Melatonin induces the expression of Nrf2-regulated antioxidant enzymes via PKC and Ca^{2+} influx activation in mouse pancreatic acinar cells.

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Short title: Melatonin and Nrf2 in the exocrine pancreas.

Abbreviations.

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ARE, antioxidant response element; CCK-8, cholecystokinin octapeptide; $[Ca^{2+}]_c$, intracellular free-Ca^{2+} concentration; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N´N´-tetraacetic acid; ER, endoplasmic reticulum; Fura-2/AM, fura-2 acetoxymethyl ester; GClc, catalytic subunit of glutamate-cysteine ligase; HO-1, heme oxygenase-1; H_2O_2, hydrogen peroxide; JNK, c-Jun NH(2)-terminal kinase; NCCE, non-capacitative Ca^{2+} entry; NQO1; NAD(P)H quinone oxidoreductase 1; Nrf2, nuclear factor erythroid 2-related factor; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

ABSTRACT.

The goal of this study was to evaluate the potential activation of the nuclear factor erythroid 2-related factor and the antioxidant-responsive element (Nrf2–ARE) signaling pathway in response to melatonin in isolated mouse pancreatic acinar cells. Changes in intracellular free-Ca^{2+} concentration were followed by fluorimetric analysis of fura-2-loaded cells. The activation of PKC and JNK were measured by western blot analysis. Quantitative reverse transcription-polymerase chain reaction was employed to detect the expression of Nrf2-regulated antioxidant enzymes. Immunocytochemistry was employed to determine nuclear location of phosphorylated-Nrf2, and the cellular redox state was monitored following MitoSOX^{TM} Red-derived fluorescence. Our results show that stimulation of fura-2-loaded cells with melatonin (1 µM to 1 mM), in the presence of Ca^{2+} in the extracellular medium, induced a slow and progressive increase of $[Ca^{2+}]_c$ towards a stable level. Melatonin did not inhibit the typical Ca^{2+} response induced by CCK-8 (1 nM). When the cells were challenged with the indoleamine in the absence of Ca^{2+} in the extracellular solution (medium containing 0.5 mM EGTA) or in the presence of 1 mM $LaCl_3$, to inhibit Ca^{2+} entry, we could not detect any change in $[Ca^{2+}]_c$. Nevertheless, CCK-8 (1 nM) was able to induce the typical mobilization of Ca^{2+}. When the cells were incubated with the PKC activator PMA (1 µM) in the presence of Ca^{2+} in the extracellular medium, we observed a response similar to that noted when the cells were challenged with melatonin 100 µM. However, in the presence of Ro31-8220 (3 µM), a PKC inhibitor, stimulation of cells with melatonin failed to evoke changes in $[Ca^{2+}]_c$. Immunoblots, using an antibody specific for phospho-PKC, revealed that melatonin induces PKCα activation, either in the presence or in the absence of external Ca^{2+}. Melatonin induced the phosphorylation and nuclear translocation of the transcription factor Nrf2, and evoked a concentration-dependent increase in the

expression of the antioxidant enzymes NAD(P)H-quinone oxidoreductase 1, catalytic subunit of glutamate-cysteine ligase and heme oxygenase-1. Incubation of MitoSOX^{TM} Red-loaded pancreatic acinar cells in the presence of 1 nM CCK-8 induced a statistically significant increase in dye-derived fluorescence, reflecting an increase in oxidation, that was abolished by pretreatment of cells with melatonin (100 µM) or PMA (1 µM). On the contrary, pretreatment with Ro31-8220 (3 µM) blocked the effect of melatonin on CCK-8-induced increase in oxidation. Finally, phosphorylation of JNK in the presence of CCK-8 or melatonin was also observed. We conclude that melatonin, via modulation of PKC and Ca^{2+} signaling, could potentially stimulate Nrf2-mediated antioxidant response in mouse pancreatic acinar cells.

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INTRODUCTION.

Oxidative stress is a condition of imbalance between reactive oxygen species (ROS) formation and antioxidant capacity, as a result of dysfunction of the cellular antioxidant system. It has been suggested that excessive amount of ROS could evolve towards an oxidative stress condition, and would damage macromolecules like proteins, DNA and lipids, finally leading to cell damage [1, 2, 3, 4]. Therefore, oxidative stress containment is paramount to protect cell physiology.

In the pancreas, changes in intracellular free-Ca^{2+} concentration ($[Ca^{2+}]_c$) represent a crucial signaling mechanism, and the major physiological processes occur downstream to Ca^{2+} mobilization [5]. It has been shown that pancreatic damage involves Ca^{2+} overload and overproduction of ROS [6]. Ca^{2+} signals are not only a result of Ca^{2+} release from intracellular stores [7], but also involve a coordinate influx from the extracellular space [8], Ca^{2+} extrusion across the plasma membrane [9], and Ca^{2+} uptake into intracellular organelles [10, 11]. Additionally, the transmission of extracellular signals into their intracellular targets is mediated by a network of interacting proteins that regulate a large number of cellular processes [12].

To counteract oxidative stress, cells have developed endogenous defense mechanisms. Among them, the redox sensitive transcription factor nuclear factor erythroid 2-related factor (Nrf2) plays a major role in the defense against oxidative stress, via the induction of antioxidant enzymes. Under non-stressing conditions, Nrf2 is sequestered by its cytosolic binding protein Kelch-like ECH Associated Protein 1 (Keap1) and targeted for proteasomal degradation. Modification of Keap1 cysteine residues leads to nuclear accumulation of Nrf2, where Nrf2 binds to the antioxidant response element (ARE). This results in the coordinated transcriptional activation of a battery of enzymes and detoxifying proteins, including antioxidant enzymes. Target

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genes include NAD(P)H quinone oxidoreductase 1(NQO1), glutamate-cysteine ligase (GCL), heme oxygenase-1 (HO-1), glutathione S-transferase (GST) or γ-glutamylcysteine synthetase (GCS), for example [13, 14]. The mechanism by which Nrf2 is stabilized and translocated to the nucleus is under current study. It has been proposed that ROS react with specific thiol groups, inducing the dissociation of the transcription factor from Keap1 and leading ultimately to Nrf2 nuclear accumulation [15]. On the other hand, experimental evidence suggests that Nrf2 phosphorylation may release Nrf2 from Keap1 [13].

Melatonin receptors are widely distributed in the body, and their expression differs among various organs [16]. Besides its well-known regulatory role on circadian rhythm, melatonin has other biological actions in various cell types and peripheral tissues. These actions control detoxification and stress response genes, thus conferring protection against a number of xenobiotics and endobiotics produced by acute and chronic noxious stimuli [17]. The presence of melatonin MT1 and MT2 type receptors in the pancreas has been reported, and evidences indicate that melatonin plays a major role in the function of the exocrine pancreas [18, 19].

In general, it has been suggested that the indole exerts a regulatory action on pancreatic enzyme secretion [20, 21] and on bicarbonate secretion [22]. The indole may interact with Ca^{2+} channels and inhibit its influx to inactivate the 5-HT pathway in the gut [23]. Melatonin may also modulate Ca^{2+} influx through a non-selective Ca^{2+} permeable cation channel in transfected CHO cells [24], and can stimulate Ca^{2+} transport across the cellular membranes [25]. In addition, melatonin exerts antioxidant [26, 27] and anti-inflammatory properties on the exocrine pancreas [26, 28-30].

