Melatonin: an endogenous negative modulator of 12-lipoxygenation in the rat pineal gland

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Major biochemical activities of the pineal gland include melatonin biosynthesis and 12-lipoxygenation. In this paper, we provide evidence *in vivo* that melatonin regulates 12-lipoxygenation via 12-lipoxygenase (LOX) expression. The relationship between these two biochemical activities was established by monitoring levels of endogenous melatonin and a 12-LOX metabolite, 12-hydroxyeicosatetraenoic acid (12-HETE), in the rat pineal gland both during the light–dark cycle and after isoproterenol injection using GC/MS with negative ion chemical ionization. As pineal melatonin production reflected a typical diurnal variation, 12-HETE levels showed an off-phase diurnal pattern in relation to melatonin levels. Intravenous administration of isoproterenol, which has been shown to elevate melatonin production, decreased the 12-HETE level significantly. The reduction of 12-HETE levels during the dark phase and after isoproterenol injection was accompanied by decreases in 12-LOX mRNA and protein levels. Direct administration of melatonin to rats by intravenous injection decreased pineal 12-

LOX protein levels significantly, indicating that melatonin plays a role in down-regulating 12-LOX expression. When pineal glands were incubated with exogenous melatonin in culture, time-dependent reduction of 12-LOX protein levels was observed. The melatonin-induced reduction in 12-LOX protein was abolished in the presence of the melatonin receptor antagonist luzindole, establishing further the role of melatonin in this process. Incubation of pineal homogenates with exogenous melatonin partially inhibited 12-LOX activity. Taken together, an inverse relationship exists in the endogenous production of 12-HETE, 12-LOX mRNA and protein with respect to melatonin production in the rat pineal gland. Melatonin decreased both 12-LOX mRNA and protein levels in addition to 12-LOX enzyme activity, indicating that melatonin is an endogenous modulator of pineal 12-lipoxygenation.

Key words: arachidonic acid, 12-hydroxyeicosatetraenoic acid (12-HETE), 12-lipoxygenase.

INTRODUCTION

The mammalian pineal gland is a prominent secretory organ that synthesizes and releases a number of endocrine substances [1,2]. Melatonin, a key member of this group of substances, is synthesized in the pineal gland from tryptophan, and secreted for circulation. As an indoleamine hormone, melatonin exerts its physiological effects on various targets by interacting with an array of specific melatonin receptors [3,4]. Increasing evidence suggests that melatonin plays a key role in seasonal control of reproduction, body mass and metabolism [1,2]. Melatonin has also been described as an immunomodulatory mediator via specific binding to human and rodent lymphoid cells [5], and a role as an antioxidant in protecting against cell and tissue damage from active free radicals has been suggested [6,7]. In addition, melatonin has been used as a pharmaceutical agent for treating sleep-related disorders [8].

In addition to the production of melatonin, the mammalian pineal gland is capable of converting polyunsaturated fatty acids into bioactive lipid mediators. Previous reports have demonstrated that the rat pineal gland expresses both 12- and 15-lipoxygenase (LOX) activities, producing 12- and 15-hydroxyeicosatetraenoic acid (12- and 15-HETE) from arachidonic acid (AA), and 14- and 17-hydroxydocosahexaenoic acid (14- and 17-HdOHE) from docosahexaenoic acid (DHA) respectively [9]. 12-Lipoxygenation is the predominant LOX activity observed in the rat pineal gland. The rat pineal gland has also been shown to

produce hepoxilins via LOX pathways [10]. Lipoxygenation in the pineal gland is unique because no other brain regions express significant LOX activities under normal physiological conditions [11].

A role for melatonin in AA metabolism has been suggested recently. It has been shown that melatonin interacts with RZR/ROR nuclear receptors, leading to the repression of 5-LOX gene expression in human B lymphocytes [12]. The down-regulation of 5-LOX gene expression by melatonin was demonstrated further in melatonin-deficient pinealectomized rats, where increased levels of hippocampal 5-LOX mRNA were observed [13]. More recently, a decreased 5-LOX mRNA content has also been observed in the rat pineal gland during the night-time, when melatonin levels are high [14], although 5-LOX activity is absent in this particular gland [9,15]. Since 12-LOX is the major LOX in the rat pineal gland, these results suggest a putative role for 12-LOX regulation by melatonin. The present study was aimed at investigating whether such an effect of melatonin exists in the rat pineal gland. The correlation *in vivo* between melatonin biosynthesis and pineal 12-lipoxygenation was first examined by determining the endogenous levels of melatonin, 12-LOX metabolites and 12-LOX mRNA/protein levels during the light–dark cycle and after intravenous administration of a β -adrenergic receptor agonist, isoproterenol. In addition, the direct effect of melatonin on 12-LOX expression was investigated via intravenous melatonin administration. Furthermore, pineal 12-lipoxygenation, as well as 12-LOX expression, were evaluated *in*

Abbreviations used: AA, arachidonic acid; DHA, docosahexaenoic acid; GC/MS-NCI, GC/MS with negative ion chemical ionization; HdOHE, hydroxydocosahexaenoic acid; HETE, hydroxyeicosatetraenoic acid; LOX, lipoxygenase; SIM, selected ion monitoring.

