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Insulin modulates norepinephrine-mediated melatonin synthesis in cultured rat pineal gland

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Abstract

The mammalian pineal gland synthesizes melatonin in a circadian manner, peaking during the dark phase. This synthesis is primarily regulated by sympathetic innervations via noradrenergic fibers, but is also modulated by many peptidergic and hormonal systems. A growing number of studies reveal a complex role for melatonin in influencing various physiological processes, including modulation of insulin secretion and action. In contrast, a role for insulin as a modulator of melatonin synthesis has not been investigated previously. The aim of the current study was to determine whether insulin modulates norepinephrine (NE)-mediated melatonin synthesis. The results demonstrate that insulin (10^{-8}M) potentiated norepinephrine-mediated melatonin synthesis and tryptophan hydroxylase (TPOH) activity in *ex vivo* incubated pineal glands. When *ex vivo* incubated pineal glands were synchronized (12h NE-stimulation, followed by 12h incubation in the absence of NE), insulin potentiated NE-mediated melatonin synthesis and arylalkylamine-*N*-acetyltransferase (AANAT) activity. Insulin did not affect the activity of hydroxyindole-*O*-methyltranferase (HIOMT), nor the gene expression of *tpoh*, *aanat*, or *hiomt*, under any of the conditions investigated. We conclude that insulin potentiates NE-mediated melatonin synthesis in cultured rat pineal gland, potentially through post-transcriptional events.

Keywords: Pineal gland; Melatonin; Insulin; Norepinephrine; Tryptophan hydroxylase; Arylalkylamine-*N*-acetyltransferase; Hydroxyindol-*O*-methyltransferase

Introduction

Melatonin, 5-hydroxytryptophan (5-HTP), serotonin (5-HT), 5-hydroxy-indolacetic acid (5-HIAA) and N-acetylserotonin (NAS) synthesis by the mammalian pineal gland are regulated in a number of ways, including sympathetic innervation via noradrenergic fibers originating in the superior cervical ganglion (Sugden, 1989; Cipolla-Neto and Afeche, 1992; Reiter, 1991). It is well established that the neurohormone melatonin is synthesized during the dark period, characterizing a typical circadian and seasonal rhythm. Rhythmicity of the pineal gland's secretory function is synchronized by the environmental light/dark cycle (LD), which is dependent on the photic information transmitted from retinal ganglion cells, via the neural retino-hypothalamic-pineal pathway (Moore et al., 1995). Melatonin synthesis is also regulated by efferents from specific diencephalic areas (Cipolla-Neto et al., 1995, 1999).

Norepinephrine (NE) released from nerves endings into the pineal perivascular space at night results in activation of β_1 and α_1 adrenoreceptors. Activation of G_s-protein-coupled β_1 adrenoceptors activates the enzyme adenylate cyclase, increasing intracellular cyclic adenosine monophosphate levels (cAMP), which in turn activates the cAMP-dependent protein kinase (PKA). At the same time, norepinephrine interaction with G_q-protein-coupled α_1 adrenoreceptor, increases inositol triphosphate (IP_3) and diacylglicerol (DAG) levels, therefore increasing intracellular Ca^{2+} and DAG levels, activating protein kinase C (PKC) isoforms. Activation of both PKA and PKC isoforms play critical roles in melatonin synthesis (Simonneaux and Ribelayga, 2003).

