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Melatonin modulates metabolic adaptation of pancreatic stellate cells subjected to hypoxia

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A B S T R A C T

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.

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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). Bradford́s 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
Β-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)phenyl-hydrazone (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

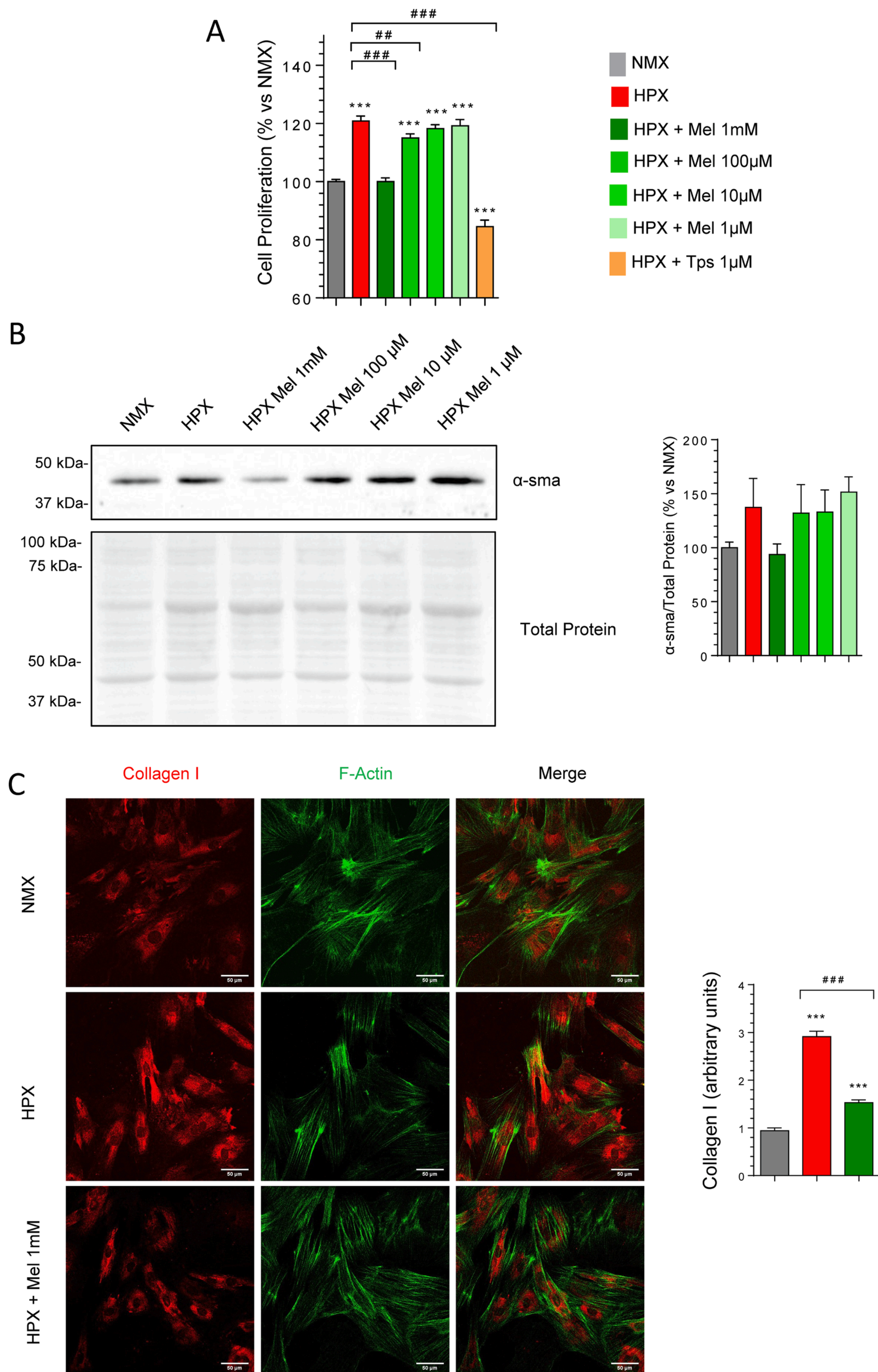

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Fig. 1. Study of fibrosis markers in PSCs subjected to hypoxia. Effect of melatonin. (A) Cells were incubated under normoxia (NMX), under hypoxia (HPX) or under hypoxia plus melatonin (1 mM, 100 μM, 10 μM or 1 μM), for 48 h and cell viability was analyzed. 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).

