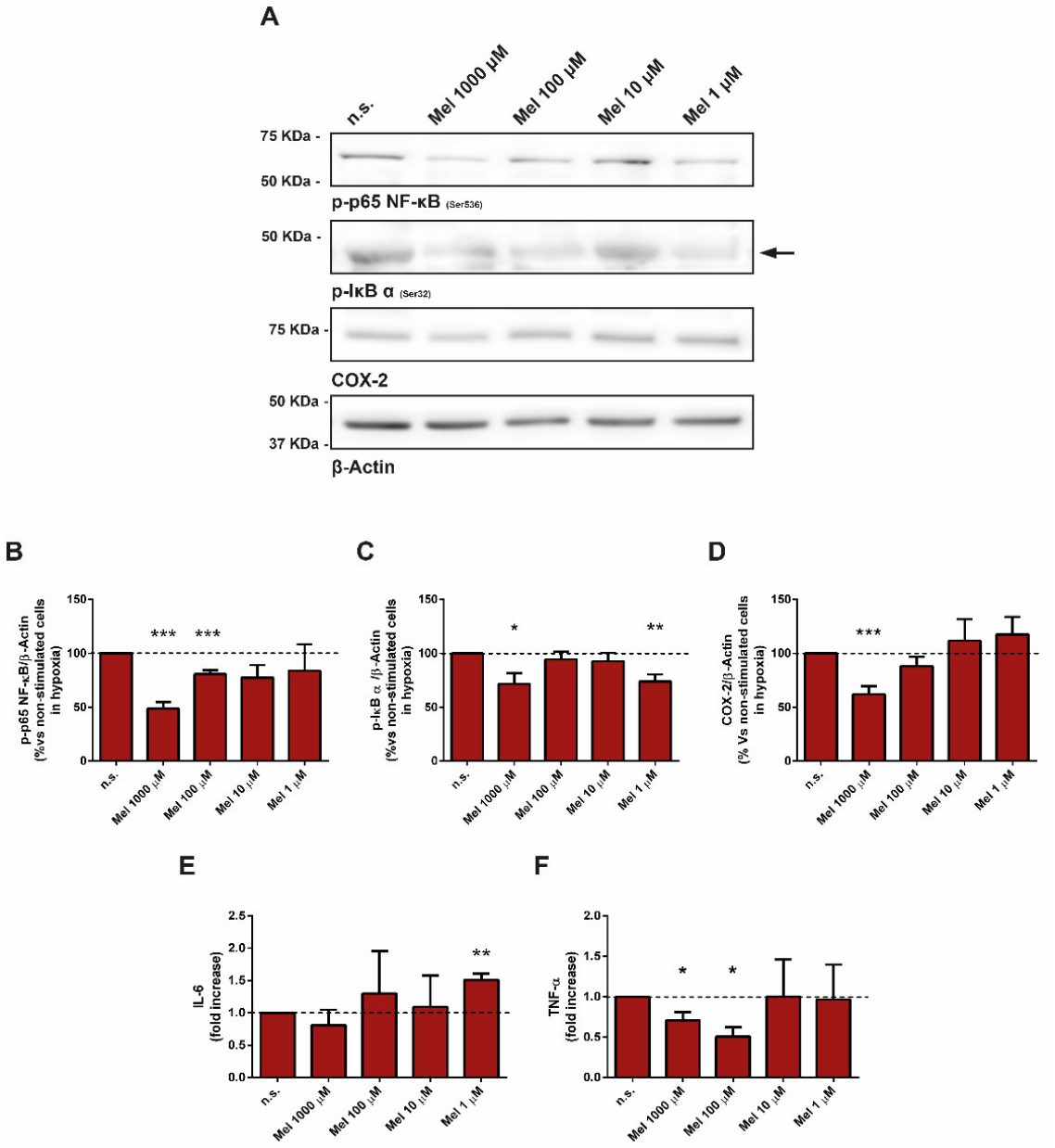

Figure 7. Effect of melatonin on major regulators of inflammation. PSC were incubated with melatonin (1000 μM, 100 μM, 10 μM, or 1 μM) for 4 h under hypoxia. (**A**) Blots showing the effect of melatonin on the phosphorylation state of NF-kB and of IκBα. The expression of COX-2 also was analyzed by Western blotting. The levels of β-actin were employed as controls to ensure equal loading of proteins. The arrow indicates the specific band of IκBα. (**B–C**) The bars show the quantification of protein phosphorylation. (**D**) The bars show the quantification of COX-2 detection. Results are the mean ± S.E.M. of normalized values, expressed as % with respect to non-treated cells. Four independent experiments were carried out. (**E**,**F**) RT-qPCR analysis was used to detect the expression of interleuquin-6 (IL-6) and of tumor necrosis factor-α (TNF-α). The bars show the fold increase of mRNA levels ± S.E.M. of each protein relative to non-treated cells (incubated under hypoxia, but in the absence of melatonin). Three different preparations were used. A horizontal dashed line represents the value observed in non-treated cells (incubated under hypoxia but in the absence of melatonin) (n.s., non-treated cells; Mel, melatonin; *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ vs. non-treated cells in hypoxia).

4. Discussion

Hypoxia is a condition that develops in the tumor microenvironment. It is a consequence of the uncontrolled proliferation of cells within the malignant tissue [5]. Hypoxia has also been related with inflammation and damage to the pancreas [32].

PSC participate in the growth and progression of pancreatic cancer [3,4]. Additionally, PSC might participate in inflammation-associated carcinogenesis [33]. Together with

tumor cells, PSC will also be subjected to the low availability of O_2 existing within the tumor. In order to survive, all cell types included in the tumor will have to adapt to these conditions. In general, the cells present in the mass are able to proliferate under hypoxia and, therefore, contribute to the growth of the cancerous tissue [8].

It is now well accepted that PSC participate in a critical manner in the development of the fibrosis that accompanies the diseases affecting the pancreas [34–36]. Moreover, fibrosis and inflammation both contribute to the creation of a microenvironment that allows tumor growth [1]. In this line, the fibrotic tissue within the tumor represents a major target in the treatment of cancer [25] and inflammation [1].

Melatonin reduced the viability of different types of cancer cells [37–39], including pancreatic cancer cells [30,40]. Melatonin also diminished the viability of PSC under normoxic conditions [22,41]. In addition, melatonin exerted anti-inflammatory actions in the pancreas [42]. Therefore, it has been postulated that treatment with melatonin might be a promising therapy for the diseases that affect the pancreas.

In the present work, we have studied the early events that occur in PSC when subjected to pharmacological concentrations of melatonin under hypoxia. We have shown that, in the presence of melatonin, PSC exhibited an initial production of ROS that might lead to the modulation of their antioxidant responses. This would protect the cells against the putative pro-oxidant conditions that we have recently related with the increased proliferation of PSC under hypoxia [7]. Consequently, these changes could influence their viability. Moreover, melatonin modulated the expression of major proteins that are involved in inflammation. This is of major interest, because it has been suggested that the microenvironment has the ability to influence tumor cells behavior. Moreover, it has been proposed that modulation of the physiology of cells that make up the stroma, rather than their elimination, might be effective in the treatment of inflammation and cancer [43].

Former works have suggested that pharmacological concentrations of melatonin (micromolar to millimolar range) induced the generation of ROS in different cellular types, including the pancreas. This effect has been related with a putative modulation of cell viability, majorly of tumor cells [30,44–47]. In a former work, we showed that melatonin stimulated ROS production in the cytosol in PSC under normoxia [29]. Additionally, previous results of our laboratory showed that hypoxia induced a prooxidant environment in PSC, without detection of cytosolic ROS generation [7]. Our results have shown that PSC, incubated under hypoxia, exhibited a concentration-dependent increase in ROS production in the presence of melatonin. The effect was stronger at the highest concentration of melatonin used (1000 μM). The ratio of GSH/GSSG, another marker of oxidative state, also was influenced by melatonin treatment in a concentration-dependent manner. A decrease in the availability of GSH was noted in the presence of the higher concentration of melatonin tested. Consistent bibliography reports that melatonin stimulates ROS production and decreases GSH in other cellular models, as we have shown in our work [48–53]. On the contrary, GSH/GSSG was increased in cells treated with 10 μM or 1 μM melatonin. Glutathione system plays a pivotal role in the mechanisms that control the oxidative state in the cell, and it is actively consumed in the presence of a pro-oxidant context [54]. In this line, melatonin might behave as a two-faced molecule that, depending on its concentration, could induce pro-oxidant or antioxidant effects. It has also been suggested that different cells may respond to the same concentrations of melatonin differently. In this line, the evidence suggests that the pro-oxidant action of melatonin is not necessarily correlated with cytotoxicity, which is concentration dependent as well as cell type dependent. Thus, how the cells manage melatonin-evoked ROS production will determine cell fate: Survival or death. This also might depend on the cell type and the context [47]. Indeed, previous works have reported pro-oxidant actions of melatonin that could be the basis of its antiproliferative effects in cancer cells [30,55,56]. Conversely, melatonin protected pancreatic acinar cells by increasing their antioxidant defenses [15].

Our results have also shown that, despite the increase observed in ROS generation in response to melatonin, the oxidation of lipids and proteins was kept to a low level. Similar

observations were obtained by Orhan et al. [57], who showed that menadione induced the oxidation of fluorescent probes, whereas no increase in the formation of protein oxidation products was observed. Therefore, upon generation or addition of ROS, it might not be necessary to detect oxidation of lipids or proteins in all cell types, despite the fluorescent probes report changes in ROS production. The final consequence will depend on how the cells manage this situation and whether the cells are able to set up antioxidant responses that will cope with the pro-oxidant condition created by a certain stimulus or drug.

In a previous work, we showed that PSC subjected to hypoxia did not exhibit cytosolic ROS production, whereas oxidation of lipids and proteins were detected. Interestingly, under these conditions, PSCs proliferated actively [7]. In the present work, the absence of protein and lipid oxidation could be explained on the basis of a stimulation of the antioxidant responses by melatonin, which would protect cellular structures against oxidation caused by hypoxia.

The transcription factor Nrf2 is an important mediator of the antioxidant response [58]. Its activation is related with the expression of several antioxidant and phase II enzymes, which play pivotal roles in the regulation of the redox status in the cell [59]. As we had observed increases in ROS generation in cells treated with melatonin, which were not accompanied by increases in the oxidation of lipids nor proteins, we examined the effect of melatonin on the antioxidant transcription factor Nrf2 and related antioxidant enzymes, in search of a putative potentiated antioxidant response in the presence of melatonin. In this regard, our results have shown that the phosphorylation of Nrf2 was increased by melatonin treatment. Concomitantly, the levels of the antioxidant enzymes GCLc, catalase, NQO1, and HO1 also were increased. It is well known that Nrf2 is regulated by the red-ox state of the cell [58]. Therefore, the resulting red-ox changes induced by melatonin could be responsible for the activation of the Nrf2-dependent pathway and the increase in the levels of the related antioxidant enzymes that we have noted.

In addition to the above-mentioned antioxidant enzymes, there are other antioxidant systems that control the oxidative state within the cell. SOD enzymes represent an additional set of antioxidant elements that provide defense against free radicals [60]. SOD1 (Cu/Zn SOD) is localized within the cytosol, whereas SOD2 (MnSOD) is localized to the mitochondria [61,62]. In a recent work, we have shown that PSC subjected to hypoxia exhibited an increase in the expression of SOD1 and SOD2 [7]. Thus, it was of interest to study the effect of melatonin on the expression of SOD. In this set of experiments, similar results to those obtained with Nrf2 were observed, i.e., melatonin increased the expression of SOD1 and SOD2. Interestingly, a previous work showed that treatment of PSC with melatonin under normoxic conditions reduced the expression of these key enzymes, which are critical for the detoxification of ROS. This could explain why protein oxidation in PSC treated with melatonin was observed under normoxia, but not under hypoxia [29].

In the presence of melatonin, an increase in TAC was noted. This could be related with the increases in the expression of Nrf2-regulated antioxidant enzymes and of SOD that we have observed. Additionally, we cannot discard that the inherent antioxidant properties of melatonin could be contributing to the increase in TAC that we have noted. Altogether, our results suggest that treatment of PSC subjected to hypoxia with melatonin reinforced their antioxidant defenses.

We have additionally shown that PKC is involved in the actions of melatonin to modulate the expression of antioxidant enzymes under hypoxia. Its inhibition resulted in a decrease in the phosphorylation of Nrf2 and in the detection of SOD1 and SOD2. The involvement of PKC in the antioxidant responses evoked by melatonin in pancreatic acinar cells has been shown previously by our group [15]. Our results provide evidence for the mechanistic action of melatonin to modulate the antioxidant responses in the exocrine pancreas. We cannot exclude that other pathways could be activated, which might lead to an increase in the expression of these antioxidant enzymes [63].

Hypoxia has been signaled as a stimulus for cell proliferation within the pancreatic tumor microenvironment, including PSC [41]. Interestingly, melatonin decreases the

viability of pancreatic cancer cells [30] and also of PSC treated under normoxia [22]. We could hypothesize that a certain level of unresolved oxidative stress might be a reason by which PSC are committed to the development of potential fibrosis in the pancreas. We have previously shown that PSC exhibited a pro-oxidant status under hypoxia, which probably led to increases in the expression of Nrf2-dependent antioxidant enzymes and of SOD. However, the activation of these critical antioxidant elements might not be enough to resolve the pro-oxidant conditions created by hypoxia and might result in the activation and proliferation of PSC [7]. In fact, PSC subjected to hypoxia alone exhibited a decrease in TAC in comparison with that noted in cells incubated under normoxia [7]. This might be the reason why, despite the hostile pro-oxidative environment created by hypoxia, PSC exhibited an increase in their proliferation. In the present case, a reinforced increase of the antioxidant defenses by melatonin could tend to counteract the production of ROS in PSC incubated under hypoxia. As a consequence, the higher level of antioxidant enzymes that is achieved in the presence of melatonin could resolve the oxidative stress induced by hypoxia. Under these circumstances, PSC could be subjected to less stress and this could decrease their proliferation (Figure 8). This is a major finding that suggests that melatonin might act as a tuner of PSC viability, avoiding an impairment of their physiology under hypoxia that could lead to uncontrolled proliferation and a concomitant fibrosis within the pancreas.

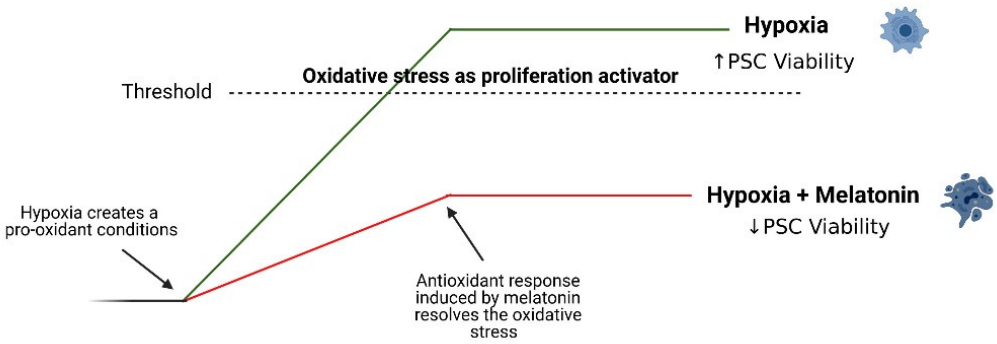

Figure 8. Potential involvement of oxidative stress-related cellular responses in melatonin effect on PSCs physiology. The hostile pro-oxidative environment created by hypoxia could induce a certain level of unresolved oxidative, which might be responsible for the development of potential fibrosis in the pancreas (green trace). On its side, melatonin reinforces the antioxidant defenses that could tend to counteract the production of ROS that we have detected under hypoxia. The consequent higher level of antioxidant enzymes that is achieved could resolve the oxidative stress induced by hypoxia and could decrease the development of fibrosis (red trace). Figure created with BioRender software (BioRender.com accessed on 1 February 2021).

The last part of our research was directed to study the effect of melatonin on putative inflammatory responses settled under hypoxia. NF-kB signaling is involved in inflammation, fibrogenesis, and cancer development [64,65]. Generally, NF-kB complexes are present in an inactive form in the cytoplasm, where they are repressed by inhibitor proteins like IκBα. Phosphorylation of the latter will allow the activation of NF-kB, which will stimulate the transcription of proinflammatory mediators [66]. On its side, IL-6 and TNF-α are important players of the inflammatory response [67,68]. It has been suggested that melatonin has a beneficial therapeutic value in the treatment of inflammation in different tissues [69,70], including the pancreas [71]. Our present results are in agreement with

those previous observations and have shown that melatonin modulated the levels of pro-inflammatory mediators in PSC subjected to hypoxia. This was reflected as a decrease in the activation of NF-kB, and decreases in the expression of COX-2 and in the transcription on IL-6 and TNF-α. Previous findings reported the involvement of NF-kB and of COX-2 in the inflammatory responses of the pancreas that were observed under simulated hypoxia [72]. The connection between oxidative stress and pancreatitis has been documented [73]. Additionally, inflammation has been related with PSC activation and the development of pancreatic fibrosis [74]. Moreover, it has been suggested that melatonin exhibits anti-inflammatory actions in the pancreas [71]. Our results are thus in agreement with these previous observations and support an anti-inflammatory role for melatonin in PSC under hypoxic conditions.