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The first enzyme involved in melatonin synthesis is tryptophan hydroxylase (TPOH), which exhibits a circadian rhythm in activity, converting tryptophan to 5-HTP. 5-HTP is decarboxylated by an L-aromatic amino acid decarboxylase (LAAD) resulting in 5-HT synthesis (Sugden, 1989; Sitaram and Lees, 1978; Ehret et al., 1991). In the rat pineal gland, cAMP and Ca^{2+} regulate arylalkylamine-*N*-acetyltransferase (AANAT) expression, controlling melatonin synthesis (Roseboom and Klein, 1995, 1995; Klein et al., 1983; Klein, 2007; Vanecek et al., 1985; Sugden et al., 1985). Once translated AANAT interacts with 14-3-3 protein forming a protector complex against proteolysis, converting 5-HT into NAS (Klein et al., 2002). The last step of melatonin synthesis is *O*-methylation of NAS, catalyzed by hydroxyindole-*O*-methyltransferase (HIOMT). Circadian rhythms of HIOMT are dependent on adrenergic stimulation, via β_1 and cAMP, whereas the regulation of the HIOMT activity rhythm seems to be dependent on post-transcriptional events induced by various neurotransmitters (Simonneaux and Ribelayga, 2003; Ribelayga et al., 1997, 1999). Additional complexity in the regulation of pineal gland synthesis of indolamines steams from influences by several peptidergic systems (Simonneaux and Ribelayga, 2003). For example, angiotensin II potentiates melatonin synthesis through AT_1 receptor mediated by tryptophan hydroxylase activation (Baltatu et al., 2002).

Melatonin has recently been shown to influence both insulin secretion and action (Lima et al., 1998; Picinato et al., 2002a,b). However, it is not known whether insulin in turn influences melatonin synthesis. Given that insulin receptors have already been localized and quantified by binding sites assay (Kar et al., 1993), with an additional amplification of insulin receptor mRNA in the rat pineal gland (Peschke et al., 2006), and that circulating insulin levels themselves exhibit circadian rhythms, we hypothesized that insulin may influence melatonin synthesis in the rat pineal gland.

Material and methods

Animals

2 months-old male Wistar rats weighing 150–180g were obtained from the Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil. The animals were kept under a 12h:12h light/dark cycle (lights on at 06:00am, ZT 0), in a temperature controlled room (21 ± 2°C), with food and water ad libitum. Ethics approval was granted by the Committee of Ethics in Animal Experimentation of the Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil.

Experimental design

Experiment 1: Standard pineal gland culture

Pineal glands were cultured as described previously (Afeche et al., 2006). Briefly after decapitation, rat pineal glands were isolated and immediately placed in ice-cold BGJb (Fitton–Jackson Modification) medium with phenol red, modified by the addition of bovine serum albumin (BSA; 1mg/mL), 2mM glutamine, 0.1mg/mL ascorbic acid and penicillin (100U/mL)-streptomycin (100μg/mL) (Gibco. Grand Island, NY, 14072, USA). Pineal glands were incubated (37°C; 95% O_2, 5% CO_2) in modified BGJb medium in 24-well plates (2 glands/well; 200μL/well) for 48h before treatment (the medium was changed after the first 24h). On the day of the treatment, all glands were placed in fresh medium for 1h and then divided into the following experimental groups (Fig. 1A) (12 glands per group, 3 full experimental blocks): Control (cultured glands without treatments), NE (10^{-6}M), NE (10^{-6}M) + Ins (10^{-9}M to 10^{-7}M) and NE (10^{-9}M to 10^{-6}M) + Ins (10^{-8}M). After 5h of incubation the glands were frozen on dry ice and kept at − 80°C prior to subsequent analysis.

Experiment 2: Norepinephrine-synchronized pineal gland culture

Animals were sacrificed and the pineal glands were isolated at 05:00 p.m. (ZT 11). In order to keep the glands in a circadian cycle very close to that occurring in the intact animal, they were immediately incubated with NE (10^{-6}M) for 12h followed by 12h without NE. This cycle was repeated, as indicated in Fig. 1 B. After 48h of culture, glands were challenged with NE (10^{-6}M) in the absence or presence of Ins (10^{-8}M) (96 glands per experimental block, 3 experimental blocks). After 5h of incubation, the glands were frozen and stored prior to subsequent analysis.

