Melatonin modifies basal and stimulated insulin secretion via NADPH oxidase

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Abstract

Melatonin is a hormone synthesized in the pineal gland, which modulates several functions within the organism, including the synchronization of glucose metabolism and glucose-stimulated insulin secretion (GSIS). Melatonin can mediate different signaling pathways in pancreatic islets through two membrane receptors and via antioxidant or pro-oxidant enzymes modulation. NADPH oxidase (NOX) is a pro-oxidant enzyme responsible for the production of the reactive oxygen specie (ROS) superoxide, generated from molecular oxygen. In pancreatic islets, NOX-derived ROS can modulate glucose metabolism and regulate insulin secretion. Considering the roles of both melatonin and NOX in islets, the aim of this study was to evaluate the association of NOX and ROS production on glucose metabolism, basal and GSIS in pinealectomized rats (PINX) and in melatonin-treated isolated pancreatic islets. Our results showed that ROS content derived from NOX activity was increased in PINX at baseline (2.8 mM glucose), which was followed by a reduction in glucose metabolism and basal insulin secretion in this group. Under 16.7 mM glucose, an increase in both glucose metabolism and GSIS was observed in PINX islets, without changes in ROS content. In isolated pancreatic islets from control animals incubated with 2.8 mM glucose, melatonin treatment reduced ROS content, whereas in 16.7 mM glucose, melatonin reduced ROS and GSIS. In conclusion, our results demonstrate that both basal and stimulated insulin secretion can be regulated by melatonin through the maintenance of ROS homeostasis in pancreatic islets.

Key Words

- ▶ melatonin
- ▶ NADPH oxidase
- ▶ superoxide
- ▶ glucose metabolism
- ▶ ROS

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Introduction

Melatonin (N-acetyl-5-methoxytryptamine) is a hormone synthesized and released by the pineal gland at night, acting as a neuroendocrine transducer, regulating the day/night cycle. Many physiological processes and functions are modulated by melatonin, including sleep–wake cycle

synchronization, organismal and cellular metabolism as well as the activity of the reproductive axis (Bartness & Goldman 1989). Melatonin receptors on the cell membrane, identified as MT1 and MT2 (Dubocovich 1995), belong to the superfamily of G-protein-coupled receptors

and are expressed in several tissues (Bartness & Goldman 1989), including pancreatic islets (Mühlbauer & Peschke 2007). The second messenger signals recruited by these receptors modulate several intracellular mechanisms, altering the activities of adenylate cyclase, guanylate cyclase, phospholipases C and A2, and potassium and calcium channels (Morgan *et al.* 1995, Barrett *et al.* 1996, Hardeland 2009).

Melatonin, which is a highly diffusible hormone, may bind to the cell nucleus and has been shown to be a powerful antioxidant for biological systems. Acting as a scavenger of reactive oxygen and nitrogen species (ROS and RNS, respectively) (Reiter *et al.* 2008), melatonin can also inhibit some pro-oxidant enzymes, such as the NADPH oxidase (Zhou *et al.* 2008) and stimulate the expression of antioxidant enzymes, including glutathione peroxidase (GpX), catalase (CAT) and superoxide dismutase (SOD) (Reiter *et al.* 2008). Importantly, in pancreatic islets, melatonin has been considered a master regulator of ROS production (Ebelt *et al.* 2000), supporting a ROS-driven role for this hormone in islet function.

Although some studies described inhibitory or stimulatory effect of melatonin in the pancreas (Peschke & Peschke 1998), there are consistent scientific evidences supporting that melatonin inhibits insulin secretion in islets (Peschke *et al.* 2000) and clonal beta cell lines (Mühlbauer & Peschke 2007). Picinato and coworkers (2002a) demonstrated that melatonin can inhibit glucose-stimulated insulin secretion (GSIS) through the MT1 receptor and its downstream signaling pathway (cAMP/PKA) in pancreatic islets (Picinato *et al.* 2002a). Studies also showed that insulin secretion can be inhibited by melatonin through MT2 receptor (Stumpf *et al.* 2008, 2009).

