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Norepinephrine activates NF-κB transcription factor in cultured rat pineal gland

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ABSTRACT

Aims: The circadian rhythm in mammalian pineal melatonin secretion is modulated by norepinephrine (NE) released at night. NE interaction with β_1-adrenoceptors activates PKA that phosphorylates the transcription factor CREB, leading to the transcription and translation of the arylalkylamine-*N*-acetyltransferase (AANAT) enzyme. Several studies have reported the interplay between CREB and the nuclear factor-κB (NF-κB) and a circadian rhythm for this transcription factor was recently described in the rat pineal gland. In this work we studied a direct effect of NE on NF-κB activation and the role played by this factor on melatonin synthesis and *Aanat* transcription and activity.

Main methods: Cultured rat pineal glands were incubated in the presence of two different NF-κB inhibitors, pyrrolidine-dithiocarbamate or sodium salicylate, and stimulated with NE. Melatonin content was quantified by HPLC with electrochemical detection. AANAT activity was measured by a radiometric assay and the expression of *Aanat* mRNA was analyzed by real-time PCR. Gel shift assay was performed to study the NF-κB activation in cultured rat pineal glands stimulated by NE.

Key findings: Our results showed that the p50/p50 homodimer of NF-κB is activated by NE and that it has a role in melatonin synthesis, acting on *Aanat* transcription and activity.

Significance: Here we present evidence that NF-κB is an important transcription factor that acts, directly or indirectly, on *Aanat* transcription and activity leading to a modulation of melatonin synthesis. NE plays a role in the translocation of NF-κB p50/p50 homodimer to the nucleus of pinealocytes, thus probably influencing the nocturnal pineal melatonin synthesis.

Introduction

Melatonin synthesis by the vertebrate pineal gland is a phenomenon that transduces the environmental light–dark cycle to a circadian rhythm of hormone production. In mammals, through the rhythmic release of norepinephrine in the pineal interstices, the internal biological clock controls the nightly hormone synthesis and secretion. The enzyme arylalkylamine *N*-acetyltransferase (AANAT) is a key regulator in this process, and its activity is elevated during the night by transcription, translation or post-translational mechanisms (Afeche et al., 2008; Simonneaux and Ribelayga, 2003).

In the rat mammalian pineal gland many genes besides *Aanat* vary in expression between day and night (Bailey et al., 2009; Klein et al., 2010). Among them, transcription factors may directly or indirectly influence the rhythmic melatonin synthesis. CREB (cAMP responsive element binding protein) is known as an important one as it initiates *Aanat* expression and consequently, melatonin synthesis (Maronde et al., 1999; Roseboom and Klein, 1995). CREB is phosphorylated (pCREB) by protein kinase A (PKA), which is activated by a β and α-adrenergic stimulation/cAMP elevation-dependent process. ICER (inducible cAMP early repressor) is another transcription factor that is stimulated by pCREB and its accumulation at the end of the night has a negative regulatory role on *Aanat* transcription (Maronde et al., 1999). In the same way, DREAM, NeuroD1 and Fra2 transcription factors interfere with melatonin synthesis influencing *Aanat* enzyme expression (Link et al., 2004). Recently, the presence of the nuclear factor κB (NF-κB) was demonstrated in the rat pineal gland (Cecons et al., 2010).

The transcription factor NF-κB can either induce or repress gene expression by its binding to DNA sequences known as κB elements (Kaltschmidt et al., 2005). Many publications have reported the NF-κB modulation on CREB activity in various cell types (F. Delfino and W.H. Walker, 1999; F.J. Delfino and W.H. Walker, 1999; Kaltschmidt et al., 2006; Yalcin et al., 2003; Zou and Crews, 2006). NF-κB is formed as homo or heterodimers of the five NF-κB family members: Rel A (p65),

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Rel B, c-Rel, p50/p105 (NF-κB1) and p52/p100 (NF-κB2) (Beinke and Ley, 2004; Massa et al., 2006). NF-κB inhibitors (IκBs) retain NF-κB complexes in the cytoplasm and, when activated, NF-κB is translocated freely to the nucleus (Hacker and Karin, 2006; Perkins, 2007).

NF-κB was described in the rat pineal gland as a transcription factor involved in inflammatory responses and showing a circadian rhythm pattern (Cecon et al., 2010; Ferreira et al., 2005). The specific role played by norepinephrine in its circadian rhythmicity is not clear since those studies were performed in vivo and reflected a condition in which several neurotransmitters and neuromodulators could be involved. Moreover, it was shown that the NF-κB rhythm does not persist in free-running conditions and that may imply that the rhythm is regulated by factors other than the central clock.

In order to investigate a direct effect of norepinephrine on NF-κB activation we studied pineal glands in culture under noradrenergic stimulation. The role played by NF-κB on melatonin synthesis and AANAT transcription and activity was also evaluated. Here we present evidences that norepinephrine activates the NF-κB p50/p50 homodimer transcription factor and that this factor modulates melatonin synthesis through the activation of AANAT mRNA expression and activity.

