Melatonin enhances leptin expression by rat adipocytes in the presence of insulin

Maria Isabel Cardoso Alonso-Vale, Sandra Andreotti, Sidney Barnabé Peres, Gabriel Forato Anhê, Cristina das Neves Borges-Silva, José Cipolla Neto, and Fabio Bessa Lima

Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil

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Melatonin is produced and secreted by the pineal gland, with a circadian rhythm characterized by low levels during the day and peak values at night (5, 36). It plays an important role as an interface among the cyclic environment, physiological rhythms, and neuroendocrine processes (22, 23). Even though melatonin has been shown to reduce body mass in diet-induced obesity and old rats (47, 63, 50), and previous studies have suggested a possible role for this hormone in maintaining optimal energy balance (9, 65), the relationship between melatonin and leptin remains to be fully clarified.

To further investigate the involvement of melatonin on leptin expression, we specifically investigated its direct action on leptin release and gene expression in isolated rat adipocytes. Considering that melatonin acts directly on adipocytes through specific pertussis toxin-sensitive G_i protein-coupled receptors (GPCR) MT_1 and MT_2 (9, 65), we hypothesized that the activation of these receptors might exert a positive modulation on leptin production by lowering cAMP levels.

To confirm this hypothesis we evaluated 1) the ability of isolated adipocytes to synthesize and release leptin under melatonin treatment, 2) how they respond to insulin and/or dexamethasone in combination with melatonin, and 3) the mechanism underlying the action of melatonin over these parameters. Our results not only revealed a new mechanism for regulation of leptin expression by melatonin acting through MT_1 receptors but also showed a potential cross talk between melatonin and insulin, where the insulin receptor and its convergent target Akt are coactivated by melatonin.

MATERIALS AND METHODS

Preparation of adipocytes. Male adult Wistar rats (8 wk old) were purchased from the Animal Resource of the Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil, and were kept on a 12:12-h light-dark cycle (lights on at 0700) in a room with controlled temperature (23 ± 1°C). Animals were housed in groups of three to four in plastic cages with food (balanced chow pellet diet, Nuvilab CR1; Nuvital, Colombo, PR, Brazil) and water ad libitum. Rats were killed at 0800 by decapitation. The abdominal wall was opened, and the peripitidymal fat pads were excised and processed for adipocyte isolation. All of the procedures followed the approved protocol in accordance with the Ethical Principles in Animal Research adopted by the Ethics Committee for Animal Research of the Institute of Biomedical Sciences (CEEA, no. 180/01). The isolation of adipocytes was performed according to Rodbell (53), with some slight modifications. Briefly, epididymal fat pads were minced with fine scissors and added to a flask (~4 g/flask) with 10 ml of sterile

LEPTIN, SECRETED BY ADIPOSE CELLS as a product of the Lep gene, is the major peripheral nutritional signal involved in the regulation of food intake and energy balance (13, 46, 66). However, the hormonal regulation of the Lep gene and leptin secretion is not completely understood. Agents that increase intracellular cAMP levels inhibit leptin expression (27, 29, 38, 52). Glucocorticoids have a potent stimulatory effect on leptin production (8, 18, 20, 54), whereas a role for insulin is more contradictory (8, 11, 16, 18, 39). In addition, it is known that circulating leptin levels are strictly related to body mass (12, 25, 32) and show a clear circadian rhythm, with increasing values at night in rodents and humans (26, 48, 56, 64).

Address for reprint requests and other correspondence: F. B. Lima, Dept. of Physiology and Biophysics, Institute of Biomedical Sciences (ICB 1 Bldg.), Univ. of São Paulo, 1524 Prof. Lineu Prestes Ave., São Paulo, SP, 05508-900, Brazil (E-mail: fabio@icbusp.br).

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Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA) containing 20 mM HEPES, 5 mM glucose, 1% BSA (fraction V; Sigma Chemical, St. Louis, MO), antibiotics (100 μg/ml penicillin and 100 μg/ml streptomycin, Invitrogen) and 1 mg/ml collagenase type II (Sigma), pH 7.4. The mixture was incubated in an orbital shaker (New Brunswick Scientific, Edison, NJ) at 150 rpm for 30 min at 37°C. The isolated adipocytes were filtered through a fine plastic mesh, transferred to a conical (50-ml) plastic tube, and washed three times in the same buffer without collagenase (25 ml/wash). After the final wash, cells were allowed to rest in the same buffer for 4 h at 37°C before being seeded as described below. According to our experience, this procedure completely abolishes any previous in vivo interference on cell metabolism and brings the cells to a similar metabolic state before the start of the following incubations. Adipocyte viability was tested with trypan blue, and the cell number was determined as previously described (21).