Considering that melatonin induced a slight drop in PSC proliferation and a decrease in the secretion of pro-inflammatory cytokines, together with the decrease in the levels of TBARS and of carbonyls, and the increase in TAC that we have noted, we could argue that melatonin could lead the cells to a somehow lower activated state, but without reaching complete quiescence. We would like to mention that the process of activation of PSC is very complex and multifaceted. Not all drugs induce changes in all parameters of activation. In the study by Estaras et al. [7], we showed that cell viability increased by 20% in comparison with that of cells incubated in normoxia. Furthermore, the levels of TBARS were increased by 104% and those of carbonyls increased by 67% with respect to the levels detected in cells incubated in normoxia. Additionally, a drop in TAC of 32% was noted. These observations are interesting because our present results are contrary to those observed in PSC subjected to hypoxia only [7] and, hence, might underlie putative pro-quiescency effects of melatonin.

The concentrations of melatonin that we have used in this study could be considered rather pharmacological than physiological, because they are higher than those found normally in blood. Nevertheless, the levels of melatonin found in blood do not necessarily indicate the concentrations of melatonin present in the extracellular space that is immediately close to the cells. This is because it has been suggested that the levels of melatonin in vivo in various body fluids and cells are not necessarily in equilibrium with those detected in blood. In addition, it has been proven that there are tissues that synthesize melatonin, which will act locally, e.g., as an autocrine or paracrine agent. Therefore, values of melatonin that are several orders of magnitude higher than the level found in blood have been found in these tissues [74–78]. Consequently, the levels of melatonin in blood cannot be strictly used to define physiological concentrations, because the local concentrations of melatonin are not defined yet.

5. Conclusions

In summary, pharmacological concentrations of melatonin evoked an increase in the production of ROS and a decrease in GSH in PSC subjected to hypoxia. Melatonin treatment led to the activation of antioxidant responses, which involved the expression of Nrf2-regulated antioxidant enzymes and of SOD, and allowed the cells to control the putative pro-oxidant conditions induced by hypoxia. PKC was involved in these responses. Treatment of cells with concentrations of melatonin in the range of millimolar and high micromolar diminished viability of PSC without inducing a massive cell death. In addition, melatonin reduced the activation of the inflammatory pathway and reduced the expression of cytokines (Figure 9). Bearing in mind the pivotal role of PSC in the fibrotic reaction that contributes to survival and development of transformed epithelia within the pancreas, the conditions created by melatonin might restrain PSC proliferation to a certain level to control the fibrotic processes that can evolve under hypoxia. Our results are in line with strategies directed to controlling the growth of fibrotic tissue within tumors, which might help in the treatment of cancer. Additionally, our findings support a probable mechanism by which melatonin modulates fibrosis within the pancreas. Moreover, we have to highlight that the actions of melatonin might be cell- and context-dependent.

Additional work will have to be carried out to investigate whether there are other metabolic pathways involved in the antiproliferative and/or antifibrotic actions of melatonin in the pancreas. Finally, in vivo studies are needed in order to corroborate the antifibrotic effects of melatonin that we have observed in vitro.

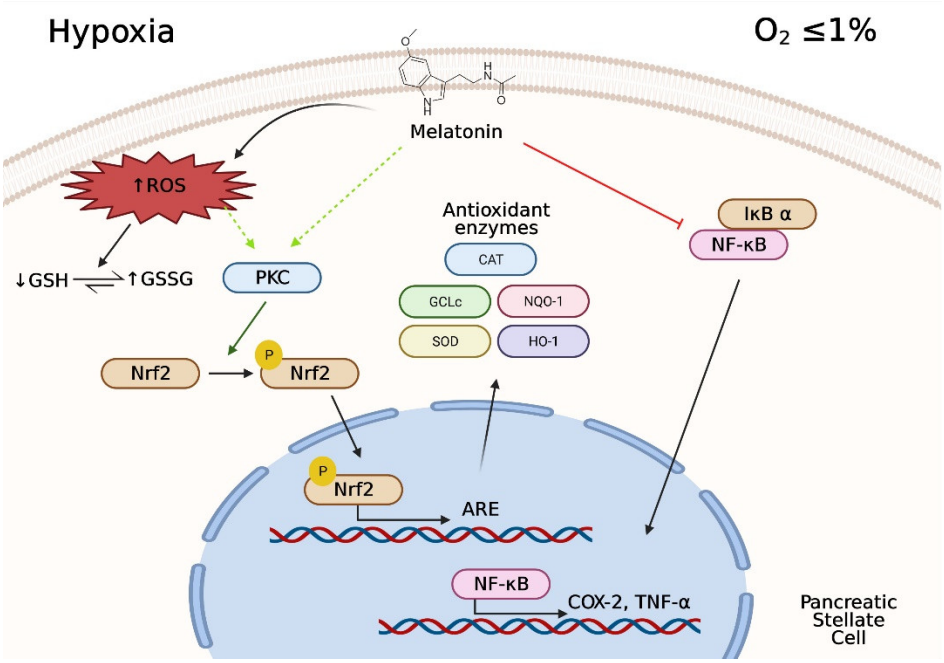

Figure 9. Summary of the major actions of melatonin to modulate PSC responses to hypoxia. The hostile pro-oxidative environment created by hypoxia is modulated by melatonin that, by terms of modulating the antioxidant response element, will lead to diminished oxidative stress. The actions of melatonin also are directed to diminish the inflammatory response settled under hypoxia. These conditions could account for an antifibrotic action of melatonin. Figure created with BioRender software (BioRender.com accessed on 1 February 2021).

Supplementary Materials: The following are available online at www.mdpi.com/2076-3921/10/4/577/s1, Figure S1. Detection of specific markers for activated PSC by confocal microscopy.

Author Contributions: A.G.—Designed the study and wrote the manuscript. M.E. (Matias Estaras), M.R.G.-P., R.M., A.G., M.E. (Mario Estevez)—Acquisition and data analysis, interpreted and discussed results, reviewed and edited the manuscript. V.R.—Interpreted and discussed results, reviewed and edited the manuscript. M.F.-B., J.M.M., D.V., G.B.-F., D.L.-G.—Suggested parts of the manuscript, reviewed and edited the manuscript. G.M.S.—Suggested discussion parts, reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data are available from the corresponding author upon reasonably request.

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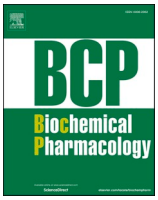

Melatonin modulates metabolic adaptation of pancreatic stellate cells subjected to hypoxia

Matias Estaras [a], Remigio Martinez [c], Alfredo Garcia [d], Candido Ortiz-Placin [a], Juan L. Iovanna [b], Patricia Santofimia-Castaño [b], Antonio Gonzalez [a,*]

[a] Instituto de Biomarcadores de Patologías Moleculares, Departamento de Fisiología, Universidad de Extremadura, Cáceres, España
[b] Centre de Recherche en Cancérologie de Marseille, INSERM U1068, CNRS UMR 7258, Aix-Marseille Université and Institut Paoli-Calmettes, Parc Scientifique et Technologique de Luminy, Marseille, France
[c] Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad de Extremadura, Cáceres, España
[d] Departamento de Producción Animal, CICYTEX-La Orden, Guadajira, Badajoz, España

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ABSTRACT

Pancreatic stellate cells (PSCs), the main cell type responsible for the development of fibrosis in pancreatic cancer, proliferate actively under hypoxia. Melatonin has received attention as a potential antifibrotic agent due to its anti-proliferative actions on PSCs. In this work, we investigated the activation of the PI3K/Akt/mTOR pathway and the metabolic adaptations that PSCs undergo under hypoxic conditions, as well as the probable modulation by melatonin. Incubation of cells under hypoxia induced an increase in cell proliferation, and in the expression of alpha-smooth muscle actin and of collagen type 1. In addition, an increase in the phosphorylation of Akt was observed, whereas a decrease in the phosphorylation of PTEN and GSK-3b was noted. The phosphorylation of mTOR and its substrate p70 S6K was decreased under hypoxia. Treatment of PSCs with melatonin under hypoxia diminished cell proliferation, the levels of alpha-smooth muscle actin and of collagen type 1, the phosphorylation of Akt and increased phosphorylation of mTOR. Mitochondrial activity decreased in PSCs under hypoxia. A glycolytic shift was observed. Melatonin further decreased mitochondrial activity. Under hypoxia, no increase in autophagic flux was noted. However, melatonin treatment induced autophagy activation. Nevertheless, inhibition of this process did not induce detectable changes in the viability of cells treated with melatonin. We conclude that PSCs undergo metabolic adaptation under hypoxia that might help them survive and that pharmacological concentrations of melatonin modulate cell responses to hypoxia. Our results contribute to the knowledge of the mechanisms by which melatonin could modulate fibrosis within the pancreas.

1. Introduction

Pancreatic cancer is one of the poorest prognosis tumors with a 5-years survival rate under 10 % [1,2]. In the recent years, the number of studies about the role of tumor microenvironment (TME) on pancreatic cancer has grown significantly. Pancreatic stellate cells (PSCs) are among the elements contributing to this TME [3]. PSCs represent a low percentage of the total cell population of the pancreatic tissue under normal physiological conditions (approximately 4–7%). These cells are preferentially placed around the acinar cells, the

Abbreviations: α-sma, alpha-smooth muscle actin; Akt, protein kinase B; AMPK, AMP-activated protein kinase; 2-DG, 2-deoxyglucose; DMEM, Dulbecco's Modified Eagle Medium; ECAR, extracellular acidification rate; FBS, fetal bovine serum; FCCP, carbonylcyanide p-(trifluoro-methoxy)phenylhydrazone; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLUT-1, glucose transporter-1; GSK3β, glycogen synthase kinase 3 beta; HBSS, Hank's balanced salts; HIF, hypoxia-inducible factor; Hpx, hypoxia; JNK, c-Jun N-terminal kinase; LDH, lactate dehydrogenase; MAPKs, mitogen-activated protein kinases; MMP, matrix metalloproteinase; 3-MA, 3-methyladenine; mTOR, mammalian target of rapamycin and mechanistic target of rapamycin; NMX, normoxia; OCR, oxygen consumption rate; OxPhos, mitochondrial oxidative phosphorylation; PFK, phosphofructokinase; p44/42, extracellular signal-regulated kinase 1/2; P70S6K, ribosomal protein S6 kinase beta-1; PTEN, phosphatase and tensin homolog; PSC, pancreatic stellate cells; RT-qPCR, quantitative reverse transcription-polymerase chain reaction; TMRM, tetramethyl rhodamine.

* Corresponding author at: Instituto de Biomarcadores de Patologías Moleculares, Departamento de Fisiología, Universidad de Extremadura, Avenida de las Ciencias s/n, E-10003, Cáceres, España.
E-mail address: agmateos@unex.es (A. Gonzalez).

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pancreatic ducts and the microvasculature present in the gland. In the healthy pancreas, the stellate cells are in an inactive state, also called quiescent, and participate in the preservation of the architecture of the pancreatic tissue and in the regulation of components of the extracellular matrix [4,5]. However, under pathological conditions, activated PSCs are the major responsible for the development of fibrosis, which is based on the secretion and accumulation of extracellular matrix elements [6]. The consequence is the development of functional changes by which PSCs exhibit an increase in proliferation, in the production of extracellular matrix proteins and metalloproteases, and in the secretion of growth factors and cytokines, which retroactively increase the activation of more PSCs [7,8]. Thus, the control of the growth of fibrotic tissue within the mass of the tumor is of relevance in the treatment of pancreatic cancer.

Hypoxia, defined as low availability of oxygen (O_2), is a recurrent condition in the development of pancreatic tumors [9]. This ambient is created as a consequence of the rapid growth of cells inside the tumor mass, the concomitant changes in the architecture of the gland that avoids the development of micro-vascular structures and the encapsulation of cancerous areas by the desmoplastic reaction [10]. In this context, the phenotypic changes exhibited by cancer cells are well-documented [9,11,12,13]. Other cellular components comprising of the tumor must also undergo adaptation to this condition. In addition, cellular energy metabolism also undergoes adaptative changes that allow fast cellular proliferation and tumor growth [14]. Phosphatase and tensin homolog (PTEN) is involved in the development of many types of cancer. Down-regulation of such tumor suppressor protein has been related with colorectal cancer [15], liver cancer [16] and/or pancreatic cancer [17]. In addition, deregulation of PTEN/PI3K/AKT/mTOR signaling has been related with the onset and/or progression of malignancy [18,19]. Moreover, PTEN gene status depicts a major role in energy metabolism in cancer and, in particular, in mitochondrial oxidative phosphorylation (OxPhos) [20].

In a recent work, we have shown that PSCs exhibited changes in cell cycle regulation and in the antioxidant defenses under hypoxia [21]. However, it is not completely understood how hypoxia-activated PSCs survive and preserve a high proliferative status. In this line, studies on PSCs metabolic adaptation to hypoxia and, in particular, involvement of PTEN/PI3K/AKT/mTOR, needs study. Moreover, it would be interesting to find maneuvers to control proliferation of PSCs in order to manage fibrosis development.

Melatonin (N-acetyl-5-methoxytryptamine) is a multi-task molecule with anti-inflammatory, antioxidant and antitumoral actions [22]. Involvement of PI3K/AKT/mTOR in the actions of melatonin has been shown in liver cancer [23] or in gallbladder cancer [24]. Moreover, melatonin activation of tumor suppressor genes and attenuation of the expression of survival genes in cancer cells has been suggested [25,26].

In vitro models have shown that proliferation and survival of pancreatic cancer cells are diminished by treatment with pharmacological concentrations of melatonin [27,28]. Moreover, melatonin potentiates the effect of conventional chemotherapies in pancreatic cancer cells [29]. Nevertheless, the troubles derived from excessive fibrosis developing in growing tumors should be resolved to avoid the chemotherapy resistance. In this sense, melatonin might represent a beneficial therapeutical solution. Studies carried out on primary cultures of human and rodent PSCs revealed that melatonin reduced cell proliferation and viability [4,30], although the exact mechanisms involved in the actions of the indolamine remain to be elucidated. Moreover, whether melatonin targets PTEN and related cellular events in order to modulate metabolic adaptation of PSCs under hypoxia is unknown.

The fundamental premise of this study was that under pathological conditions, activated PSCs play a major role in the development of fibrosis. Interestingly, control of the growth of fibrotic tissue within the mass of the tumor is of relevance in the treatment of pancreatic cancer. In this research, we aimed at providing further insights into the adaptations exhibited by PSCs under hypoxia. For this purpose, we investigated the activation of signaling pathways involved in cell proliferation and the adaptation of glycolytic metabolism under conditions that mimic the low O_2 availability in the tumor mass. Additionally, we analyzed whether melatonin could reverse or reduce the changes observed, in order to be considered a useful tool for the treatment of fibrosis.

Table 1
List of primers used in the study.

Primer	Forward	Reverse
Glut-1c	5́-ATCCTTATTGCCCAGGTGTT-3́	5́-CAGAAGGGCAACAGGATACA-3́
Ldha	5́-GCAGGTGGTTGACAGTGCAT-3́	5́-ACCCGCCTAAGGTTCTTCAT-3́
Pfkp	5́-GACAAGATCCCCAAGAGCAA-3́	5́-AGCCGTCATAGATTGCGAAC-3́
Gapdh	5́-GGGTGTGAACCACGAGAAAT-3́	5́-CCTTCCACGATGCCAAAGTT-3́

RT-qPCR was performed for 30 min at 48 °C. PCR conditions were 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C plus 1 min at 55 °C using the primers listed above. The abundance of *Gapdh* mRNA in each sample was used for normalization (n = 3 independent experiments).

2. Materials and methods

2.1. Chemicals

Collagenase CLSPA was obtained from Worthington Biochemical Corporation (Labclinics, Madrid, Spain). Antimycin A, cell Lytic for cell lysis and protein solubilization, crystal violet, carbonylcyanide p-(trifluoro-methoxy)phenylhydrazone (FCCP), 2-deoxyglucose, 3-methyladenine, melatonin, oligomycin, rotenone, thapsigargin and Tween®-20 were obtained from Sigma Chemicals Co. (Madrid, Spain). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), Hank's balanced salts (HBSS), horse serum, medium 199 and SuperSignal™ West Pico PLUS Chemiluminescent Substrate were purchased from Invitrogen (Fisher Scientific Inc., Madrid, Spain). Polystyrene plates for cell culture and primers for RT-qPCR were purchased from Thermo Fisher Sci. (Madrid, Spain). L-glutamine and penicillin/streptomycin was obtained from BioWhittaker (Lonza, Basel, Switzerland). Bradford's reagent, Tris/glycine/SDS buffer (10×) and Tris/glycine buffer (10×) were from Bio-Rad (Madrid, Spain). U0126 were obtained from Tocris (Biogen Científica, Madrid, Spain). All other analytical grade chemicals used were obtained from Sigma Chemicals Co. (Madrid, Spain).