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vitro using pineal homogenates and cultured pineal glands respectively.

EXPERIMENTAL

Chemicals

Authentic HETEs and ^2H -labelled standards, [5,6,8,9,11,12,14,15- ^2H]HETEs (d_8 -HETEs), were purchased from Biomol Research Laboratory (Plymouth Meeting, PA, U.S.A.). Melatonin was obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.) and d_4 -melatonin [*N*-acetyl-5-methoxy(a,a,b,b- d_4)tryptamine] was generously given by Dr. Sandy P. Markey (National Institute of Mental Health, NIH, Bethesda, MD, U.S.A.). [^3H]AA (55 mCi/mmol) was purchased from NEN Research Products (Boston, MA, U.S.A.). Luzindole was obtained from Torcris (Ballwin, MO, U.S.A.).

Animals and experimental designs

Male Sprague–Dawley rats were used throughout this study according to NIH protocol #LMBB-OC-07. Typically, rats (200 ± 10 g body mass) were housed in pairs and conditioned for 4–10 days upon arrival with a controlled light cycle (light from 07:00h to 19:00h) and free access to a rat chow diet (NIH-07) and water. The animals were then divided into groups for subsequent treatments.

For studies performed during the regular light–dark cycle, a group of six animals was killed and the pineal glands were collected at each specified time point. Day (or light) and night (or dark) studies were carried out between 10:00h and 12:00h (day) and from 00:00–02:00h (night) respectively, according to diurnal variations in the melatonin production (see Figure 2).

Isoproterenol (Sigma), a potent β -adrenergic receptor agonist, was used to stimulate melatonin production during the daytime according to the procedures reported previously [16,17]. A total of 16 rats were divided into three groups: pre-injection controls (four animals), vehicle [0.9% (w/v) NaCl] controls (six animals) and the isoproterenol injection group (six animals). Isoproterenol was dissolved in 0.9% (w/v) NaCl solution (5 mg/ml) and injected into the tail vein (5 mg/kg). The injection was performed between 10:00 and 12:00h, since the stimulation of melatonin production by isoproterenol would be more pronounced during the daytime. Rat restrainers (Lab Products, Inc., Seaford, DE, U.S.A.) were used during the injection. After 3 or 6 h, rats were killed by decapitation and the pineal glands were collected. Similarly, melatonin (0.5 mg/kg) was dissolved in saline solution containing 2% (v/v) DMSO (5 mg/ml) and injected into the tail vein. The injection was performed between 10:00h and 12:00h using six animals for each group. After 1 h, rats were killed and pineal glands were collected.

Extraction of pineal substances

Rat pineal glands were collected immediately after the animals were decapitated and placed in 1 ml of cold methanol containing 50 $\mu\text{g}/\text{ml}$ butylated hydroxytoluene on dry ice or stored at -80°C for later analysis. The whole collection procedure took less than 40 s for each pineal gland, and this rapid process has previously been shown to yield consistent results [18]. Each pineal gland was homogenized in butylated hydroxytoluene/methanol, and pineal lipids, LOX metabolites and melatonin were then extracted by the method of Bligh and Dyer [19]. Internal standards for chemical analyses, such as tricosanoic acid (23:0) and ^2H -labelled standards (d_8 -12-HETE, d_8 -15-HETE and d_4 -melatonin), were added before homogenization.

Melatonin analysis

Aliquots of pineal extracts (typically one-fifth or one-third of the total extract of one pineal gland) were dried down under nitrogen, and derivatives of melatonin were formed with pentafluoropropionic anhydride in ethyl acetate [18,20]. The reaction mixture was then dried down with nitrogen and partitioned into 0.5 ml of acetonitrile and 1 ml of hexane by vigorous vortex-mixing [21]. After the removal of the hexane layer, the acetonitrile layer was dried, redissolved in 6 μl of ethyl acetate, and 2–4 μl was then analysed by GC/MS. Quantitative determination of melatonin was performed using the selected ion monitoring (SIM) mode, focusing on the ions at m/z 320 (melatonin) and m/z 323 (d_4 -melatonin) [18,21].