Incubations of adipocytes. Incubations were set up in six-well culture plates. Before the cells were incubated, the adipocytes were washed three times, and the medium was completely aspirated. From the resultant adipocyte pack, an aliquot of cells was added to wells containing 4 ml of incubation buffer (similar to the digestion buffer, except that collagenase type II was absent and 10% fetal bovine serum was added) to obtain a final cell concentration of 4 × 10⁵ cells/ml, followed by the addition, where indicated, of 1 nM melatonin,1 5 nM insulin,2 7 nM dexamethasone,3 75 ng/ml pertussis toxin (PTX; Calbiochem-Novabiochem, La Jolla, CA), 25 μM forskolin (or varying concentrations), or melatonin receptor antagonists: 10 μM luzindole (2-benzyl-N-acetyltryptamine) or 10 μM 4P-PDOT (4-phenyl-2-propionamidotetrane; Tocris Cookson, Ellisville, MO). All other drugs were purchased from Sigma. The incubation was carried out at 37°C in a 5% CO₂ atmosphere, usually for 6 h. At the end, aliquots of the medium were taken out and stored at −20°C for later determination of the leptin released, and the cells were collected for the analysis of leptin mRNA and the insulin intracellular signaling.

RT-PCR assay for leptin gene expression. All reagents were purchased from Invitrogen. Total RNA was extracted from isolated adipocytes by use of guanidine isothiocyanate-based TRIzol solution, according to the manufacturer’s specifications (17), and quantified spectrophotometrically at 260 nm with acceptable 260/280 ratios of 1.7 to 2.0. The RNA quality was also checked by 1% agarose gel electrophoresis stained with 1 g/ml ethidium bromide. Superscript II reverse transcriptase (RT) was used to reverse transcribe 5 μg of total RNA isolated using an oligo(dT)₁₀ primer in a total reaction volume of 50 μl. After the RT reaction, 4-μl aliquots were used as cDNA template for polymerase chain reaction (PCR) amplifications with Taq DNA polymerase and 2.5 mM MgCl₂. The thermal cycling parameters used were 25 cycles for 45 s at 95°C, 45 s at 60°C, and 30 s at 72°C, using a Gradient Mastercycler (Eppendorf, Hamburg, CA). Aliquots of the supernatant containing 3 mg of total protein were used for immunoprecipitation with anti-insulin receptor β-subunit antibody (α-IRβ) and protein A-Sepharose 6MB before sample treatment with Laemmli buffer and 6.5% SDS-PAGE, as described elsewhere (15). The nitrocellulose blots were incubated for 4 h at 22°C with α-IRβ or anti-phosphotyrosine antibody (α-pTyr) diluted in blocking buffer (3% nonfat dry milk). For Akt serine phosphorylation studies, equal amounts of protein (30 μg) were subjected to 8% SDS-PAGE and immunoblotted with anti-phospho(Ser473)-Akt antibody (α-pAkt). Electrotransfer of proteins from the gel to nitrocellulose membrane was performed for 90 min at 120 V (constant). The filter was preincubated overnight at 4°C in blocking buffer (5% nonfat dry milk, 10 mM Tris, 150 mM NaCl, and 0.02% Tween 20) to reduce nonspecific protein binding to the nitrocellulose. To visualize the autoradiogram, we used the commercial enhanced chemiluminescence reagents (ECL, Amersham Biosciences, São Paulo, SP, Brazil) according to the manufacturer’s specifications (17), and quantified densitometry (Eagle Eye-Stratagene, model 401304, software Eagle version 3.0, for Windows (GraphPad Software, San Diego, CA). The level of significance was set at P = 0.05. Data were presented as means ± SE.

RESULTS

Time course of leptin release in insulin- and/or dexamethasone-stimulated isolated adipocytes. Figure 1 demonstrates the time course effects of insulin, dexamethasone, and the combination of the two hormones on the leptin release in isolated adipocytes. A significant increase was evident after 6 h of incubation with glucocorticoid alone or associated with insulin (2.5-fold the control value). Insulin alone did not stimulate the adipocytes or synergize the effect of dexamethasone. On the basis of these results, the 6-h incubation was chosen for the remaining assays.

Effects of melatonin, insulin, and dexamethasone on leptin release and mRNA expression. After 6 h of incubation, melatonin or insulin alone did not change the amount of leptin released by adipocytes compared with control cells (Fig. 2).