Data from our group demonstrate that ROS production through NADPH oxidase (NOX) complex (Rebelato *et al.* 2012, Graciano *et al.* 2013) has a significant role in the process of insulin secretion, negatively regulating GSIS in pancreatic islets and modulating glucose metabolism (Morgan *et al.* 2009, Rebelato *et al.* 2011). Briefly, NOX consists of two membrane-bound subunits, gp91^{phox} and p22^{phox}, which form the flavocytochrome b₅₅₈ enzymatic core. Upon activation, this complex associates with the cytosolic subunits p47^{phox}, p67^{phox}, p40^{phox} and the small GTPase Rac1 (Babior 1999). Recruitment of the cytosolic components to the membrane-bound subunits is critical for NOX activity (Miyano & Sumimoto 2007).

Therefore, considering the effects of melatonin on insulin secretion and its antioxidant properties, as well as the role of NADPH oxidase in islet ROS production,

the aim of this study was to investigate the relationship between melatonin and NOX-derived ROS involved in the process of insulin secretion and pancreatic islets function.

Material and methods

Ethics statement

All experiments were performed under the guidelines of the Brazilian College for Animal Experimentation (COBEA) and the guidelines of the Animal Experimental Committee of the Institute of Biomedical Sciences, University of São Paulo.

Reagents

The reagents for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were obtained from Bio-Rad. Tris, EDTA, PMSF, aprotinin, dithiothreitol (DTT), Tween 20, Triton X-100, glycerol and collagenase were purchased from Sigma-Aldrich. Hydroethidine was obtained from Molecular Probes. The antibodies anti-p47^{phox} and anti-gp91^{phox} were obtained from Upstate Biotechnology (Upstate Biotechnology, Temecula, CA, USA), and anti- α -tubulin was purchased from Invitrogen. The enhanced chemiluminescence reagent kit (ECL) was obtained from GE Healthcare. VAS2870, a specific inhibitor of NO, was obtained from Enzo Life Science (New York, EUA).

Animals

Male Wistar rats (200–250 g) were kept on a 12 h light:12 h darkness (LD) cycle (lights on at 07:00 h) in a temperature controlled room (23 ± 2°C) and had access to food and water *ad libitum*. Animals were assigned to two experimental groups: control (CTL) and pinealectomized (PINX).

Surgical procedures

Rats were subjected to pinealectomy (PINX) or SHAM (control) surgery as described previously (Hoffman & Reiter 1965). All the experiments were performed after 45 days of pinealectomy, during which melatonin levels were absent (0 pg/mL for PINX group *n*=10 and 22 ± 3 pg/mL for the control group *n*=10), according to blood sample assays using ultrahigh-performance liquid

chromatography (Dionex UHPLC Ultimate 3000) with electrochemical detection (ESA Coulochem III) and an autosampler (WPS-3000TSL with sample thermostetting) running Chromeleon software. These results confirmed that the PINX method completely removes melatonin from the circulation. In addition, PINX was confirmed post-mortem by careful visual inspection. Our group already demonstrated the efficacy of PINX procedure in previous publications (Agez *et al.* 2009, Fisher & Sugden 2010, Jaworek *et al.* 2010, Nogueira *et al.* 2011).

Pancreatic islets isolation

Pancreatic islets were isolated using collagenase digestion method, as described previously by Lacy and Kostianovsky (1967). Briefly, after distension via pancreatic duct injection with collagenase (0.68 mg/mL), pancreas was removed and digested in a shaking water bath at 37°C. Pancreatic islets were isolated from PINX and sham-operated animals to confirm the chronic effects of the absence of melatonin. In addition, islets were also obtained from control animals to determine the acute *in vitro* effects of melatonin.