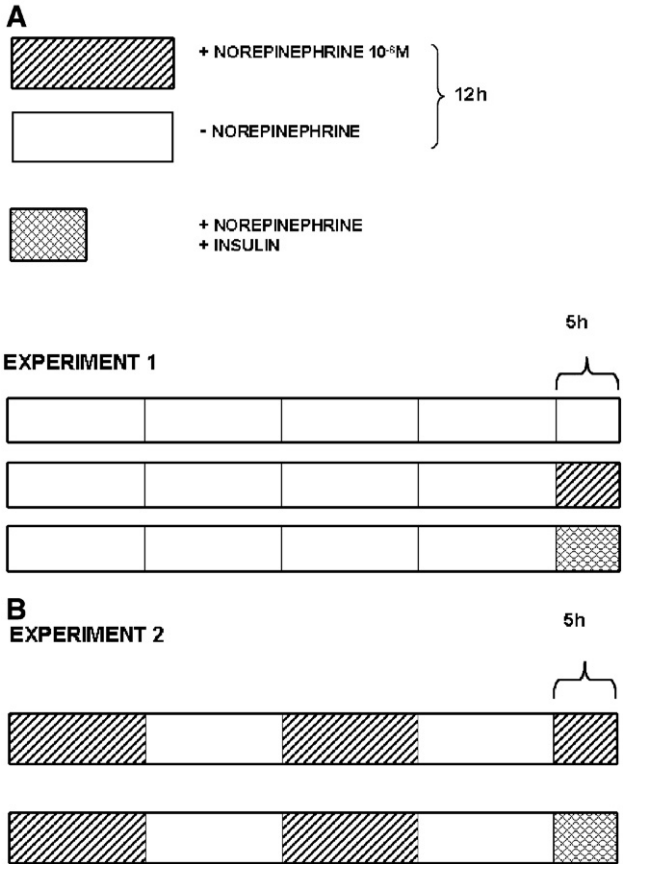

Fig. 1. Pineal gland culture experimental design. ▨ + Norepinephrine, □ − Norepinephrine, ▩ + Norepinephrine + insulin. Standard pineal gland culture (A). Norepinephrine-synchronized pineal gland culture (B).

Measurements of melatonin levels

Pineal gland melatonin levels were measured by high performance liquid chromatography (HPLC) with electrochemical detection running Empower software (Waters System, Milford, MA, USA). Melatonin was separated on a Resolve C18 column (5μm, 150 × 3.9mm). The chromatographic system was isocratically operated with the following mobile phase: 0.1M sodium acetate, 0.1M citric acid, 0.15mM EDTA, 30% methanol, pH 3.7, at a flow rate of 1mL/min. The electrochemical detector potential was adjusted to + 900mV. The elution time for melatonin was about 6min. Each gland was sonicated (Microson XL 2005, Heat System Inc., Farmingdale, NY, USA) in a solution of 0.1M perchloric acid (120μL), containing 0.02% EDTA and 0.02% sodium bisulfate. After centrifugation (2min, 13,000*g*, Eppendorf 5415C centrifuge, Brinkman Instruments Inc., Westbury, NY, USA), 40μL of the supernatant was injected into the chromatographic system (Injector Mod. 7125, 20μL loop, Rheodyne Inc., San Francisco, CA, USA).

Enzymatic assays

TPOH activity determination

Each pineal gland was sonicated in sodium phosphate buffer (2mM, pH 7, 100μl). To each sample the following were added: HEPES (50mM, pH 7), catalase (100μg/ml), tryptophan (50μM), dithiothreitol (5mM), Fe $(NH_4)_2$ $(SO_4)_2$ (10μM), 6-MPH_4 (500μM) and 1μl of [^{3}H]tryptophan (1mCi/ml-previously dried under nitrogen) (Sitaram and Lees, 1978; Ehret et al., 1991). The material was incubated at 37°C for 10min. After a charcoal activated solution had been added (7.5% in 1M HCl) to terminate the reaction, 200μl of the supernatant was transferred to scintillation tubes, liquid scintillation was added and radioactivity was evaluated with a Beckman LS6500 β counter.

AANAT activity determination

AANAT activity was measured by a radiometric assay (Afeche et al., 2006; Deguchi and Axelrod, 1972; Parfitt et al., 1975). Briefly, 100μl of 0.1M sodium phosphate buffer, pH 6.8, containing 40mM tryptamine and [^{3}H]-acetyl coenzyme A (2mM, final specific activity = 4mCi/mmol) were added to a microcentrifuge tube containing one gland kept at 4°C. The glands were sonicated and then incubated at 37°C for 20min. The reaction product *N*-[^{3}H]-acetyltriptamine was extracted with chloroform (1ml). Samples of 500μl were evaporated until dry in a scintillation vial and radioactivity was determined with a Beckman LS6500 β counter.