Redox-sensitive components are included in the protection mechanisms activated by melatonin, which involve transcriptional response dependent on Nrf2. By

these pathways, melatonin stimulates the expression of antioxidant and detoxification genes [17]. In this line, it has been suggested that melatonin antagonizes oxidative injury through the activation of the Keap1-Nrf2-ARE signaling pathway, and can thus activate enzymes downstream to Nrf2, therefore reversing oxidative injury in the nervous system [31]. This effect has also been suggested in the pancreas, where the indoleamine ameliorates cerulein-induced pancreatitis involving the activation of Nrf2 and NF_KB [32]. Recent evidence additionally suggests that the antioxidant action of both, melatonin and proteasome inhibitors, involves the Keap1-ARE pathway via the up-regulation of Nrf2. Melatonin and proteasome inhibitors suppress the degradation of Nrf2 and also enhance its nuclear translocation [33]. Additionally, it has been shown that Nrf2-regulated antioxidant response is activated by protein kinase C (PKC) [13, 34], and that the physiological function of melatonin is coupled to PKC to modulate cell physiology in Purkinje neurons [35], in photoreceptors [36], in fibroblasts [37], and in myometrial cells [38], for example.

However, the mechanisms whereby melatonin exerts antioxidant effects in the exocrine pancreas are not completely understood. Specially, the involvement of Ca^{2+} signaling in melatonin-evoked effects needs deep attention, because most cellular activity in the pancreas is initiated by changes in $[Ca^{2+}]_c$. In the present study, we aimed to investigate the mechanisms by which melatonin protects the exocrine pancreas against oxidative stress, paying attention to Nrf2 activation and the involvement of Ca^{2+} homeostasis.

MATERIALS AND METHODS.

Animals and chemicals.

Adult male Swiss mice, weighing between 25 and 30 g, were used in the present study. Animals were obtained from the animal house of the University of Extremadura (Caceres, Spain). Animals were humanely handled and sacrificed in accordance to the institutional Bioethical Committee. Collagenase CLSPA was obtained from Worthington Biochemical Corporation (Lakewood, NJ, U.S.A.). Cell Lysis Reagent for cell lysis and protein solubilization, (Tyr[SO_3H][27]) cholecystokinin fragment 26–33 amide (CCK-8), ethylene glycol-bis(2-aminoethylether)-N,N,N´N´-tetraacetic acid (EGTA), melatonin, Tween®-20, propidium iodide, phorbol 12-myristate 13-acetate (PMA), protease inhibitor cocktail (Complete, EDTA-free) and thapsigargin were obtained from Sigma Chemicals Co. (Madrid, Spain). Fura-2-AM, hydrogen peroxide (H_2O_2) and MitoSOX™ Red were obtained from Life Technologies (Invitrogen, Barcelona, Spain). Bradford reagent, Tris/glycine/SDS buffer (10×) and Tris/glycine buffer (10×) were from Bio-Rad (Madrid, Spain). Enhanced chemiluminescence detection reagents were obtained from Pierce (Fisher Scientific Inc., Madrid, Spain). Antibodies against phospho-PKCα and α-tubulin were purchased from Santa Cruz Biotechnology Inc. (Quimigranel S.A., Madrid, Spain). Anti phospho-Nrf2 antibody was obtained from Bioss (Interchim, Montlucon, France). Antibody against phospho-SAPK/JNK (Thr183/Tyr185) was purchased from Cell Signaling (IZASA Biolabs, S.A., Barcelona, Spain). Secondary antibodies rabbit anti-goat IgG-HRP, goat anti-rabbit IgG-HRP and goat anti-rabbit IgG FITC-conjugate antibody were purchased from Pierce (Fisher Scientific Inc., Madrid, Spain). All other analytical grade chemicals used were obtained from Sigma Chemicals Co. (Madrid, Spain).

Preparation of isolated pancreatic acinar cells.

A suspension of mouse pancreatic single cells and small acini was prepared as described previously [10]. Briefly, after mice had been killed by severing of the vertebral column, the pancreas was removed rapidly and placed in a physiological Na-HEPES solution (composition -in mM-: NaCl 130; KCl 4.7; $CaCl_2$ 1.3; $MgCl_2$ 1; KH_2PO_4 1.2; glucose 10; HEPES 10) containing 0.01% trypsin inhibitor (soybean) and 0.2% bovine serum albumin (BSA) and pH adjusted to 7.4 with NaOH. This buffer was supplemented with 30 units/mL collagenase CLSPA from Worthington. Tissues were incubated for 10 min at 37 ºC, after which the cell suspension was gently pipetted through tips of decreasing diameter for mechanical dissociation of the cells. After centrifugation at $30 \times g$ for 5 min at 4 ºC, cells were resuspended in Na-HEPES buffer without collagenase. Cell viability was not significantly changed by the isolation procedure, as assayed by trypan blue exclusion test, and was greater than 95%.

Fura-2 loading and determination of dye-derived fluorescence.

Freshly isolated mouse pancreatic acinar cells, resuspended in Na-HEPES buffer, were loaded with fura-2-AM (4 μM) at room temperature (23–25 ºC) for 40 min, as described previously [39]. Then, cells were centrifuged at $30 \times g$ for 5 min at 4 ºC and resuspended in Na-HEPES buffer without trypsin inhibitor and BSA.

For monitorization of changes of fura-2-dependent fluorescence, related to changes in $[Ca^{2+}]_c$, small aliquots of dye-loaded cells were transferred to a coverslip mounted on an experimental perfusion chamber, and placed on the stage of an epifluorescence inverted microscope (Nikon Diaphot T200, Melville, NY, USA).

The cells were continuously superfused with a control Na-HEPES buffer containing (in mM): 140 NaCl, 4.7 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 Hepes, 10 glucose (pH adjusted to 7.4). When Ca^{2+} free conditions were applied, the Na-HEPES buffer contained no added Ca^{2+}

and was supplemented with 0.5 mM EGTA. In order to determine fura-2-derived fluorescence, an image acquisition system was employed (Hamamatsu Photonics, Hamamatsu, Japan). Cells were excited alternatively at 340/380 nm, with light from a xenon arc lamp passed through a high-speed monochromator (Polychrome IV, Photonics, Hamamatsu, Japan). Fluorescence emission at 505 nm was detected using a cooled digital CCD camera (Hisca CCD C-6790, Hamamatsu, Japan) and recorded using dedicated software (Aquacosmos 2.5, Hamamatsu Photonics, Hamamatsu, Japan).

The ratio of fura-2-emitted fluorescence closely reflects changes in $[Ca^{2+}]_c$ [40]. In this series of experiments, results are expressed in units of fluorescence (u. f.) as the ratio of fluorescence emitted at 340 nm and 380 nm excitation wavelengths, normalized to the basal fluorescence. All fluorescence measurements were made from areas considered individual cells.

Cell viability was not changed by the isolation procedure, as determined using the trypan blue exclusion test, and was > 95%. After loading with the fluorescent dye, cells were kept at 4 ºC until use, and experiments were performed within 4 h. In experiments in which Ca^{2+}-free medium was used, Ca^{2+} was omitted and 0.5 mM EGTA was added to the extracellular solution. In the experiments in which the effect of La^{3+} was tested, neither Ca^{2+} nor EGTA were added to the extracellular solution. No apparent morphological changes of cells were observed that could yield errors in Ca^{2+} measurements.

All stimuli were dissolved in the extracellular Na-HEPES buffer, with or without Ca^{2+}, and applied directly to the cells in the perfusion chamber. Experiments were performed at room temperature (23–25 ºC), and different batches of cells were used for the studies.

Western blot analysis.