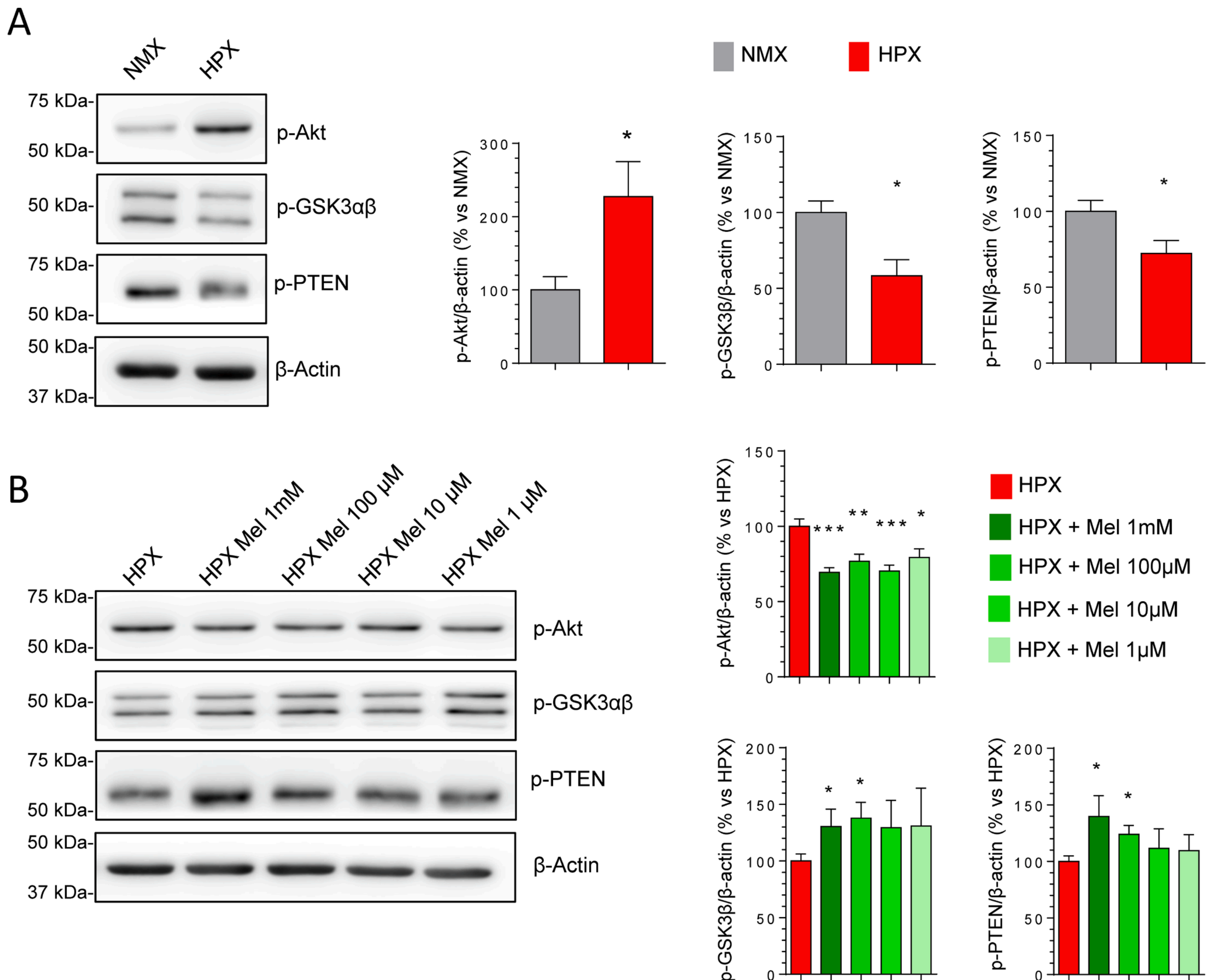

Fig. 2. Phosphorylation of Akt, GSK3 and PTEN in PSCs incubated under hypoxia. Effect of melatonin. (A) Separate batches of cells were incubated for 4 h under normoxia (NMX) or under hypoxia (HPX). The blots show the level of the phosphorylated state of Akt, GSK3 and PTEN. The levels of β-actin were 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

