J. Pineal Res. 2008; 44:88–94
Doi:10.1111/j.1600-079X.2007.00493.x

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Journal of Pineal Research

Activation of insulin and IGF-1 signaling pathways by melatonin through MT1 receptor in isolated rat pancreatic islets

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Abstract: Melatonin diminishes insulin release through the activation of MT1 receptors and a reduction in cAMP production in isolated pancreatic islets of neonate and adult rats and in INS-1 cells (an insulin-secreting cell line). The pancreas of pinealectomized rats exhibits degenerative pathological changes with low islet density, indicating that melatonin plays a role to ensure the functioning of pancreatic beta cells. By using immunoprecipitation and immunoblotting analysis we demonstrated, in isolated rat pancreatic islets, that melatonin induces insulin growth factor receptor (IGF-R) and insulin receptor (IR) tyrosine phosphorylation and mediates the activities of the PI3K/AKT and MEK/ERKs pathways, which are involved in cell survival and growth, respectively. Thus, the effects of melatonin on pancreatic islets do not involve a reduction in cAMP levels only. This indoleamine may regulate growth and differentiation of pancreatic islets by activating IGF-I and insulin receptor signaling pathways.

Key words: melatonin, isolated islets, insulin signaling, IGF-1

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Received June 19, 2007;
accepted August 2, 2007.

Introduction

Melatonin is a pineal secretory product whose synthesis and release is related to the light–dark cycle [1–5]. In mammals, melatonin is involved with several physiological processes. These processes include regulation of the immune system [6, 7], cardiovascular functions [8], carbohydrate metabolism [9, 10], and inhibition of tumor growth [11], among others [12–14]. The effects of melatonin on cellular processes are of two types: receptor- and/or non-receptor-mediated. Because of the fact that melatonin can permeate the cell membrane, it is possible to demonstrate that some of its effects are mediated by direct molecular interaction with calcium–calmodulin [15], or regulating several mitochondrial physiological processes [16], in addition to its ability to interact with several oxygen and nitrogen radicals protecting cells against deleterious oxidation processes [17].

In addition to nuclear receptors of the RZR-ROR type, several effects of melatonin are mediated by specific high-affinity G-protein-coupled receptors (GPCRs). In mammals, two distinct receptor subtypes were cloned and named MT1 (Mel1a) and MT2 (Mel1b) [18–21]. Functional, autoradiographic and molecular investigations as well as binding studies proved that the melatonin effects through MT1 receptor are mediated by reduction in cAMP formation [22, 23]. Recently, melatonin receptors were found in rat pancreatic B cells and in INS-1 cells (an insulin-secreting cell line) [24, 25] and human pancreas [26]. In pancreatic islets of neonate rats, B cells from adult rats and in INS-1 cells, melatonin diminished insulin release [21, 24, 25]. This is in agreement with our observations that melatonin decreases PKA activity in rat pancreatic islets [9].

Morphometric analysis of the pancreas from pinealectomized rats showed degenerative pathological processes with large islet area and low islet density [27]. In addition, diabetic animals and humans have shown reduced serum melatonin levels [28]. These observations indicate that melatonin may play an important role to ensure the functioning of pancreatic B cells.

The effect of melatonin on cell proliferation, growth and differentiation has been reported to involve protein phosphorylation [29–31]. MT1 and MT2 receptors stimulate c-Jun N-terminal kinase that is pertussis toxin (PTX)-sensitive, Ras/Rac-dependent, and may involve tyrosine kinase activity of the Src-family proteins [32]. Recently, it was demonstrated that melatonin induces rapid tyrosine phosphorylation and activation of the insulin receptor β-subunit tyrosine kinase in rat hypothalamus [33].

The early steps of insulin growth factor-1 (IGF-1) and insulin receptor-signaling pathways involve tyrosine phosphorylation of insulin receptor substrates 1 and 2 (IRS-1 and IRS-2) and Shc [34–37]. Tyrosine phosphorylation of IRS proteins triggers signaling pathways inducing PI3K/AKT and MAPK, which regulate essential processes (e.g. intermediary metabolism and growth) in mammalian cells including pancreatic islet cells [38, 39].