Table 1
Primer and probe sequences for rat *tpoh*, *aanat* and *hiomt*, real-time quantitative RT-PCR assays

Gene	Primer/Probe	Sequence
Tpoh	Forward	5′-GCTGAACCCAGTTTTGCTCA-3′
	Reverse	5′-TTGCTTGCACAGTCCAAACTC-3′
	Probe	5′6-FAM-AAGTAGCACGTTGCCAGT-TTCTGAACCG-TAMRA-3′
Aanat	Forward	5′-CCACCAGTGCGTTTGAGATT-3′
	Reverse	5′-GACACAGGGTGAGGAAGTGC-3′
	Probe	5′6-FAM-AGCGCGAAGCCTTTATCT-CAGTCTCG-TAMRA-3′
Hiomt	Forward	5′-GGGCAAGACCCAGTGTGAG-3′
	Reverse	5′-GGGCAAGAATGAAGAGGTCAG-3′
	Probe	5′6-FAM-TTTGTCGCTGGTGACTTC-TTCCGTTC-TAMRA-3′

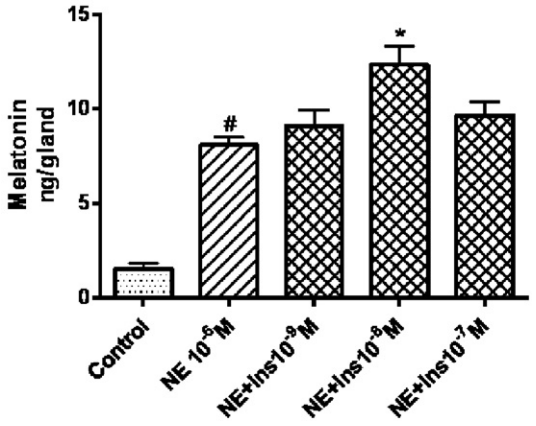

Fig. 2. NE acute stimulation of standard pineal gland culture in association to different concentrations of insulin (10^{-9} M to 10^{-7} M). Melatonin content expressed in ng/gland. One-way ANOVA, Bonferroni's Multiple Comparison Test, #$P<0.001$ vs Control and *$P<0.01$ vs NE 10^{-6} M, plotted as means±SEM.

HIOMT activity determination

The pineal glands were sonicated in phosphate buffer (0.05M, pH 7.9, 50μl) and soon afterwards 150μl of a solution containing ^{14}C-S-adenosyl-L-methionine (activity 43.8mCi) and N-acetylserotonin (1mM) was added (Ribelayga et al., 1997; Axelrod and Weissbach, 1961; Ceinos et al., 2004). The homogenates were incubated for 30min at 37°C. The reaction was interrupted by adding 200μl of sodium borate buffer (12.5mM, pH 10) and 1ml of chloroform saturated in water. The tubes were centrifuged at 13,000*g* rotation for 5min at 4°C. The product (^{14}[C] melatonin) was extracted in 800μl of chloroform, which was subsequently evaporated, and the radioactivity evaluated with a Beckman LS6500 β counter.

RNA extraction and quantitative real-time RT-PCR

RNA extraction and quantitative RT-PCR, including the standard RNA preparation for all assays by T7 polymerase method (allowing absolute quantification of gene expression, Ambion, Austin, Texas), were performed like previously described (Young et al., 2002; Gibson et al., 1996; Chomczynski and Sacchi, 1987; Depre et al., 1998). The correlation between the C_t (the number of PCR cycles required for the fluorescent signal to reach a detection threshold) and the amount of standard was linear over at least a 5-log range of RNA for all assays (data not shown). Specific quantitative assays were designed from rat sequences available in GenBank. Taqman assays for rat *tpoh*, *aanat* and *hiomt* are presented in Table 1. Gene expression data are represented as mRNA molecules per ng total RNA.