For western blotting analysis cells were incubated with the appropriate stimulus, centrifuged and then washed twice with PBS. After washing, cells were sonicated for 5 s at 4 ºC in 100 µL of lysis buffer, supplemented with protease inhibitor cocktail and 0.2 mM Na_3VO_4. The homogenates were clarified by centrifugation at $10000 \times g$ (15 min, 4 °C) and the supernatant was used for analysis of protein concentration followed by dilution with 4× SDS sample buffer. Protein lysates (30 µg/well) were fractionated by SDS-PAGE using 10% polyacrylamide gels and transferred to nitrocellulose membranes. After blocking, membranes were incubated overnight at 4 ºC with the specific primary antibody. The following day, membranes were washed twice and incubated for 45 min at 25 °C with IgG-HRP conjugated secondary antibody. Membranes were then washed again, incubated with enhanced chemiluminescence detection reagents, and, finally, exposed to Hyperfilm ECL films (Amersham, Arlington Heights, IL). The intensity and molecular weight of the bands which appear were quantified using the software Image J (http://imagej.nih.gov/ij/). The experiments were carried out employing different batches of cells, prepared on different days.

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

This was performed in total RNA samples, purified from isolated mouse pancreatic cells using a commercially available kit (Sigma, Madrid, Spain), utilizing the Power SYBR Green RNA-to-C_T^{TM} 1-Step kit (Applied Biosystems, Township, USA). Reverse transcription was performed for 30 min at 48 ºC, and PCR conditions were 10 min at 95 ºC followed by 40 cycles of 15 s at 95 ºC plus 1 min at 55 ºC using the following forward and reserve primers, respectively (Thermo Scientific, Offenbach,

Germany), 5´ -AGCACAGGGTGACAGAAGAG-3´ and 5´ -GAGGGACTCTGGTCTTTGTG-3´ (*Gclc*), 5´ -GGCACAAGGACGTGCTCAAGT-3´ and 5´ -TGCAGAGTTTCAAGAACATCG-3´ (*Ho-1*), 5´ -GGGGACATGAACGTCATTCTCT-3´ and 5´ -AAGACCTGGAAGCCACAGAAGC-3´ (*Nqo-1*), 5´ -GGGTGTGAACCACGAGAAAT-3´ and 5´ -CCTTCCACGATGCCAAAGTT-3´ (*Gapdh*). 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 $\Delta\Delta$Ct method, and were expressed as the fold change between sample and calibrator.

Immunocytochemistry.

This procedure was employed to determine phosphorylated-Nrf2 location. Briefly, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and incubated with the specific primary antibody. After washing out the first antibody, cells were incubated with a FITC-labeled secondary antibody. Monitorization of FITC-derived fluorescence signals was performed employing confocal laser-scanning microscopy (Bio-Rad, MRC 1024; American Laser Corp., Salt Lake City, UT). Using a $\times$ 60 oil immersion objective fluorescence, images of 256 $\times$ 256 pixels with a resolution of 0.287 mm/pixel were recorded. Samples were excited at 488 nm from a 100 mW argon ion laser and emitted fluorescence was collected through a 522/35-nm band-pass filter employing a photomultiplier. Propidium iodide was excited with the 543-nm line from a 25-mW helium–neon laser, and emission was collected through a 605/32-nm band-pass filter. The software used for the imaging was Laser Sharp MRC-1024 Version 3.2 (Bio-Rad, Deisenhofen, Germany). The absence of non-specific staining was assessed by processing the samples without primary antibody.

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Determination of oxidation.

For the determination of the redox state, cells were incubated in the presence of MitoSOX^{TM} Red (2.5 mM) for 15 min. at 37 ºC. Generation of ROS was determined by measuring cellular fluorescence at 510 nm/580 nm (excitation/emission) using an ELISA spectrofluorimeter (Tecan Infinite M200, Grödig, Austria). Data show the mean increase of fluorescence expressed in percentage ± S.E.M. (n) with respect to control (non-stimulated) cells, where n is the number of independent experiments.

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 Student's *t* test, and only *P* values < 0.05 were considered statistically significant.

RESULTS.

Changes in $[Ca^{2+}]_c$ in response to melatonin.

Stimulation of fura-2-loaded cells with 1 nM CCK-8, in the presence of extracellular Ca^{2+} (1 mM), induced an initial increase in $[Ca^{2+}]_c$ that was followed by a decrease towards a plateau value over the prestimulation level (n = 4 experiments/97 of 97 total cells studied; Fig. 3A, continuous line). When the cells were incubated with CCK-8 (1 nM) in the absence of extracellular Ca^{2+} (medium containing 0.5 mM EGTA) a similar response was observed; however, the recovery of $[Ca^{2+}]_c$ was faster and

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decreased towards the prestimutation level (n = 3 experiments/60 of 60 total cells studied; Fig. 1A, dashed line).

In the next set of experiments the cells were incubated with different concentrations of melatonin (1 μM to 1 mM), in the presence of Ca^{2+} in the extracellular medium (Fig. 1B-E). The pattern of Ca^{2+} mobilization that we observed consisted of a slow and progressive increase of $[Ca^{2+}]_c$ towards a stable level. In occasions, single and short amplitude spikes in $[Ca^{2+}]_c$ could be noted. At the end of each experiment cells were stimulated with 1 nM CCK-8, and we obtained the typical Ca^{2+} response induced by the agonist. Throughout the following determinations, we chose the concentration of 100 μM melatonin, a concentration that has shown clear effects in recent studies [21, 41].

To find out the source of the increase in $[Ca^{2+}]_c$ that we observed when the cells were incubated with melatonin, cells were challenged with the indoleamine in the absence of Ca^{2+} in the extracellular solution (medium containing 0.5 mM EGTA). Under these conditions we could not detect any change in $[Ca^{2+}]_c$ when the cells were challenged with melatonin. At the end of the experiment, cells were stimulated with 1 nM CCK-8, which evoked a typical mobilization of Ca^{2+} (Fig. 2A). These observations suggest that the responses of $[Ca^{2+}]_c$ noted in the presence of melatonin could have an extracellular origin.

To further study the extracellular origin of the responses evoked in $[Ca^{2+}]_c$ by melatonin, cells were incubated in the presence of 1 mM $LaCl_3$. It has been shown that $LaCl_3$ blocks the entry of Ca^{2+} through the plasma membrane [42, 43]. Now, any increase of $[Ca^{2+}]_c$ would be a consequence of its release from intracellular stores. Thus, the cells were incubated with melatonin (100 μM) and in the presence of 1 mM $LaCl_3$ in the extracellular solution (Fig. 2B). We could not observe any change in $[Ca^{2+}]_c$ when

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the cells were challenged with melatonin. At the end of the experiment, cells were stimulated with 1 nM CCK-8, which evoked a typical mobilization of Ca^{2+}. These observations support the hypothesis that the responses of $[Ca^{2+}]_c$ noted in the presence of melatonin have an extracellular origin.

Involvement of PKC in melatonin-evoked changes in $[Ca^{2+}]_c$.

Stimulation of PKC has been related to the activation of Ca^{2+} entry [44, 45]. Therefore, we investigated whether activation of PKC was related to the Ca^{2+} entry that we observed in the presence of melatonin. For this purpose, fura-2-loaded cells were challenged with 1 μM PMA, a PKC activator [44], in the presence of Ca^{2+} in the extracellular medium (Fig. 3A; n = 5 experiments/100 of 100 total cells studied). Under these conditions, we observed a slow and progressive increase of $[Ca^{2+}]_c$ towards a stable level, similar to that noted when the cells were challenged with melatonin. When the indoleamine was included in the extracellular solution we could not observe further increases in $[Ca^{2+}]_c$. At the end of the experiment, stimulation of cells with 1 nM CCK-8 evoked the typical Ca^{2+} response induced by the agonist.