Materials and methods

Animals

Male Wistar rats (200–220 g) were kept under a 12:12 h light–dark cycle (lights on at 06:00 h) in a temperature controlled room (21 ± 2 °C) with water and food ad libitum. The animals were killed by decapitation between 09:00 and 10:00 h. All experiments were performed in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA) and approved by the Committee of Ethics in Animal Experimentation of the Institute of Biomedical Sciences (CEUA — Permit number: 091), University of São Paulo, São Paulo, Brazil.

Pineal gland culture

Pineal glands were cultured as previously described (Afeche et al., 2006). Briefly, pineal glands were maintained at 37 °C, in an atmosphere of 95% O_2 and 5% CO_2 in BGJb medium (Fitton-Jackson Modification, Gibco, Grand Island, NY, USA) with phenol red, bovine serum albumin (1 mg/mL), 2 mM glutamine, 0.1 mg/mL ascorbic acid and penicillin (100 U/mL)–streptomycin (100 μg/mL), in 24-well plates (2 glands/well; 200 μL/well) for 48 h (the medium was changed after the first 24 h). After this period, all glands were placed in fresh medium for 1 h before the pre-incubation with two different NF-κB inhibitors: pyrrolidine-dithiocarbamate (PDTC 300 μM) or sodium salicylate (SS 1 mM) for 30 min. Then, NE (1 μM) was added for additional 30 min [recommended time to NF-κB activation assessment (Glezer et al., 2003)] or 5 h (to the other assays). After that, the glands were frozen on dry ice and kept at −80 °C prior to subsequent analysis. All culture media components and drugs were purchased from Sigma (St. Louis, MO, USA).

Isolated pinealocyte culture

Pinealocytes were obtained by papain digestion (Papain Dissociation System, Worthington Biochemical Corporation, Freehold, NJ, USA). The glands were excised and immediately placed in ice-cold Dulbecco's Modified Eagle's Medium (DMEM) (glucose: 1000 mg/L, HEPES: 5.9 g, sodium bicarbonate: 3.7 g) (Sigma, St. Louis, MO, USA). The glands were incubated at 37 °C for 45 min in papain (0.01%) and DNase (0.01%) solution. After removal of papain and its blockage by ovomucoid (2 mg/mL), the cells were mechanically dispersed and suspended in DMEM supplemented with 10% fetal calf serum (FCS) and 1% penicillin–streptomycin. Cells in culture medium were cultivated in 75 cm^2 culture flasks, at 37 °C, in 5% CO_2/95% air, for 16 h. After this period, the cells in suspension were predominantly pinealocytes, which were separated after removal of all the culture medium content. The cells were centrifuged and suspended in DMEM at the concentration of 2×10^5 cells/mL, transferred to cell culture flasks and kept at 37 °C in 5% CO_2/95% air for 1 h prior to the pharmacological treatments. Pinealocytes were stimulated in the presence or absence of NE (1 μM) for 30 min. Then, the cells were centrifuged (300 *g*, 5 min, 20 °C) and the pellet was suspended according to the electrophoretic mobility shift assay protocol.

Isolated astrocyte culture

After removal of the pinealocytes in suspension, the astrocytes remained attached to the culture flasks and were kept in culture for one week. After this period, the cells were washed twice with Hanks solution and 6 mL of trypsin 0.25% (Cultilab, Campinas, SP, Brazil) was added for 5 min. Trypsin action was blocked with DMEM supplemented with 10% FCS and the cells were centrifuged at 300 *g* for 5 min at 20 °C. The pellet was suspended in DMEM, transferred to culture flasks and kept at 37 °C in 5% CO_2/95% air for 1 h prior to the pharmacological treatments. Treated astrocytes were stimulated with NE (1 μM) for 30 min and control astrocytes remained in plain medium for the same time. Then, the cells were centrifuged (300 *g*, 5 min, 20 °C) and the pellet was suspended according to the electrophoretic mobility shift assay protocol.

Melatonin determination

Pineal gland melatonin concentration was 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 (5 μm, 150 × 3.9 mm). The chromatographic system was isocratically operated with the following mobile phase: 0.1 M sodium acetate, 0.1 M citric acid, 0.15 mM EDTA, 30% methanol, pH 3.7, at a flow rate of 1 mL/min. The electrochemical detector potential was adjusted to +900 mV. The elution time for melatonin was about 6 min. Each gland was sonicated (Microson XL 2005, Heat System Inc., Farmingdale, NY, USA) in a solution of 0.1 M perchloric acid, containing 0.02% EDTA and 0.02% sodium bisulfate. After centrifugation (2 min, 13,000 *g*), 40 μL of the supernatant was injected into the chromatographic system (Injector Mod. 7125, 20 μL loop, Rheodyne Inc., San Francisco, CA, USA). All the reagents were obtained from Merck (Frankfurter, Darmstadt, Germany).

AANAT activity determination

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

Real-time quantitative PCR

Total RNA was extracted from pineal glands using guanidine isothiocyanate based Trizol reagent (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's specifications. Superscript III reverse transcriptase (RT) (Invitrogen Corporation, Carlsbad, CA, USA) was used to reverse transcribes 5 μg of total RNA. AANAT gene