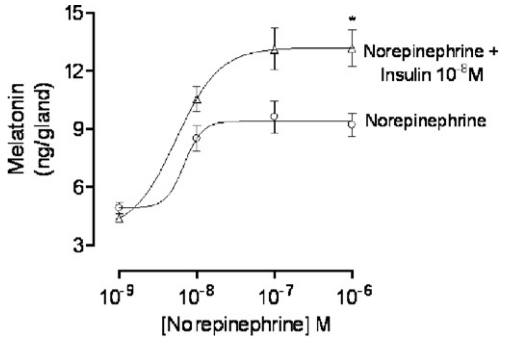

Fig. 3. A dose response curve to NE (10^{-9} M to 10^{-6} M) ○ and Insulin (10^{-8} M)+NE(10^{-9} M to 10^{-6} M) △. Values expressed in logarithmic scale of [NE] (M). One-way ANOVA, Bonferroni's Multiple Comparison Test *P=0.002 vs NE 10^{-6} M, plotted as mean±SEM.

Statistics

The HPLC (ng/gland), enzymatic assay (pmol/gl/h) and RT-PCR (molecules/ng total RNA) results were plotted as the mean ± SEM. One-way ANOVA with Bonferroni's post test was performed using GraphPad Prism version 5.01 for Windows, GraphPad Software, San Diego California USA. When it was appropriated Student's *t*-test was applied.

Results

Experiment 1 — standard pineal gland culture

Fig. 2 shows the effects of different concentrations of insulin (10^{-9}M to 10^{-7}M) on NE-mediated (10^{-6}M) melatonin synthesis. A significant potentiating effect of insulin was observed at the concentration of 10^{-8}M when compared to the norepinephrine positive control group (NE 10^{-6}M). Insulin (10^{-9}M to 10^{-7}M) alone had no effect on melatonin synthesis (data not shown).

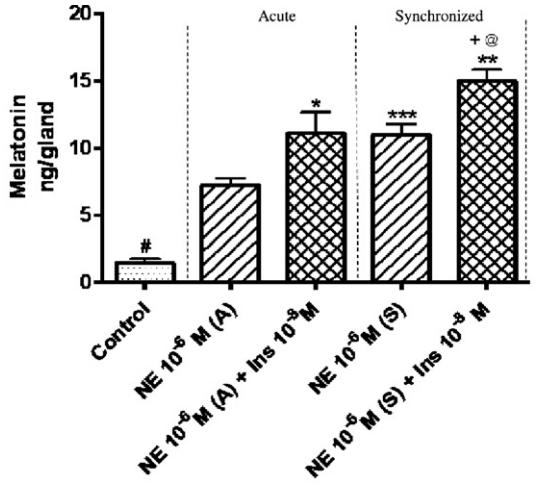

Fig. 4. Acute (A) and Synchronized (S) protocol stimulation. Melatonin values are expressed in ng/gland. One-way ANOVA, Bonferroni's multiple comparisons test, #P<0.001 vs all groups, *P<0.05 vs NE 10^{-6} M (A), ***P<0.01 vs NE 10^{-6} M (A), **P<0.001 vs NE 10^{-6} M (A),+P<0.01 vs NE 10^{-6} M (S), @P<0.05 vs NE 10^{-6} M (A)+Ins 10^{-8} M, plotted as means±SEM.