1 The melatonin concentration used is close to its nocturnal peak (51, 65).
2 The insulin concentration is close to its maximal effect for glucose transport stimulation (30).
3 The dexamethasone concentration is close to its seminaximal inhibitory effect on glucose transporter gene expression (24).

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However, when adipocytes were incubated with insulin in the presence of melatonin, a significant increment on leptin release (120%; Fig. 2A) and mRNA content (50%; Fig. 2B) was observed. Dexamethasone alone increased leptin release (105%) and gene expression (50%). Simultaneous treatment with melatonin and dexamethasone did not result in any significant increment on leptin release and mRNA content compared with dexamethasone alone. Similarly, the addition of insulin did not result in any synergism with dexamethasone. However, when dexamethasone was associated with insulin and melatonin, a further increase in leptin release (250%) and in gene expression (100%) was obtained.

Melatonin prevented forskolin-induced inhibition of leptin release and gene expression. Considering that elevated cytoplasmatic cAMP levels inhibit leptin synthesis, forskolin, a potent stimulator of adenylyl cyclase and cAMP synthesis, was added to the incubations to further characterize whether the effects of melatonin on leptin expression is a cAMP-mediated event. A dose-response curve is depicted in Fig. 3, where a clear, sigmoid-shaped decay curve is seen in adipocytes treated with forskolin (IC50 = 6.1 ± 0.78 μM). In the same figure, the dashed curve shows the attenuating effect of melatonin. As noted, a rightward shift was observed in the forskolin decaying curve in the presence of melatonin (IC50 = 80.5 ± 2.6 μM). The next experiments (Fig. 4, A and B) were performed under a concentration of 25 μM forskolin, which was enough to elicit a nearly maximal inhibitory effect in non-melatonin-treated adipocytes and, on the other hand, was almost entirely suppressed in the presence of melatonin (1 nM).

In the absence of melatonin, forskolin treatment promoted an overall reduction (between 50 and 95%) in the leptin release. Melatonin addition completely antagonized this effect. Moreover, even in the presence of forskolin, melatonin was effective in its positive interaction with insulin alone (80%) or combined with dexamethasone (125%; Fig. 4A). The expression of leptin mRNA was also assessed in cells incubated with forskolin (Fig. 4B). These experiments revealed that forskolin was able to reduce basal (38%) as well as insulin/dexamethasone-induced (39%) leptin expression. Both effects were prevented by the addition of melatonin.

Effect of melatonin on insulin-induced leptin release was completely blocked by PTX. As mentioned above, melatonin may act directly on adipocytes through specific MT1 and MT2 receptors. Both receptors are coupled to a PTX-sensitive G1 protein that inhibits the forskolin-induced cAMP formation. To reinforce the hypothesis that melatonin action on leptin release is a receptor-mediated event, we tested whether PTX was able to block the stimulatory effect of a physiological concentration of melatonin (1 nM). Figure 5 shows that PTX (75 ng/ml) alone did not interfere with the basal leptin release but completely blocked the synergistic effect of melatonin plus insulin.

**Fig. 1.** Time course of insulin (Ins) and/or dexamethasone (Dex) treatment on leptin release. Adipocytes from epididymal fat pads were isolated from male Wistar rats. Cells were incubated at 37°C for 6 h in the absence or presence of 5 nM insulin and/or 7 nM dexamethasone. Aliquots of the medium were collected at 0, 3, and 6 h for determination of leptin content by RIA. Data represent means ± SE of 4 independent experiments performed in duplicate. *P < 0.05 vs. control (−) after 6 h of incubation.

**Fig. 2.** Effects of melatonin (Mel), insulin, and dexamethasone on leptin release (A) and gene expression (B). Isolated adipocytes were incubated at 37°C for 6 h in the absence or presence of 1 nM melatonin, associated or not with 5 nM insulin and/or 7 nM dexamethasone. A: aliquots of the medium were collected for determination of leptin content by RIA. Two sets of experiments are depicted in the graph. The 1st shows cell treatments without melatonin; the 2nd shows the same treatments in the presence of melatonin. Data represent means ± SE of 5–6 independent experiments and are expressed as arbitrary units [1 AU = mean value of control (−)]. *P < 0.05 vs. control (−); *P < 0.05 vs. correspondent treatment in the absence of melatonin.
MT₁ but not MT₂ antagonist blocked the effect of melatonin on insulin-induced leptin release. To determine the involvement of MT₁ or MT₂ melatonin receptor subtypes on melatonin action on leptin release, we assessed the ability of 10 μM luzindole (a nonselective melatonin receptor antagonist) and 10 μM 4P-PDOT (a selective MT₂ receptor antagonist) to antagonize the effect of melatonin on insulin-induced leptin release. 4P-PDOT did not show such an effect, whereas luzindole completely blocked the positive interaction of melatonin and insulin on leptin release. In addition, 4P-PDOT or luzindole alone had no effect on leptin release in control adipocytes (Fig. 6).