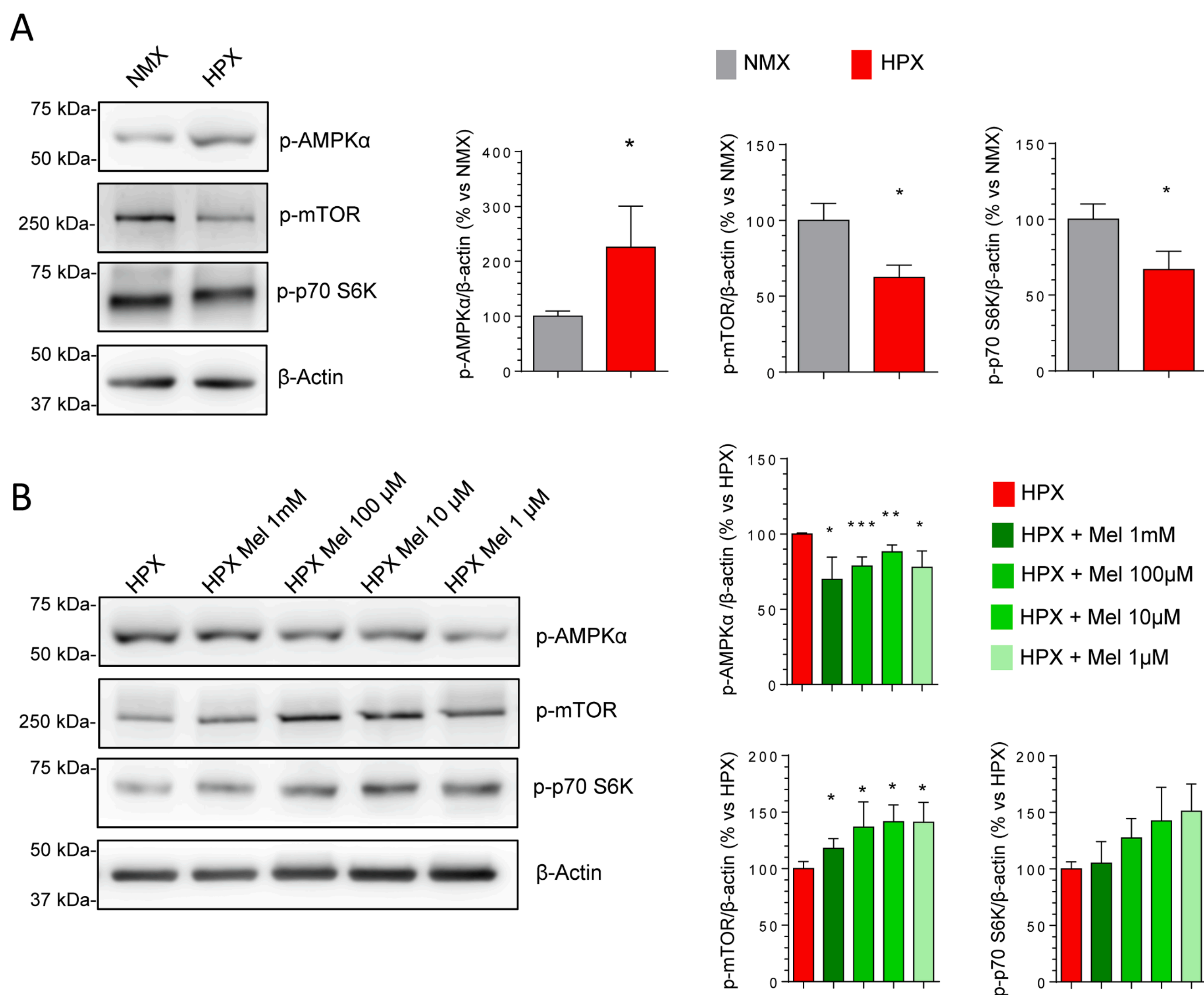

Fig. 3. Phosphorylation of AMPK, mTOR and p70 S6 kinase in PSCs incubated under hypoxia. Effect of melatonin. (A) Separate batches of cells were incubated for 4 h under normoxia (NMX) or under hypoxia (HPX). The blots show the level of the phosphorylated state of AMPK, mTOR and p70 S6 kinase. The levels 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