2.2. Preparation of cell cultures.

Cultures of pancreatic stellate cells (PSCs) were prepared following methods used in our laboratory. With this procedure activated PSCs are obtained [30]. *Wistar* rat pups (4–5 days after birth) were used in agreement with the protocols and guidelines approved by the Ethical Committee for Animal Research of the University of Extremadura (identification code 44/2016) and by the Institutional Committee of the Junta de Extremadura (identification code 20160810). The tests were carried out employing batches of cells obtained from different preparations.

2.3. Induction of hypoxia

Cells in culture were subjected to hypoxia (1% O_2), employing previously described methods [21]. Temperature (37 °C), humidity (95%) and air atmosphere (content of 1% O_2/5% CO_2/94 % N_2) were continuously controlled in an incubator chamber (Okolab; Izasa Scientific, Madrid, Spain).

2.4. Quantitative reverse transcription-polymerase chain reaction (RT-qPCR) analysis

Total RNA samples were purified using a commercially available kit

Table 2
Primary antibodies used in the study.

Antibody	Dilution	Supplier
B-Actin HRP-Conjugated	1:50000	Thermo Fisher
Beclin-1	1:1000	Santa Cruz Biotechnology
LC3 AB I-II	1:1000	Cell Signaling
p-Akt (Ser473)	1:2000	Cell Signaling
p-AMPKα (Thr172)	1:1000	Cell Signaling
p-GSK-3αβ (Tyr279/Tyr216)	1:1000	Santa Cruz Biotechnology
p-mTOR (Ser2448)	1:1000	Cell Signaling
p-PTEN (Ser380)	1:1000	Cell Signaling
p-p70 S6K (Thr389)	1:1000	Cell Signaling
p-p44/42 (Thr202/Tyr204)	1:2000	Cell Signaling
Collagen 1	1:500	Thermo Fisher
α-sma	1:1000	Thermo Fisher

List of primary antibodies used for detection of the desired protein. Western blotting and immunofluorescence analysis were used as described in Materials and methods section. The corresponding secondary HRP-conjugated or fluorescent-labeled specific antibody was employed. Thermo Fisher (Madrid, Spain); Santa Cruz Biotechnology (Quimigen S.L., Madrid, Spain); Cell Signaling (C-Viral, Madrid, Spain).

(Sigma, Madrid, Spain), utilizing the Power SYBR Green RNA-to-CTTM 1-Step kit (Applied Biosystems, Township, USA). RT-qPCR analysis were carried out as described previously [31]. The primers used are listed in Table 1. The relative mRNA levels were calculated and were expressed as the fold change between each sample and the calibrator.

2.5. Cellular viability and proliferation

The effect of the different treatments on cellular viability and proliferation was studied using Crystal Violet assay, as previously described [21]. Results are expressed in percentage as the mean ± S.E.M. (n) of the change of absorbance of each sample *vs* non-treated cells, which were batches of cells incubated in the absence drugs and under hypoxia (n is the number of independent experiments).

2.6. Western blotting analysis

Detection of protein expression and/or phosphorylation was carried out by Western blotting analysis, following methods used previously [32], with slight modifications. To detect proteins with high molecular weight, wet transfer was used. The primary antibodies employed were species-specific and are listed in Table 2. Quantification of the intensity of the bands was carried out employing Image J software (http://imagej.nih.gov/ij/). Graphics show the mean ± SEM of values expressed as % *vs* non-treated cells, which were batches of cells incubated in the absence drugs and under hypoxia.

2.7. XF cell mito stress test (OXPHOS measurement)

PSCs were seeded at a density of 30,000 cells per well in 24-well SeaHorse® plates. The next day, cells were incubated under hypoxia in the absence or in the presence of melatonin for 24 h. In a different plate, cells were cultured under normoxic condition for 24 h. Before the measurement of Oxygen Consumption Rate (OCR), culture medium was replaced by DMEM culture medium without phenol red, which was supplemented with 2 mM L-glutamine, 10 mM glucose and 1 mM pyruvate. Cells were then incubated at 37 °C in a non-CO_2 incubator for 1 h. OCR was measured under basal conditions and in response to 1 μM oligomycin, 1.5 μM carbonylcyanide p-(trifluoro-methoxy)phenylhydrazone (FCCP) or 0.5 μM rotenone plus 0.5 μM antimycin A. The OCR data are expressed as absolute values normalized with respect to the number of cells, which was checked with crystal violet staining.

2.8. XF glycolysis stress test (Glycolysis experiment)

PSCs were seeded at a density of 30,000 cells per well in 24-well SeaHorse® plates. The next day, cells were incubated under hypoxia in the absence or in the presence melatonin for 24 h. In a different plate, cells were cultured under normoxic condition for 24 h. Before the measurement of extracellular acidification rate (ECAR), culture medium was replaced by DMEM culture medium without phenol red, which was supplemented with 2 mM L-glutamine. Cells were then incubated at 37 °C in a non-CO_2 incubator for 1 h. ECAR was measured under basal conditions and in response to 10 mM glucose, 1 μM oligomycin, and 100 mM 2-Deoxyglucose (2-DG). Data are expressed as absolute values normalized with respect to the number of cells, which was checked with crystal violet staining.

2.9. Immunofluorescence and confocal microscopy studies

This procedure was carried out following previously described methods [33]. Briefly, the cells, grown on a glass cover slip, were subjected to the treatments. Afterwards, the cells were fixed with paraformaldehyde (4%) and permeabilized with Triton × 100 (0.1%). Cells were sequentially incubated with the specific primary antibody and, after washing, with the corresponding secondary antibody. The antibodies employed are listed in Table 2. For F-actin staining we have employed the dye Fluorescein Isothiocyanate Labeled (FITC)-phalloidin. Monitorization of antibody-derived fluorescence signal was performed employing a confocal laser system, Zeiss LSM 980, connected to an AxioObserver7 microscope (Carl Zeiss Iberia S.L., SSC Madrid, Spain). Cells were observed with a 25 × oil immersion objective. Fluorescence images of 256 × 256 pixels were recorded. The lines used were 488 nm for FITC-labeled phalloidin and 546 nm for Alexa 546-labeled antibody. The same conditions were used for acquisition of images of cells subjected to the treatments mentioned. Quantification of fluorescence was performed employing Image J software. The mean intensity of fluorescence was calculated, after subtraction of background fluorescence. Five to eight different cells were randomly chosen within three aleatory fields in 3 different preparations for each treatment. Data are expressed as absolute values of fluorescence. The absence of non-specific staining was assessed by processing the samples without primary antibody.

2.10. Statistical analysis

Statistics between individual treatments was performed employing the Student's *t* test. Additionally, one-way analysis of variance (ANOVA) followed by Tukey *post hoc* test was used. Only P values < 0.05 were considered statistically significant.

3. Results

3.1. Effects of melatonin on hypoxia-induced changes in fibrosis markers

Upon activation, PSCs contribute to the development of fibrosis. As a consequence, PSCs exhibit an increase in cell proliferation. Additional markers of PSCs activation is the expression of alpha-smooth muscle actin (α-sma) and the increase in collagen 1 deposition [6]. Therefore, we first studied cell viability and proliferation in PSCs. For this purpose, cells were incubated during 48 h under normoxia, under hypoxia or under hypoxia plus melatonin (Mel; 1 mM, 100 μM, 10 μM or 1 μM). Separate batches of cells were incubated in the presence of thapsigargin (Tps, 1 μM), a cell death inducer [34]. Viability was increased in PSCs subjected to hypoxia, compared with the values obtained in cells incubated in normoxia (Fig. 1A). When PSCs were incubated in the presence of melatonin and under hypoxia, a statistically significant drop of cell viability was noted, compared with that of cells incubated in the absence of melatonin and under hypoxia (Fig. 1A). In the presence of Tps a decrease in cell viability was observed (Fig. 1A). We next evaluated the

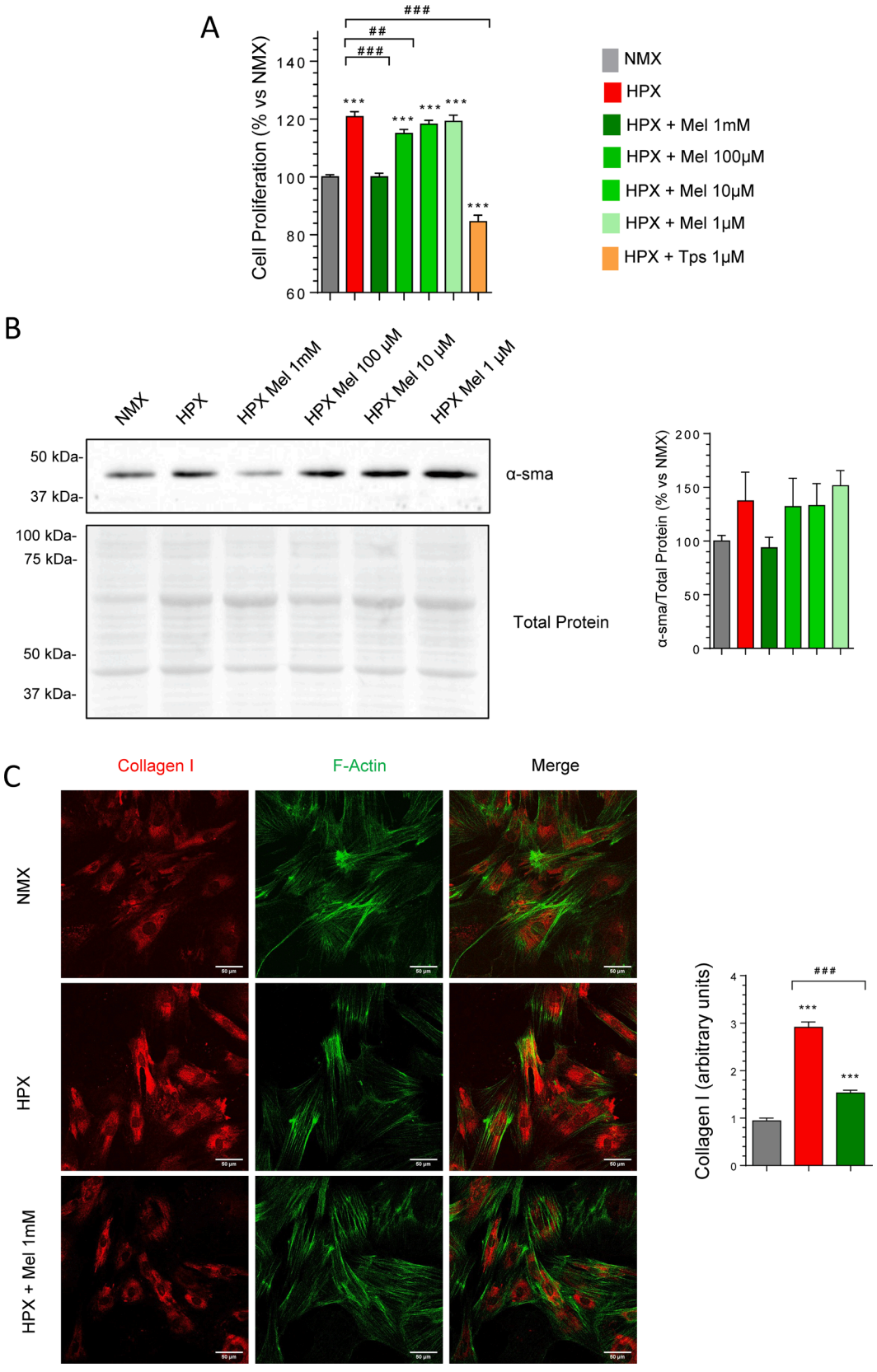

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Fig. 1. Study of fibrosis markers in PSCs subjected to hypoxia. Effect of melatonin. (A) Cells were incubated under normoxia (NMX), under hypoxia (HPX) or under hypoxia plus melatonin (1 mM, 100 μM, 10 μM or 1 μM), for 48 h and cell viability was analyzed. Separate batches of cells were incubated in the presence of 1 μM thapsigargin (Tps). Results are expressed in % as the mean ± S.E.M. (n) of cell viability for each treatment *vs* non-treated cells (incubated under normoxia or under hypoxia and in the absence of melatonin). Data are representative of three experiments (***, P < 0.001 *vs* non-treated cells under normoxia; ##, P < 0.01 *vs* non-treated cells under hypoxia; ###, P < 0.001 *vs* non-treated cells under hypoxia). (B) Separate batches of cells were incubated for 48 h under normoxia (NMX), under hypoxia (HPX) or under hypoxia plus melatonin (1 mM, 100 μM, 10 μM or 1 μM). The blots show the level of expression of alpha-smooth muscle actin (α-sma). The total protein levels were employed as controls to ensure equal loading of proteins. The bars show the quantification of protein detection for each treatment. Data show the mean ± S.E.M. of normalized values, expressed as % with respect to cells incubated in normoxia. Four independent experiments were carried out. C) Immunofluorescence studies showing the detection of collagen type 1 (red) and F-actin (green). Quantification of fluorescence is shown in bar chart. Data are expressed as absolute values of fluorescence. Data are representative of three different preparations (***, P < 0.001 *vs* non-treated cells under normoxia; ###, P < 0.001 *vs* non-treated cells under hypoxia).

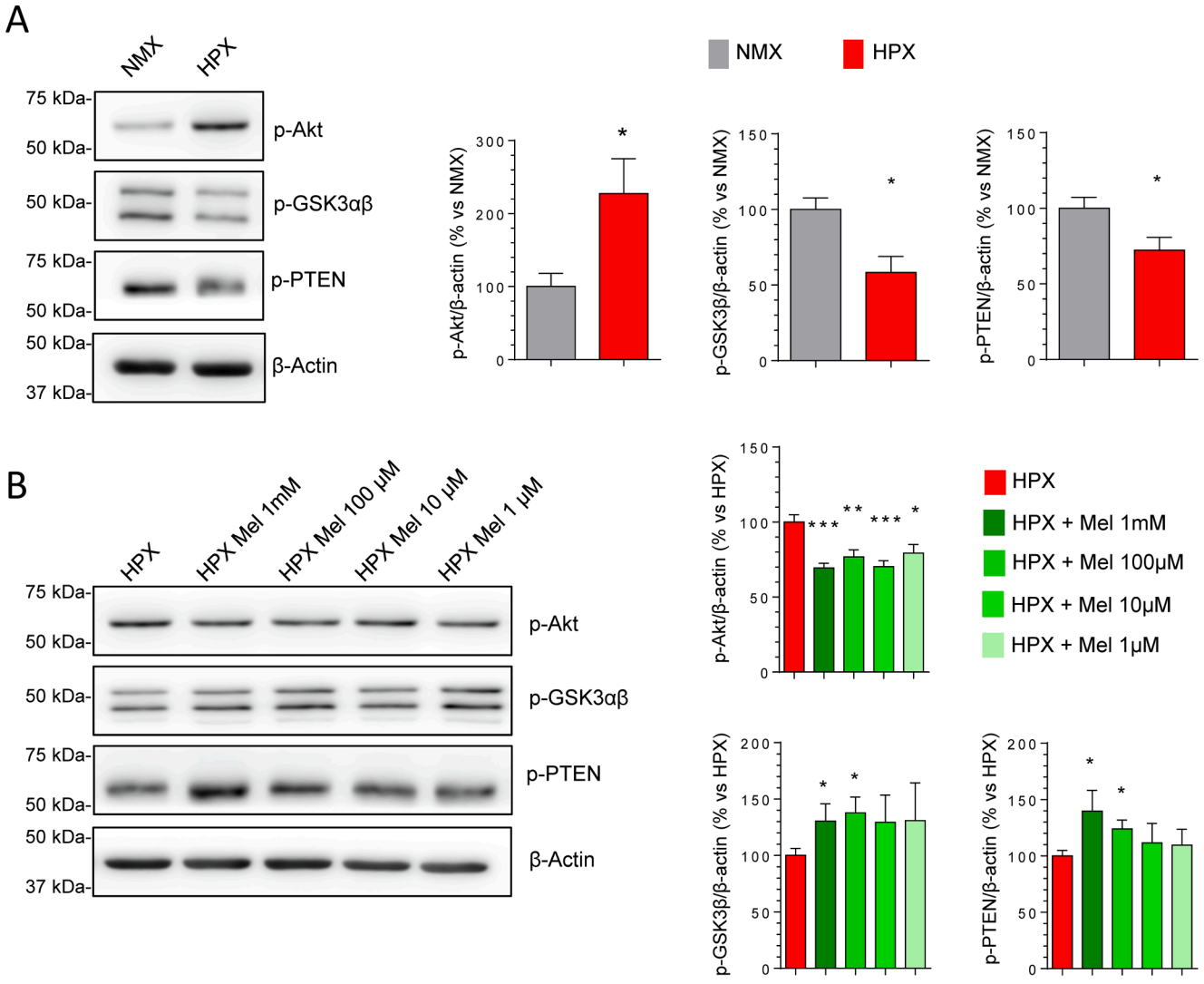

Fig. 2. Phosphorylation of Akt, GSK3 and PTEN in PSCs incubated under hypoxia. Effect of melatonin. (A) Separate batches of cells were incubated for 4 h under normoxia (NMX) or under hypoxia (HPX). The blots show the level of the phosphorylated state of Akt, GSK3 and PTEN. The levels of β-actin were employed as controls to ensure equal loading of proteins. The bars show the quantification of protein phosphorylation for each treatment. Data show the mean ± S.E.M. of normalized values, expressed as % with respect to cells incubated in normoxia. Four independent experiments were carried out (*, *P* < 0.05; and **, *P* < 0.01 *vs* cells incubated in normoxia). (B) Cells were incubated with melatonin (1 mM, 100 μM, 10 μM or 1 μM) during 4 h under hypoxia. The blots show the effect of melatonin on the phosphorylation state of Akt, GSK3 and PTEN. The levels of β-actin were employed as controls to ensure equal loading of proteins. The bars show the quantification of protein phosphorylation for each treatment. Data show the mean ± S.E.M. of normalized values, expressed as % with respect to non-treated cells (incubated under hypoxia and in the absence of melatonin). Four independent experiments were carried out (Mel, melatonin; *, *P* < 0.05; and ***, *P* < 0.001 *vs* non-treated cells).