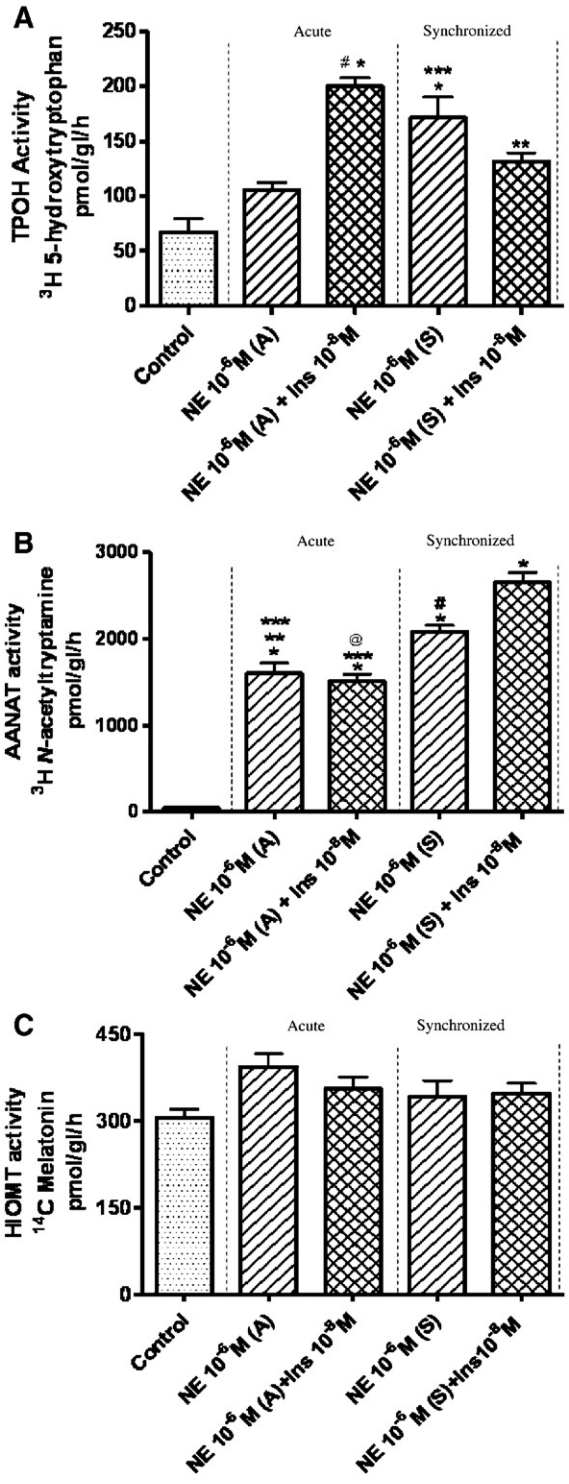

Fig. 5. Pineal gland enzymatic activities, acute (A) and synchronized (S) protocol stimulation. (5A) Analysis of TPOH activity. *P<0.001 vs Control; #P<0.001 vs NE 10^{-6} M (A), ***P<0.01 vs NE 10^{-6} M (A), **P<0.05 vs Control. (5B) Analysis of AANAT activity. *P<0,001 vs Control, **P<0.01 vs NE 10^{-6} M (S), ***P<0.001 vs NE 10^{-6} M (S)+Ins 10^{-8} M, @P<0.001 vs NE 10^{-6} M (S), #P<0.01 vs NE 10^{-6} M (S)+Ins 10^{-8} M. (5C) Analysis of HIOMT activity. In all experiments One-way ANOVA, Bonferroni's Multiple Comparisons Test was applied; values were expressed in pmol/gl/h, and plotted as means±SEM.

Since it was determined that the physiological dose of 10^{-8}M of insulin show the best potentiating effect and that, in the culture condition used, 10^{-6}M of norepinephrine was a concentration that determines a maximal response on melatonin production, the next step was to study the effects of 10^{-8}M of insulin in an entire range of norepinephrine concentrations (10^{-9}M to 10^{-6}M). The concentration × effect curves (Fig. 3) show that the effect of insulin is best seen on the higher range of norepinephrine concentrations and well characterized by the analysis of the maximal response of the fitted sigmoidal curves (9.41 ± 0.63 vs. 13.20 ± 0.80ng/gland; $P = 0.002$). On the other hand, it seems that the sensitivity of the glands to noradrenaline is not changed since there is no statistical difference between the EC_{50}s (6.9×10^{-8} vs. 5.5×10^{-8}M; $P > 0.05$).