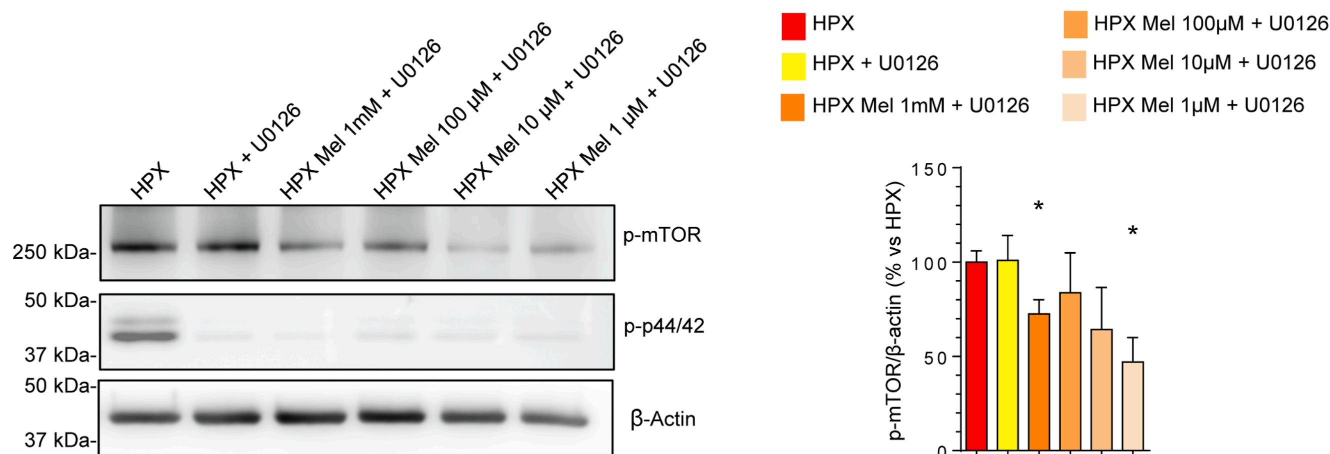

Fig. 4. Involvement of p44/42 MAPK in mTOR activation in PSCs subjected to hypoxia. Different batches of cells were incubated for 5 min. in the presence of U0126, an inhibitor of p44/42 MAPK, and under hypoxia. Thereafter, the cells were incubated for 4 h with melatonin (1 mM, 100 μM, 10 μM or 1 μM), 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).