expression of α-sma. In PSCs incubated under hypoxia during 48 h, an increase in the detection of α-sma was noted, in comparison with the levels detected in cells incubated under normoxia, although the differences were not statistically significant (Fig. 2B). Separate batches of PSCs were treated with melatonin (Mel; 1 mM, 100 μM, 10 μM or 1 μM) and subjected to hypoxia (48 h treatement). Under these conditions, a decrease in the detection of α-sma was noted in PSCs incubated with 1 mM melatonin, compared with the levels detected in cells subjected to hypoxia only. Moreover, the level detected was similar to that noted in cells incubated under normoxia. No differences were observed in PSCs

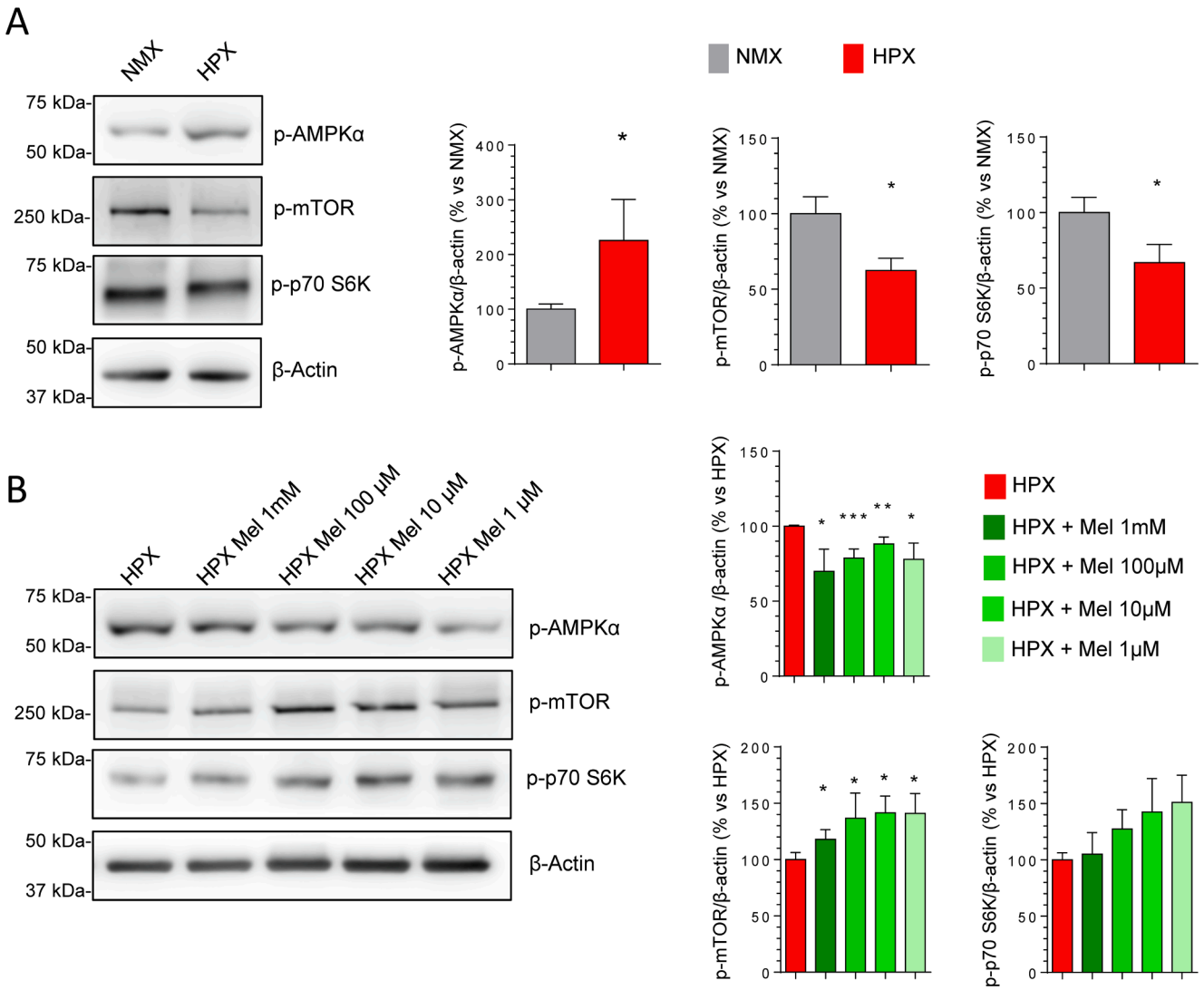

Fig. 3. Phosphorylation of AMPK, mTOR and p70 S6 kinase in PSCs incubated under hypoxia. Effect of melatonin. (A) Separate batches of cells were incubated for 4 h under normoxia (NMX) or under hypoxia (HPX). The blots show the level of the phosphorylated state of AMPK, mTOR and p70 S6 kinase. The levels of β-actin were employed as controls to ensure equal loading of proteins. The bars show the quantification of protein phosphorylation for each treatment. Data show the mean ± S.E.M. of normalized values, expressed as % with respect to cells incubated in normoxia. Four independent experiments were carried out (*, $P < 0.05$; and ***, $P < 0.001$ *vs* cells incubated in normoxia). (B) Cells were incubated with melatonin (1 mM, 100 μM, 10 μM or 1 μM) during 4 h under hypoxia. The blots show the effect of melatonin on the phosphorylation state of AMPK, mTOR and p70 S6 kinase. The levels of β-actin were employed as controls to ensure equal loading of proteins. The bars show the quantification of protein phosphorylation for each treatment. Data show the mean ± S.E.M. of normalized values, expressed as % with respect to non-treated cells (incubated under hypoxia and in the absence of melatonin). Four independent experiments were carried out (Mel, melatonin; *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ *vs* non-treated cells).

incubated with the other concentrations of melatonin (Fig. 2B). We further studied the effect of hypoxia on the levels of collagen type 1. In this set of experiments PSCs were incubated during 48 h under normoxia, under hypoxia or under hypoxia plus 1 mM melatonin. Immunocytochemistry studies revealed that the detection of collagen type 1 was significantly increased in cells subjected to hypoxia, in comparison with the levels noted in cells incubated under normoxia (Fig. 1C). When PSCs were treated with 1 mM melatonin and under hypoxia, a statistically significant decrease in the detection of collagen type 1 was observed, in comparison with the levels noted in PSCs incubated under hypoxia and in the absence of melatonin. The levels detected of collagen type 1 dropped dramatically, although the level detected was still over that noted in cells subjected to normoxia (Fig. 1C).

3.2. Effects of melatonin on hypoxia-induced changes in the phosphorylation of Akt, GSK3 and PTEN

The phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway is pivotal for cell survival and comprises of major positive and negative regulators of growth, survival, and proliferation. These proteins are considered key components in cancer development and progression [35]. The pathway is negatively regulated by the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and also involves the serine/threonine protein kinase glycogen synthase kinase 3 (GSK3) [36]. In first instance, we were interested in analyzing whether PSCs subjected to hypoxia exhibited changes in major proteins involved in this pathway. For this purpose, PSCs were incubated under hypoxia during 4 h. For comparisons, separate batches of cells were incubated under normoxic conditions. The analysis of samples revealed that cells subjected to hypoxia exhibited a significant increase in the phosphorylation of Akt, whereas phosphorylation of GSK3 and PTEN was

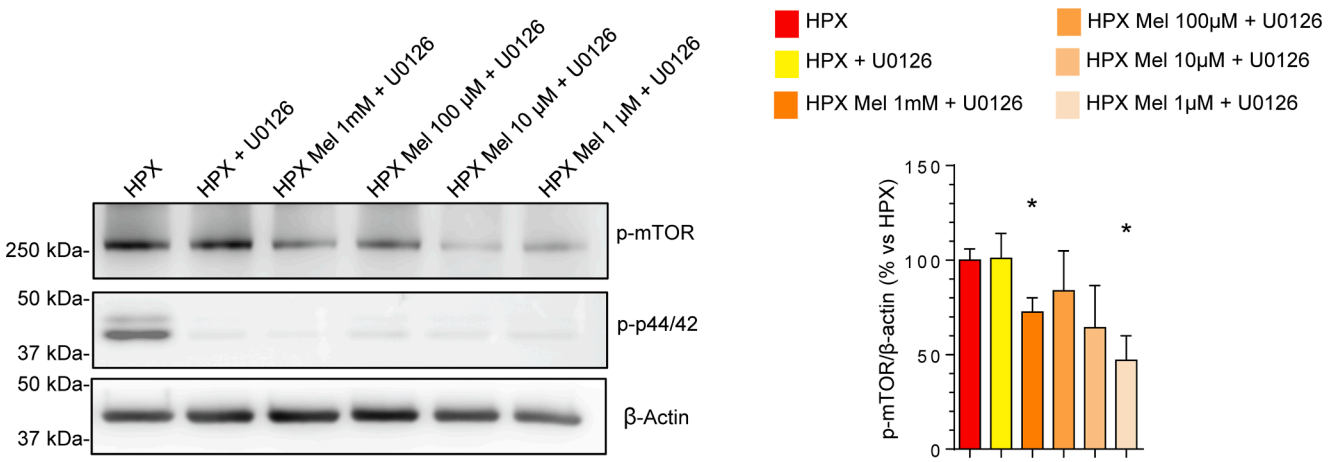

Fig. 4. Involvement of p44/42 MAPK in mTOR activation in PSCs subjected to hypoxia. Different batches of cells were incubated for 5 min. in the presence of U0126, an inhibitor of p44/42 MAPK, and under hypoxia. Thereafter, the cells were incubated for 4 h with melatonin (1 mM, 100 µM, 10 µM or 1 µM), under hypoxia and in the presence of U0126. The blots show the level of the phosphorylated state of p44/42 MAPK and mTOR. The levels of β-actin were employed as controls to ensure equal loading of proteins. The bars show the quantification of protein phosphorylation for each treatment. Data show the mean ± S.E.M. of normalized values, expressed as % with respect to non-treated cells (incubated under hypoxia only). Four independent experiments were carried out (Mel, melatonin; *, $P < 0.05$ *vs* non-treated cells in hypoxia only).

diminished, compared with cells incubated in normoxia (Fig. 2A).

Bearing in mind the role of the above mentioned proteins in cancer and taking into account former observations which showed that melatonin decreased the proliferation of PSCs [30,37,38], we next investigated whether melatonin induced any changes in the phosphorylation state of these proteins. PSCs were incubated in the presence of melatonin (1 mM, 100 µM, 10 µM or 1 µM) during 4 h under hypoxia. The treatment with melatonin induced statistically significant decreases in the phosphorylation of Akt, in comparison with the levels noted in non-treated cells, which were incubated under hypoxia but in the absence of melatonin (Fig. 2B). On the contrary, phosphorylation of GSK3 and PTEN was increased when compared with the levels detected in non-treated cells (Fig. 2B). The effects did not seem to depend on the concentration of melatonin used.

3.3. Effects of melatonin on hypoxia-induced changes in the phosphorylation of AMPK, mTOR and p70 S6 kinase

Mammalian target of rapamycin (mTOR)-ribosomal protein S6 kinase beta-1 (p70S6 kinase) pathway and AMP-activated protein kinase (AMPK) play major roles in the regulation of cellular metabolism [39]. In this part of our research, we were interested in investigating whether hypoxia induced changes in these pivotal proteins.

In a first instance, PSCs were incubated during 4 h under hypoxia. Separate batches of cells were incubated under normoxia. Hypoxia induced a statistically significant increase in the phosphorylation of AMPK, compared with the level detected in cells incubated under normoxia (Fig. 3A). However, the phosphorylation of mTOR and p70 S6 kinase was significantly decreased under hypoxia, in comparison with that noted in cells incubated under normoxia (Fig. 3A).

We next investigated whether melatonin induces any changes in the phosphorylation state of these proteins. Therefore, PSCs were incubated during 4 h in the presence of melatonin (1 mM, 100 µM, 10 µM or 1 µM) and under hypoxia. Treatment with melatonin induced a decrease in the phosphorylation of AMPK, whereas phosphorylation of mTOR and p70 S6 kinase was increased, in comparison with the levels noted in cells incubated under hypoxia but in the absence of melatonin (Fig. 3B).

3.4. Melatonin activates mTOR signaling in a p44/42 MAPK-dependent pathway

One of the major targets of the classical PI3K/Akt signaling is mTOR. Additionally, mTOR is subjected to inputs from different intracellular signaling pathways. Mitogenic signaling through activation of Ras/MEK/p44/42 MAPK also activates mTOR [40]. In this line, it has been proposed that mTOR is a substrate for the protein kinase p44/42 [41]. Moreover, melatonin induces phosphorylation of p44/42 MAPK in PSCs [42]. Because we had observed that treatment of cells with melatonin led to a decrease in Akt phosphorylation that was not accompanied by a decrease in the phosphorylation of mTOR, we investigated the involvement of the crucial regulator of cell signaling p44/42 MAPK in mTOR phosphorylation. Thus, PSCs were incubated during 5 min under hypoxia and in the presence of the p44/42 MAPK inhibitor U0126 (10 µM), prior to addition of melatonin. Thereafter, cells were further incubated during 4 h under hypoxia and in the presence of melatonin (1 mM, 100 µM, 10 µM or 1 µM), with the inhibitor present in the culture medium. The inhibitor did not induce detectable changes in the phosphorylation of mTOR. However, in the presence of U0126 the phosphorylation of mTOR in the presence of melatonin was decreased (Fig. 4). Moreover, in the presence of the inhibitor the basal phosphorylation of p44/42 MAPK, which was noted under hypoxia, was abolished. Additionally, the inhibitor blunted phosphorylation of p44/42 MAPK in the presence of melatonin (Fig. 4).

3.5. Effect of hypoxia and melatonin treatment on mitochondrial functionality

We have previously reported that, when subjected to hypoxia, PSCs proliferate more actively than under normoxia [21]. This increase should be expected to be accompanied by a metabolic reprograming, employing the metabolites and fuels available under hypoxia. Both in healthy and pathological conditions, mitochondria respiration through oxidative phosphorylation represents one of the more powerful sources of energy for the cell. In this part of the research, we decided to study the contribution of mitochondrial respiration to the bioenergetics status of PSCs. Separate batches of cells were subjected to normoxia or to hypoxia. Moreover, additional sets of cells were subjected to hypoxia and were further incubated in the presence of melatonin (1 mM, 100 µM, 10 µM or 1 µM). Using XF Cell Mito Stress, we analyzed several parameters related with oxidative phosphorylation.

Basal and maximal respiration, ATP production by oxidative phosphorylation, spare capacity and proton leak were significant decreased in PSCs subjected to hypoxia in comparison with cells incubated under normoxia (Fig. 5A).