In another set of experiments, cells were incubated in the presence of the PKC inhibitor Ro31-8220 [46]. In the presence of the PKC inhibitor (3 μM), stimulation of cells with melatonin (100 μM) failed to evoke a change in $[Ca^{2+}]_c$. Again, stimulation of cells with 1 nM CCK-8 at the end of the experiment evoked a typical Ca^{2+} response (Fig. 3B; n = 6 experiments/127 of 138 total cells studied). These results suggest that melatonin induces Ca^{2+} entry from the extracellular medium via PKC activation.

Members of the PKC family play a key regulatory role in a variety of cellular functions, including cell growth and differentiation, gene expression, hormone secretion and membrane function. PKC isoforms can be activated through phosphorylation.

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Confirmation of PKC activation by melatonin was carried out by western blot. Immunoblots using an antibody specific for phospho-PKCα were performed. Pancreatic acinar cells were incubated with 1 μM - 100 μM melatonin for 1 hour, in the presence of Ca^{2+} in the extracellular medium. Then, cell lysates were analyzed to determine the level of PKCα phosphorylation. The data indicate that incubation of pancreatic acinar cells with melatonin resulted in a concentration-dependent increase in the level of phosphorylated PKCα. PMA (1 μM) also evoked a significant increase in the phosphorylation of the kinase (Fig. 4A and B).

To ascertain whether Ca^{2+} entry was necessary for melatonin-induced phosphorylation of PKCα, cells were challenged with melanonin (1 μM – 100 μM) or PMA (1 μM) in the absence of Ca^{2+} in the extracellular medium. Under these conditions, incubation of cells with the stimuli also induced phosphorylation of PKCα (Fig. 4A and C). The results show that melatonin induces phosphorylation of the kinase either in the presence or in the absence of Ca^{2+} in the extracellular medium.

Activation by melatonin of crucial Nrf2-dependent antioxidant enzymes.

Nrf2 is a transcription factor that functions as the key controller of the redox homeostatic gene regulatory network. Upon activation, the Nrf2 signaling enhances the expression of a multitude of antioxidant and phase II enzymes that restore redox homeostasis [14]. To study whether melatonin could stimulate the transcriptional activation of certain antioxidant enzymes through the activation of Nrf2, RT-qPCR of the relative mRNA abundance was performed. Results reveal that melatonin (1 hour incubation; 1 μM - 100 μM) evoked a concentration-dependent and statistically significant increase in the expression of GCLc, HO-1 and NQO1 (Fig. 5). However, we could not detect any significant increase in the expression of the mentioned antioxidant

enzymes when the cells were incubated during 1 hour in the presence of the pancreatic secretagogue CCK-8 (1 nM). To evaluate the implication of PKC in the actions of melatonin, cells were incubated for 1 hour in the presence of 1 μM PMA. The PKC activator induced a statistically significant increase in the expression of the mentioned antioxidant enzymes (Fig. 5). On the contrary, inhibition of PKC by preincubation of cells during 1 hour with Ro31-8220 (3 μM) inhibited the effect of melatonin (100 μM; Fig. 5). Moreover, to ascertain if the Ca^{2+} influx activated by melatonin contributed to the activation of the Nrf2-dependent antioxidant enzymes, we carried out the experiments in the absence of extracellular Ca^{2+} (we employed a nominally Ca^{2+} free medium, i.e., no Ca^{2+} was added to the extracellular solution, that was prepared employing milli-Q water). Under these conditions, melatonin (100 μM) failed to induce any increase in the expression of the mentioned antioxidant enzymes (Fig. 5). As a control, cells were incubated in the presence of H_2O_2 (100 μM), a known Nrf2 activator [47]. The oxidant triggered the expression of all three antioxidant enzymes studied (Fig. 5).

Having determined that melatonin induced changes in the expression of antioxidant enzymes regulated by Nrf2, it was of interest to analyze whether melatonin influences the phosphorylation and nuclear translocation of the transcription factor. For this purpose we performed immunocytochemical studies, employing a specific antibody against the phosphorylated form of Nrf2. In non-treated cells, we did not observe nuclear staining for the transcription factor (Fig. 6A). In the presence of the indoleamine (100 μM; 1 hour incubation), phosphorilated Nrf2 could be detected in the nucleus (Fig. 6B). A similar effect was observed when the cells were incubated with PMA (1 μM; Fig. 6C). However, we could not observe nuclear translocation of Nrf2 when the cells

were stimulated with melatonin in the absence of extracellular Ca^{2+} (Fig. 6D) or after previous inhibiton of PKC by incubation of cells with Ro31-8220 (3 μM) (Fig. 6E).

These results provide evidences that melatonin induces the nuclear translocation of the transcription factor Nrf2, and stimulates the expression of crucial antioxidants enzymes that might regulate the cellular redox status.

Effect of melatonin on the oxidative state of pancreatic acinar cells.

Bearing in mind that melatonin stimulated Nrf2-dependent antioxidant enzymes expression, we investigated the effect of the indoleamine on the cellular oxidative state. For this purpose we employed the redox-sensitive fluorescent dye MitoSOX[TM] Red, and we studied the protective effect of melatonin against CCK-8-evoked oxidation. This is a pancreatic agonist that induces oxidation in the exocrine pancreas [38]. Incubation during 1 hour of MitoSOX[TM] Red-loaded pancreatic acinar cells in the presence of 1 nM CCK-8 induced a statistically significant increase in dye-derived fluorescence, suggesting oxidation of the dye (Fig. 7). Melatonin (100 μM; 1 hour incubation) did not increase MitoSOX[TM] Red -derived fluorescence compared with that observed in control (non-stimulated) cells (Fig. 7). When the cells were preincubated with melatonin (100 μM; 5 min. preincubation) and then were stimulated with CCK-8 (1 nM), the oxidation induced by CCK-8 was blunted. A similar effect was observed when the cells were preincubated in the presence of the PKC activator PMA (1 μM). On the contrary, inclusion of the PKC inhibitor Ro31-8220 (3 μM) blocked the protective effect of melatonin on CCK-8-induced increase in MitoSOX[TM] Red fluorescence (Fig. 7). As a control, we tested the effect of H_2O_2, a well known oxidant. In the presence of H_2O_2 (100 μM) an increase in MitoSOX[TM] Red -derived fluorescence was observed (Fig. 7).

These data suggest that activation of PKC by melatonin is involved in the protective effect of the indoleamine against CCK-8-induced oxidation in mouse pancreatic acinar cells. The results further support a putative relationship between the effects of melatonin on Nrf2 and the activation of cellular antioxidant response.

Effect of melatonin on JNK.

In the exocrine pancreas, agonists can activate several signaling pathways which may directly or indirectly influence ROS formation. In a former work, performed in a cellular model of pancreatitis, it has been shown that cerulein induced ROS formation involving c-Jun NH(2)-terminal kinase (JNK) activation [49].

To analyze whether JNK is involved in melatonin-evoked responses in pancreatic acinar cells, we carried out immunoblots using an antibody specific for phospho-SAPK/JNK. Pancreatic acinar cells were incubated for 1 hour with 100 μM melatonin, 1 nM CCK-8 or 1 μM PMA, in the presence of Ca^{2+} in the extracellular medium. Then cell lysates were analyzed to determine the level of SAPK/JNK phosphorylation. The data indicate that incubation of pancreatic acinar cells with melatonin resulted in an increase in the level of phosphorylated SAPK/JNK. CCK-8 (1 nM) and PMA (1 μM) also evoked significant increases in the phosphorylation of the kinase (Fig. 8).