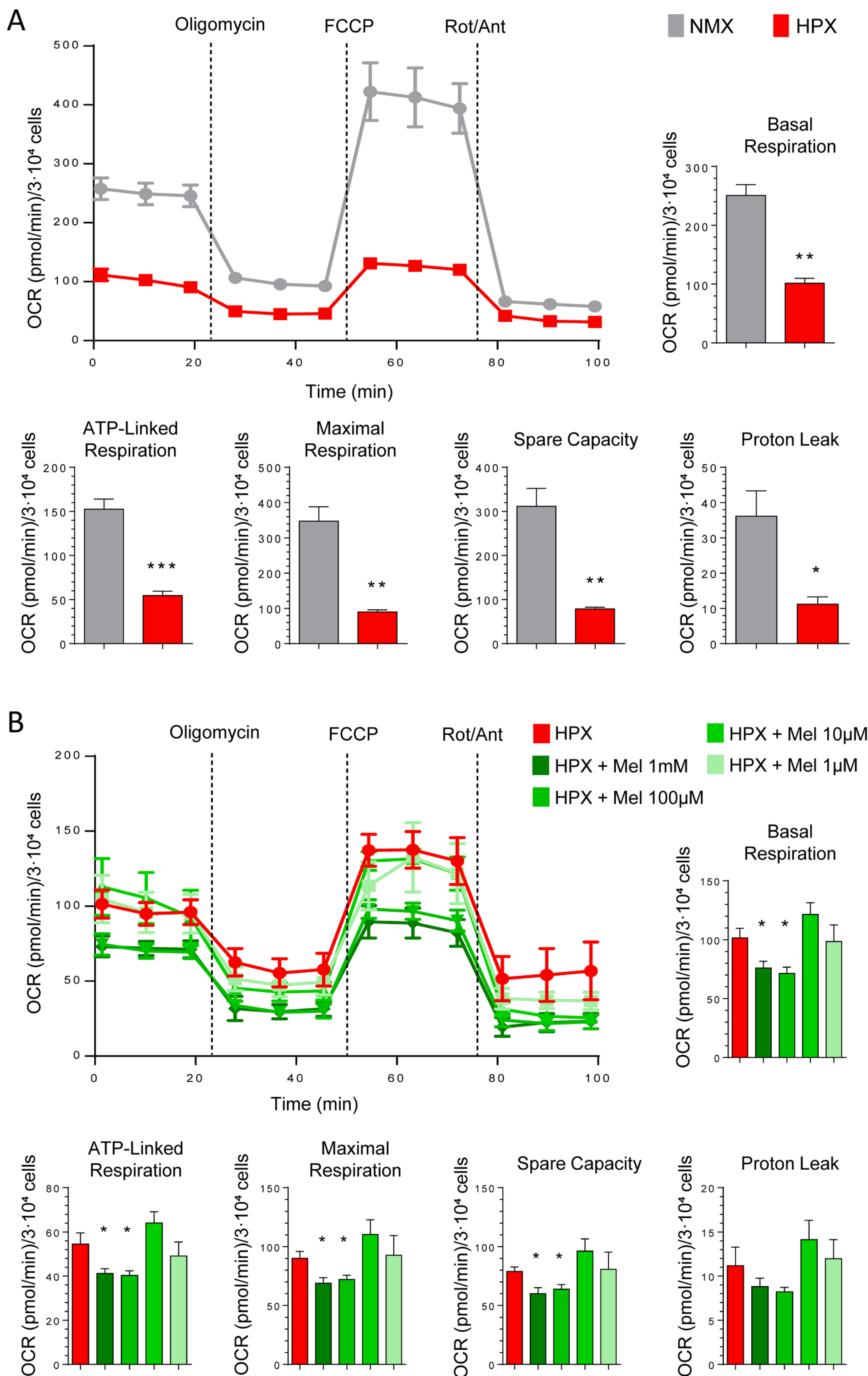

Fig. 5. Analysis of mitochondrial respiration in PSCs under hypoxia. Effect of melatonin. (A) Cells were incubated under normoxia (NMX) or under hypoxia (HPX) for 24 h. Then, oxygen consumption rate (OCR) was measured using the XF Cell Mito Stress Test Kit. The line chart shows the mean of OCR ± S.E.M. 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

