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In vivo activation of insulin receptor tyrosine kinase by melatonin in the rat hypothalamus

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Abstract

Melatonin is the pineal hormone that acts via a pertussis toxin-sensitive G-protein to inhibit adenylate cyclase. However, the intracellular signalling effects of melatonin are not completely understood. Melatonin receptors are mainly present in the suprachiasmatic nucleus (SCN) and *pars tuberalis* of both humans and rats. The SCN directly controls, amongst other mechanisms, the circadian rhythm of plasma glucose concentration. In this study, using immunoprecipitation and immunoblotting, we show that melatonin induces rapid tyrosine phosphorylation and activation of the insulin receptor β-subunit tyrosine kinase (IR) in the rat hypothalamic suprachiasmatic region. Upon IR activation, tyrosine phosphorylation of IRS-1 was detected. In addition, melatonin induced IRS-1/PI(3)-kinase and IRS-1/SHP-2 associations and downstream AKT serine phosphorylation and MAPK (mitogen-activated protein kinase) phosphorylation, respectively. These results not only indicate a new signal transduction pathway for melatonin, but also a potential cross-talk between melatonin and insulin.

Keywords: AKT, GPCR, hypothalamus, insulin receptor, melatonin, tyrosine phosphorylation.

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Melatonin (5-methoxy-N-acetyltryptamine) is produced and secreted by the pineal gland in a circadian fashion. The production of melatonin occurs at night and the duration of its secretion is proportional to the length of the dark period. Melatonin, thus, acts as a neuroendocrine transducer of circadian and seasonal photoperiodic information. In mammals, the physiological functions of melatonin are, among other mechanisms, regulation of the circadian clock in the hypothalamic suprachiasmatic nucleus (SCN), seasonal reproduction, and inhibition of dopamine release from the retina (Morgan *et al*. 1994; Vanecek 1998). Experiments with pinealectomized animals demonstrated that melatonin also plays a role in energy expenditure, body mass regulation, and insulin peripheral action and secretion (Margraf and Lynch 1993; Lima *et al*. 1998; La Fleur *et al*. 1999; Picinato *et al*. 2002). It has been demonstrated that the biological effects of melatonin are mediated, at least in part, by specific high-affinity G protein-coupled receptors (GPCRs). In mammals, two distinct high-affinity membrane-bound receptors, the MT_1 and MT_2 receptors, have been described. These receptors are encoded by separate genes (Reppert *et al*. 1994, 1995, 1996). Both melatonin receptors inhibit adenylyl cyclase via pertussis toxin-sensitive Gi proteins (Yung *et al*. 1995; Barret *et al*. 1999). The MT_1 receptor is localized in the hypothalamic SCN and hypophyseal pars tuberalis (Sugden *et al*. 1999; revised by Dubocovich *et al*. 2003). MT_2 is localized in the SCN and retina (Reppert *et al*. 1995; Willians *et al*. 1995; Hunt *et al*. 2001).

There is a consistent body of evidence demonstrating that insulin acts in the hypothalamus to suppress feeding behaviour (Woods *et al*. 1979; Brüning *et al*. 2000). Insulin receptors (IR) display distinct patterns of expression in the hypothalamus, pituitary and olfactory bulb (Baskin *et al*. 1994; Folli *et al*. 1994). IR is a protein tyrosine kinase that catalyzes the phosphorylation of several intracellular

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Abbreviations used: AII, angiotensin II; FFA, free fat acids; GPCRs, G protein-coupled receptors; IR, insulin receptor; IRS, insulin receptor substrate; JAK2, Janus kinase 2; LH, luteinizing hormone; MAPK, mitogen-activated protein kinase; PDK, phosphoinositide-dependent kinase; PH, pleckstrin homology; PI(3)K, phosphatidylinositol-3-OH kinase; PMSF, phenylmethylsulfonylfluoride; SCN, suprachiasmatic nucleus; SDS–PAGE, sodium dodecyl sulfate – polyacrylamide gel electrophoresis; SHP-2, SH2-containing phosphotyrosine phosphatase.