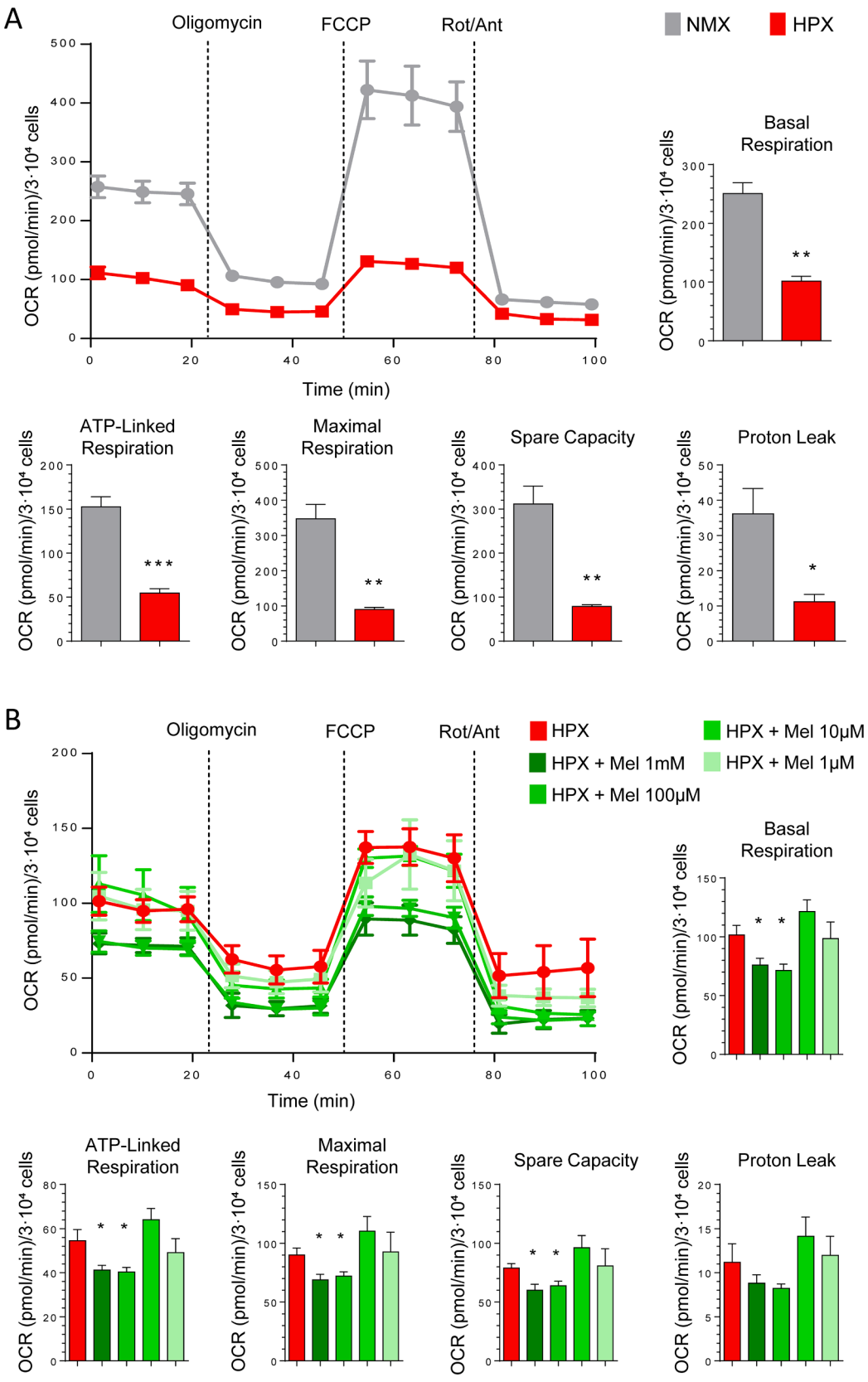

Fig. 5. Analysis of mitochondrial respiration in PSCs under hypoxia. Effect of melatonin. (A) Cells were incubated under normoxia (NMX) or under hypoxia (HPX) for 24 h. Then, oxygen consumption rate (OCR) was measured using the XF Cell Mito Stress Test Kit. The line chart shows the mean of OCR ± S.E.M. of normalized values of each measurement time point (n = 9). The OCR was measured under basal conditions or following the addition of oligomycin, carbonylcyanide p-(trifluoro-methoxy)phenylhydrazone (FCCP), or rotenone plus antimycin A (Rot/Ant). The bar graphs show the quantification of basal respiration, ATP-linked respiration, maximal respiration, spare capacity and proton leak, respectively. OCR data were normalized with respect to the number of cells, using crystal violet test. Results are expressed as the mean of OCR ± S.E.M. of normalized values. Three independent experiments were carried out (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ *vs* cells incubated in normoxia). (B) Cells were incubated with melatonin (1 mM, 100 μM, 10 μM or 1 μM) during 24 h under hypoxia and then the XF Cell Mito Stress Test Kit was performed. The line chart shows the mean of OCR ± S.E.M. of normalized values for each time point measurement (n = 9). The OCR was measured under the same conditions mentioned above. The bar graphs show the quantification of basal respiration, ATP-linked respiration, maximal respiration, spare capacity and proton leak, respectively. OCR data were normalized with respect to the number of cells, using crystal violet test. Results are expressed as the mean of OCR ± S.E.M. of normalized values. Three independent experiments were carried out (Mel, melatonin; *, $P < 0.05$ *vs* non-treated cells under hypoxia).

We have shown previously that treatment of cells with melatonin reverses the effect of hypoxia on PSCs proliferation [42]. At this stage, we were interested in investigating if melatonin could exert any changes in the metabolic profile of PSCs subjected to hypoxia, which could be the basis of the drop induced by melatonin on cellular viability under hypoxia [42]. Therefore, we evaluated the OXPHOS in PSCs treated with melatonin under hypoxic conditions. In the presence of the highest concentrations of melatonin (1 mM and 100 μM) statistically significant

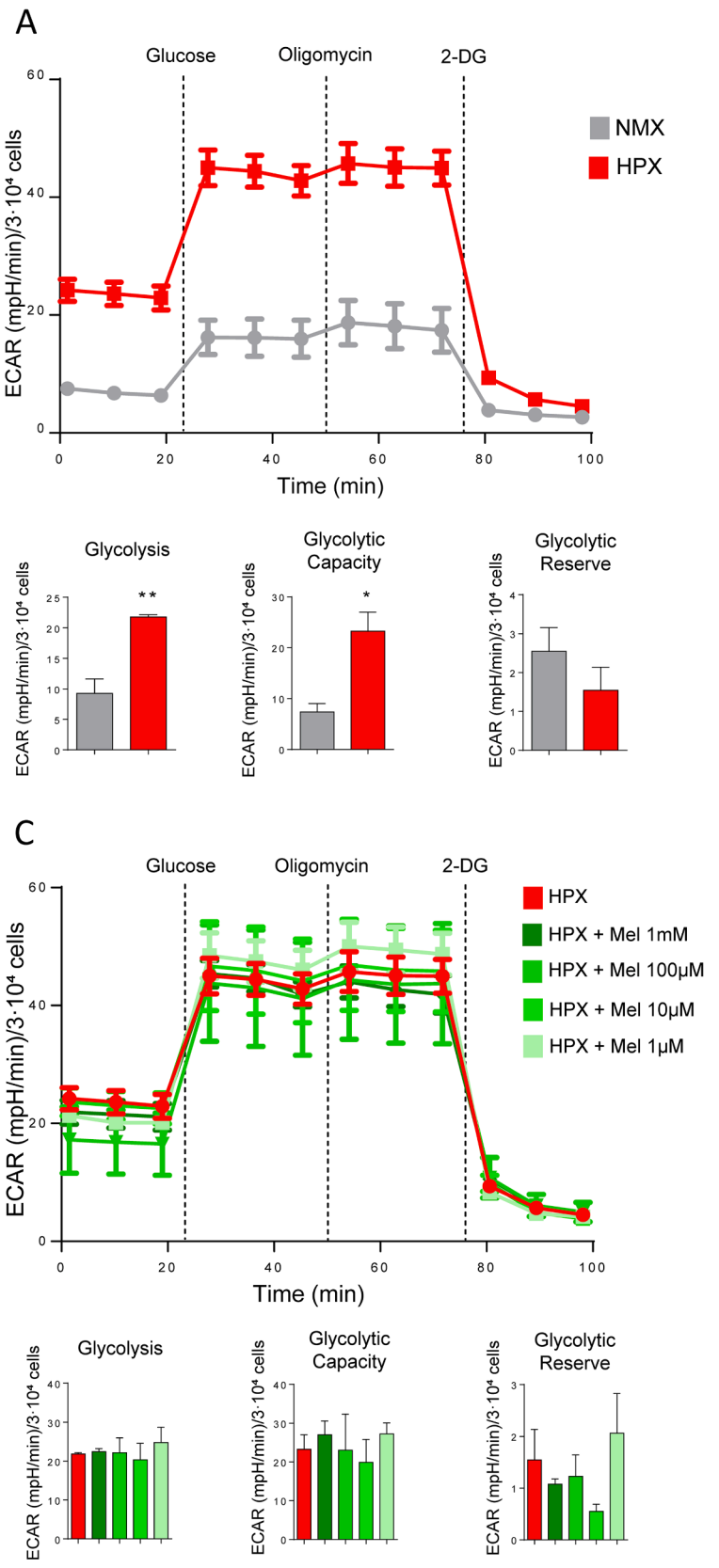

Fig. 6. Study of glycolytic metabolism in PSCs subjected to hypoxia. Effect of melatonin. (A) Cells were incubated under normoxia (NMX) or under hypoxia (HPX) for 24 h. Then, extracellular acidification rate (ECAR) was measured using the XF Glycolysis Stress Test Kit. The line chart shows the mean of ECAR ± S.E.M. of normalized values of each time point measurement (n = 9). The ECAR was measured under basal conditions or following the addition of glucose, oligomycin or 2-deoxy-glucose (2-DG). The bar graphs show the quantification of glycolysis, glycolytic capacity and glycolytic reserve, respectively. ECAR data were normalized with respect to the number of cells using crystal violet test. Results are expressed as the mean of ECAR ± S.E.M. of normalized values. Three independent experiments were carried out (*, $P < 0.05$ and **, $P < 0.01$ *vs* cells incubated in normoxia). (B) Separate batches of cells were incubated for 24 h under normoxia or under hypoxia. Then, RT-qPCR analysis was used to detect the mRNA levels of glucose transporter-1 (GLUT-1), phosphofructokinase (PFK) and lactate dehydrogenase (LDH). The bars show the fold increase of mRNA levels ± S.E.M. of each mRNA relative to cells incubated under normoxia (*, $P < 0.05$ and **, $P < 0.01$ *vs* cells in normoxia). (C) Cells were incubated with melatonin (1 mM, 100 μM, 10 μM or 1 μM) during 24 h under hypoxia and then the XF Glycolysis Stress Test Kit was performed. The line chart shows the mean of ECAR ± S.E.M. of normalized values of each measurement time point (n = 9). The ECAR was measured under the same conditions mentioned above. The bar graphs show the quantification of glycolysis, glycolytic capacity and glycolytic reserve, respectively. ECAR data were normalized with respect to the number of cells, using crystal violet test. Results are expressed as the mean of ECAR ± S.E.M. of normalized values. Three independent experiments were carried out (Mel, melatonin). (D) Cells were treated with melatonin under hypoxia and the levels of GLUT-1, PFK and LDH mRNA were analyzed by RT-qPCR. The bars show the fold increase of mRNA levels ± S.E.M. of each mRNA relative to cells incubated under hypoxia and in the absence of melatonin (Mel, melatonin; *, $P < 0.05$ *vs* non-treated cells in hypoxia).

decreases in the basal and maximal respiration and in the ATP production by the mitochondria were noted, in comparison with those observed in cells incubated in the absence of melatonin but under hypoxia (Fig. 5B). In addition, melatonin significantly decreased the spare capacity, which is a parameter that describes the maximum effort exhibited by mitochondria to produce ATP under stressful conditions (Fig. 5B). Similar effects were noted in the proton leak, although the differences were not statistically significant with respect to the values

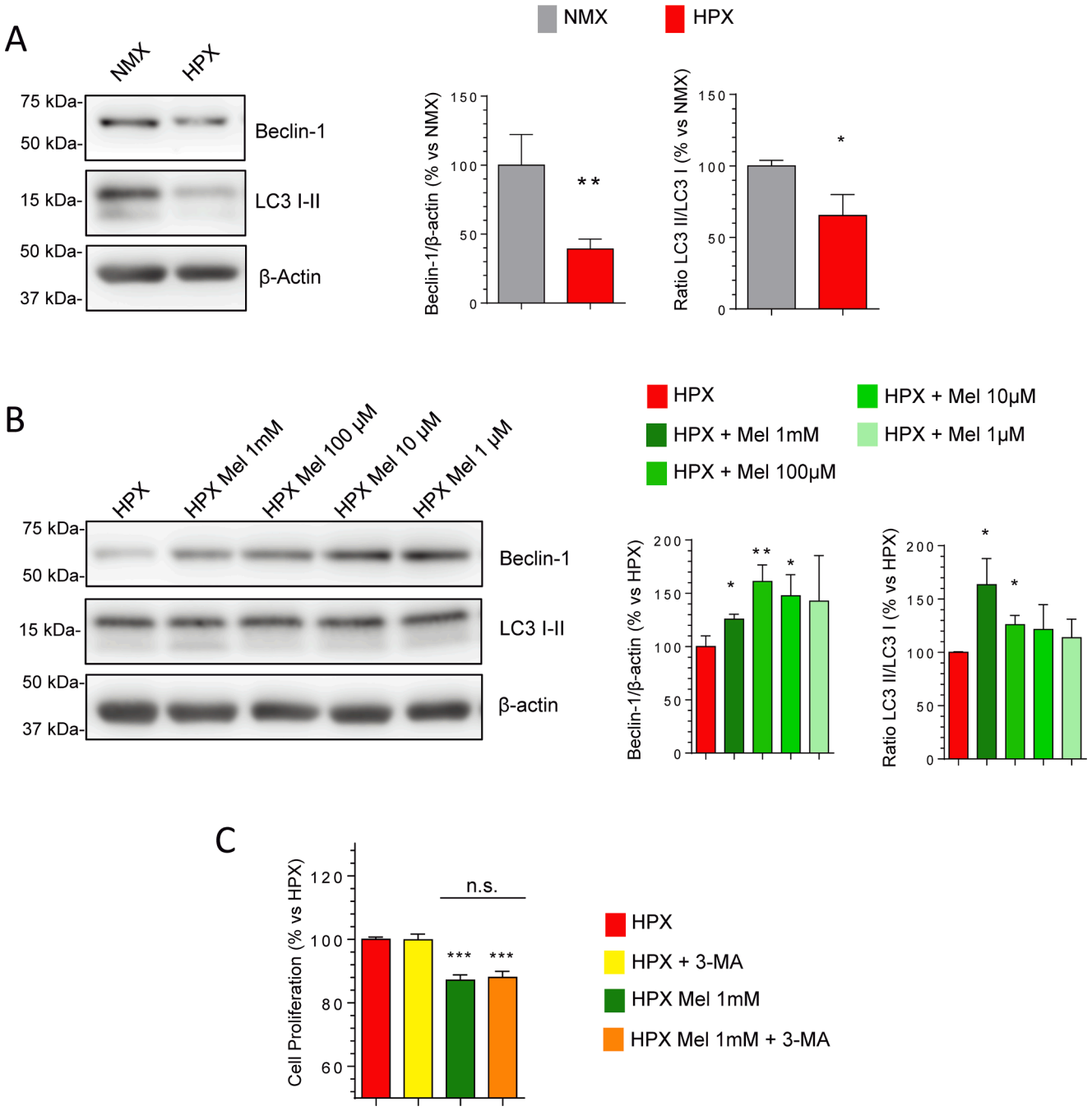

Fig. 7. Study of autophagy in PSCs subjected to hypoxia. Effect of melatonin. (A) Separate batches of cells were incubated for 4 h under normoxia (NMX) or under hypoxia (HPX). The blots show the level detected for Beclin-1 and LC3 I-II. The levels of β-actin were employed as controls to ensure equal loading of proteins. The bars show the quantification of protein detection for each treatment. Data show the mean ± S.E.M. of normalized values, expressed as % with respect to cells incubated in normoxia. Four independent experiments were carried out (*, $P < 0.05$; and ***, $P < 0.001$ *vs* cells incubated in normoxia). (B) Cells were incubated with melatonin (1 mM, 100 μM, 10 μM or 1 μM) during 4 h under hypoxia. The blots show the effect of melatonin on the level of Beclin-1 and LC3 I-II. The levels of β-actin were employed as controls to ensure equal loading of proteins. The bars show the quantification of protein detection for each treatment. Data show the mean ± S.E.M. of normalized values, expressed as % with respect to non-treated cells (incubated under hypoxia and in the absence of melatonin). Four independent experiments were carried out (Mel, melatonin; *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ *vs* non-treated cells in hypoxia). (C) Cells were incubated with 1 mM melatonin alone or in combination with 1 mM 3-methyl-adenine (3-MA), under hypoxia, for 48 h and cell viability was analyzed. Results are expressed in % as the mean ± S.E.M. (n) of cell viability for each treatment *vs* non-treated cells (incubated under hypoxia and in the absence of melatonin and 3-MA). Data are representative of three experiments (n.s., non-significative; ***, $P < 0.001$ *vs* non-treated cells).

achieved in cells subjected to hypoxia in the absence of melatonin (Fig. 5B). No statistically significant differences were observed in these parameters in cells incubated under hypoxia with 10 μM or 1 μM melatonin with respect to cells incubated in its absence and under hypoxia (Fig. 5B).