The ability of melatonin to induce phosphorylation of IGF-R, IR, IRS-1, AKT/PKB, ERK1/2, STAT3 and PTP-1B as well as IR/PTP-1B association were examined in incubated rat pancreatic islets. Additionally, the PTP-1B

content was determined. The involvement of MT1 or MT2 melatonin receptors was also investigated by using specific inhibitors of these receptors.

Materials and methods

Reagents

The reagents for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were obtained from Bio-Rad (Richmond, CA, USA). Tris, ethylenediaminetetraacetic acid (EDTA), aprotinin, phenylmethylsulfonylfluoride (PMSF), dithiothreitol (DTT), Triton X-100, Tween 20, glycerol, melatonin and collagenase were purchased from Sigma-Aldrich (St Louis, MO, USA). Melatonin receptor inhibitors, luzindole and 4-phenyl-2-propionamidotetraline (4P-PDOT), were obtained from Tocris Cookson Inc (Ellisville, MO, USA). Anti-IGF-R, anti-IR, anti-IRS-1, and anti-phosphotyrosine antibodies were from Santa Cruz Technology (Santa Cruz, CA, USA). Phospho-AKT (Ser473), phospho-ERK1/2 MAPK (Thr202/Tyr204), and phospho-STAT3 (Ser727) antibodies were from Cell Signaling Technology (Danvers, MA, USA). PTP-1B antibody was from Upstate Biotechnology (Lake Placid, NY, USA) and anti-phosphoserine was from Chemicon International (Temecula, CA, USA). The enhanced chemiluminescence reagent kit, ECL, was from Amersham-Pharmacia Biotech (Buckinghamshire, UK).

Animals

Six- to 8-week-old female albino rats (150–200 g) were used. The experiments were performed following the guidelines of the Animal Experimental Committee of the Institute of Biomedical Sciences, University of Sao Paulo. The rats were kept in groups of five at 23°C in a room with a light/dark cycle of 12/12 hr (lights on at 07:00 hours).

Isolation of pancreatic islets

Islets of Langerhans were isolated after collagenase digestion [40]. Briefly, rat islets were isolated by distension of the pancreas via the pancreatic duct with collagenase (0.68 mg/mL). The pancreas was then removed and digested in a shaking water bath at 37°C.

Western blot analysis

Batches of 300 islets were incubated for 30 min at 37°C in the Krebs–Henseleit (KH) buffer (139 mM Na^+, 5 mM K^+,