substrates, including the insulin receptor substrate (IRS) proteins and the src-homology-2/α collagen related protein, Shc (reviewed by Saltiel and Kahn 2001). The IRS proteins, once phosphorylated at distinct tyrosine residues, act as docking sites for proteins containing Src homology 2 (SH2) domains, including phosphatidylinositol-3-OH kinase [PI(3)K] and the SH2-containing phosphotyrosine phosphatase, SHP-2 (reviewed by Saltiel and Kahn 2001). A downstream substrate of PI(3)K activity is the serine/threonine protein kinase B or AKT (Coffer *et al*. 1998; Lawlor and Alessi 2001). Upon insulin receptor tyrosine kinase activation and autophosphorylation, there is also recruitment of Shc protein and the adaptor molecule, Grb2, leading to activation of the extracellular signal-regulated protein kinase (p42/44 MAP kinase, ERK) pathway (Skolnik *et al*. 1993; De Fea and Roth 1997).

It has been recently demonstrated that melatonin can induce MAP kinase activation in cultured cells (Chan *et al*. 2002). Furthermore, other hormones that act through GPCRs, including angiotensin II (AII), vasopressin, and luteinizing hormone (LH) can induce tyrosine phosphorylation of cytoplasm proteins (Marrero *et al*. 1995; Saad *et al*. 1995; Velloso *et al*. 1996; Gallo-Payet and Guillon 1998; Carvalho *et al*. 2003). AII and LH activate the cytosolic protein tyrosine kinase Janus kinase 2 (JAK2) and probably use this kinase to induce intracellular protein tyrosine phosphorylation.

In this study, we evaluated the ability of melatonin to induce the tyrosine phosphorylation of IRS-1, as well as IRS-1/PI(3)K and IRS−1/SHP2 associations, and the phosphorylation of AKT/PKB and p42/44MAPK in the rat hypothalamus *in vivo*. We also identified a kinase that was activated by melatonin, the insulin receptor, and assessed a possible cross-talk between the melatonin and insulin signalling pathways. The present data reveal a new signal transduction pathway for melatonin and demonstrate, for the first time, that the insulin receptor is co-activated by a GPCR-binding hormone.

Materials and methods

Reagents

The reagents and apparatus for sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE) and the nitrocellulose membranes were from Bio-Rad (Richmond, CA, USA). Tris, phenylmethylsulfonylfluoride (PMSF), aprotinin, dithiothreitol, and melatonin were from Sigma-Aldrich (St Louis, MO, USA). Sodium amobarbital and human recombinant insulin (Humulin R) were purchased from Eli Lilly Co. (Indianapolis, IN, USA). The melatonin receptors antagonists, MT_1/MT_2 non-selective luzindole and the MT_2-seletive 4-phenyl-2-propionamidotetraline (4P-PDOT), were obtained from Tocris Cookson (Ballwin, MO, USA). Anti-IR, anti-IRS-1, and anti-phosphotyrosine antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-PI3-kinase was from Upstate Biotechnology (Lake Place, NY, USA). Phospho-AKT (Ser473) and Phospho-p42/44 MAPK (Thr202/Tyr204) antibodies were from New England BioLabs (Beverly, MA, USA). The enhanced chemiluminescence reagent kit, ECL, and the protein A Sepharose 6MB were from Amersham-Pharmacia Biotech (Buckinghamshire, UK).

Animals and surgical procedures

Adult male Wistar rats (250–300 g) were used in all experiments in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA). After an overnight fast, the rats were anaesthetized with sodium thiopental (5 mg/100 g body weight, i.p.) and positioned on a David Kopf stereotaxic apparatus using head and chin holders. The lateral, anteroposterior and dorsoventral coordinates of the lateral ventricle were obtained from Paxinos and Watson (1986). A cannula was positioned and fixed to the skull with cranioplastic cement. The rats were studied 6 days after cannula implantation into the lateral ventricle.