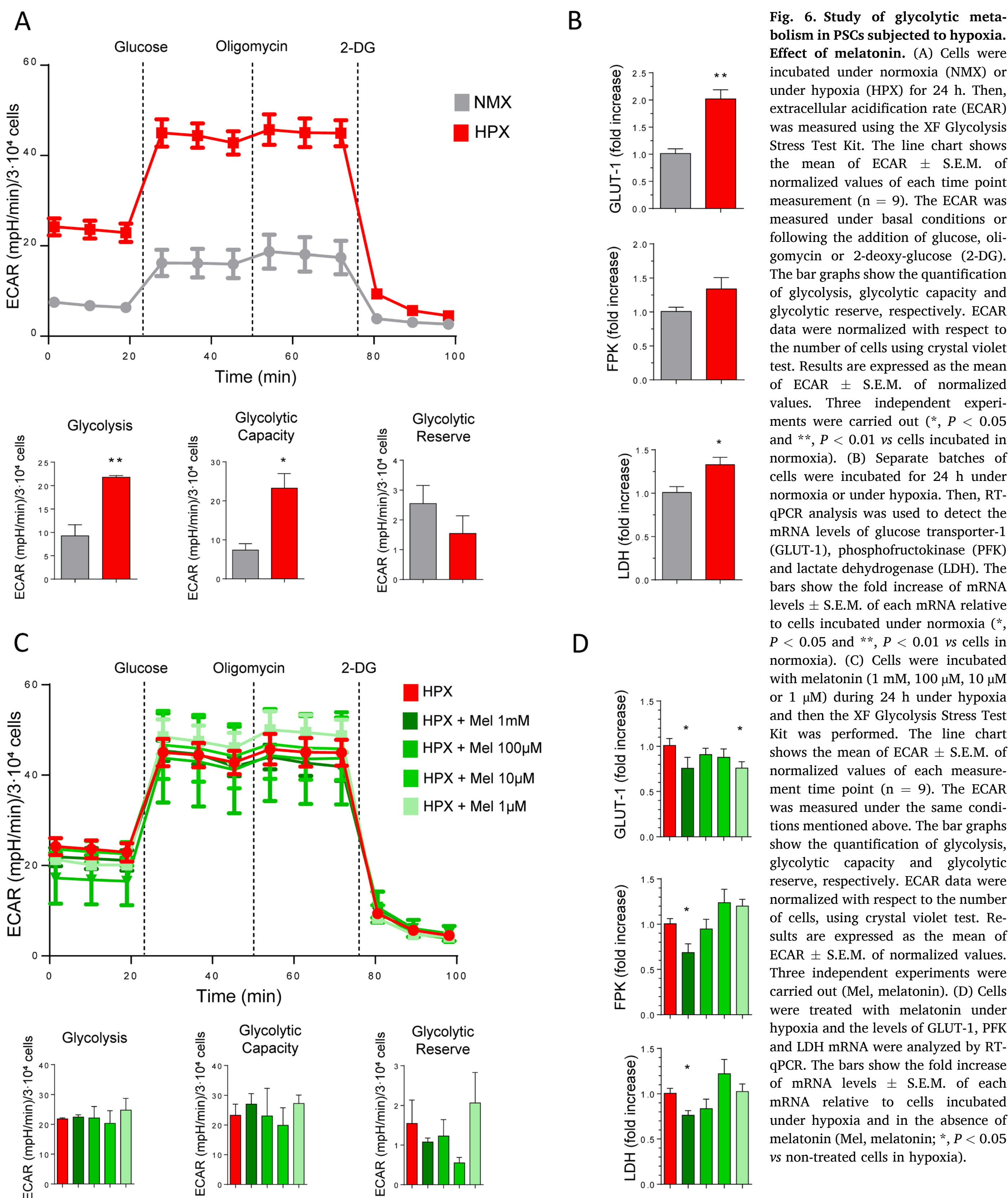

Fig. 6. Study of glycolytic metabolism in PSCs subjected to hypoxia. Effect of melatonin. (A) Cells were incubated under normoxia (NMX) or under hypoxia (HPX) for 24 h. Then, extracellular acidification rate (ECAR) was measured using the XF Glycolysis Stress Test Kit. The line chart shows the mean of 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