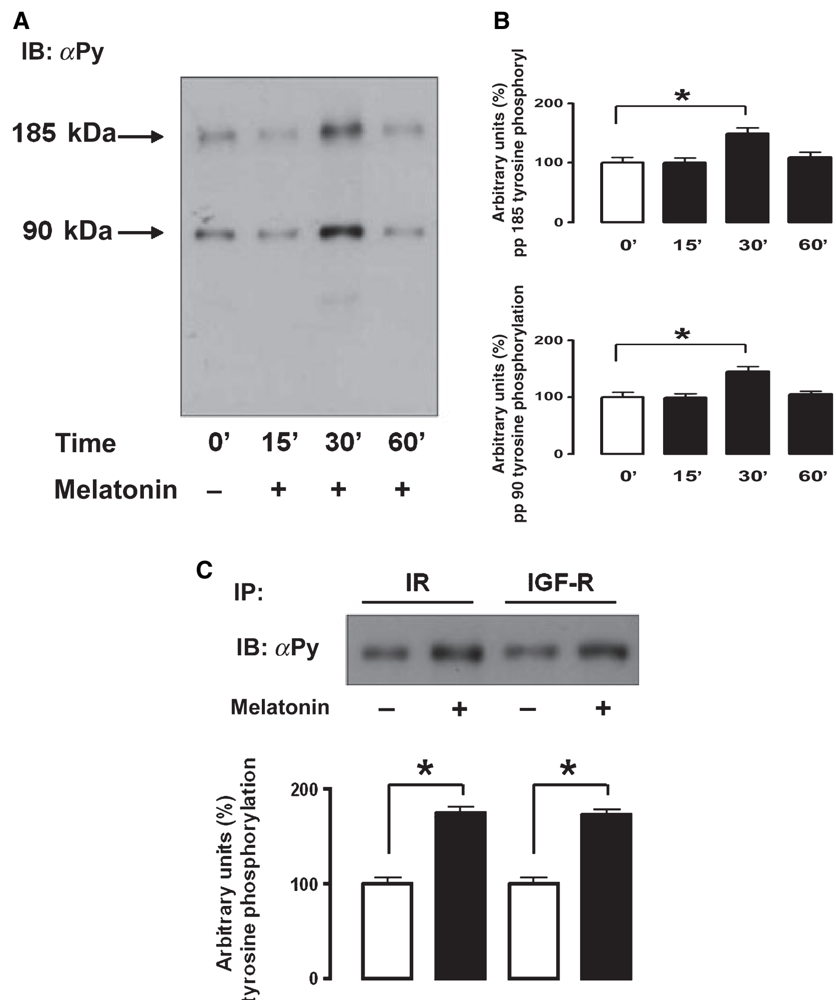

Fig. 1. Time-course of melatonin-induced tyrosine phosphorylation and Induction of IGF-R and IR tyrosine phosphorylation by melatonin treatment. Isolated pancreatic islets were incubated in KB buffer containing 2.8 mM glucose, in the presence (+) of 0.1 μM melatonin for 0 (basal), 15, 30 and 60 min. Islet proteins were extracted and processed as described in Materials and Methods. The nitrocellulose membranes containing the soluble proteins were submitted to immunoblotting with antiphosphotyrosine monoclonal antibody. (A) Representative autoradiogram from time-course experiments; (B) quantification of pp90 and pp185 tyrosine phosphorylation; (C) proteins extracted from pancreatic islets incubated for 30 min in the presence of 2.8 mM glucose and 0.1 μM melatonin (+) were immunoprecipitated with anti-IGF-R and anti-IR antibodies and then submitted to immunoblotting with antiphosphotyrosine. The phosphorylation levels are shown as mean ± S.E.M. of the scanning densitometric analysis of four distinct experiments.*$P < 0.05$ versus basal condition.

1 mM Ca^{2+}, 1 mM Mg^{2+}, 124 mM Cl^{-}, 24 mM HCO_3^{-}) in presence or absence of 0.1 μM melatonin or melatonin receptor inhibitors as indicated in the figure legends. Thereafter, solubilization buffer containing 100 mM Tris, pH 7.6, 1% Triton X-100, 0.01 mg/mL aprotinin, 2 mM PMSF, 10 mM Na_3VO_4, 10 mM NaF, 10 mM $Na_4P_2O_7$, and 10 mM EDTA was added and the isolated pancreatic islets were sonicated to disrupt the cells. Insoluble material was removed by 30 min of centrifugation at 12,800 g at 4°C. The supernatant was used for immunoprecipitation with anti-IGF1-R, anti-IR, anti-IRS-1, anti-PTP-1B and protein A. Sepharose 6 MB was added before sample treatment with Laemmli buffer and 8% SDS-PAGE as described elsewhere [41, 42]. For whole-tissue extracts, similar-sized aliquots (80 μg protein) were subjected to SDS-PAGE and immunoblotted with anti-phosphotyrosine, anti-phospho-AKT, anti-phospho-p42/44 MAP kinase, anti-STAT3 antibodies. Electrotransfer of proteins from the gel to nitrocellulose was performed for 90 min at 120 V (constant). To reduce non-specific protein binding to the nitrocellulose, the filter was preincubated overnight at 4°C in a blocking buffer (5% nonfat dry milk, 10 mM Tris, 150 mM NaCl and 0.02% Tween 20). The nitrocellulose blots were incubated for 4 hr at 22°C with the specific antibodies described in the figure legends diluted in blocking buffer (3% nonfat dry milk). To visualize the autoradiogram, enhanced chemiluminescence reagents exposed to photographic film were used. Quantitative analysis of the blots was performed using Scion Image software (Frederick, MD, USA).