Intracerebroventricular infusions

Anaesthetized 6-h fasted rats (sodium thiopental 5 mg/100 g body weight, i.p.) received an acute infusion of 5 μL of saline containing, or not, insulin (60 μg) or melatonin (100 ng) through the cannula. Following the infusion, and after the indicated time in the figure legend, animals were decapitated by guillotine, the skulls were immediately opened, and the suprachiasmatic region of the hypothalamus was rapidly excised under a surgical microscope and was immediately homogenized as described below. Histological analysis of the remaining brain showed that the removed area included the posterior part of the medial pre-optic area, the suprachiasmatic nucleus and the most anterior and ventral portions of the anterior hypothalamic nucleus. The extent of the lesion never exceeded the basal retrochiasmatic area.

Western blot analysis

Hypothalami from three different animals were treated as pool samples. The removed tissue was immediately homogenized in approximately 100 μL of ice-cold 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, using a Polytron PTA 2100 generator operated at maximum speed for 30 s. Insoluble material was removed by centrifugation for 30 min at 12 800 *g* in a Beckman 70.1 Ti rotor (Palo Alto, CA, USA) at 4°C. The supernatants of the samples were used for immunoprecipitation with anti-phosphotyrosine, or anti-IR, or anti-IRS-1, and protein A-Sepharose 6MB. The precipitated proteins were treated with Laemmli sample buffer (Laemmli 1970) and run on SDS–PAGE (8% bis-acrylamide) in a Bio-Rad miniature lab gel apparatus (Mini-Protean, Bio-Rad). To determine the phosphorylation status of AKT and p42/44MAPK, the solubilized proteins from hypothalami samples were treated with Laemmli sample buffer containing 100 mM dithiothreitol and heated in a boiling water bath for 4 min. Similar sized aliquots (80 μg protein) were subjected to SDS–PAGE (8% bis-acrylamide). M_r standards were myosin (210 kDa), β-galactosidase (110 kDa), BSA (85 kDa) and ovalbumin (49 kDa).

Electrotransfer of proteins from the gel to nitrocellulose was performed for 90 min at 120 V (constant) in the miniature transfer apparatus (Mini-Protean). To reduce non-specific protein binding to the nitrocellulose, the filter was pre-incubated overnight at 4°C in blocking buffer (5% non-fat dry milk, 10 mM Tris, 150 mM NaCl and

0.02% Tween 20). The nitrocellulose blots were incubated for 4 h at 22°C with the specific antibodies described in the figure legends diluted in blocking buffer (3% non-fat dry milk). To visualize the autoradiogram, commercial enhanced chemiluminescence reagents exposed to photographic film were used. Quantitative analysis of the blots was done using Scion Image software (Frederick, MD, USA).

IR kinase assay

Insulin receptor tyrosine kinase activity was measured by autophosphorylation. A low dose of melatonin was infused into the lateral ventricle in order to stimulate IR autophosphorylation and partial receptor activation. The insulin receptor was then immunoprecipitated and allowed to autophosphorylate *in vitro* in the presence of exogenous ATP (15 μM). Tyrosine autophosphorylation was measured by immunoblotting with anti-phosphotyrosine antibody.

Data analysis

Results are presented as direct comparisons of bands present in the autoradiographs from saline or melatonin injected rats. Band intensities were quantified by optical densitometry using the Scion Image software (Frederick, MD, USA). Student's *t*-test for unpaired data was employed.

Results

Melatonin-induced tyrosine phosphorylation of IRS-1 and insulin receptor β-subunit

Figure 1 illustrates a typical immunoblot with antibody against phosphotyrosine-containing proteins from samples previously immunoprecipitated with anti-phosphotyrosine antibody. Figure 1(a) shows that melatonin induces tyrosine phosphorylation of two distinct bands, an upper broad band migrating at 180–140 kDa and a lower band migrating between 90 and 100 kDa. The maximal tyrosine phosphorylation occurred at 5 min after melatonin infusion and decreased dramatically thereafter. Parallel experiments were performed in which the rats received an infusion of insulin as a positive control for induced tyrosine phosphorylation (Carvalheira *et al*. 2001) (Fig. 1b).

By re-incubating the membranes with specific antibodies, we detected that most of the 180–140 kDa band corresponded to the insulin receptor substrate-1, IRS-1. The 90–100-kDa band corresponded to the 95 kDa β subunit of the insulin receptor (IR) (data not shown).