DISCUSSION.

Melatonin is produced in the mammalian pineal gland and retina following a circadian rhythm with high levels being released from the pineal in the blood at night, and regulates an array of physiological processes [19, 50, 51]. Melatonin is also released from the gastrointestinal tract, where the amount of this indole is greater than

the content of melatonin in the central nervous system [52]. Moreover, the indole is present in plant foods [53].

Melatonin MT1 and MT2 receptors are widely expressed throughout the body (gastrointestinal tract included) [54], and it exhibits antioxidant and anti-inflammatory functions. Actually, active research is carried out in order to clarify the signaling pathway activated by melatonin in the induction of antioxidant response. Existing data support that the actions of the indoleamine are related to its effects on intracellular cAMP [55], reactive oxygen species (ROS) production [56], via the cGMP signaling pathway [57], or involving Ca^{2+} signaling [18, 21, 58, 59]. Recently, it has been suggested that melatonin protects the pancreatic damage via the decrease of oxidative stress and an increase of the activities of antioxidant enzymes [60]. In the latter work, the total antioxidant status and glutathione peroxidase activity were determined *in vivo*. However, the intracellular mechanisms involved in melatonin-evoked antioxidant responses in the exocrine pancreas remain to be elucidated. Our work has evaluated the probable mechanisms by which melatonin sets on the antioxidant response and protects the exocrine pancreas against oxidative stress.

Ca^{2+} is a second messenger involved in the regulation of pancreatic exocrine function [5]. Therefore, investigation of the effects of melatonin on $[Ca^{2+}]_c$ would provide important information on the role of melatonin in the regulation of pancreatic acinar cells physiology. Our results suggest that the indole is stimulating the entry of Ca^{2+} from the extracellular space. Melatonin-evoked Ca^{2+} signals were not detected if extracellular Ca^{2+} was omitted, and also in the presence of La^{3+}. This effect resembles the characteristics of a non-capacitative Ca^{2+} entry (NCCE). The movement of Ca^{2+} into the cell via this pathway has been previously described [61, 62]. Moreover, Ca^{2+} influx evoked by the indoleamine involved PKC activation. Indeed, melatonin induced

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phosphorylation of the kinase either in the presence or in the absence of Ca^{2+} in the extracellular medium. Thus, our results suggest that PKC is activated by melatonin, and afterwards Ca^{2+} entry takes place. This is consistent with our observations, in which we could not detect Ca^{2+} entry in cells stimulated with melatonin in the presence of the PKC inhibitor Ro31-8220. Our results support previous findings that report the involvement of PKC in melatonin-induced Ca^{2+} signals [63], and in the activation of NCCE [64].

Cellular redox homeostasis mediates a plethora of cellular pathways that determine life and death events. The balance between oxidative stress and protective mechanisms predisposes the daily evolution of cellular physiology. Imbalances between them can produce aberrant redox homeostasis with resultant pathologies. It has been proposed that melatonin upregulates the expression of antioxidant enzymes. Recent evidence suggests that the antioxidant action of melatonin involves the Keap1-ARE pathway via the upregulation of Nrf2 [65]. The action of melatonin on Nrf2 pathway has been shown in several tissues. For example, melatonin markedly promoted the translocation of Nrf2 protein from the cytoplasm to the nucleus, increased the expression of Nrf2-ARE pathway-related downstream factors, and prevented the decline of antioxidant enzyme activities, attenuating brain injury [66]. Melatonin enhanced the expression of HO1 via NF-κB, p38 MAPK and Nrf2 cascade signaling pathways in murine peritoneal macrophages [67]. Additionally, melatonin treatment increased Nrf2 accumulation in the nuclear fraction, and increased the expression of HO-1 in the cytosolic fraction, attenuating cisplatin-induced nephrotoxicity [68], or protecting endothelial cells [69] and liver [70] against ischemic injury.

In view of our previously published results, which show that melatonin does not stimulate amylase release in pancreatic acinar cells [20], we might speculate that the

route for Ca^{2+} entry activated by melatonin might selectively direct Ca^{2+} to processes that mediate activation of the ARE in pancreatic acinar cells.

Our results support this hypothesis. Here we have shown that incubation of pancreatic acinar cells with melatonin induced nuclear translocation of phosphorylated Nrf2. Moreover, in the presence of melatonin, we detected an increase in the expression of the Nrf2-regulated antioxidant enzymes NQO1, GCLc and HO-1. Interestingly, extracellular Ca^{2+} and PKC activation were involved in the expression of such enzymes. Finally, melatonin protected pancreatic acinar cells against CCK-8-induced oxidation, and PKC was involved in this effect.

Sledzinski et al. [49] suggested that CCK-8 can activate several signaling pathways which directly or indirectly may influence ROS formation. The authors showed that activation of JNK was involved in an iron-dependent ROS formation. We could speculate with a putative effect of melatonin on JNK-phsophorylation state to modulate ROS formation in pancreatic acinar cells. Taking into account the mentioned work, a decrease in ROS formation would be explained by an inhibition of JNK in the presence of melatonin. However, we have observed an increase in the phosphorylation of JNK in the presence of melatonin, despite we did not detect any increase in ROS formation in cells incubated with the indole. We also observed an increase in phosphorylated JNK when the cells were challenged with CCK-8 or PMA. However, we noted a decrease in ROS formation when the cells were incubated with melatonin or PMA plus CCK-8 compared with the effect of CCK-8 alone. Our results rather suggest that the effect of melatonin on the cellular redox status seems not to involve inactivation of JNK. Therefore, changes in antioxidant enzyme genes expression might be of major relevance in the actions of melatonin to modulate redox status in our cellular model.

In conclusion, our observations suggest that melatonin potentially stimulates Nrf2-mediated antioxidant enzyme expression in mouse pancreatic acinar cells. The mechanisms that underlie such action seem to involve the activation of PKC and Ca^{2+} entry from the extracellular space. This is of the greatest significance, because induction of antioxidant enzyme synthesis through Nrf2 is a fundamental process in cellular response to stress, letting the cell compensate for the damages caused by oxidants. Thus, targeting the Nrf2 defense pathway by melatonin may represent a mechanism by which the indoleamine might prevent oxidative stress-induced damage in the exocrine pancreas. The mechanisms by which melatonin is coupled to PKC deserves future study.

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Legends to figures.

Figure 1. Changes in $[Ca^{2+}]_c$ in response to cholecystokinin (CCK)-8 and melatonin (1 μM – 1 mM). (A) Time-course of changes in $[Ca^{2+}]_c$ in fura-2-loaded mouse pancreatic acinar cells stimulated with 1 nM CCK-8 alone in the presence (continuous line; n = 4 expt./97 of 97 total cells studied) or in the absence of Ca^{2+} in the extracellular medium (dashed line; n = 3 expt./60 of 60 total cells studied; e.c., extracellular Ca^{2+}). (B-E) Cells were incubated in the presence of 1 μM (n = 8 expt./66 of 128 total cells studied), 10 μM (n = 9 expt; 90 of 146 total cells studied), 100 μM (n = 21 expt; 325 of 485 total cells studied), or 1 mM (n = 8 expt./48 of 110 total cells studied) melatonin. In the presence of melatonin, cells were further stimulated with 1 nM CCK-8. The horizontal continuous bars indicate the time during which stimuli were applied to the cells. A thin dashed horizontal line indicates the level corresponding to basal $[Ca^{2+}]_c$.