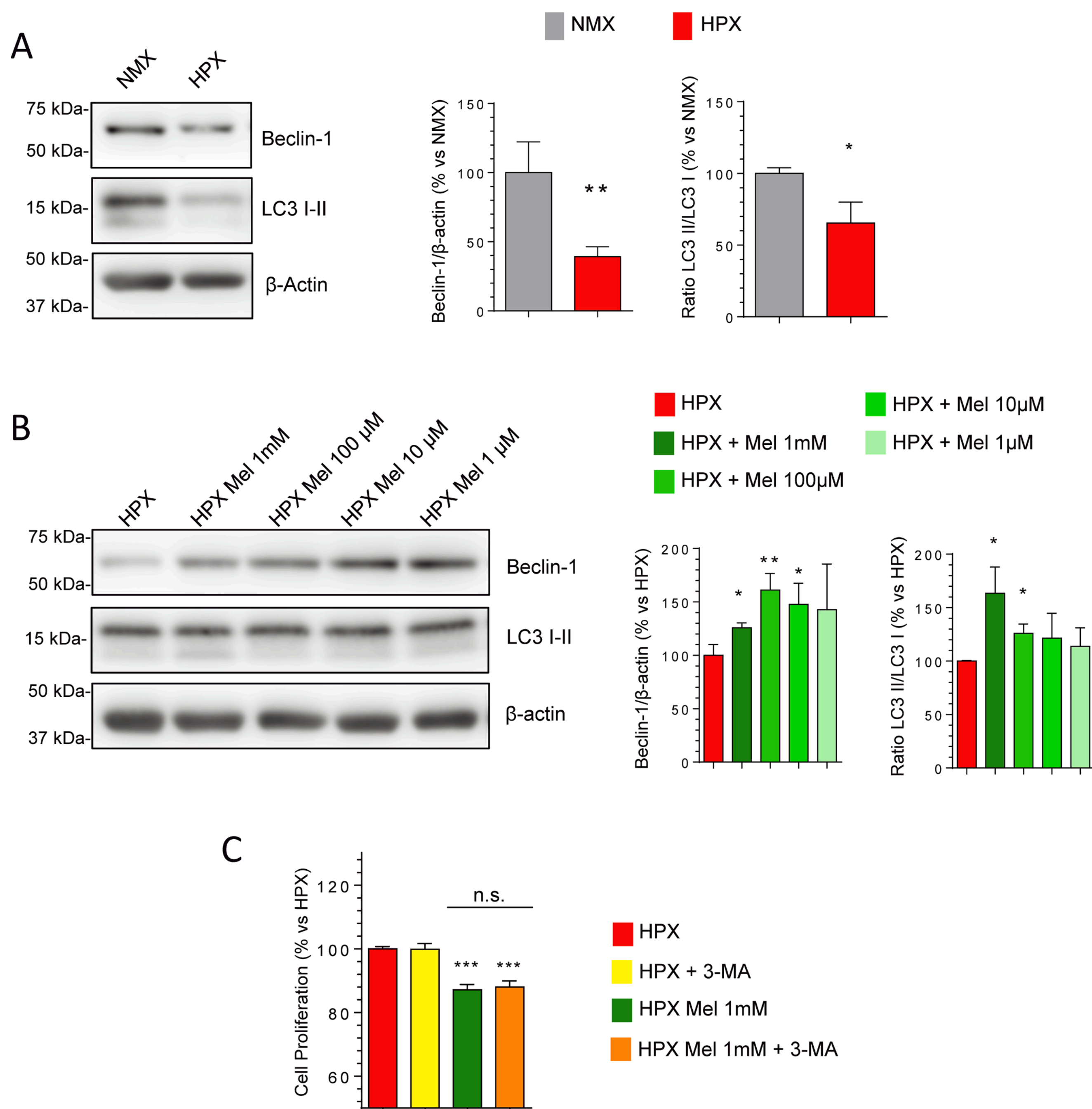

Fig. 7. Study of autophagy in PSCs subjected to hypoxia. Effect of melatonin. (A) Separate batches of cells were incubated for 4 h under normoxia (NMX) or under hypoxia (HPX). The blots show the level detected for Beclin-1 and LC3 I-II. The levels of β-actin were employed as controls to ensure equal 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