Experiment 2 — norepinephrine-synchronized pineal gland culture

In a second experiment the potentiating effect of insulin was studied now in a culture condition more similar to the natural exposition cycle of the pineal glands to norepinephrine stimulation (Drijfhout et al., 1996). First of all, Fig. 4 shows that the proper effect of norepinephrine on melatonin synthesis is higher when the glands were kept under a circadian pattern of stimulation with this neurotransmitter. Moreover, in this kind of in vitro culture the potentiating effect of insulin (10^{-8}M) was again seen and is approximately of the same magnitude of that obtained in the standard type of pineal gland culture.

Enzymatic assays and quantitative RT-PCR

The addition of insulin, concomitantly to norepinephrine, increased ($P < 0.001$) TPOH activity by 40% only in the acute stimulation condition. The noradrenergic stimulation only evoked an increase on the TPOH activity in the norepinephrine-synchronized group, although a trend to an augmentation was observed in the NE standard stimulation group (Fig. 5A). In the norepinephrine-synchronized culture there was no additional effect of insulin on the TPOH activity, without any significant and correlated increase in gene expression of both standard and synchronized conditions (Fig. 6A).

The AANAT activity (Fig. 5B), as expected, increased in the presence of norepinephrine (10^{-6} M) in the standard culture condition. This increase of activity is associated to the expected stimulation of AANAT mRNA expression (Fig. 6B). In the standard experimental condition, the addition of insulin did not change the effects due to norepinephrine alone. However, it should be stressed that the increase of AANAT activity stimulated by norepinephrine was almost 100% bigger in the norepinephrine-synchronized culture condition without any additional effect on the AANAT gene expression. Moreover, in this experimental condition the addition of insulin promoted an additional increase of AANAT activity without affecting its gene expression.

As far as the activity of HIOMT is concerned (Fig. 5C), no differences were observed in gene expression (Fig. 6C). The