Static insulin secretion

Groups of 5 islets were preincubated at 37°C for 30 min in 0.5 mL Krebs–Henseleit (KH) with 0.2% bovine serum albumin (BSA) in 5.6 mM glucose and incubated for 1 h in KH with 0.2% BSA in different concentrations of glucose (2.8 and 16.7 mM). At the end of the experiment, the medium was collected, and insulin was measured by radioimmunoassay (RIA) using human insulin as a standard. For total insulin content, islets in each well were disrupted in 500 µL acid ethanol solution (52 ethanol: 17 water; 1 chloridic acid) and sonicated (3 pulses of 5 s). Insulin was measured by RIA. Insulin secretion was calculated as the insulin secreted per total insulin content in the islets. Radioactive insulin was purchased from PerkinElmer.

Analysis of superoxide content in pancreatic islets

Superoxide production was determined by dihydroethidium (DHE) fluorescence evaluation. The reaction between anion superoxide and DHE results in the formation of an oxidized product that intercalates into DNA, staining a red fluorescence in the nucleus (Zhao *et al.* 2003).

Groups of 20 islets were preincubated at 37°C for 30 min in 0.5 mL KH with 0.2% BSA in 5.6 mM glucose and incubated for 1 h in KH with 0.2% BSA in the presence of 2.8 and 16.7 mM glucose. After incubation, DHE was added to a final concentration of 50 µM. Samples, protected from light, were then incubated for an additional 20 min at room temperature. Islets were disrupted with trypsin and gentle pipetting and resuspended in 200 µL of RPMI-1640 medium and placed in a 96-well plate for analysis in flow cytometer (Guava EasyCyte, Millipore). Fluorescence was assessed, and averages were normalized to glucose 2.8 mM averages. Data were expressed as mean fluorescence intensity (MFI).

Measurement of [¹⁴C]-glucose decarboxylation

Groups of islets were incubated in 1.2 mL of KH buffer containing BSA 0.2% at 37°C in glass vials containing a filter paper and 400 µL of phenylethylamine, diluted 1:1 v/v in methanol, in a separate compartment. The incubation buffer contained 36 µCi/mmol of [U-¹⁴C]-glucose. In the experiments performed to measure [U-¹⁴C]-glucose oxidation, islets were incubated in the presence of 2.8 and 16.7 mM glucose for 1 h. The incubation stopped by the addition of 400 µL HCL (10 mol/L), and the vials were shaken for additional 90 min. The filter paper with phenylethylamine was transferred to a plastic tube with 1.8 mL of biodegradable scintillation liquid (Amersham Pharmacia, Uppsala, Sweden), and the adsorbed ¹⁴CO₂ was measured using a scintillation counter (Beckman-LS 5000TD, Beckman Instruments, Fullerton, CA, USA).

Western blot analysis

Groups of 350 islets were resuspended in 80 µL extraction buffer (100 mM Tris, 1% Triton X-100, 0.01 mg/mL aprotinin, 2 mM PMSE, 10 mM Na₃VO₄, 10 mM NaF, 10 mM Na₄P₂O₇ and 10 mM EDTA). The samples were boiled for 5 min and centrifuged for 20 min at 4°C (10,000g). The protein content of the supernatant was determined by the Bradford method (1976). For immunoprecipitation (IP), pancreatic islet extracts were centrifuged for 20 min at 4°C (12,000g). The supernatant (3 mg of protein) was used for IP with anti-p47phox and protein A-Sepharose 6MB before Laemmli sample buffer containing 100 mM dithiothreitol. After running gel electrophoresis (10% gel) and transferring to PVDF membrane, expression of the subunits of NADPH oxidase

Table 1 Rabbit and mouse antibodies and respective dilutions.

Antibody	Animal origin	Dilution
Polyclonal anti-p47 ^{PHOX} (Upstate Cell Signalling Solutions)	Rabbit	1:500
Polyclonal anti-gp91 ^{PHOX} (Upstate Cell Signalling Solutions)	Rabbit	1:1000
Monoclonal anti- α Tubulin (Zymed)	Mouse	1:2000
Rabbit anti-IgG/peroxidase (Amersham)	Mouse	1:10,000
Mouse anti-IgG/peroxidase (Amersham)	Rabbit	1:10,000

was assessed using the antibodies described in Table 1. The enhanced chemiluminescence reagents – ECL – (GE Healthcare) were used, and the membrane was exposed to photographic film or Image Quant apparatus, GE Healthcare (GE Healthcare AS). The intensity of the bands was quantified by optical densitometry using ImageJ software (Wayne Rasbond, NIH, Bethesda, MD, USA). Quantification of protein expression was normalized by protein α -tubulin.