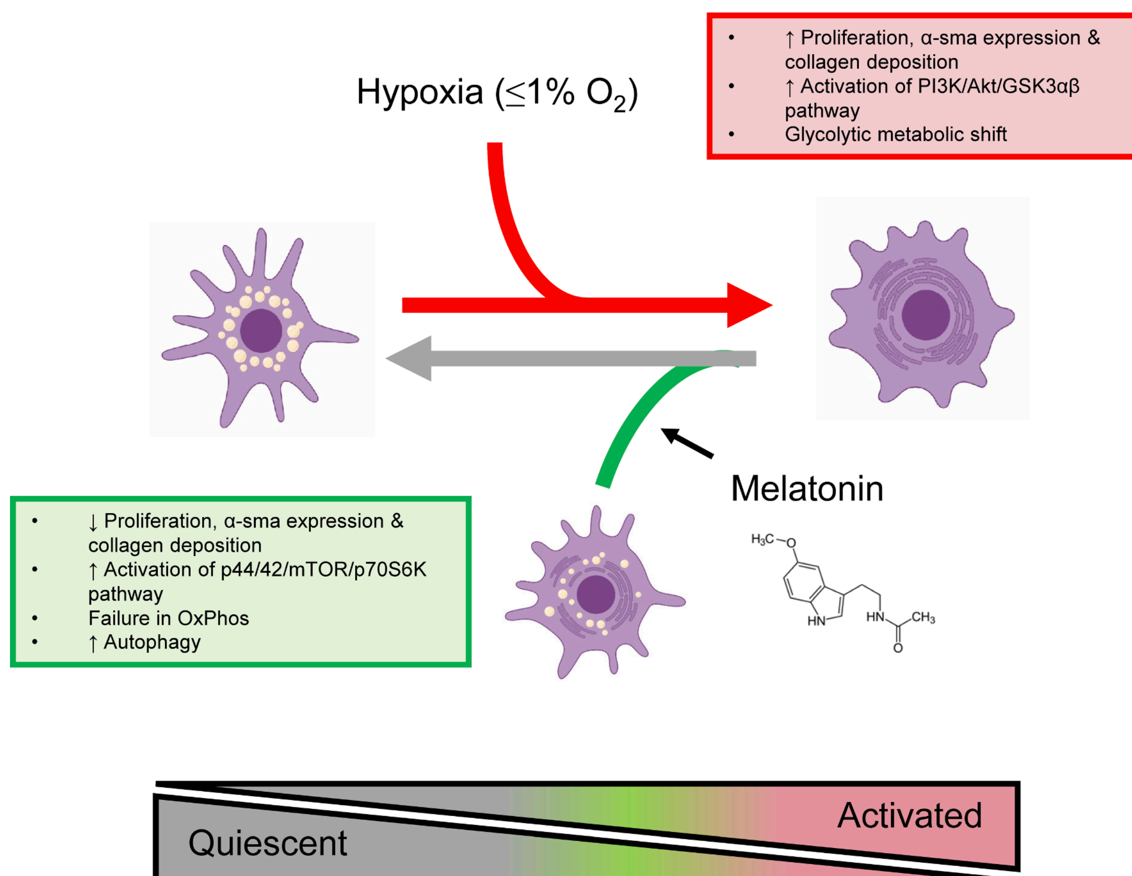

Fig. 8. Melatonin modulates the responses of PSCs to hypoxia. The low availability of O_2 is a recurrent condition in pancreatic fibrosis. Under this ambient, activated PSCs, which are considered the major responsible for the production of fibrotic tissue, proliferate more actively and exhibit increases 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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