3.6. *Effect of hypoxia and melatonin on the glycolytic metabolism*

Under hypoxic conditions, the ability of cells to obtain energy from mitochondrial metabolism is decreased in comparison with normoxic state. Therefore, it would be expected that cells will use other sources of

energy under hypoxia. We next examined the contribution of glycolysis to cell metabolism in PSCs. Separate batches of cells were incubated in normoxia or in hypoxia. Moreover, other batches of cells were subjected to hypoxia and were additionally incubated in the presence of melatonin (1 mM, 100 μM, 10 μM or 1 μM). Using XF Glycolysis Stress Test we observed that the glycolysis and the glycolytic capacity were significantly increased in PSCs subjected to hypoxia, in comparison with cells incubated under normoxia (Fig. 6A). We also noted a drop in the glycolytic reserve under hypoxia *vs* normoxia. This was probably due to the high consumption of this source of energy under hypoxia (Fig. 6A). Furthermore, we evaluated the expression of the glucose 1 transporter (Glut-1), phosphofructokinase (PFK) and lactate dehydrogenase (LDH) in PSCs incubated under hypoxia during 24 h. RT-qPCR of the relative mRNA abundance revealed increases in the expression of *Glut-1*, *LDH* and *PFK-1* in cells incubated under hypoxia, in comparison with the values noted in cells incubated in normoxia (Fig. 6B). In a next step, we investigated the effects of melatonin on these parameters in cells subjected to hypoxia. We did not observe significant changes in glycolysis and in the glycolytic capacity in PSCs treated with melatonin with respect to cells incubated in its absence and under hypoxia. Nevertheless, the glycolytic reserve was decreased in the presence of melatonin, although the differences were not statistically significant (Fig. 6C). The expression of Glut-1 was slightly decreased in cells incubated the presence of melatonin *vs* cells incubated in its absence and under hypoxia, being statistically significant at the concentration of 1 mM and 1 μM melatonin (Fig. 6D). The expression of PFK and LDH also was decreased in the presence of melatonin 1 mM, whereas no detectable changes were noted with the other concentrations of melatonin tested with respect to values noted in cells incubated in the absence of melatonin and under hypoxia (Fig. 6D).

3.7. Effects of melatonin on hypoxia-induced changes in the levels of beclin-1 and LC3

Autophagy is a regulated process by which cells dispose of intracellular proteins and organelles with the involvement of lysosomes. This process plays a major role in cancer. However, activation of autophagy and the concomitant cell fate is context-dependent. To some extent, autophagy could be considered a way by which cells obtain energy in order to survive [43]. Beclin-1 and microtubule-associated protein 1 light chain 3 (LC3) are major constituents of the autophagy pathway [44].

In this part of our study, we decided to investigate the effect of hypoxia on the expression of beclin-1 and on the conversion of LC3-I to LC3-II. For this purpose, PSCs were incubated during 4 h under hypoxia. For comparisons, separate batches of cells were incubated under normoxia. Under hypoxia, the level of beclin-1 was lower with respect to that detected in cells incubated in normoxia. Similar results were noted when the levels of LC3 conversion were studied (Fig. 7A).

Additional experiments were carried out to investigate the effect of melatonin on the levels of these proteins in PSCs subjected to hypoxia. In this set of experiments, cells were incubated during 4 h in the presence of melatonin (1 mM, 100 μM, 10 μM or 1 μM) and under hypoxia. In the presence of melatonin, we detected increases in the levels of beclin-1 and in the conversion of LC3-I to LC3-II, in comparison with the levels noted in cells incubated under hypoxia but in the absence of melatonin (Fig. 7B).

Activation of autophagy can exert cytoprotective or cytotoxic effects, depending on the cellular context [45]. We have formerly reported that melatonin diminished PSCs viability under hypoxia [42] and here we observed that melatonin increased autophagy (Fig. 7B). Thus, we next evaluated the effect of melatonin-induced autophagy on PSCs viability subjected to hypoxia. For this purpose, we set up a viability experiment in the presence of 3-methyladenine, an autophagy inhibitor [46]. Separate batches of cells were treated with 3-methyladenine and were additionally incubated in the presence of melatonin (1 mM) and under hypoxia. Treatment of PSCs with 1 mM 3-methyladenine (3-MA) under hypoxia did not induce detectable changes on cell viability (Fig. 7C). As expected, incubation of PSCs in the presence of 1 mM melatonin under hypoxia induced a statistically significant decrease in cell viability with respect to cells incubated in its absence (Fig. 7C). However, incubation of PSCs in the presence of 3-MA (1 mM) plus melatonin (1 mM) did not revert nor increase the effect of melatonin (Fig. 7C).

4. Discussion

PSCs represent a low proportion within the cellular mass of the pancreas. Nevertheless, PSCs contribute to the preservation of the extracellular matrix and the pancreatic architecture under physiological conditions [5,7]. Interestingly, under stress conditions or in response to messengers derived from other cell types (cytokines, growth factors, …), these cells acquire a state referred to as activated state. The latter is a condition that is detrimental for pancreatic physiology, because progressive fibrosis can be developed within the gland [7,8]. Fibrosis will contribute to the formation of the tumor stroma. Fibroblasts, immune cells, and vasculature present in the gland also will support the development of fibrosis [47]. Activated PSCs, in turn, could influence neighboring cells, both pancreatic acini as well as those tumor cells that could be developing within the gland [8]. In this line, it has been proposed that progressive fibrosis occurs in the process of transition from acute to chronic pancreatitis, which would result in pancreatic insufficiency and in the development of cancerous processes in the gland [48]. PSCs participate actively in the development of the stroma through their proliferation and through the deposition of an extensive extracellular matrix [49].

The main consequence of the growth of fibrotic tissue is that cancer cells will be protected. Within the cell mass, conditions will be created that ease their growth and which might help the growing tumor tissue acquire resistance to chemotherapy and radiation. The stroma can undergo changes during malignancy transition and might help to growth, invasion, and metastasis of pancreatic cancer. Therefore, it is of major interest to find tools that reduce the amount of fibrotic tissue within the tumor, in order to facilitate the treatment of cancer [8,50].

On the other hand, a low availability of O_2 is a common condition occurring in solid tumors, including pancreatic tumors. All the cellular components bounded in the mass will exhibit adaptations that will help them survive. In fact, hypoxia is one of the key components leading to clinical resistance in cancer treatment [9].

Melatonin is emerging as a potential tool for cancer treatment. Notably, because melatonin alleviates the secondary effects of chemotherapy and radiation therapy [51,52] and, in addition, because of its antiproliferative effects in different types of cancers [53–57], including pancreatic cancer [27,58]. Moreover, melatonin modulates PSCs proliferation and viability and is emerging as a potential antifibrotic agent [4,30,37,38].

In a recent work, we have shown that proliferation of PSCs subjected to hypoxia is increased with respect to that of cells incubated under normoxic conditions. This could be explained by certain adaptative antioxidant responses that are activated in PSCs due to the prooxidative conditions created by cell metabolism under the low environmental O_2 availability [21]. However, additional mechanisms could be underlying PSCs adaptation to hypoxia, which deserve study. Moreover, how melatonin modulates adaptation of PSCs to hypoxia in order to exert its antiproliferative actions is not completely known.

Here we showed that PSCs subjected to hypoxia exhibited increases in proliferation and in the expression of fibrosis markers, and adaptative responses in major regulators of energy metabolism, which were counteracted by treatment with melatonin. Furthermore, treatment of PSCs with melatonin was followed by a decrease in cell viability.

The PI3K/Akt signaling is pivotal for the regulation of cell growth, survival and proliferation, and comprises of components that play a key role in cancer, one of which is GSK3 [35]. Additionally, PTEN exerts a

negative regulatory role [36]. Our first objective was to study the effect of hypoxia on the PI3K/Akt pathway in PSCs and to analyze whether melatonin exerts any modulatory effect. Our results showed that, in comparison with PSCs incubated under normoxia, in hypoxia the phosphorylated state of Akt was increased, whereas the phosphorylation of GSK3 and PTEN were decreased. On the contrary, treatment of cells with melatonin decreased the phosphorylation of Akt, whereas that of GSK3 and PTEN was increased. Thus, our results suggest that melatonin induces opposite changes to those evoked by hypoxia in the Akt/GSK3/PTEN signaling, which might modulate cell fate. Our results point towards important roles of Akt/GSK3/PTEN in the antiproliferative actions of melatonin. In relation with our findings, it has been shown that proliferation of hepatocarcinoma cells is decreased by GSK3 activation [59]. On its side, upregulation of PTEN has been related with inhibition of proliferation of renal cancer cells [60]. These effects were achieved respectively in response to treatment of cells with bryostatin-1, a macrolide lactone, and with scutellarin, a flavone glycoside, that exhibited antiproliferative effects. Interestingly, involvement of GSK3 [59] and PTEN [61] in the proteolysis of cyclin D1 and subsequent cell cycle arrest has been shown. This is of major interest, because PSCs subjected to hypoxia exhibited increases in the expression of cyclin D1 [21] and melatonin decreased its expression [37].

Our results also suggest that the effects of hypoxia on AMPK/mTOR/p70S6 kinase are counteracted by melatonin. mTOR is a canonical effector of the PI3K/Akt pathway and has been related with cell proliferation and metabolism [39]. Our results also have shown that the phosphorylation of mTOR was decreased in PSCs subjected to hypoxia, despite the phosphorylation of Akt was increased. Phosphorylation of the substrate of mTOR, p70S6kinase, also was decreased under hypoxia treatment. Moreover, melatonin induced an increase in the phosphorylation of mTOR and of p70S6kinase, whereas phosphorylation of Akt occurred in the opposite way (described above). This might reflect putative modulatory effects of melatonin on energetic metabolism in PSCs subjected to hypoxia.

It is well accepted that PI3K/Akt/mTOR network is the classical way for mTOR activation. Moreover, it is one of the most frequently deregulated signaling pathways in cancer [62]. However, our results suggest that, in PSCs, mTOR might not be connected to Akt phosphorylation. In this line, mTOR could be activated by p44/42 MAPK, as it has been proposed previously [40,41]. Our results are in agreement with this assumption and are supported by the fact that blockade of p44/42 by U0126 blunted the increase that we have observed in the phosphorylation of mTOR in the presence of melatonin. Therefore, our results suggest that activation of mTOR by melatonin in PSCs subjected to hypoxia occurs through p44/42 activation, rather than through Akt. This could be an interesting and/or novel mechanism by which p44/42 MAPK is involved in mTOR activation in PSCs in response to melatonin.

AMPK is a master bioenergetic sensor in the cell that it is activated when there is an increase in the ratio ADP/ATP or AMP/ATP [63]. This means that AMPK is activated when the cell undergoes a rise in energy demand. This protein plays major roles in the regulation of cellular metabolism during hypoxia [64,65]. Here we have shown that PSCs subjected to hypoxia exhibited an increase in the phosphorylation of AMPK, which was reverted by treatment of cells with melatonin. In the next steps of our investigation, we further analyzed two of the main sources of energy supply to the cells: the oxidative phosphorylation in mitochondria and the glycolytic pathway.

Oxidative phosphorylation, carried out in the mitochondrial inner membrane, is the most efficient source of ATP production in the cell [66]. O_2, the final electron acceptor in the electron transport chain, is a limiting factor under hypoxic conditions. However, it has been shown that mitochondria can continue to function at low O_2 concentrations in pancreatic cancer [67]. This was the reason why we studied the contribution of mitochondria to energy production in PSCs subjected to hypoxia. Our results showed that all parameters related with mitochondrial functionality exhibited statistically significant decreases in PSCs subjected to hypoxia, in comparison with those noted in cells incubated in normoxia. These results suggest that mitochondrial respiration is modulated by melatonin, which might result in a low availability of energy supply.

On the other hand, using SeaHorse technology, we observed that glycolysis and glycolytic capacity were increased in cells subjected to hypoxia with respect to normoxia. In addition, the expression of the glucose transporter Glut-1 was increased. This fact could allow the cells to count with an increase in the amount of glucose that could be available for cell metabolism and energy supply.

We additionally observed that the expression of the enzyme LDH was upregulated. Anaerobic fermentation of lactate increases energy production from glucose, since it is not metabolised to pyruvate in the Krebs cycle. This metabolic switch to a more glycolytic profile is a hallmark of cancer cells and plays a major role in the adaptation of cellular metabolism to a low availability of O_2 in cancer [68,69].

We have previously reported that PSCs exhibited an increase in the expression of hypoxia inducible factors 1 and 2 (HIF-1/2) when subjected to hypoxia [21]. This transcription factor, activated by low O_2 availability, controls the balance of oxygen supply and demand [70]. HIF-1/2 also modulates PI3K/Akt/mTOR system [71], is negatively correlated with PTEN [72] and, further, modulates the expression of several metabolic enzymes involved in glycolysis [13]. Here we have shown that PSCs subjected to hypoxia undergo metabolic adaptation, as it may occur in the tumour microenvironment. Our observations could be related with the expression of HIF-1/2 in PSCs that we have formerly noted [21]. Interestingly, melatonin might modulate targets downstream to HIF-1/2.

Our results also show that melatonin, at the higher concentrations tested, further reduced the functionality of the mitochondria. In previous work, we have shown that this compound increased the production of reactive oxygen species, induced mitochondrial membrane depolarisation and evoked calcium mobilisation from different sources in PSCs [38]. Interestingly, our results have shown that the activity and the glycolytic capacity of PSCs treated with melatonin under hypoxia does not differ significantly from that observed in cells subjected to hypoxia in the absence of melatonin. Nevertheless, melatonin decreased the mRNA expression of GLUT-1, PFK and LDH in PSCs subjected to hypoxia, whereas the mRNA levels were increased in cells incubated under hypoxia and in the absence of melatonin. We could thus assume that under hypoxia, when an actively proliferating cell cannot obtain energy from the oxidative phosphorylation pathway, the up-regulation of another metabolic pathway could compensate for the low availability of energy, which in this case points towards glycolysis. Our results further showed that the mitochondrial activity was decreased by the additional treatment of cells with melatonin. However, the glycolytic activity was not up-regulated. A possible explanation for this observation is that the cells are not proliferating actively in the presence of the indolamine. In fact, we have previously shown that melatonin diminishes the proliferation of PSCs [38,43]. Altogether, our results provide evidence for putative metabolic adaptations of PSCs under hypoxia. Moreover, our results suggest that melatonin targets the oxidative metabolism in PSCs, which was upregulated under hypoxia. As a consequence, the metabolic adaptation of PSCs to the low availability of energy supply from mitochondria under hypoxia is counteracted by melatonin. This could represent a maneuver for melatonin to modulate PSCs proliferation.