RNA isolation

Total RNA was isolated from fresh islets, using TRIzol (Life Technologies) according to the manufacturer's instructions. RNA concentration was determined by spectrophotometry using NanoDrop2000, whereas its integrity was evaluated in a 2% (w/v) agarose gel containing ethidium bromide.

Real time-PCR

Total RNA (3 μ g) obtained from isolated pancreatic islets was reverse transcribed to cDNA. Glucose transporter 2

(*Glut2*), glucokinase (*Gck*), superoxide dismutase (*Sod1*), catalase (*Cat*) and glutathione peroxidase (*Gpx*) mRNA expressions were evaluated by real-time PCR using ROTOR-GENE 3000 equipment (Corbett Research, Mortlake, Australia) and SYBR GREEN (Invitrogen) as fluorescent dye. Gene expression was evaluated by the 2(-Delta CT) method (Livak & Schmittgen 2001) using the ribosomal protein L37 α (*Rpl37a*) or hypoxanthine guanine phosphoribosyl transferase (*Hprt*) gene expression as an inner control. The Ct value is the calculated cycle number where the fluorescence signal was emitted significantly above the background levels. The sequences of the used primers, manufactured by Integrated DNA Technologies (Coralville, IA, USA), are described in Table 2.

Statistical analysis

Results are presented as mean $\pm$ S.E.M. Two-way ANOVA, followed by Bonferroni's post hoc test was applied. Statistical analyses were also performed using the unpaired Student's *t*-test when appropriate, and *P* < 0.05 was considered to be statistically significant. GraphPad Prism software was used in the data analyses (GraphPad Software v4.03).

Results

Pinealectomy results in an increase of ROS content in isolated pancreatic islets

ROS generation was first evaluated in isolated pancreatic islets from control (CTL) and pinealectomized (PINX) rats. All experiments were performed using low (2.8 mM)

Table 2 Primer sequences for rat qPCR assays.

Gene/GenBank accession #	Primer sequence	Product length (bp)	Temperature (°C)
<i>rSlc2a2</i> /NM_012879.2	5'-CAGGGTGAAGACCAGGACCA-3' 5'-CCTCTGCTTCCAGTACATTGC-3'	80	58.1 61.0
<i>rGck</i> /NM_001270849.1	5'-CCGTTTCGTGTCAAGTGGA-3' 5'-ATATGTGCTCCGCAG-3'	156	60.7 58.0
<i>rSod1</i> /NM_017050.1	5'-CCGGTGCAGGGCGTC-3' 5'-TCCTGTAATCTGTCCTGACACCA-3'	74	59.2 58.2
<i>rCat</i> /NM_012520.1	5'-CATTGAGCCCAGCCCG-3' 5'-GGCGGTGAGTGTCTGGGTAA-3'	64	52.2 59.6
<i>rGpx</i> /NM_030826.3	5'-CCTGGTGGTCTCGGTTT-3' 5'-TCGGACATACTTGAGGGAATTCA-3'	90	58.1 59.4
<i>rRpl37a</i> /NM_001108801	5'-TTGAAATCAGCCAGCACGC-3' 5'-TGCCAACGCCTCGTCTCT-3'	74	59.5 59.1
<i>rCyba</i> /NM_024160.1	5'-GAGGTCCGCAAGAAGCCAAG-3' 5'-GAAACTCAAGCAGGAGCCACTG-3'	120	56.2 55.5
<i>rHprt</i> /S79292.1	5'-GCTGAAGATTGGAAAAGGTGT-3' 5'-ACAGAGGGCCACAATGTGAT-3'	112	54 54