Figure 2. Changes in $[Ca^{2+}]_c$ in response to melatonin (100 μM) in the absence of an extracellular source for Ca^{2+} influx. (A) Time-course of changes in $[Ca^{2+}]_c$ in fura-2-loaded mouse pancreatic acinar cells incubated with 100 μM melatonin in the absence of extracellular Ca^{2+} (e.c.; medium containing 0.5 mM EGTA) (n = 20 expt./397 of 504 total cells studied). As a control, and in the presence of melatonin, cells were further stimulated with 1 nM CCK-8. (B) Cells were incubated in the presence of 1 mM LaCl$_3$ and then were stimulated with 100 μM melatonin. At the end of the experiment cells were stimulated with 1 nM CCK-8. The experiments were carried out in a medium nominally free of Ca^{2+}, i.e., no Ca^{2+} was added and EGTA was omitted in order to avoid competition with La^{3+} (n = 7 expt./136 of 156 total cells studied). The horizontal

continuous bars indicate the time during which stimuli were applied to the cells. A thin dashed horizontal line indicates the level corresponding to basal $[Ca^{2+}]_c$.

Figure 3. Involvement of PKC activation in melatonin-evoked changes in $[Ca^{2+}]_c$. (A) Time-course of changes in $[Ca^{2+}]_c$ in fura-2-loaded mouse pancreatic acinar cells incubated with 1 μM PMA in the presence of extracellular Ca^{2+}. Following preincubation of cells with the PKC activator, melatonin (100 μM) was included in the extracellular solution. At the end of the experiment, cells were further stimulated with 1 nM CCK-8 (n = 5 experiments/100 of 100 total cells studied). (B) Cells were incubated in the presence of the PKC inhibitor Ro31-8220 (3 μM) and then were stimulated with 100 μM melatonin. At the end of the experiment cells were stimulated with 1 nM CCK-8 (n = 6 expt./127 of 138 total cells studied). The horizontal continuous bars indicate the time during which stimuli were applied to the cells. A thin dashed horizontal line indicates the level corresponding to basal $[Ca^{2+}]_c$.

Figure 4. Activation of PKC by melatonin. (A) Representative blots showing the phosphorylation status of PKCα evaluated with a phosphospecific antibody, in the presence or in the absence of Ca^{2+} in the extracellular medium. To ensure equal loading of proteins, the levels of α-tubulin were employed as controls under the tested conditions. Cells were incubated during 1 hour with the appropriate stimulus, in the presence or in the absence of extracellular Ca^{2+}. The experiment shown is representative of three others. (B and C) The graphs show the quantification of protein phosphorylation. Values are the mean ± S.E.M. of normalized values expressed as % of phosphorylation in non-stimulated cells (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ *vs* non-stimulated cells).
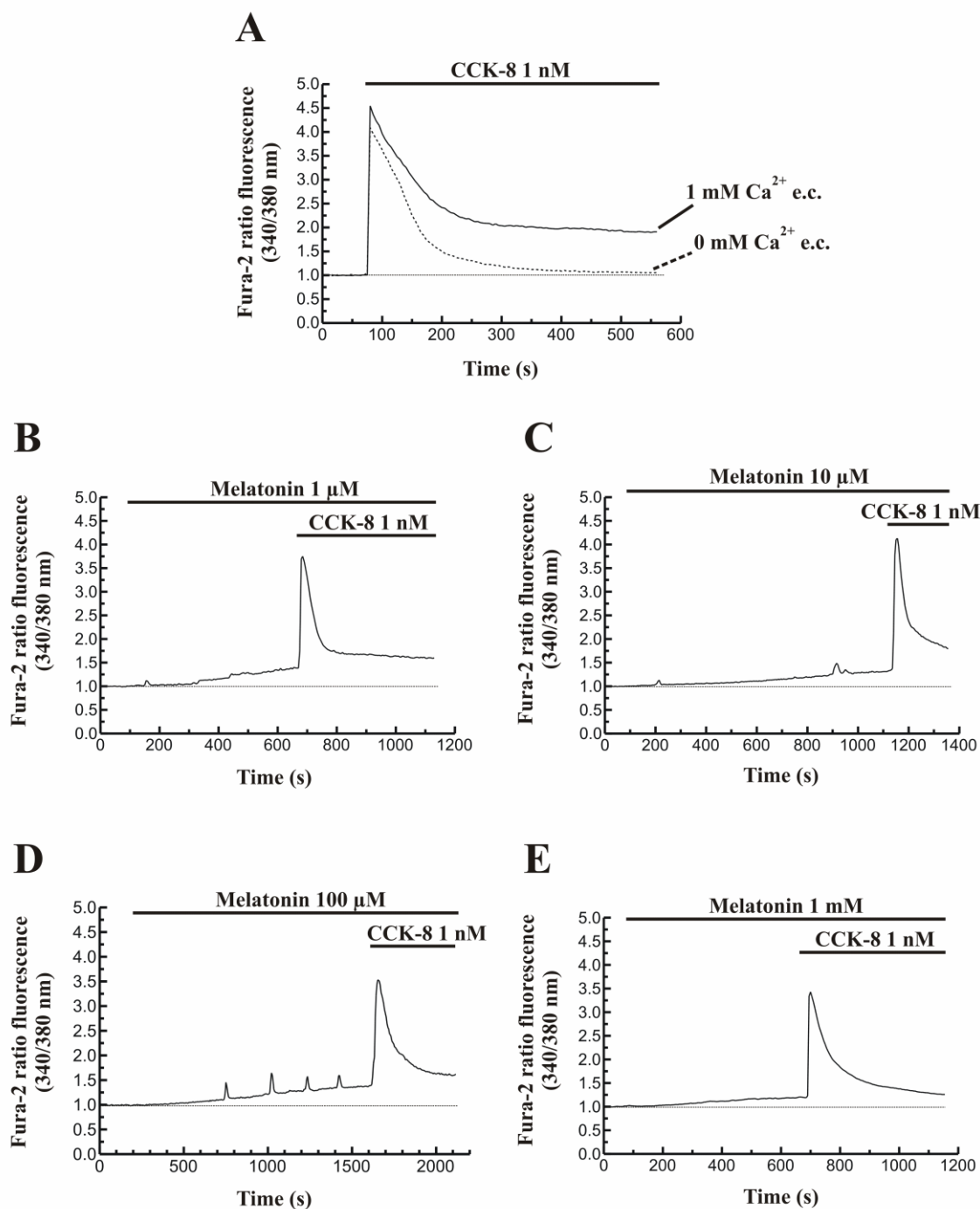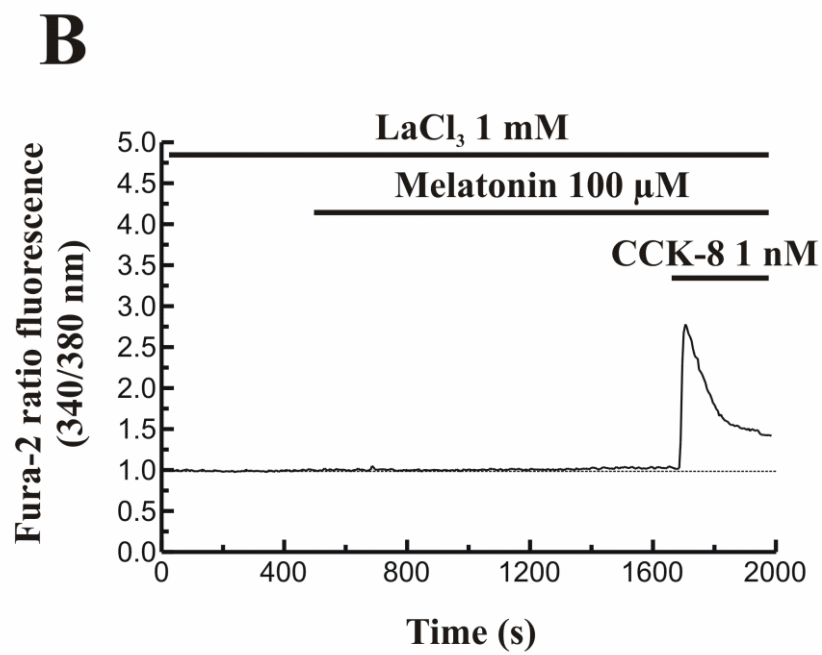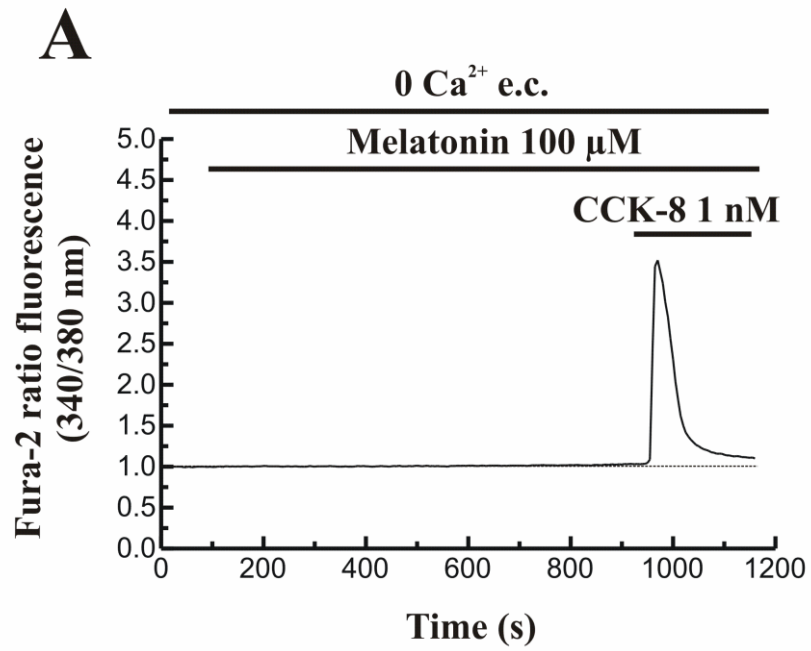
Figure 5. Quantitative real-time PCR (RT-qPCR) analysis of the relative mRNA abundance of Nrf2-dependent antioxidant enzymes in mouse pancreatic acinar cells. RT-qPCR analysis of Nrf2-target genes: glutamate cysteine ligase-catalytic subunit (GClc; A), heme-oxygenase-1 (HO-1; B) and (NQO1; C), reveals statistically significant increases in the levels of Nrf2-dependent antioxidant enzymes in cells incubated during 1 hour in the presence of melatonin. When the cells were challenged in the presence of CCK-8 (1 nM; 1 hour incubation), no statistically significant changes in the expression of the antioxidant enzymes could be noted. Incubation of cells for 1 hour with the PKC activator PMA (1 μM) also evoked an increase in the expression of all three antioxidant enzymes. Preincubation of cells during 1 hour in the presence of the PKC inhibitor Ro31-8220 (3 μM) or in the absence of extracellular Ca^{2+} blunted the effect of melatonin (the concentration of indoleamine employed was 100 μM). *Gapdh* mRNA was used for normalization. Data are expressed as the mean ± S.E.M. of the change relative to non-treated cells (n = 3 independent experiments; *, p < 0.05).