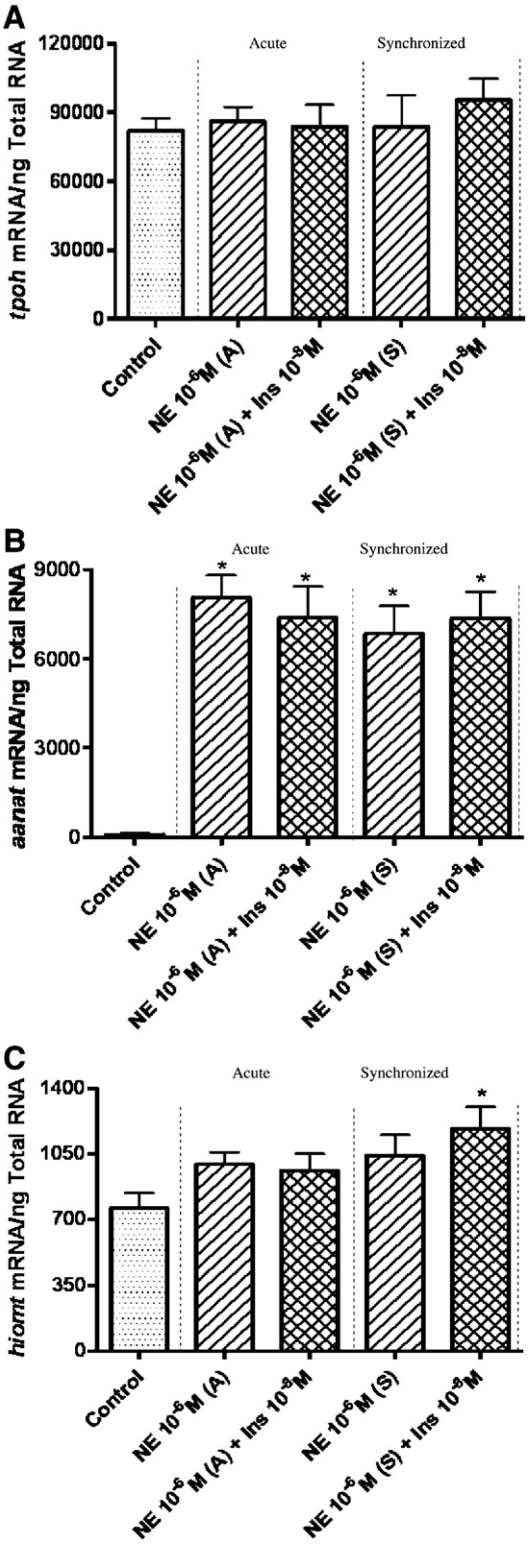

Fig. 6. Pineal gland gene expression analysis, acute (A) and synchronized (S) protocol stimulation. (6A) Gene expression of *tpoh*. (6B) Gene expression of *aanat*, *$P < 0.001$ vs Control. (6C) Gene expression of *hiomt*, *$P < 0.05$ vs Control. In all experiments One-way ANOVA, Bonferroni's Multiple Comparisons Test was applied; values were expressed in mRNA/ng Total RNA, plotted as means ± SEM.

addition of insulin did not change this picture in any stimulated groups.

Discussion

The purpose of the present study was to investigate whether insulin influences NE-mediated melatonin synthesis in cultured rat pineal gland. We report that insulin potentiates NE-mediated melatonin synthesis, an effect that is likely due to alterations in TPOH and AANAT activities, through post-transcriptional mechanisms. These are the first studies showing a role for insulin in the regulation of melatonin synthesis.

Initially we found, in the standard pineal gland culture condition, that the optimum insulin concentration that is able to potentiate the norepinephrine-mediated melatonin synthesis was 10^{-8} M. This physiological concentration that is well known to act on insulin receptors, avoids potential cross-activation of IGF receptors, which both type I and II have been characterized in pineal glands (De Keyser et al., 1994; Smith et al., 1988).

In an attempt to mimic the LD cycle stimulatory conditions in vitro, isolated pineal glands were synchronized through repetitive cycles of 12 h of NE stimulation and 12 h absence of norepinephrine. The results showed that in this synchronized condition, norepinephrine is much more effective in stimulating melatonin synthesis in consequence of an additional increase of the activity of AANAT enzyme. Moreover, in this experimental condition, insulin maintained its ability to potentiate NE-mediated melatonin synthesis on the top of the already high level of norepinephrine-stimulated production.

Trying to identify the potential mechanism(s) by which insulin potentiated NE-mediated melatonin synthesis under acute stimulations, we measured both the activity and gene expression of key enzymes involved in this process (i.e. TPOH, AANAT and HIOMT). Data obtained suggests that the increase of TPOH activity by insulin may play a role in the potentiation of NE-mediated melatonin synthesis in the standard pineal gland culture condition.

Extensively studies have shown that TPOH activation can occur through CaMK — and/or PKC (Ribelayga et al., 1999), which also can be induced by insulin cascade signaling (Saltiel and Kahn, 2001), PKA-dependent (Ehret et al., 1991; Johansen et al., 1995), as well as tyrosine phosphorilation. In the later case, Angiotensin II has been shown to activate TPOH in a tyrosine kinase dependent manner (Baltatu et al., 2002; Doan et al., 2001). TPOH has also been shown to be regulated at a transcriptional level, an effect mediated by the MAP kinase pathway (Wood and Russo, 2001). However, consistent with activation of TPOH activity through post-transcriptional mechanisms, we found that insulin is without effect on gene expression.

We also investigated the potential mechanisms by which insulin influences melatonin synthesis in NE-synchronized pineal glands. In this condition, the effect of insulin was associated with augmentation of AANAT activity, as opposed to TPOH. This effect of insulin on AANAT activity again appears to be mediated by post-transcriptional events. Previous studies have shown that AANAT is stabilized through interaction with 14-3-3 protein, when in a phosphorilated state (Klein et al., 2002). Recently study has shown that 14-3-3 protein interacts with Akt substrate in an insulin-stimulated manner (Ramm et al., 2006). However, it remains to be tested whether insulin-stimulation of 14-3-3/AANAT interaction is the phenomenon responsible for increased AANAT activity observed.

Conclusions

The present study showed for the first time that insulin potentiates NE-mediated melatonin synthesis in cultured rat pineal gland, potentially through increased TPOH and/or AANAT activity. These effects appear to occur through post-transcriptional mechanisms.

Acknowledgements

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