Figure 1

PINX effect on ROS content. Mean fluorescence intensity by dihydroethidium (DHE) in isolated pancreatic islets after 60 min of incubation with 2.8 mM (A) and 16.7 mM (B) glucose in the presence or absence of 20 μ M VAS2870 (VAS), an inhibitor of NADPH oxidase. Results are presented as mean $\pm$ S.E.M. ($n=8$). Two-way ANOVA, Bonferroni's post hoc test. (A) * $P<0.05$; @ $P<0.05$ vs CTL 2.8 mM; # $P<0.05$ vs 2.8 mM PINX 2.8 mM; (B) * $P<0.05$ vs 16.7 CTL. CTL, control; PINX, pinealectomy.

and high (16.7 mM) glucose concentrations to explore the minimum and maximum responses of pancreatic islets to glucose. An increase of 29% in intracellular ROS content after 1 h of incubation with 2.8 mM glucose was observed in PINX rats compared with CTL (Fig. 1A). To verify whether NOX was the source of ROS in our model, a well-known NOX inhibitor, VAS2870 (Wind *et al.* 2010, Wingler *et al.* 2011, Kahles & Brandes 2012, Altenhofer *et al.* 2015), was used. In low glucose, a reduction in ROS content was observed in both CTL and PINX groups (36 and 24.4%, respectively) after VAS2870 treatment, but PINX-derived islets still presented higher ROS content than CTL islets (Fig. 1A). Under high glucose, there was no significant difference between groups, despite a 25.2% reduction in ROS levels in CTL group after treatment with NOX inhibitor (Fig. 1B).

Pinealectomy increases NADPH oxidase assembly

The effect of PINX on the expression of NOX components, p47^{phox} and gp91^{phox}, was investigated in isolated pancreatic islets by Western blot. The analysis showed no changes in total protein content of these subunits (Fig. 2A and B). Association of the cytosolic NOX subunit,

Figure 3

PINX effect on [¹⁴C]-glucose decarboxylation and mRNA expression of *Glut2* and *Gsk* in isolated pancreatic islets. The islets were incubated for 60 min in 2.8 mM (A) or 16.7 mM (B) glucose. Total glucokinase mRNA (*Gsk*) expression (C) and *Glut2* mRNA expression (D) were evaluated in islets obtained from pinealectomy (PINX) and control (CTL) animals after sacrifice. Results are presented as mean $\pm$ S.E.M. ($n=5$). Two-way ANOVA, Bonferroni's post hoc test. * $P<0.05$, ** $P<0.01$.

p47^{phox}, to the membrane-bound gp91^{phox} is a key step in the activation of the oxidase and subsequent superoxide generation (Bedard & Krause 2007). We, therefore, assessed this association by immunoprecipitation with anti-p47^{phox} followed by immunoblotting against gp91^{phox} in 2.8 mM glucose. Data revealed an 18% increase in gp91^{phox}-p47^{phox} association in the PINX group compared with CTL animals, indicative of increased enzymatic activation (Fig. 2C).

Measurement of [¹⁴C]-glucose decarboxylation and *Gsk* and *Glut2* mRNA expression in PINX animals

In an attempt to estimate the metabolic flux by the glycolytic pathway, which can be linked to the insulin secretion process, [¹⁴C] glucose decarboxylation was assessed in PINX and CTL rats. Under 2.8 mM glucose, a reduction of 41.4% in glucose decarboxylation was observed in PINX group (Fig. 3A). However, a high

Figure 2

PINX effect on protein expression of p47^{phox} and gp91^{phox} and NADPH oxidase activity in isolated pancreatic islets. (A) p47^{phox} protein expression, (B) gp91^{phox} protein expression and (C) immunoprecipitated IP: p47^{phox} IB: gp91^{phox} after 30 min of incubation with 2.8 mM glucose. Results are presented as mean $\pm$ S.E.M. ($n=3$). Student's *t*-test * $P<0.05$, CTL, control; PINX, pinealectomy.