Melatonin-induced IRS-1 association with PI (3) kinase and with SHP-2

In order to confirm that the upper band contains at least the protein named IRS-1, and that the lower band contains the β-subunit of the insulin receptor, some excised hypothalami samples from saline or acutely melatonin-treated rats were immunoprecipitated with anti-IR or anti-IRS-1 antibodies, followed by immunoblotting with anti-phosphotyrosine antibody (Figs 2a and 3a, respectively).

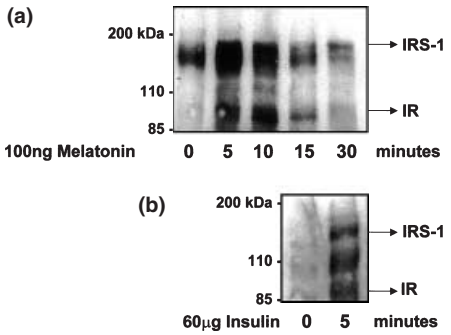

Fig. 1 Effect of melatonin i.c.v. infusion on cellular protein tyrosine phosphorylation in hypothalamus of rats. The effect of melatonin infusion through a lateral ventricle cannula was examined. Saline (0), or melatonin (100 ng) at the time indicated (a), or 60 μg insulin (b) were administered into the lateral ventricle of anaesthetized male normal rats. Following the infusion, after the indicated times, the rats were decapitated; the crania were immediately opened to excise the hypothalami. The excised pre-optic hypothalamic areas of three different animals were immediately homogenized, as described in Material and methods. The soluble proteins were immunoprecipitated with anti-phosphotyrosine antibody and immunoprecipitated proteins were resolved by 8% SDS–PAGE. Immunoblotting of the resolved proteins was performed with anti-phosphotyrosine antibody in order to visualize the effect of melatonin infusion on tyrosine phosphorylation of some soluble proteins. The insulin infusion was used as a positive control, and the hypothalami were excised after 5 min of insulin infusion.

In the hypothalami samples previously immunoprecipitated with anti-insulin receptor antibody and subsequently immunoblotted with anti-phosphotyrosine antibody, melatonin was shown to induce an approximately 3-fold increase ($p < 0.02$) in insulin receptor tyrosine phosphorylation above basal (Fig. 2a), with no difference in the amount of immunoprecipitated insulin receptor β-subunit (Fig. 2b).

In samples from hypothalami previously immunoprecipitated with anti-IRS-1 antibody and immunoblotted with anti-phosphotyrosine antibody, there was a 10-fold increase ($p < 0.05$) in the melatonin-stimulated IRS-1 phosphorylation (Fig. 3a), without any change in the amount of IRS-1 immunoprecipitated, demonstrated by subsequent immunoblotting of the same nitrocellulose sheets with anti-IRS-1 antibody (Fig. 3b). Previously published data demonstrate a co-immunoprecipitation between IRS-1 and some SH2-containing proteins, such as the 85-kDa subunit of PI(3)K and SHP2, a phosphotyrosine phosphatase with approximately 78 kDa (reviewed by Saltiel and Kahn 2001). When the same blots were subsequently incubated with antibodies directed against the 85-kDa subunit of PI(3)K, there was a faint band in the basal state (saline infusion). After melatonin infusion, the intensity of this band displayed a significant ($49 \pm 9\%$, $p < 0.05$) increase compared with the basal state (Fig. 3c).