Melatonin upregulates insulin-induced IRβ and Akt phosphorylation. To gain further insight into the molecular basis by which melatonin and insulin interact to induce leptin release and mRNA expression, we investigated the insulin-signaling cascade in the presence of melatonin. Figure 7A shows a representative blot of the immunoprecipitated IRβ protein content, which did not present significant differences among the groups. Figure 7B shows that insulin alone induced an expected (61) and significant rise (2.5-fold the control value) in IRβ tyrosine phosphorylation. However, when the adipocytes were treated with insulin plus melatonin, IRβ tyrosine phosphorylation was significantly increased (2.3-fold) compared with insulin alone. To investigate whether the modulation of this early step in insulin cell signaling was transmitted to downstream convergent targets, we accessed Akt serine phosphorylation (Fig. 7B). Similarly, Akt serine phosphorylation was significantly upregulated (2-fold) by the treatment with the two hormones compared with insulin alone.

**DISCUSSION**

Glucocorticoids and insulin have been proposed as potential upregulators of leptin synthesis. In fact, as demonstrated earlier (8, 18, 20, 54), our results confirmed the ability of glucocorticoids to stimulate leptin gene expression and release. The effect of insulin, however, is more controversial. In humans, many studies (40, 55, 60) have found no acute stimulatory effect, whereas long-lasting hyperinsulinemic clamps resulted in increased serum leptin levels in some (33, 59), but not all (19, 28), studies. Direct effects of insulin on leptin gene expression are also unclear, since increase (54), decrease (18, 52), and no change (27) have been reported. In rodents, insulin has been reported to increase leptin levels (6, 8, 11, 16, 39). However, some authors showed that insulin was effective in stimulating leptin production at very high concentrations (6, 8) or after a long time of stimulation (39). In addition, others reported that insulin at a physiological range has no acute stimulatory effect on leptin secretion from isolated rat adipocytes (16, 39). Our findings demonstrated that insulin by itself had no direct stimulatory effect on leptin expression but inter-
acted synergistically with melatonin to produce such an effect. Interestingly, a synergism between insulin and dexamethasone occurred in the presence of melatonin. Thus the present work clearly demonstrates that melatonin confers to adipocytes the ability to synthesize leptin in response to insulin stimulation and, in this way, establishes a synergism between insulin and dexamethasone. In addition, it is remarkable that in the presence of the three hormones the leptin expression was maximal. In rats, melatonin, insulin, and corticosterone show daily rhythms, with maximal plasma concentrations at night (1, 26, 49), when they might interact on the adipose tissue to magnify the daily amplitude of leptin production.

We (31) have already investigated the interaction between melatonin and insulin, focusing on metabolic responses of adipocytes; melatonin in vitro elicited an enhancement in the adipocyte sensitivity to insulin. In addition, the absence of melatonin as a consequence of pineal ablation decreased insulin-stimulated glucose uptake in adipocytes in both fed (1, 30) and fasted animals (2). During fasting, pinealectomy has also revealed alterations in insulin-stimulated glucose oxidation and incorporation into lipids (2). Taken together, these data and the present study suggest that the response to insulin might be modulated by melatonin, and this interaction not only underlies the metabolic effects but can also be extrapolated to other adipocyte functions, like its ability to produce leptin.

Recent studies have reported contradictory results on the possible relationship between melatonin and leptin secretion. In humans, for example, no major effect was reported in postmenopausal women supplied with melatonin during daytime (10), although a recent work with nocturnal-life style adult humans reported a simultaneous decrease in melatonin and leptin levels (49). Middle-aged rats, together with a decline in melatonin production, displayed higher absolute serum leptin compared with young rats (45, 50, 63). This observation could be attributed to increased aging-related adiposity (48, 62), as circulating leptin is strictly and positively correlated with the amount of body fat (12, 25, 32). However, when adjusted for body fat percentage, leptin levels are, in fact, significantly lower in aged rats (37, 43, 48). Moreover, it was shown that the daily rhythm in serum leptin levels is abolished with aging (48). In this way, it is possible to conclude that the well-known reduction of melatonin production in aged rats might be the cause of the observed reduction in leptin serum levels.