Statistical analysis

Results are presented as mean ± S.E. (ANOVA). Student–Newman–Keuls comparison test was used to verify if significance were appropriate, with confidence levels set at $P < 0.01$. The level of significance was set at $P < 0.05$.

Results

A representative immunoblot with anti-phosphotyrosine antibody of soluble intracellular proteins from freshly isolated pancreatic islets incubated in KB buffer containing 2.8 mM glucose plus 0.1 μM melatonin for 15, 30 and 60 min is shown in Fig. 1. In the basal state and absence of melatonin, two faint bands were detected. One upper broad band migrated at 170–140 kDa band and a lower band migrated between 90 and 100 kDa (Fig. 1A). The maximal tyrosine phosphorylation in both bands occurred at 30 min of incubation. Phosphotyrosine-containing proteins were increased by 1.5-fold above basal levels as indicated by densitometric analysis (Fig. 1B, bar graph). Posterior incubation of nitrocellulose sheets with specific insulin receptor substrate 1 (IRS-1) antibody confirmed that the upper 170–140 kDa band corresponded mainly to IRS-1 (data not shown).

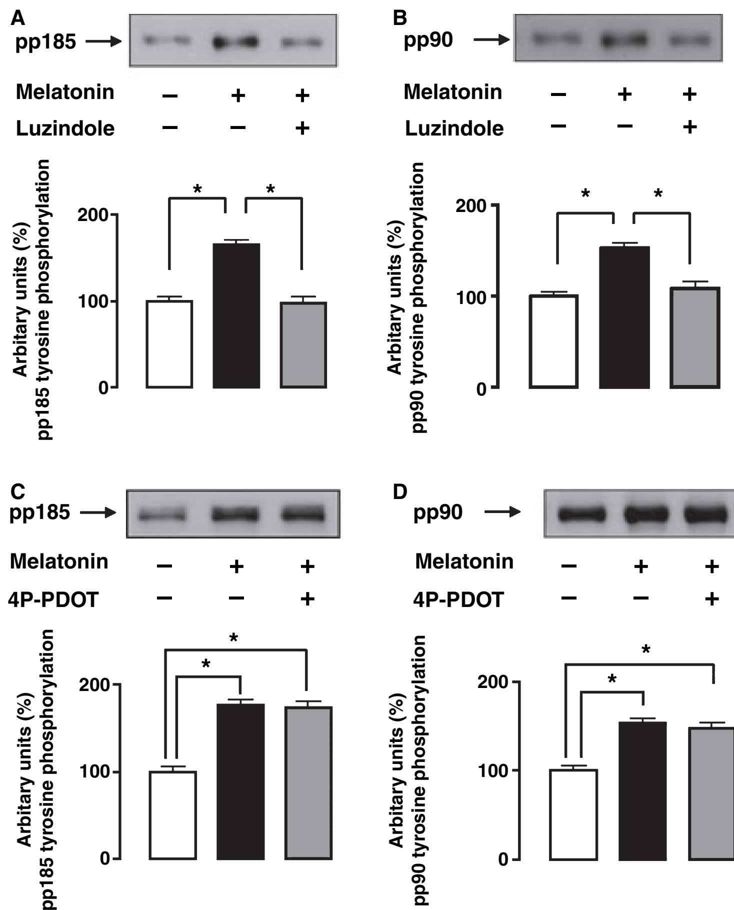

Fig. 2. Effect of MT receptors inhibitors, luzindole (A) and PPDOT (B), on the melatonin-induced tyrosine phosphorylation of IRS-1 and IR in isolated pancreatic islets. Isolated pancreatic islets were pré-incubated for 30 min in the presence of 2.8 mM glucose and 0.1 μM luzindole (A,B) or 10 μM PPDOT (C,D) and then incubated with 0.1 μM melatonin. Islet proteins were extracted and processed as described in Materials and Methods. The nitrocellulose membranes containing the soluble proteins were submitted to immunoblotting with anti-phosphotyrosine monoclonal antibody of four distinct experiments. The tyrosine phosphorylation levels are shown as mean ± S.E.M. of the scanning densitometric analysis of four distinct experiments. *$P < 0.05$ as indicated in the figure.