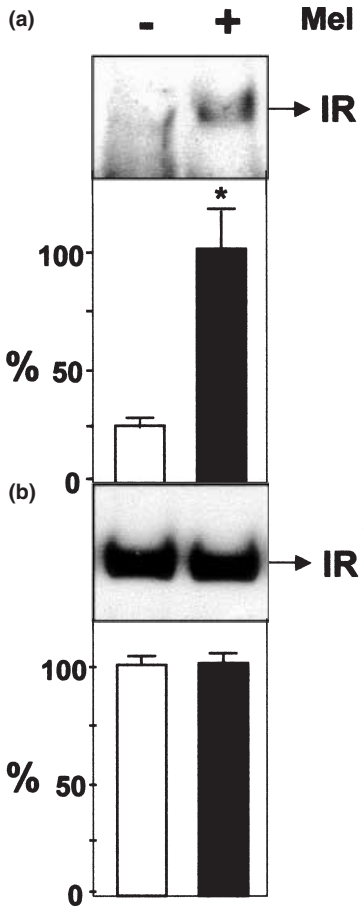

Fig. 2 Melatonin-induced insulin receptor β-subunit tyrosine phosphorylation. Pooled hypothalami extracts from three different rats, as described in legend to Fig. 1, that received saline (–) or 100 ng melatonin (+) (Mel) were immunoprecipitated with anti-IR antibody and then immunoblotted with anti-phosphotyrosine (a) and anti-IR (b). Scanning densitometry was performed on autoradiograms from four experiments. The values are expressed as mean ± SEM. $^*p < 0.05$.

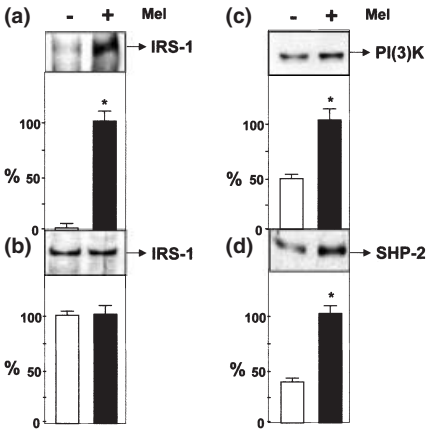

Fig. 3 Melatonin-induced IRS-1 tyrosine phosphorylation and association with PI(3)K and SHP-2. Pooled hypothalami extracts from three different rats, as described in legend to Fig. 1, that received saline (–) or 100 ng melatonin (+) (Mel) were immunoprecipitated with anti-IRS-1 antibody and then immunoblotted with anti-phosphotyrosine (a), anti-IRS-1 (b), anti-PI3-kinase (c) and anti-SHP-2 (d) antibodies. Scanning densitometry was performed on autoradiograms from four experiments. The values are expressed as mean ± SEM. $^*p < 0.05$.

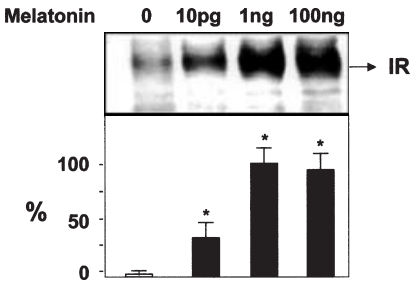

Fig. 4 Dose–response curve for melatonin-stimulated tyrosine phosphorylation of insulin receptor (IR). After injection of the indicated doses of melatonin (Mel), pooled pre-optic hypothalamic area extracts from three different rats were immunoprecipitated with anti-IR antibodies. To analyze the tyrosine phosphorylation status, the immunoblots were performed with anti-phosphotyrosine. This is a representative autoradiograph from three different experiments. The values in the bar graph are expressed as mean ± SEM. $^*p < 0.05$.

The incubation of the same blots with anti-SHP2 antibody demonstrated that melatonin induced a 53 ± 8% increase ($p < 0.05$) in the IRS-1/SHP−2 association (Fig. 3d). These results are consistent with the formation of a stable association between IRS-1 and each of the demonstrated proteins, PI(3)K and SHP-2.

Melatonin dose–response curve for tyrosine phosphorylation of insulin receptor β-subunit

Figure 4 demonstrates a dose–response experiment. After 5 min of 5 μL infusion containing 10 pg, 1 ng or 100 ng melatonin into the lateral ventricle, the hypothalami from different animals were excised and the soluble proteins were precipitated with the anti-insulin receptor antibody. Melatonin-stimulated insulin receptor tyrosine phosphorylation was dose-dependent. The phosphorylated proteins were detectable after injection of as little as 10 pg melatonin and maximal stimulation occurred when 1 ng was infused.