Hypoxia can induce autophagy, a pathway that leads to the formation of energy precursors that are necessary to sustain cell metabolism and survival [73]. Interestingly, our results showed that PSCs subjected to hypoxia exhibited decreases in the levels of beclin-1 and in the conversion on LC3-I in LC3-II, which are major proteins involved in autophagy. The reason why the autophagic flux was not increased under hypoxia might be that it was not necessary, because the cells were obtaining enough energy to meet their needs to maintain proliferation via glycolysis. Possibly, the activation of autophagy in PSCs under hypoxia could require a more severe nutrient deprivation. This could

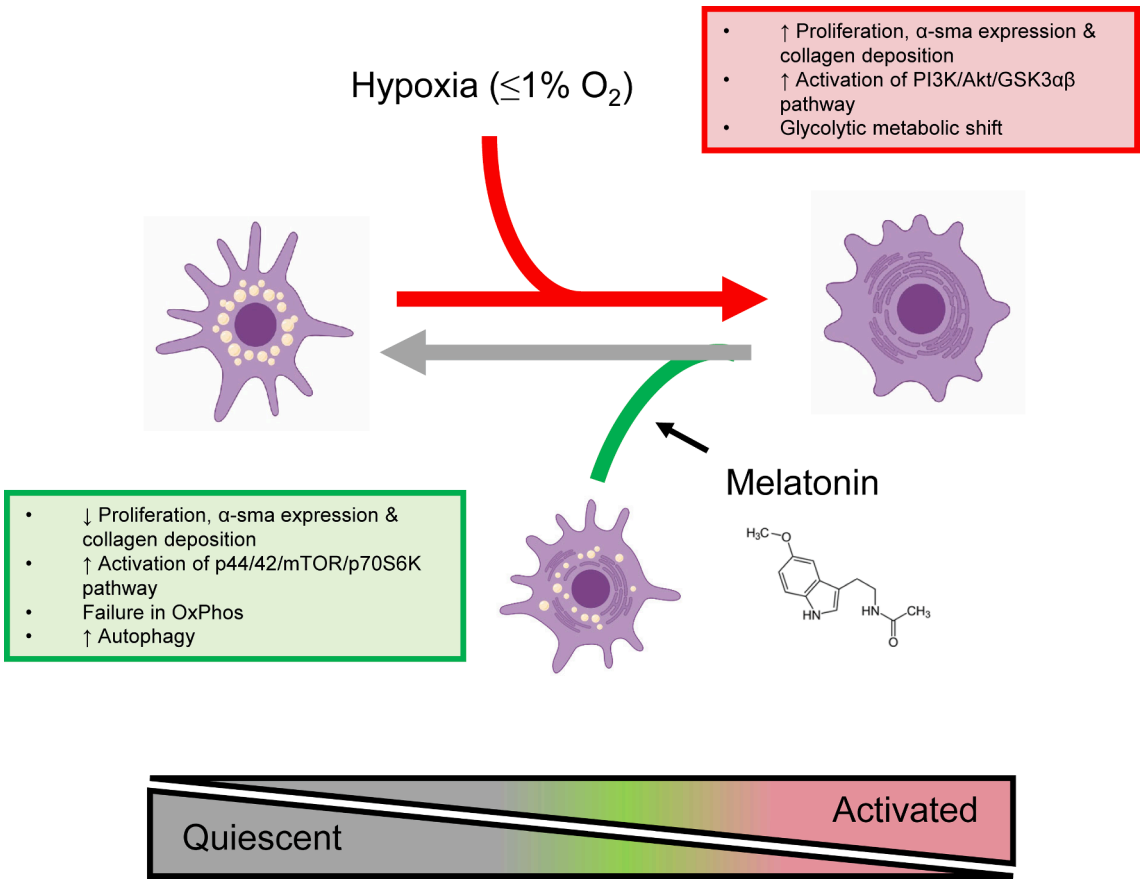

Fig. 8. Melatonin modulates the responses of PSCs to hypoxia. The low availability of O_2 is a recurrent condition in pancreatic fibrosis. Under this ambient, activated PSCs, which are considered the major responsible for the production of fibrotic tissue, proliferate more actively and exhibit increases in the expression of several activation markers, such as α-sma or collagen type 1. To maintain this proliferative rate under hypoxia, PSCs must undergo adaptations in their physiology. In this study, we have shown that the PI3K/Akt pathway was upregulated in PCS subjected to hypoxia. Moreover, the low availability of O_2 induced a metabolic shift towards a glycolytic profile. Treatment with melatonin suppressed the increased proliferation of cells that had been observed under hypoxia. Additionally, melatonin decreased the expression of the classical activation markers α-sma and collagen type 1. Melatonin modulated the pathways that we noted are activated under hypoxia. However, other signaling pathways such as the p44/42/mTOR/p70 S6K pathway, and/or processes such as autophagy, also were activated in response to melatonin. All in all, our results suggest that melatonin could be an effective tool to control the proliferation of PSCs under conditions that mimic pathological states of the pancreas (figure created with BioRender.com).

occur under conditions of chronic hypoxia, which could be taking place in the TME. Therefore, our results suggest that autophagy is not a critical pathway by which PSCs obtain their energy under hypoxia, and that PSCs rather undergo adaptations in the glycolytic metabolism to support cell survival. Interestingly, beclin-1 and the conversion on LC3-I in LC3-II were increased upon treatment of cells subjected to hypoxia with melatonin. At this point, we could hypothesize that activation of autophagy might be involved in melatonin-induced drop of cell viability. However, the autophagy inhibitor 3-methyladenine did not change the effect of melatonin on cell viability. These results therefore suggest that autophagy inhibition does not play a role in melatonin-induced cytotoxicity in PSCs subjected to hypoxia. A possible explanation for this observation is that activation of autophagy occurs as a compensatory mechanism that, however, is not responsible for the decrease of PSCs viability in the presence of melatonin.

All these results point towards putative antifibrotic actions of melatonin. In fact, we have shown in a former work that PSCs subjected to hypoxia exhibited increases in the expression of matrix metalloproteinases (MMP) 2, 3, and 13 [42]. MMPs are a multigene family of endopeptidases that are involved in remodeling of extracellular matrix during fibrosis and are involved in pathological processes, including inflammation and cancer [74]. Our former results further showed that treatment with melatonin diminished the expression of MMPs [42]. Bearing in mind that MMPs play a pivotal role in fibrosis and in cancer, together with the modulation of energy supply that we observed in the present work, our results suggest potential actions of melatonin to reduce the progression of abnormal tissue within the pancreas under hypoxia. Moreover, our hypothesis is reinforced by the finding that, in the presence of melatonin, the expression of alpha-smooth muscle actin (α-sma) and of collagen type 1 was diminished (Fig. 1).

In conclusion, our results provide evidence for the activation of the PI3K/Akt pathway in PSCs subjected to hypoxia. Under these conditions, these cells have a high energy demand that is maintained by a metabolic shift towards anaerobic glycolysis. These changes might support the high proliferative rate of PSCs under hypoxia. On the other hand, melatonin, for which we have described antiproliferative actions on these cells under hypoxia, counteracts the activation of PI3K/Akt pathway. In addition, melatonin decreases mitochondrial activity. In this context, activation of the p44/42/mTOR/p70 S6K pathway and of autophagy occurs, which could be considered as compensatory mechanisms for the effects of the indolamine. However, these mechanisms do not allow the cells to recover the high proliferative rate that has been observed under hypoxia. Thus, it could be feasible that there might be a relationship between fibrosis and the activation of the PI3K/Akt/mTOR pathway under hypoxia, which might be modulated by melatonin. Also, melatonin could modulate concomitant metabolic adaptations that PSCs undergo under hypoxic conditions. Taken together, our results provide further evidence for the mechanisms employed by melatonin to

modulate PSCs proliferation and to putatively modulate fibrosis within the pancreas. Fig. 8 shows a summary of the changes that PSCs undergo under hypoxia and which could be modulated by melatonin.

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Authors Contribution

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Matias Estaras, Patricia Santofimia-Castaño, Remigio Martinez, Alfredo García and Cándido Ortiz-Placin. The experimental studies were designed by Patricia Santofimia-Castaño, Juan L. Iovanna and Antonio Gonzalez. The first draft of the manuscript was written by Antonio Gonzalez. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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7. DISCUSIÓN

Nuestro grupo de investigación lleva años investigando los efectos de la melatonina sobre la fisiología del páncreas. Los primeros trabajos se centraron en el estudio de los efectos antiproliferativos de esta molécula sobre células tumorales pancreáticas [201]. Posteriormente, las investigaciones continuaron sobre la población celular mayoritaria del páncreas, las células acinares. En ellas, la indolamina tenía un claro efecto protector sobre el daño inducido por la sobreestimulación con agonistas fisiológicos [191, 192]. En nuestro último proyecto, nuestros esfuerzos se han centrado en estudiar el papel de esta molécula sobre otra población celular del páncreas, distinta a las anteriores, las CEP.

7.1. Efecto de la melatonina y/o la hipoxia sobre la proliferación de las CEP

Las CEP tienen una función esencial en el establecimiento de la fibrosis pancreática, una marca fisiopatológica de enfermedades como la pancreatitis y el cáncer pancreático [36]. La activación de este grupo celular cambia el fenotipo de esta población incrementando su proliferación y capacidad de producir en exceso proteínas de MEC Esto contribuye al desarrollo del estroma y a la pérdida del parénquima pancreático[21]. Es por ello, que controlar la proliferación de este tipo celular puede contribuir al manejo terapéutico de la fibrosis pancreática.

La melatonina ha mostrado su efecto como agente antiproliferativo en distintos modelos celulares entre los que se incluyen cáncer de mama [202], cáncer de colon [203], cáncer de pulmón [204] o el cáncer de páncreas [205]. Nuestros resultados muestran que la melatonina, a las mayores concentraciones empleadas (1 mM y 100 µM), reduce la proliferación de las CEP. Sin embargo, esta reducción de la viabilidad no es tan dramática como en el caso de las células tumorales. Es bien conocido que esta molécula puede actuar sobre una gran variedad de tipos celulares, atribuyéndole tanto efectos citoprotectores como citostáticos o citotóxicos [206]. En CEP tratadas con melatonina se ha observado la activación de caspasa-3, un claro indicativo de la activación de la apoptosis. Curiosamente, esta activación de caspasa-3 se produce hasta en concentraciones donde la viabilidad celular no se ve comprometida como a 1 µM. Esto podría ser un indicador de que el tratamiento con melatonina produce en las CEP un desequilibrio entre las fuerzas pro-apoptóticas y anti-apoptóticas, cuya consecuencia, en el caso de altas concentraciones, son el descenso observado en la población de CEP en cultivo.

Además, aunque el efecto citotóxico de la melatonina se ha encontrado en algunas líneas tumorales, el efecto de esta molécula sobre la proliferación celular más ampliamente descrito en la bibliografía es el oncostático o citostático [207–209]. El tratamiento con melatonina reduce la expresión de la ciclina D1 en las CEP. Esta ciclina es un regulador fundamental del ciclo celular y controla la transición entre la fase G1 hacia la fase de síntesis (S) del ciclo celular. La expresión de esta proteína se encuentra aumentada en diferentes tipos de cáncer, incluyendo el cáncer de páncreas [210]. Su desregulación se ha relacionado con los efectos citostáticos en diferentes líneas celulares [211,212].

La hipoxia, un elemento característico del microambiente tumoral pancreático, constituye un estrés para las células ante el cual tienen que adaptar su fisiología para sobrevivir y poder proliferar activamente [134]. Nuestros resultados muestran que bajo condiciones de hipoxia las CEP incrementan su proliferación, en comparación con el estado de normoxia. Las células cultivadas en una atmosfera que contenía un 1% de O_2 incrementaron su número (determinado mediante la técnica del cristal violeta), la incorporación de BrdU y la expresión de ciclina D1.

La hipoxia cambia el contexto y el fenotipo celular, y con ello, puede modular la sensibilidad de las células a las drogas [138]. Por ello, analizamos el efecto de la melatonina sobre la viabilidad de las CEP cultivadas bajo condiciones de hipoxia. La melatonina disminuyó la viabilidad de las CEP cultivadas en hipoxia, especialmente a las concentraciones más altas testadas. El efecto más fuerte fue encontrado con la concentración de melatonina 1 mM, la cual inhibe completamente el incremento proliferativo inducido por la hipoxia, revertiendo a las células al estado proliferativo de la normoxia. La expresión de ciclina D1 en las CEP también se redujo con el tratamiento de melatonina bajo condiciones de hipoxia.

7.2. Efecto de la melatonina y/o la hipoxia sobre la vía de las MAPKs en las CEP.

Nuestro siguiente objetivo fue monitorizar los cambios producidos en la activación de las MAPKs. Esta familia de proteínas cinasas juegan un papel central en distintas vías de señalización, participando en un gran número de procesos celulares como la proliferación y supervivencia celular, la migración, el metabolismo, la diferenciación celular o la respuesta a estrés [56]. Los cambios en la activación de estas proteínas pueden constituir biomarcadores del estado de activación de las CEP, pues son fieles reflejos del estado proliferativo de las células.

Nuestros resultados muestran que, en presencia de melatonina, la fosforilación de JNK y p44/42 disminuyó significativamente en las concentraciones mayores

empleadas. Sin embargo, la fosforilación de p38 se encontraba incrementada en las CEP por efecto de la indolamina. La desactivación de JNK y p44/42 se ha relacionado con una disminución de la proliferación y supervivencia celular en células tumorales pancreáticas [213,214]. La activación de p38 se ha reportado en respuesta a estrés celular como el estrés oxidativo [215]. Nuestros resultados están en concordancia con estas observaciones y sugieren que la modulación de las MAPK podría ser uno de los mecanismos empleados por la melatonina para el control de la proliferación celular.

A continuación, evaluamos el efecto de la hipoxia sobre la activación de las MAPKs en las CEP. Esta condición indujo un incremento en la fosforilación de JNK y una reducción en la activación de p44/42 y p38. Además, el tratamiento de las CEP con el inhibidor de JNK SP600125 abolió el incremento proliferativo inducido por la hipoxia. La activación de JNK contribuye a la proliferación de células de adenocarcinoma de pulmón, potencia el carcinoma hepatocelular y la progresión y metástasis del cáncer colorectal [216–219]. Además, la activación de esta cinasa en el estroma tumoral pancreático se ha relacionado con la progresión tumoral [220]. Nuestras observaciones sugieren que la señalización de JNK está implicada en la adaptación y proliferación de las CEP en condiciones de hipoxia.

Finalmente, analizamos el efecto de la melatonina sobre la activación de esta familia de proteínas en condiciones de hipoxia. En este contexto, las concentraciones más altas de melatonina disminuyeron la fosforilación de JNK, hecho que podemos relacionar con la caída observada en la proliferación en presencia de la indolamina y en hipoxia. En contraposición con los efectos observados de la indolamina en condiciones de normoxia, la fosforilación de p44/42 aumentó tras el tratamiento con melatonina bajo condiciones de hipoxia. Se ha observado en la línea tumoral HepG2, que la melatonina estimulaba la activación de p44/42, mientras se producía un descenso de la viabilidad celular [221]. Este efecto está en concordancia con nuestras observaciones. Desde nuestro punto de vista, hipotetizamos que esta activación de p44/42 puede ejercerse como un mecanismo protector o compensatorio al daño inducido por la melatonina. La cinasa p38 también incrementa su activación tras el tratamiento con melatonina en hipoxia. La activación de esta MAPK está relacionada con la muerte celular [222]. Creemos que la activación de esta proteína en respuesta a la melatonina bajo condiciones de normoxia e hipoxia podría mediar la activación de caspasa-3 que se observa tras el tratamiento.

7.3. Efecto de la melatonina y/o la hipoxia sobre la vía de PI3K/Akt en las CEP.

La vía de señalización de PI3K/Akt/mTOR es una ruta fundamental en el control del crecimiento celular, la supervivencia, la proliferación, la autofagia y el metabolismo [72]. Esta ruta de señalización ha sido considerada un componente clave en el desarrollo y progresión de diferentes tipos de células tumorales, incluyendo el cáncer pancreático [223]. Nuestros resultados mostraron que bajo el tratamiento con las concentraciones mayores de melatonina (1 mM y 100 μM) se produjo un incremento en la fosforilación de AMPK y un descenso en Akt y mTOR. Es decir, la melatonina reduce la activación de esta vía de señalización. Este efecto de la melatonina también ha sido observado en las células HT-29, una línea celular de cáncer colorrectal [224].

La hipoxia indujo una incremento significativo en la fosforilación de Akt y una reducción de la fosforilación de PTEN y GSK3β. PTEN es un regulador negativo de la vía de PI3K/Akt y GSK3β es un sustrato de fosforilación de Akt, que es inhibido por esta modificación [225]. Este incremento en la activación de la vía de PI3K/Akt se correlacionaría con el incremento del estado proliferativo inducido por la hipoxia. mTOR es un efector canónico de la vía de PI3K/Akt. Sin embargo, la hipoxia reduce la fosforilación de mTOR y su sustrato p70S6 cinasa, e incrementa la fosforilación de AMPKα, un regulador negativo de mTOR.

La melatonina, bajo condiciones de hipoxia, indujo efectos contrarios a los cambios inducidos por la hipoxia en la fosforilación de las proteínas de esta ruta. Akt disminuyó su fosforilación, mientras que encontramos incrementos en la fosforilación de PTEN y GSK3β. Esta desactivación de la ruta de PI3K/Akt está en consonancia con la reducción de la proliferación observada por el efecto de la melatonina. Sin embargo, mTOR y p70S6K incrementaron su fosforilación bajo el tratamiento con la indolamina en hipoxia. Nuestros resultados sugieren que en condiciones de hipoxia la señalización mediada por mTOR no se encuentra ligada a las acciones de la vía de PI3K/Akt. Investigaciones previas habían propuesto a p44/42 como otro posible mediador de la activación de mTOR[226,227]. Nuestros resultados están de acuerdo con estas observaciones. En condiciones de hipoxia, la melatonina incrementa la fosforilación de p44/42 y mTOR, y la inhibición de la cinasa p44/42 con el inhibidor U0126 abolió el incremento en la fosforilación de mTOR inducido por esta hormona.

7.4. Efecto de la melatonina y/o la hipoxia sobre el estado redox de las CEP.

El mantenimiento de un adecuado equilibrio redox es fundamental para la fisiología celular [228]. La melatonina es bien conocida por su papel como molécula antioxidante. Sin embargo, estos efectos sobre el estado redox son dependientes del contexto y la concentración en la que esta hormona se encuentre [229].