To examine if the increased tyrosine phosphorylation of the 90–100 kDa band was due to activation of IR or IGF-R, samples from melatonin-treated islets were used for immunoprecipitation with anti-IR or anti-IGF-1R antibodies, followed by immunoblotting with anti-phosphotyrosine antibody. Melatonin induced a twofold increase ($P < 0.01$) in insulin receptor tyrosine phosphorylation and a similar effect was observed on IGF-R phosphorylation (Fig.1C).

In the presence of 2.8 mM glucose and luzindole (0.1 μM), an inhibitor of both MT1 and MT2 receptors, melatonin induction of tyrosine phosphorylation of IRS-1 and IR was abolished (Fig. 2A,B). Conversely, 4P-PDOT (10 μM), a specific MT2 receptor inhibitor, did not interfere with the induction of tyrosine phosphorylation by melatonin (Fig. 2C,D).

The effect of melatonin on AKT serine phosphorylation (Fig. 3A), ERK1/2 threonine and tyrosine phosphorylation was then investigated (Fig. 3B). Melatonin (at 0.1 μM) increased the phosphorylation state of these proteins by 1.6-fold above basal level. By using an anti-phospho-STAT3 (Ser 727)-specific antibody it was found that melatonin induced a 1.7-fold increase above basal values of STAT3 serine phosphorylation (Fig. 3C).

The phosphotyrosine phosphatase, known as PTP-1B, plays a role in the regulation of IR tyrosine phosphorylation [43]. Melatonin induced a twofold increase in PTP-1B tyrosine phosphorylation with no further increase in PTP-1B serine phosphorylation (Fig. 4A,B). Using PTP-1B antibody in membranes containing immunoprecipitated IR, it was observed that melatonin induces a 53% reduction ($P < 0.05$) in the IR/PTP-1B association (Fig. 4C).

Discussion

The present study demonstrates, for the first time, in rat pancreatic islets, that melatonin induces IGF1-R and IR tyrosine phosphorylation and activates two intracellular signaling pathways: the PI3 K/AKT, which is mainly involved with cell metabolism, and MEK/ERKs that participates in cell proliferation, growth and differentiation. To investigate if the effect of melatonin-induced tyrosine phosphorylation of IR and IRS-1 was due to activation of one or both membrane melatonin receptors (MT1 and/or MT2), isolated islets were incubated with 0.1 μM luzindole, an inhibitor of MT1 and MT2 melatonin receptors at this concentration [44]; 10.0 μM 4P-PDOT, a specific MT2 receptor inhibitor was also used in our experiments [44]. Complete inhibition of melatonin action was observed with luzindole, whereas 4P-PDOT did not influence tyrosine phosphorylation induced by melatonin. These results suggest that the effect of melatonin on tyrosine phosphoryla-