Effect of melatonin on AKT and MAP kinase phosphorylation

Melatonin infusion induced a 3-fold increase in AKT serine phosphorylation at 10 min, and then returned to basal levels after 15 min (Fig. 5a). This result was confirmed in three distinct experiments, as demonstrated in the representative figure, and the bar graphs indicate the densitometry analysis.

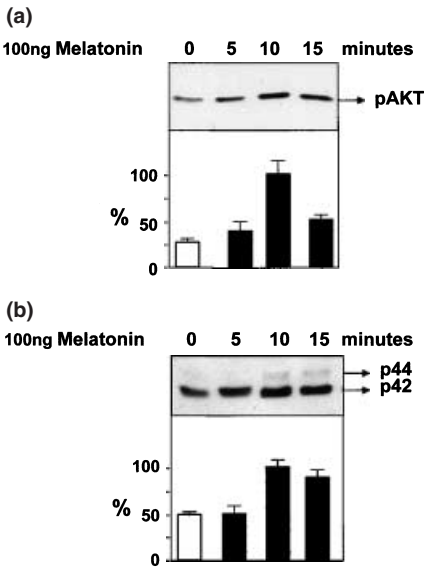

Fig. 5 Melatonin-stimulated AKT and p42MAP kinase phosphorylation in hypothalami of rats. Saline (0) or 5 μL (100 ng) of melatonin was infused into the lateral ventricle of anaesthetized male rats. The animals were decapitated after the hormone infusion at the indicated time. The crania were opened and excised hypothalami of three different animals were treated together. After centrifugation, aliquots containing 80 μg of proteins were resolved on 8% SDS–PAGE, transferred to nitrocellulose, and detected with (a) anti-phospho-AKT (Ser473) antibody (pAKT) or with (b) anti-phospho-p42/44 MAPK (Thr202/Tyr204) antibody (p44 and p42). This is a representative autoradiogram from three different experiments. The values in the bar graph are expressed as mean ± SEM. $^*p < 0.05$.

Figure 5(b) shows that melatonin induces increased phosphorylation of p42MAP kinase at 10 min, which remains phosphorylated above basal level for 15 min after melatonin infusion.

Melatonin-induced insulin receptor tyrosine kinase activity

To determine whether the melatonin-induced insulin receptor phosphorylation was due to the induction of its tyrosine kinase activity, we performed an *in vitro* kinase assay based on the dose–response results (Fig. 4). After the infusion of 10 pg melatonin *in vivo,* the hypothalami were excised and submitted to immunoprecipitation with anti-insulin receptor antibody and protein A. The immunoprecipitated proteins were incubated with exogenous ATP (15 μM) to allow autophosphorylation to occur if the insulin receptor kinase activity was activated by melatonin intracerebroventricular (i.c.v.) infusion.

Immunoblotting of these assays with anti-phosphotyrosine antibody demonstrated that there was a clear increase in IR autophosphorylation (Fig. 6) of approximately 3-fold when compared with basal plus ATP.

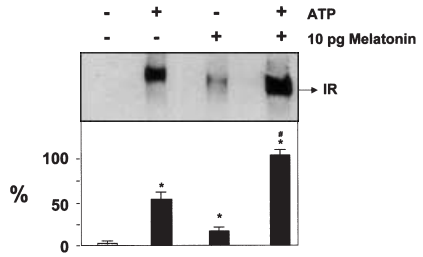

Fig. 6 Melatonin-induced tyrosine kinase activity. Insulin receptor tyrosine kinase activity was measured by autophosphorylation *in vitro* in the absence (–) or presence (+) of 15 μM ATP before and after the injection of a submaximal dose of melatonin (10 pg). The material was submitted to 8% SDS–PAGE and an increase in IR autophosphorylation were observed after melatonin infusion plus the addition of ATP *in vitro*. The values in the bar graph are expressed as mean ± SEM. $^*p < 0.05$ compared with basal.

Effect of the melatonin receptor inhibitors, luzindole and 4P-PDOT, on melatonin-induced insulin receptor tyrosyl phosphorylation

In order to analyze whether the effect of melatonin detected on insulin receptor autophosphorylation was due to melatonin membrane receptors, MT_1 and MT_2, experiments using luzindole, a non-selective melatonin receptor antagonist, and 4P-PDOT, a selective MT_2 receptor antagonist were performed.