Figure 4

PINX effect on static insulin secretion in pancreatic islets. Pancreatic islets were isolated and incubated for 60 min with 2.8 mM (A) and 16.7 mM (B) glucose and 20 μ M VAS2870 (VAS), an inhibitor of NADPH oxidase. Results are presented as mean $\pm$ S.E.M. ($n=5$). Two-way ANOVA, Bonferroni's post hoc test. * $P<0.05$. CTL, control; PINX, pinealectomy.

glucose concentration raised the glucose decarboxylation in the PINX group by 33.3% compared to CTL (Fig. 3B). Furthermore, it was observed that PINX induced a greater expression of two essential genes involved in glucose metabolism, *Gck* and *Glut2* (Fig. 3C and D).

Pinealectomy dysregulates basal and glucose-stimulated insulin secretion (GSIS)

To estimate the functionality of pancreatic islets under the absence of melatonin, basal and glucose-stimulated insulin secretion assays were performed. In PINX group, a decrease of 63% in insulin secretion was observed at 2.8 mM glucose in comparison with CTL, and the inhibition of NOX by VAS2870 at this same basal condition promoted an increase in insulin secretion in both groups (Fig. 4A). Under 16.7 mM glucose (stimulated condition), pinealectomy caused an increase of 49% in insulin compared with CTL. Inhibition of NOX by VAS2870 at this glucose concentration, however, had no effect on insulin secretion (Fig. 4B).

Pinealectomy and antioxidant transcripts

To test whether antioxidant enzymes were changed due to pinealectomy, mRNA expression of *Sod1*, *Gpx* and *Catalase* was assessed by qPCR on control and PINX-isolated pancreatic islets. We observed a decrease in *Sod1*

Figure 6

Melatonin effects on ROS content and p22^{phox} mRNA expression in islets obtained from control animals. Mean fluorescence intensity by dihydroethidium (DHE) in isolated pancreatic islets after 60 min of incubation with 2.8 mM (A) and 16.7 mM (B) glucose, 20 μ M VAS2870 (VAS) an inhibitor of NADPH oxidase and 100 nM melatonin. (C) p22^{phox} mRNA expression in 2.8 mM and (D) p22^{phox} in 16.7 mM glucose and 100 nM melatonin. Results are presented as mean $\pm$ S.E.M. ($n=5$). Two-way ANOVA, Bonferroni's post hoc test. (A) * $P<0.05$ (B) * $P<0.05$, @ $P<0.05$, (C) and (D) * $P<0.05$.

(Fig. 5A) expression, but no differences in *Gpx* (Fig. 5B) and *Catalase* (Fig. 5C) gene expression were found between the two groups.

Melatonin treatment decreases ROS content and p22^{phox} subunit mRNA expression in pancreatic islets

Figure 6 shows the acute effect of melatonin treatment (100 nM), NADPH oxidase inhibitor (VAS2870-20 μ M) and their combination on superoxide content in pancreatic islets obtained from control animals at different glucose concentrations (2.8 and 16.7 mM). Under 2.8 mM of glucose, VAS2870 decreased 46%, melatonin 54% and the combination of melatonin and VAS2870 70% compared with baseline (Fig. 6A). Under 16.7 mM glucose, VAS2870, melatonin and the association of melatonin and VAS2870

Figure 5

Analysis of mRNA expression of antioxidant enzymes in pancreatic islets. (A) *Sod1*, (B) *Gpx* and (C) *Catalase* gene expressions in islets obtained from pinealectomy (PINX) and control (CTL) animals after sacrifice. Results are presented as mean $\pm$ S.E.M. normalized by the housekeeping gene *Rpl37a* ($n=5$). Student's *t*-test, * $P<0.05$.

Figure 7

Melatonin effects on static insulin secretion in pancreatic islets. Pancreatic islets were isolated and incubated for 60 min with 2.8 mM (A) and 16.7 mM (B) glucose and 20 μ M VAS2870 (VAS), an inhibitor of NADPH oxidase and 100 nM melatonin. Results are presented as mean $\pm$ S.E.M. ($n=5$). Two-way ANOVA, Bonferroni's post hoc test. (A) * $P<0.05$ vs 2.8 mM CTL, (B) @ $P<0.05$ vs 16.7 mM.

induced a reduction of ROS content to 41, 36 and 73%, respectively. We next evaluated the expression of p22^{phox} mRNA, a subunit of NADPH oxidase, after supplementation with melatonin. The challenge with melatonin promoted a significant reduction of p22^{phox} mRNA expression under 2.8 and 16.7 mM glucose concentrations (Fig. 6C and D).