Bajo condiciones de normoxia, la melatonina incrementó la producción de ERO totales y mitocondriales en las CEP. Además, se observó un incremento en la oxidación de proteínas y del consumo de glutatión reducido. Estos efectos se acompañaron de una disminución de la expresión y actividad de las enzimas antioxidantes SOD-1 y SOD-2, y una disminución de la capacidad antioxidante total de la célula. Todos estos datos indicaban que la melatonina inducía un estado pro-oxidante en las CEP, el cual podría contribuir a la disminución de la viabilidad celular y el estado proliferativo de estas células. En otros experimentos observamos que bajo el tratamiento con melatonina se producía un incremento en la expresión de diferentes enzimas antioxidantes como la catalasa, la hemo oxigenasa 1, la N-quinona oxidorreductasa 1 o la subunidad catalítica de la glutamato-cisteína ligada. Ante estas condiciones pro-oxidantes, inducidas por la melatonina, hipotetizamos que el incremento en la expresión de las enzimas antioxidantes dependientes de Nrf2 se produciría como un mecanismo compensatorio.

Nuestros resultados muestran que en las CEP cultivadas bajo hipoxia se produjo un incremento en la oxidación de lípidos y proteínas, en la producción de ERO mitocondriales, en el consumo del glutatión reducido y una disminución en la capacidad antioxidante total. Es decir, la hipoxia crea un ambiente prooxidante en las CEP. Sin embargo, bajo estas condiciones prooxidantes, las células proliferan más activamente que en condiciones de normoxia, por lo que deben tener adaptaciones que les permitan este fenotipo. Por ello, analizamos la respuesta antioxidante de las CEP bajo condiciones de hipoxia. Los datos reportaron un incremento en la fosforilación de Nrf2 y en la expresión de diferentes enzimas antioxidantes. Estas adaptaciones de la respuesta antioxidante pueden ser responsables, al menos en parte, de los cambios que le permiten a las CEP proliferar más activamente bajo condiciones de hipoxia.

Finalmente, analizamos el estado redox de las CEP cultivadas bajo hipoxia y tratadas con melatonina. Observamos que el tratamiento con melatonina incrementaba la producción de ERO; Sin embargo, se reducía la oxidación de lípidos y proteínas inducida por la hipoxia. La capacidad antioxidante total de las CEP bajo hipoxia se incrementó en respuesta al tratamiento con melatonina, al contrario de lo ocurrido en condiciones de normoxia. Además, la concentración mayor de melatonina indujo una reducción de la ratio GSH/GSSG, lo que consideramos indicativo del consumo de este sistema antioxidante. Si bien la condición de hipoxia ya incrementaba la expresión de diferentes enzimas antioxidantes, el tratamiento con melatonina incrementó aún más la expresión de estas proteínas. Estos datos nos indican que la melatonina, en condiciones de hipoxia, podría actuar como una molécula antioxidante, a diferencia de su efecto prooxidante bajo condiciones de normoxia. Esta acción antioxidante estaría implicada

en resolver el estrés oxidativo inducido por la hipoxia, y con ello, reducir la proliferación evocada por esta situación de estrés celular

7.5. Efecto de la melatonina y/o la hipoxia sobre la función mitocondrial y el metabolismo glucolítico en las CEP.

El último objetivo que nos planteamos para esta tesis doctoral fue estudiar la función mitocondrial y el metabolismo glucolítico de las CEP bajo los tratamientos con melatonina y/o hipoxia. Estos elementos representan las principales fuentes de obtención de energía para las células y su modulación podría contribuir a los cambios observados en la proliferación celular.

La mitocondria, la principal fuente de energía del metabolismo celular, ha sido descrita como una de las dianas celulares de la melatonina [230]. En las CEP, la melatonina reduce el potencial de membrana mitocondrial e incrementa la producción de ERO mitocondriales. La alteración de estos dos parámetros es un indicativo de efecto mitocondrial, que podría ser el resultado de la alteración en su funcionamiento [231]. Por ello, analizamos la respiración mitocondrial, una de las principales funciones de este orgánulo. Observamos que la melatonina reducía la respiración basal y máxima, así como la producción de ATP ligada a la fosforilación oxidativa. Además, el tratamiento con esta molécula disminuyó la *spare capacity*, que es un parámetro que representa la capacidad de las células para hacer frente a un estrés energético. Estos datos indicaban que la melatonina inducía un daño en la función mitocondrial, el cual podría contribuir al efecto antiproliferativo de esta molécula. En respuesta a este daño mitocondrial, ciertos mecanismos compensatorios podrían activarse. En este sentido, observamos que la melatonina incrementaba la expresión del complejo I de la cadena de transporte de electrones. Otros mecanismos como la fusión mitocondrial o la mitofagía no parecen contribuir a paliar los efectos inducidos en este orgánulo.

Dado que la principal fuente de obtención de energía se encontraba alterada, estudiamos la actividad glucolítica de las CEP bajo el tratamiento con melatonina. En este apartado, observamos que las altas concentraciones de esta molécula disminuían la glucólisis y la capacidad glucolítica. Además, en presencia de melatonina detectamos cambios en la expresión de Glut-1, PFK y LDH, que son proteínas involucradas en el metabolismo glucolítico. Bajo la acción de la melatonina, la energía no producida por la actividad mitocondrial no parecía estar compensada por un aumento en la actividad glucolítica.

En el siguiente paso, nos propusimos estudiar los cambios que se producían en la actividad mitocondrial y la glucolisis bajo la condición de hipoxia en las CEP. El O_2, el aceptor final de electrones de la cadena de transporte de electrones, es un factor limitante bajo hipoxia. Sin embargo, ha sido demostrado que la mitocondria puede continuar sus funciones a bajas concentraciones de O_2 en el cáncer de páncreas [232]. Por este motivo, decidimos estudiar la función mitocondrial bajo la condición de hipoxia. Nuestros resultados mostraron que todos los parámetros relacionados con la actividad mitocondrial se encontraban disminuidos con respecto al estado de normoxia. Sin embargo, este déficit de la actividad mitocondrial era compensado por un incremento de la glucolisis y la capacidad glucolítica. Además, se producía un incremento en la expresión de genes relacionados con el metabolismo glucolítico, como Glut-1, PFK o LDH. Este efecto probablemente tiene relación con la activación de HIF que hemos reportado en las CEP en condiciones de hipoxia.

Finalmente, analizamos el efecto de la melatonina bajo hipoxia sobre la actividad mitocondrial y glucolítica. Observamos que la melatonina reducía, aún más, la respiración de las células bajo hipoxia. La actividad glucolítica no mostró cambios significativos en las CEP tratadas con melatonina bajo hipoxia, en comparación con las CEP únicamente sometidas a hipoxia. Sin embargo, el tratamiento de las células con la indolamina en condiciones de hipoxia redujo la expresión de las enzimas glucolíticas anteriormente mencionadas. Una posible explicación para estas observaciones podría ser que las CEP tratadas con melatonina bajo hipoxia no proliferan tan activamente y, por lo tanto, su demanda energética disminuye, por lo que no sería necesario compensar el déficit energético que supone la reducción de la actividad mitocondrial inducida por la melatonina.

7.6. Limitaciones y perspectivas sobre el estudio y el uso de la melatonina como agente anti-fibrótico.

Nuestro proyecto de investigación se ha centrado en entender los efectos de la melatonina en las CEP, las principales productoras del tejido fibrótico en el páncreas. Entender cuáles son los efectos de la melatonina y cuáles son los mecanismos moleculares responsables de estos efectos, es fundamental para poder desarrollar y mejorar las herramientas terapéuticas de las patologías que afectan a la glándula. Para poder escalar este conocimiento hasta el uso clínico en humanos, son necesarios estudios adicionales.

El siguiente paso que nos podríamos plantear serían los estudios *in vivo*. Recientes investigaciones han mostrado el potencial efecto anti-fibrótico de la melatonina

empleando un modelo de inducción de fibrosis pancreática en rata [233]. Este trabajo pone de manifiesto que este papel anti-fibrótico de la melatonina está relacionado con su capacidad antioxidante y antiinflamatoria. Nuestros resultados muestran cuales son los efectos de la melatonina sobre la fisiología de las CEP.

Nuestras investigaciones no han mostrado que la melatonina ejerza un grado de muerte muy elevado en las CEP, sino más bien una ralentización o desactivación parcial. A las mismas concentraciones de melatonina usadas en células tumorales y en PSC, se han observado efectos protectores sobre las células acinares sanas, mientras que sobre las células tumorales la melatonina produce un grado de muerte celular más elevado que el observado en las CEP [190]. Este hecho, que no produzca un efecto citotóxico tan dramático en las CEP, puede ser positivo. Las CEP, aunque participan en procesos patológicos, no son células tumorales que tienen una alta carga de mutaciones en su ADN. Si la melatonina produjera una elevada mortalidad en esta población celular, otras poblaciones de células tipo-fibroblásticas o estrelladas de otros órganos podrían verse afectadas por esta molécula. Hipotetizamos que la melatonina, tanto a nivel fisiológico como farmacológico, podría desempeñar un papel como “guardián de la salud” o “Cancerbero de la salud”. Cancerbero era el perro del dios griego del inframundo Hades y su función era separar a los vivos de los muertos y evitar que los primeros entraran en el mundo de los segundos y viceversa. De la misma forma, la melatonina trataría de proteger a las células sanas y de erradicar a las células “enfermas”.

Respecto a las concentraciones de melatonina que hemos empleado en nuestro estudio, oscilando del rango milimolar al micromolar, todas ellas han sido descritas como concentraciones farmacológicas, más que fisiológicas [234]. En animales, las concentraciones de melatonina empleadas con efectos farmacológicos se encuentran en el rango 25-50 mg/kg de peso del animal [196,233]. En humanos, las dosis administradas en los ensayos clínicos varían desde 3-20 mg/día [197]. Una ventaja del uso de esta molécula como herramienta terapéutica es que apenas se han descrito efectos secundarios. Sin embargo, en los últimos años, sobre todo en EE.UU., se ha incrementado mucho el consumo de esta molécula sin prescripción médica. Esto ha incrementado el número de noticias en la prensa alertando sobre los problemas derivados de su sobreconsumo, como es la alteración del ritmo circadiano o alteración de la tolerancia a la glucosa. En los ensayos clínicos, se han establecido diferentes protocolos de administración de la melatonina (vías de administración, horas de administración, etc). Todos estos datos sobre la farmacodinámica, farmacocinética y el

uso responsable deben ser evaluados en los ensayos clínicos para mostrar su potencial anti-fibrótico en las enfermedades del páncreas.

8. CONCLUSIONES

De los resultados presentados en esta Tesis Doctoral, podemos obtener las siguientes conclusiones:

- La melatonina reduce la viabilidad y la proliferación de las CEP. Este efecto se produce tanto en normoxia como en hipoxia, condición en la que estas células incrementan su estado proliferativo.
- Las MAPKs son moduladas por los tratamientos de melatonina y/o hipoxia en las CEP. La activación de la cinasa JNK promueve el estado proliferativo de las CEP. La melatonina reduce la activación de esta cinasa en condiciones de normoxia e hipoxia, lo que estaría relacionado con su efecto anti proliferativo.
- En condiciones de normoxia, la melatonina reduce la activación de la vía de PI3K/Akt/mTOR. La hipoxia incrementa la activación de Akt y reduce la activación de mTOR, mientras que la melatonina, bajo condiciones de hipoxia, revierte estos cambios.
- En condiciones de normoxia, la melatonina crea un ambiente prooxidante en el que las CEP incrementan la expresión de las enzimas antioxidantes dependientes de Nrf2. La hipoxia también crea un ambiente prooxidante, frente al cual adaptan su respuesta antioxidante las CEP para poder mantener un estado proliferativo elevado. Bajo condiciones de hipoxia, la melatonina resuelve el estrés oxidativo inducido por la hipoxia y reduce la proliferación celular.
- La melatonina altera la fisiología mitocondrial bajo condiciones de normoxia e hipoxia, modulando el metabolismo de la glucosa La condición de hipoxia induce un cambio metabólico en las CEP desde una producción de energía mitocondrial a la producción de energía por la vía glucolítica anaeróbica.

Conclusión general:

La melatonina muestra un efecto antiproliferativo en las CEP en condiciones de normoxia. Entre los mecanismos que emplea esta molécula para conseguir esta disminución de la viabilidad celular se encuentran cambios en el ciclo celular, la activación de proteínas apoptóticas, la modulación de vías de señalización como las MAPKs o PI3K/Akt/mTOR, la alteración del estado redox celular y el daño de la función mitocondrial.

Por otro lado, la hipoxia incrementa la proliferación de esta población celular. Pero para poder sobrevivir y proliferar activamente, estas células tienen que sufrir adaptaciones de su respuesta antioxidante y/o en su metabolismo energético.

Finalmente, la melatonina también muestra un efecto antiproliferativo en las CEP cultivadas bajo hipoxia. Como el contexto celular cambia, algunos de los efectos de la melatonina difieren de los observados en condiciones de normoxia.

8. CONCLUSIONS

The following conclusions can be obtained from the results presented in this doctoral thesis:

- Melatonin diminished the viability and proliferation of PSC. This effect was observed both in normoxia and hypoxia, the latter being a condition in which these cells exhibited an increase in their proliferative state.
- MAPKs were modulated under melatonin and/or hypoxia treatments. Activation of JNK kinase promoted the proliferative state of PSC. Melatonin reduced the activation of this kinase under normoxic and hypoxic conditions, which could be related to its anti-proliferative effect.
- Under normoxic conditions, melatonin reduced the activation of the PI3K/Akt/mTOR pathway. Hypoxia increased Akt activation and reduced mTOR activation, whereas melatonin, under hypoxic conditions, reverted these changes.
- Under normoxic conditions, melatonin created a prooxidant environment under which PSC increased the expression of Nrf2-dependent antioxidant enzymes. Hypoxia also created a prooxidant environment, to which PSC adapted their antioxidant response. This was considered a maneuver directed to maintain a high proliferative state. Under hypoxic conditions, melatonin resolved hypoxia-induced oxidative stress and reduced cell proliferation.
- Melatonin altered mitochondrial physiology under normoxic and hypoxic conditions, modulating glucose metabolism. The hypoxic condition induced a metabolic shift in PSC from mitochondrial energy production to production by anaerobic glycolytic pathway.

General conclusion:

Melatonin shows an antiproliferative effect on PSC under normoxic conditions. Among the mechanisms employed by this molecule to achieve this decrease in cell viability, we can mention changes in the cell cycle, activation of apoptotic proteins, modulation of signalling pathways such as MAPKs or PI3K/Akt/mTOR, alteration of the cellular redox state and a tuning or, to some extent, impairment of mitochondrial function.

On the other hand, hypoxia increases the proliferation of this cell population. But in order to survive and to actively proliferate, these cells have to undergo adaptations in their antioxidant response and/or energy metabolism.

Finally, melatonin also shows an anti-proliferative effect in PSC subjected to hypoxia. As the cellular context changes, some of the effects of melatonin differ from those observed under normoxic conditions.

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- Estaras M y cols. Melatonin controls cell proliferation and modulates mitochondrial physiology in pancreatic stellate cells. J Physiol Biochem. 2022 Nov 5. doi: 10.1007/s13105-022-00930-4.
- Gonzalez A y cols. Melatonin modulates red-ox state and decreases viability of rat pancreatic stellate cells. Sci Rep. 2020 Apr 14;10(1):6352. doi: 10.1038/s41598-020-63433-6.
- Estaras M y cols. Melatonin Induces Apoptosis and Modulates Cyclin Expression and MAPK Phosphorylation in Pancreatic Stellate Cells Subjected to Hypoxia. Int J Mol Sci. 2021 May 24;22(11):5555. doi: 10.3390/ijms22115555.
- Estaras M y cols. Melatonin Modulates the Antioxidant Defenses and the Expression of Proinflammatory Mediators in Pancreatic Stellate Cells Subjected to Hypoxia. Antioxidants (Basel). 2021 Apr 8;10(4):577. doi: 10.3390/antiox10040577.
- Estaras M y cols. Melatonin modulates metabolic adaptation of pancreatic stellate cells subjected to hypoxia. Biochem Pharmacol. 2022 Aug;202:115118. doi: 10.1016/j.bcp.2022.115118.

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- Estaras M y cols. Melatonin modulates proliferation of pancreatic stellate cells through caspase-3 activation and changes in cyclin A and D expression. J Physiol Biochem. 2020 May;76(2):345-355. doi: 10.1007/s13105-020-00740-6.
- Estaras M y cols. Pancreatic stellate cells exhibit adaptation to oxidative stress evoked by hypoxia. Biol Cell. 2020 Oct;112(10):280-299. doi: 10.1111/boc.202000020.

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