When the rats were pre-treated for 10 min with 10 μM luzindole or 300 nM 4P-PDOT, there were marked reductions in the melatonin-induced tyrosine phosphorylation of the insulin receptor in extracts immunoprecipitated with anti-insulin receptor antibody (Fig. 7).

Discussion

In the present study, the action of melatonin upon various tyrosine kinase pathways was examined in the hypothalamus. Our results show that melatonin induces a rapid MT_1/MT_2 membrane receptor-dependent tyrosine phosphorylation and activation of the insulin receptor β-subunit tyrosine kinase (IR).

Upon IR activation, tyrosine phosphorylation of IRS-1 was detected. In addition, melatonin induces IRS-1/PI(3)K and IRS-1/SHP-2 associations and downstream AKT serine phosphorylation and p42MAPK (also known as ERK-2) phosphorylation, respectively. The present report is the first to demonstrate that melatonin stimulates tyrosine phosphorylation of insulin receptor and IRS-1. These data suggest that IRS-1 phosphorylation may play a role as a converging target in insulin- and melatonin-stimulated signalling pathways and also as a probable link for both hormones in the control of body weight (Le Gouic *et al.* 1996; Rasmussen *et al.* 1999) and carbohydrate metabolism (Alquier *et al.* 2003).

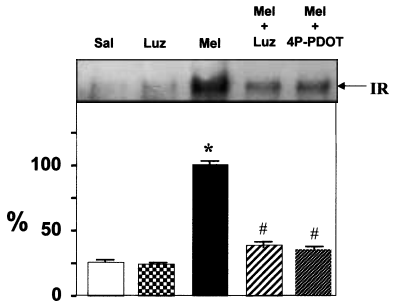

Fig. 7 Effect of melatonin receptors inhibitors, luzindole and 4P-PDOT, on melatonin-induced tyrosine autophosphorylation of the insulin receptor. Rats were pre-treated by i.c.v. infusion of 10 μM/L luzindole or 300 nM 4P-PDOT 10 min prior to 100 ng melatonin (Mel) infusion through a lateral ventricle cannula. The excised hypothalami, as described before, were then submitted to immunoprecipitation with anti-IR antibody and protein A sepharose followed by immunoblotting with anti-phosphotyrosine antibody. The melatonin-induced insulin receptor tyrosine phosphorylation was detected in samples from rats pre-treated or not with each one of the melatonin receptors antagonists. The autoradiogram is representative of three different experiments and the bar graph represents the mean scanning densitometry analysis of them. *$p < 0.05$, basal (saline) versus melatonin. #$p < 0.05$ melatonin versus each inhibitor plus melatonin.

The major lipid product of PI(3)K activity is phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3 has binding affinity for a sequence called the pleckstrin homology (PH) domain. Thus, PH-domain-containing proteins are localized to membrane-associate signalling complexes (Coffer *et al.* 1998; Lawlor and Alessi 2001). Some targets of PI 3-kinase lipid products are AKT/PKB and its upstream activator, phosphoinositide-dependent kinase (PDK). AKT/PKB activates various enzymes involved in cell growth and in the inhibition of apoptosis, in addition to other functions. These data, taken together with our results of melatonin-induced AKT phosphorylation, suggest that AKT may participate in a novel mechanism by which melatonin acts as a putative modulator of feeding behaviour associated to seasonal variations of body weight and metabolism (Bartness and Wade 1985; Rosenthal *et al.* 1987).

Evidence from distinct source indicates that SHP-2 activity has a positive role in the activation of MAP kinase members and in mitogenic signalling by insulin, EGF, and interleukin-6 (Xiao *et al.* 1994; Hausdorff *et al.* 1995; Deb *et al.* 1998; Cunnick *et al.* 2000). We showed that melatonin is able to induce tyrosine phosphorylation of IRS-1 and its simultaneous association with SHP-2 and a later tyrosine/threonine phosphorylation of p42MAP kinase in the hypothalamus of intact rats. In this regard, our data may be of physiological importance, as MAPK activation is related to light-induced clock entrainment (Butcher *et al.* 2002).