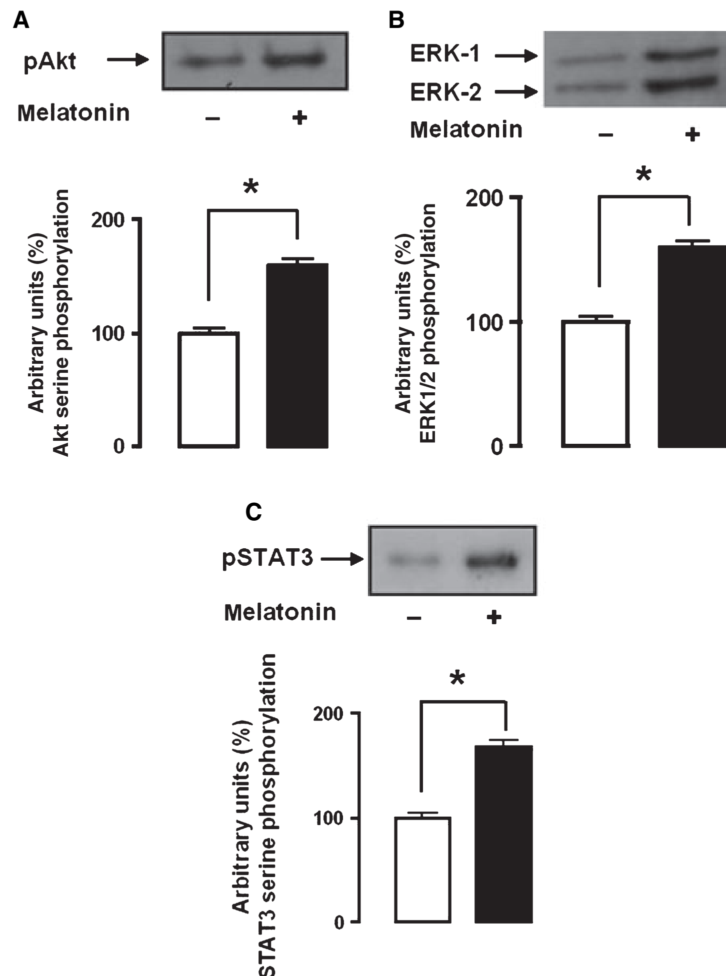

Fig. 3. Induction of AKT, ERK1/2 and STAT3 activation by melatonin treatment. Isolated pancreatic islets were incubated in KB buffer containing glucose 2.8 mM, in the presence (+) of 0.1 μM melatonin, for 30 min. The proteins from the islets were extracted and processed as described in Materials and Methods. The nitrocellulose membranes containing the soluble proteins were submitted to immunoblotting with (A) anti-phospho-AKT(Ser473) antibody; (B) antiphosphoERK1/2 antibody and (C) anti-phospho-STAT3 (Ser727) antibody. The phosphorylation levels are shown as means ± S.E.M. of the scanning densitometric analysis of four distinct experiments. *$P < 0.05$ due to melatonin effect.