Melatonin treatment does not change basal insulin secretion and reduces glucose-stimulated insulin secretion (GSIS)

To verify the capacity of the pancreatic islets to secrete insulin after acute supplementation of melatonin, VAS2870 and the combination of melatonin and VAS2870, an insulin secretion assay was performed. The inhibition of NOX by VAS2870 induced a high insulin secretion at 2.8 mM glucose compared with that at 2.8 mM baseline, whereas melatonin treatment did not change the basal insulin secretion and the combination of VAS2870 and melatonin induced a greater insulin release at 2.8 mM glucose (Fig. 7A). Under 16.7 mM glucose, the challenge with melatonin promoted a significant reduction in glucose-stimulated insulin secretion. The VAS2870 group, as well as the combination of VAS2870 and melatonin, did not alter the insulin secretion at 16.7 mM glucose (Fig. 7B).

Discussion

The present work shows that NADPH oxidase-derived ROS oxidase plays a significant role in the regulation of

basal and GSIS in isolated pancreatic islets, and melatonin is an important modulator of this mechanism.

The chronic absence of melatonin in PINX rats raised the levels of ROS in isolated islets under basal levels of glucose (2.8 mM). This high ROS content was associated with an increased interaction of the cytosolic NOX subunit p47^{phox} to the membrane-bound gp91^{phox}, a key step in the activation of the oxidase system, and subsequent ROS generation. Islets treated with VAS2870 caused a reduction in ROS content at 2.8 mM glucose, demonstrating that NOX possesses a high activity at low glucose levels (Munhoz *et al.* 2016). Previous works have also demonstrated that melatonin can directly regulate ROS generation and modulate pro-oxidant enzymes expression, such as the NADPH oxidase (Zhou *et al.* 2008).

Conversely, under high glucose exposure, inhibition of NOX did not reduce ROS levels in PINX animals. Melatonin can affect many systems in the cell, such as the mitochondrial metabolism (Ramis *et al.* 2015); hence, it is plausible that the absence of melatonin may induce ROS production not only through NOX but also through other oxidant sources, such as the mitochondria. However, it is important to consider that DHE is a specific probe for the detection of cytosolic ROS content (Zhao *et al.* 2003, Dikalov 2011), and previous publications have already shown that most of the ROS products generated after glucose stimulation are superoxide, which was confirmed using SOD linked to polyethylene glycol (PEG-SOD) (Graciano *et al.* 2013).

It was observed that under 2.8 mM glucose, the increased ROS generation in PINX rats was accompanied by a significant decrease in glucose metabolism. In a study by Rebelato and coworkers (2010), low doses of hydrogen peroxide impaired glucose metabolism, as well as basal and GSIS (Rebelato *et al.* 2010). ROS are known to decrease the activity of both glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (glycolytic pathway) (Lind *et al.* 1998) and aconitase (Bulteau *et al.* 2003) (Krebs cycle), thereby reducing the formation of [U¹⁴CO₂] under low glucose concentration. Our results with [U¹⁴CO₂] are consistent with these findings.

Under 16.7 mM glucose, PINX group showed a higher glucose metabolism capacity than the controls, possibly because ROS can decrease GAPDH and aconitase activity and induce an increase in glucose metabolism, which was also observed by Picinato and coworkers (2002a). Also, PINX induced a higher mRNA expression of *Glut2* and *Gck*, which are involved in the transport and phosphorylation of glucose inside the cell.

Bazwinsky-Wutschke and coworkers (2014) observed that MT1/MT2 receptor knockdown increased the expression of Glut2 (Bazwinsky-Wutschke *et al.* 2014), possibly through the lower activation of the transcriptional factor CREB, which is also modulated by melatonin (Bazwinsky-Wutschke *et al.* 2012). Taken together, the defect of melatonin signaling can lead to an increase in Glut2 expression and contributes to increased glucose metabolism in PINX animals.