The implications of intracellular interactions between melatonin and insulin signalling systems are interesting. In addition to body weight and food intake modulation, insulin is able to promote sharp decreases in elevated plasma FFA levels in fasted rats through a direct effect on the CNS (Coimbra and Migliorini 1986). Furthermore, there is a growing body of evidence indicating that melatonin, through its MT_1 and MT_2 receptors, is involved in the neuronal mechanism regulating circadian rhythms, in particular, those of the glucose blood level (La Fleur *et al.* 1999, 2001).

Melatonin regulates SCN functions through direct activation of MT_1 and MT_2 receptors (Masana and Dubocovich 2001). Activation of MT_1 acutely inhibits the neuronal firing rate (Liu *et al.* 1997; Gillette and Mitchell 2002; Jin *et al.* 2003), while activation of the MT_2 phase shifts circadian rhythms of activity *in vivo* via activation of PKC (Dubocovich *et al.* 1998; McArthur *et al.* 1997; Hunt *et al.* 2001).

The concentrations used for Luzindole and 4P-PDOT are in the range for non-specific blockage of MT_1 and MT_2, in the case of Luzindole and specific blockage of MT_2 receptor in the case of 4P-PDOT (Hunt *et al.* 2001). Since the melatonin-induced IR tyrosine phosphorylation was blocked, at same magnitude, in both cases, it is possible to conclude that the melatonin receptor involved is, most probably, of the MT_2 type.

The molecular mechanisms by which the melatonin receptor, which lacks intrinsic tyrosine kinase activity, couples to tyrosine phosphorylation events are not known. MT_2 (and MT_1), through Gi protein activation, could control the tyrosine kinase capacity of the IR, by modulating the

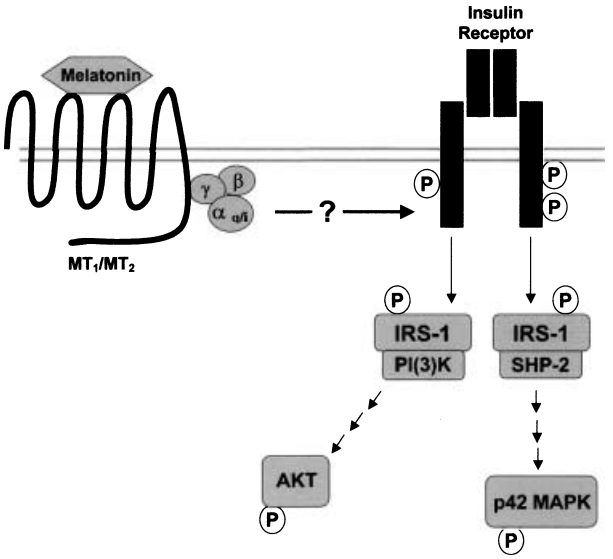

Fig. 8 Summary of the findings. Melatonin activation of the insulin receptor is mediated by a membrane receptor (MT_1/MT_2). Insulin receptor phosphorylation and activation leads to IRS1 tyrosine phosphorylation. Once phosphorylated, IRS1 binds to and activates PI(3)K and SHP-2. PI(3)K activation promotes downstream activation of AKT. SHP-2 activation is one of the proteins recruited for the insulin receptor to induce p42 MAPK activation.

activity of specific phosphotyrosine phosphatases that respond to the intracellular cAMP levels. This possibility is supported by recent evidence obtained with transgenic mice, which over express protein Gi. The Gi-transgenic mice have an enhanced insulin-induced IR and IRS-1 tyrosine phosphorylation mediated by the suppression of protein-tyrosine phosphatase-1B activity and expression (Tao *et al*. 2001). However, further studies will be required to confirm this hypothesis.

In summary, our study demonstrates the existence of an additional intracellular signalling mechanism stimulated by melatonin in the rat hypothalamus (Fig. 8). Intracellular cross-talk between melatonin and insulin signalling may have a role in the intracellular mechanism controlling body weight, feeding behaviour and blood glucose circadian rhythm.

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