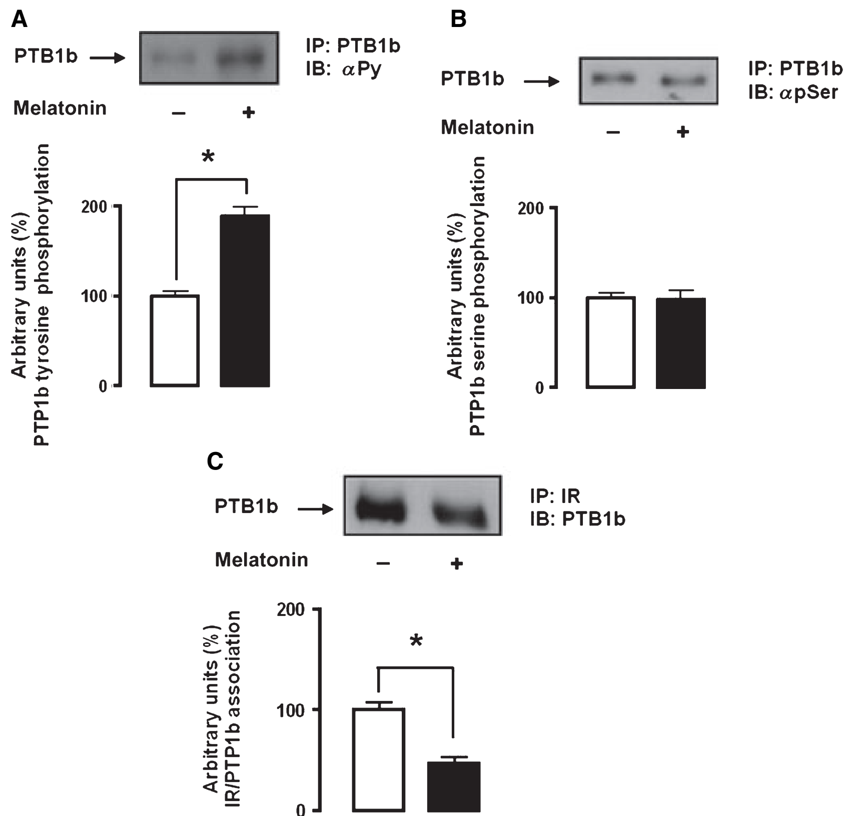

Fig. 4. Induction of PTP-1B tyrosine phosphorylation and reduced association with IR by melatonin. Isolated pancreatic islets were incubated in KB buffer containing glucose 2.8 mM, in the presence (+) of 0.1 μM melatonin for 30 min and were immunoprecipitated with anti-PTP-1B antibody and then submitted to immunoblotting with (A) anti-phosphotyrosine antibody, (B) anti-phosphoserine antibody or (C) anti-IR antibody. Scanning densitometric analysis was performed in autoradiograms from four experiments. The values are expressed as mean ± S.E.M. *$P < 0.05$ due to melatonin effect.

tion of IR and IRS-1 in pancreatic B-cells is, probably, mediated by the MT1 receptor, a GPCR. Similar observations were described in our previous studies [9] and by Peschke et al. [21].

Studies on cell proliferation have shown that GPCRs activate proteins with tyrosine kinase activity including those of the MAPK pathway [45–47]. The effects of melatonin on cell proliferation and differentiation are reported to be mediated by the MAPK signaling cascade [48, 49]. Melatonin increases MEK and ERK1/2 phosphorylation as shown in MT1-CHO cells and GT1-7 neurons [50, 51]. Cells from pars tuberalis treated with IGF-I after incubation with melatonin show a marked increase (10-fold) in phosphorylation status of p42/p44 (also known as ERK1/2 activity) [39]. The molecular mechanisms by which the MT1 receptor, which lacks intrinsic tyrosine kinase activity, couples with tyrosine phosphorylation are not known. MT1, through Gi protein activation, could control tyrosine kinase activity of the IR by modulating the activity of specific phosphotyrosine phosphatases that respond to intracellular cAMP levels.

Treatments of adipocytes with agents that elevate intracellular cAMP levels partially abolish the ability of Gi2 to suppress PTP-1B activity [51]. Gi2 activates the insulin-signaling pathway by suppression of PTP-1B activity [51]. The results presented herein show that melatonin increases tyrosine phosphorylation of PTP-1B, which was accompanied by reduced association of this protein with IR. The decreased activity of PTP-1B was not accompanied by a change in its context since serine phosphorylation was not altered. Several MAPK family members have been identified as kinases capable of phosphorylating STAT3 serine including extracellular regulated kinase (ERK) [52–54]. Espanel et al. [55] have suggested that STATs are PTP-1B substrates. The absence of PTP-1B in embryonic fibroblasts results in GH-dependent hyperphosphorylation of JAK2 and activates STAT3 and STAT5. In the present study, melatonin also induced STAT3 serine phosphorylation.

In conclusion, MT1 receptors in addition to decreasing cAMP, activates additional signaling pathways acting on IGF-R, IR, IRS-1, AKT, ERK1/2 and STAT3 phosphorylation in pancreatic islets. This effect probably involves a reduction in PTP1B activity indicated by higher tyrosine phosphorylation and lower association with IR. Thus melatonin inhibits insulin secretion by reducing cAMP levels but may regulate growth and differentiation of pancreatic islets by activating signaling pathways of IGFI and insulin receptors.

Acknowledgments

The authors are indebted to Marlene S. Rocha for technical assistance. This research was supported by FAPESP and CNPq.

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