Under 2.8 mM glucose concentration, the absence of melatonin induced an impairment of basal insulin secretion. Furthermore, a reduction in glucose metabolism and an increase in NOX-derived ROS were observed. The decrease of basal insulin secretion was entirely reversed by the NOX inhibitor VAS2870, indicating that NOX-derived ROS can modulate basal insulin secretion. Under 16.7 mM glucose, PINX presented an increase in glucose-stimulated insulin secretion, which may be linked to the increased glucose metabolism and a decrease in ROS content. It has been shown that in high glucose, i.e., 16.7 mM, there is an increase of antioxidant enzymes activity, such as superoxide dismutase (SOD) (Oliveira *et al.* 1999), which can also contribute to decrease the ROS content. Due to the fact that the antioxidant defense system is probably in their optimal activation state under high glucose concentration, the presence of VAS2870 did not cause a significant change in ROS levels, as observed in basal condition (Oliveira *et al.* 1999, Munhoz *et al.* 2016).

Recent evidence shows that insulin secretion is inversely related to ROS concentration (Rebelato *et al.* 2011, Graciano *et al.* 2013). Under low glucose, high ROS content limits insulin secretion to basal levels (Munhoz *et al.* 2016). On the other hand, in high glucose concentration, such as 16.7 mM, ROS levels are reduced, promoting high insulin secretion (Rebelato *et al.* 2011). As melatonin can modulate ROS production (Reiter *et al.* 2008, Zhang & Zhang 2014), it would be expected that its absence could dysregulate ROS content, as well as basal insulin secretion and GSIS. This is consistent with our current findings, which demonstrate that PINX modifies the relationship between ROS and insulin secretion at different glucose concentrations.

To investigate the acute effects of melatonin supplementation on ROS production and insulin secretion, we incubated isolated islets with 100 nM of melatonin. Melatonin treatment was able to reduce ROS production in both 2.8 and 16.7 mM glucose concentrations, possibly due its antioxidant properties

(Reiter *et al.* 2008) and the combination of melatonin and VAS2870 further inhibits ROS production. The challenge with melatonin also reduced the expression of the membrane-bound p22^{phox} subunit in both glucose concentrations. The p22^{phox} subunit is critical for NOX activity (Bedard & Krause 2007), and its reduction can be associated with the observed low ROS production in our experiments.

Under 2.8 mM glucose level, melatonin *per se* did not change basal insulin secretion, even when ROS content was reduced, showing that at least acutely, melatonin pleiotropic effects overwhelm the regulation promoted by ROS on control of insulin secretion (Peschke *et al.* 2000). In cell lines, such as INS-1E (Stumpf *et al.* 2008, 2009), and isolated pancreatic islets (Picinato *et al.* 2002b), melatonin may inhibit insulin secretion through MT1 and MT2 receptors, which modulate AMPc and GMPc, respectively. Activation of melatonin receptors reduces the level of these second messengers, which are necessary for the amplification of secretory response via PKA and PKG (Peschke *et al.* 2000, Stumpf *et al.* 2008, 2009). On the other hand, the reduction in ROS content, promoted by the inhibition of NOX, induced an increase in basal insulin secretion.

A similar response was found in 16.7 mM glucose. Melatonin treatment reduced glucose-stimulated insulin secretion despite the reduction in intracellular ROS content. As mentioned previously, receptor-mediated melatonin effects also seem to override the modulation of insulin secretion by ROS in this condition. Under high glucose, VAS2870 treatment lowered ROS production, but did not promote additional insulin release, probably for the reason that cells were already on their maximum secretory capacity due to glucose stimulation (Rebelato *et al.* 2011).

As a whole, many studies have shown the relevance of intracellular ROS for GSIS regulation in pancreatic islets (Rebelato *et al.* 2011, Li *et al.* 2012). However, the underlying mechanisms of pancreatic β -cell function modulated by ROS are not fully understood. Here, we provided evidence that melatonin can modulate NADPH oxidase-derived ROS production, and through this pathway be an important regulator of glucose metabolism, basal insulin secretion and GSIS.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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