Figure 6. Immunocytochemistry study of cells showing intracellular localization of phosphorylated-Nrf2. (A) Nuclear translocation of phosphorylated-Nrf2 could not be detected in non-treated cells. (B) Cells were incubated for 1 hour in the presence of 100 μM melatonin. (C) Treatment of cells during 1 hour with the PKC activator PMA (1 μM) induced a similar effect to that noted with melatonin. (D) Preincubation of cells in the absence of extracellular Ca^{2+} (nominally free Ca^{2+} medium) blocked the effect of melatonin. (E) We could not detect nuclear staining for phosphorylated-Nrf2 if cells were preincubated for 1 hour in the presence of the PKC inhibitor Ro31-8220 (3 μM),

prior to the treatment of cells with melatonin. Images are representative of three different preparations (N, nucleus).

Figure 7. Study of the oxidative state of pancreatic acinar cells loaded with the redox-sensitive dye MitoSOX^{TM} Red. Cells were incubated during 1 hour in the presence of different stimuli, and the effect on **MitoSOX^{TM} Red** oxidation was studied. Results report the oxidative state of treated cells compared with that of control (non-treated) cells. Data are representative of six independent experiments (**, $P < 0.01$; and ***, $P < 0.001$ *vs* non-treated cells. †, $P < 0.05$; ††, $P < 0.01$; †††, $P < 0.001$ *vs* CCK-8).

Figure 8. Effect of melatonin on JNK. (A) Representative blots showing the phosphorylation status of JNK evaluated with a phosphospecific antibody. To ensure equal loading of proteins, the levels of α-tubulin were employed as controls under the tested conditions. Cells were incubated during 1 hour in the presence of the appropriate stimulus. The experiment shown is representative of three others. (B) The graphs show the quantification of protein phosphorylation. Values are the mean ± S.E.M. of normalized values expressed as % of phosphorylation in non-stimulated cells (*, $P < 0.05$ *vs* non-stimulated cells).

Highlights

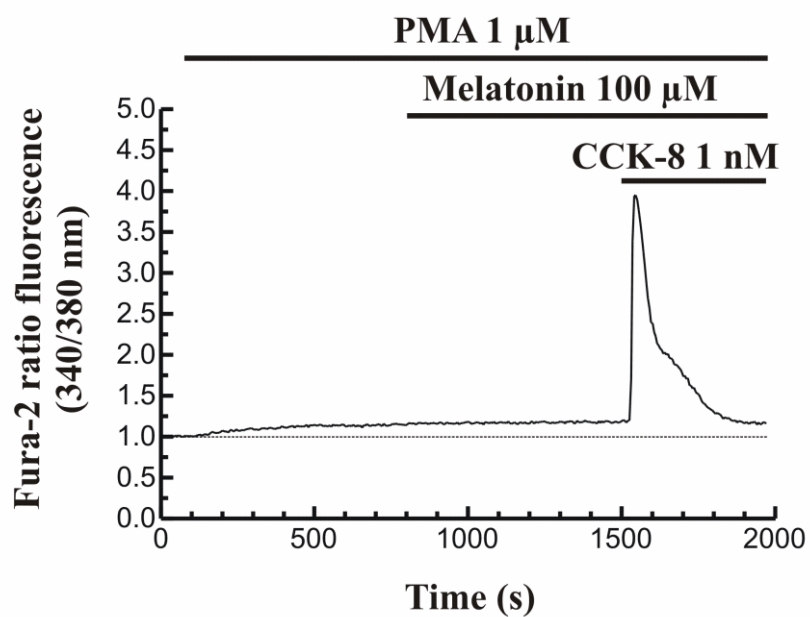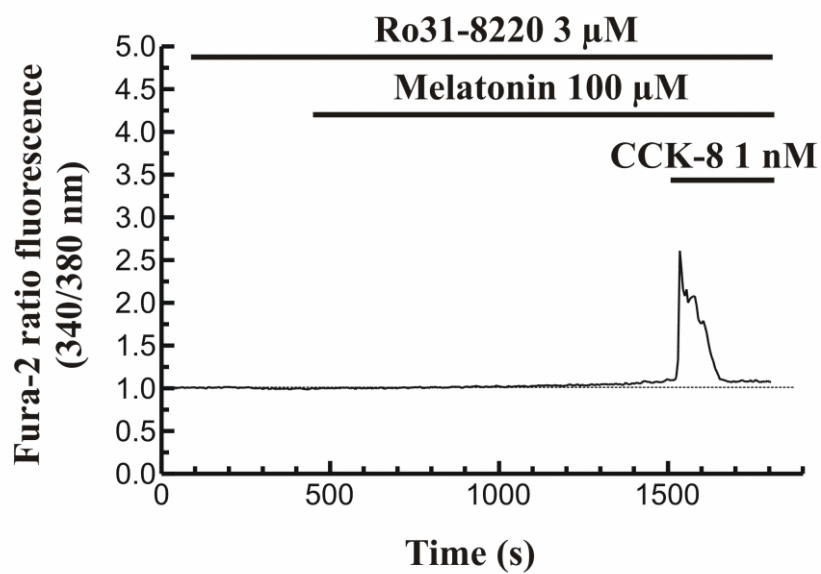Melatonin induces PKCα activation in mouse pancreatic acinar cells