Results that are apparently contradictory were demonstrated when pinealectomy model was adopted. Although melatonin administration suppressed increased leptin levels after pinealectomy (14), a recent study (42) showed no differences in leptin levels between pinealectomized and intact obese rats. We have described that pinealectomy has no influence on plasma leptin levels in fed rats measured at three different times of the day (1) but interacts synergistically with fasting to provoke a more accentuated fall in leptinemia (2). Studies using melatonin supplementation showed both an increase in leptin levels in some mammalian species [Mustella vison (41) and garden dormouse (3)] and a decrease in middle-aged rats [in parallel with body weight reduction (50, 63)].

All of these controversies may be due not only to the use of different species but also to the use of different models in vivo that results in some interferences: 1) changes in glucocorticoid levels [melatonin treatment shows both an increase in leptin expression by 10.220.32.247 on October 29, 2017 http://ajpendo.physiology.org/ Downloaded from
To clarify the mechanisms on melatonin action, we have shown that melatonin totally prevented the inhibitory effects of forskolin on both leptin synthesis and secretion, suggesting that its action occurs via a negatively coupled adenyl cyclase receptor and cAMP formation. Besides, when adipocytes were treated with PTX (75 mg/ml; which ADP-ribosylates some G_{i} proteins, thereby uncoupling them from their associated receptors and inhibiting the signaling pathway), the effect of melatonin was completely abolished, reinforcing the hypothesis that melatonin modulates the synthesis and the secretion of leptin through a PTX-sensitive G_{i} protein-coupled membrane receptor.

As mentioned before, MT_{1} and MT_{2} melatonin receptors were recently described in adipocytes. By using a subtype-selective MT_{2} receptor antagonist (4P-PDOT) and a nonselective receptor antagonist [luzindole (34)], we demonstrated that the effect of melatonin acting synergistically with insulin to induce leptin secretion is an event dependent on MT_{1} melatonin receptor 7 subtype.

It was recently reported (27) that processes linked to G_{i} protein stimulation might regulate the release of leptin in adipocytes. Those authors showed that insulin alone was unable to increase leptin release in isolated adipocytes from massively obese human subjects. However, by using GPCR agonists, they demonstrated that insulin induced a twofold increase in leptin secretion from the isolated adipocytes. These events were inhibited by PTX. Therefore, these findings are in agreement with ours, as melatonin is also a G_{i} protein agonist, and the stimulatory effect of insulin was obtained only in the presence of melatonin. Moreover, it was shown that G_{i2} protein improves insulin signaling through the suppression of phosphotyrosine phosphatase 1B (57).

To gain further insight into the molecular basis of the permissive effect of melatonin on insulin-induced leptin expression, we investigated the insulin-signaling pathway after melatonin incubations. Interestingly, our data show that melatonin is able to upregulate insulin-induced IR{beta} tyrosine phosphorylation without modifying the protein content and the stimulatory effect of insulin was obtained only in the presence of melatonin. Moreover, it was shown that G_{i2} protein improves insulin signaling through the suppression of phosphotyrosine phosphatase 1B (57).

Fig. 7. Effect of melatonin on insulin receptor-β (IRβ) protein content, IRβ tyrosine phosphorylation, and Akt serine phosphorylation.

A

IP: α-IRβ; IB: α-IRβ

B

IP: α-IRβ; IB: α-pTyr

C

IB: α-pAKT (Ser)

To gain further insight into the molecular basis of the permissive effect of melatonin on insulin-induced leptin expression, we investigated the insulin-signaling pathway after melatonin incubations. Interestingly, our data show that melatonin is able to upregulate insulin-induced IR beta tyrosine phosphorylation without modifying the protein content. This effect seems to be transmitted through the intracellular insulin cascade, since the serine phosphorylation of the downstream Akt was also affected by melatonin. The ability of melatonin to activate insulin signaling has been recently described in the hypothalamus of rats, and it has been shown to involve the activation of its membrane receptors (4). Another study showed that a constitutively active version of the Ser/Thr kinase Akt induces leptin production in 3T3-L1 adipocytes (7). Taken together, these studies and the present data suggest a connection between the ability of melatonin to potentiate the insulin signaling and its permissive effect on insulin-induced leptin expression, as both are mediated by melatonin membrane receptors.

This is the first work that correlated melatonin and leptin expression by an in vitro approach, and here we emphasize the relevance of its association with insulin. In conclusion, melatonin exerts a permissive role on insulin-stimulated leptin production. These effects are caused by melatonin binding to the G_{i} protein-coupled MT_{1} membrane receptors and might be linked to its ability to potentiate insulin-induced phosphorylations of insulin receptors and Akt.
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