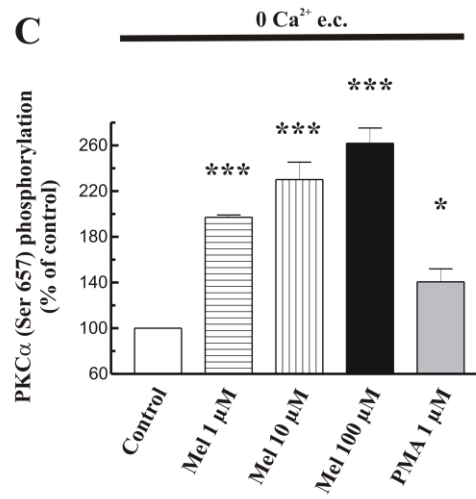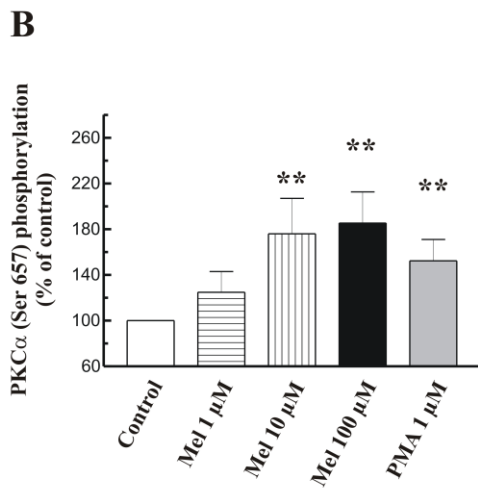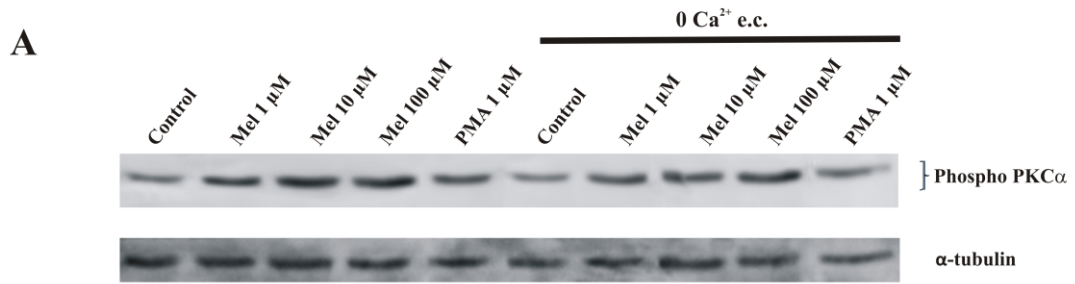Non-capacitative Ca2+ influx is observed in the presence of melatonin

Melatonin induces nuclear translocation of Nrf2
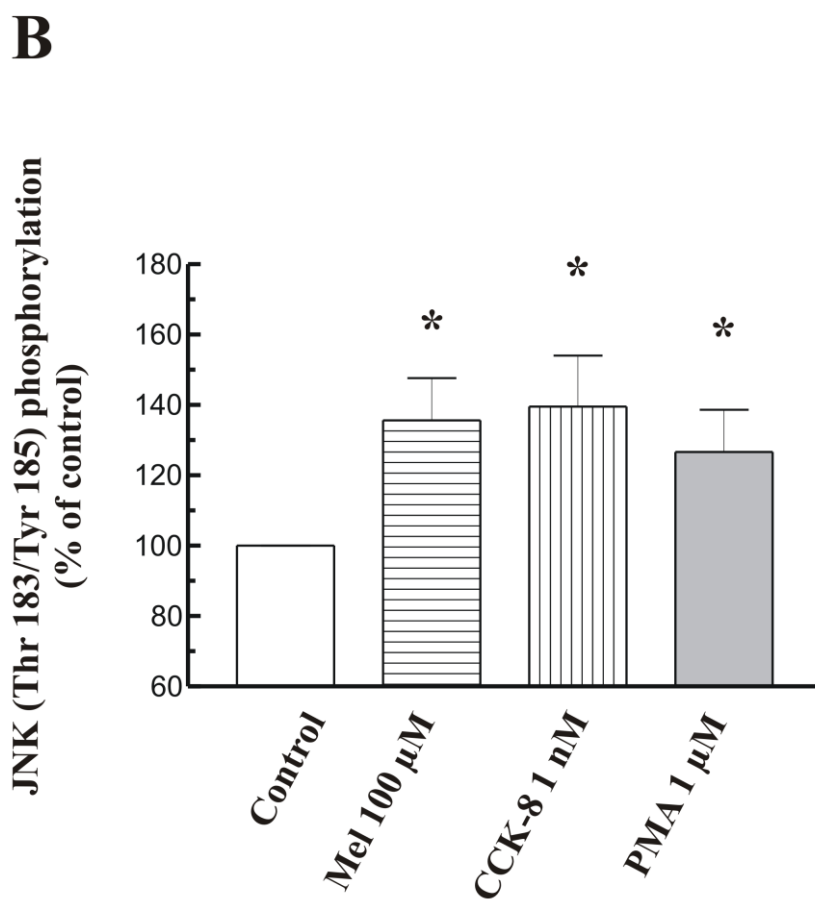
Melatonin induces the expression of antioxidant enzymes

A

0 Ca^{2+} e.c.

Melatonin 100 μM

CCK-8 1 nM

Fura-2 ratio fluorescence (340/380 nm)

Time (s)

B

LaCl$_3$ 1 mM

Melatonin 100 μM

CCK-8 1 nM

Fura-2 ratio fluorescence (340/380 nm)

Time (s)

A

B

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A P.I. (nucleus staining)   Anti-phospho-Nrf2   Merged   Transmitted light image

B

C

D

E

A

Control   Mel 100 µM   CCK-8 1 nM   PMA 1 µM

Phospho JNK

α-tubulin

B

JNK (Thr 183/Tyr 185) phosphorylation (% of control)

Graphical abstract