Analysis of pineal LOX metabolites

LOX metabolites were separated from bulk pineal lipids by solid-phase extraction using C_{18} SPE columns, as described by Powell [22]. Derivatives of pentafluorobenzyl esters were then formed from these partially purified metabolites [9,23]. Following platinum oxide-catalysed hydrogenation [23], the hydroxy groups of these hydrogenated pentafluorobenzyl esters were converted into trimethylsilyl ethers [9,18]. Finally, the fully derivatized LOX metabolites were analysed by GC/MS with negative ion chemical ionization (GC/MS-NCI) with an HP 5989 GC/MS spectrometer. The spectrometer was operated in the SIM mode to record ions of m/z 399 (HETEs) and m/z 407 (d_8 -HETEs) [9,18].

Lipoxygenation *in vitro*

To investigate if melatonin has a direct effect on lipoxygenation in pineal homogenates, fresh rat pineal glands were collected during the daytime (10:00–11:00h) and homogenized in 50 mM Tris/HCl buffer (pH 7.4). Aliquots of pineal homogenates (200 μl) were incubated with 1 μCi of [^3H]AA (55 mCi/mmol) in the presence of melatonin ranging from 0 (control) to 10 μM . After 20 min, incubations were terminated by the addition of 100 μl of 15% (v/v) formic acid, and LOX metabolites were extracted with dichloromethane as described previously [23]. LOX metabolites derived from [^3H]AA were analysed using reversed-phase HPLC coupled with a radioactivity detector (Flow One Beta; Radiomatic, Tampa, FL, U.S.A.). The effects of isoproterenol and cAMP on 12-lipoxygenation were also determined using pineal homogenates.

12-LOX mRNA determination via reverse transcriptase (RT)-PCR

Total rat pineal RNA was isolated from each single gland using TriZOL reagent (Life Technologies, Gaithersburg, MD, U.S.A.) according to the manufacturer's protocol and quantified spectrophotometrically. Isolated RNA (1 μg) was used for first-strand cDNA synthesis after treatment with DNase I to remove any contaminating DNA. Briefly, the treated RNA was incubated with 0.5 μg of oligo(dT) (12–18) primer for 10 min at 70°C and then reverse-transcribed by using 200 units of Superscript II RT (Muloney Murine Reverse Transcriptase; Life Technologies) in 20 μl of reaction buffer containing 2 μl of $2 \times$ PCR buffer, 25 mM MgCl_2 , 1 μl of 10 mM dNTP mixture and 2 μl of 0.1 M dithiothreitol. The mixture was placed in a Perkin–Elmer 2400 Gene Amp PCR system, set at 42°C for a 50 min cycle followed by a 15 min incubation at 70°C and a 4°C soak. After the reaction, the mixture was centrifuged for 10 s at 8160 g (10000 rev./min), and RNA was digested by incubation with 1 μl of RNase H (2 units) for 20 min at 37°C .

For 12-LOX mRNA synthesis, 1–2 μl of template cDNA was

placed in a PCR reaction buffer (PCR master mix; Boehringer Mannheim, Indianapolis, IN, U.S.A.). In a total of 25 μ l of 2 $\times$ PCR buffer containing 10 mM Tris/HCl, 50 mM KCl, 1.5 mM MgCl₂ and 1.25 units of *Taq* polymerase in Brij35 (0.005 %, v/v), 0.2 mM each of dATP, dCTP, dGTP and dTTP were added along with 1 μ l (40 pmol) each of an upstream primer 5'-TGG GGC AAC TGG AAG GAT-3' (an 18-mer) and a downstream primer 5'-AGC AAA GAG CTC AGC CAT CAA-3' (a 21-mer) as described by Hada et al. [24]. These primers were obtained from Lofstrand Lab, Ltd. (Gaithersburg, MD, U.S.A.). PCR was then performed using 35 cycles programmed as follows: initial denaturation for 2 min at 94 °C and 15 s at 94 °C, annealing for 30 s at 55 °C, and primer extension for 1 min at 72 °C. Each of the glyceraldehyde-3-phosphate dehydrogenase primers (1 μ l, or 20 pmol) (Clontech, Palo Alto, CA, U.S.A.) was used as a positive control, providing a 0.45 kb band. 12-LOX mRNA bands were made visible by using ethidium bromide (Sigma) staining after 8 μ l of the PCR product was electrophoresed with a 2 % (w/v) agarose gel using 1 $\times$ TBE (45 mM Tris/borate/1 mM EDTA) buffer.

Western blot analysis of 12-LOX protein

To determine 12-LOX protein levels under various conditions, each rat pineal gland was collected in a sterilized 1 ml Eppendorf centrifuge tube on dry ice. The pineal gland was homogenized in 100 μ l of RIPA buffer (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), and the supernatant was collected after a 20 min centrifugation at 16000 *g* (14000 rev./min). Protein assays were performed using the bicinchoninic acid (BCA) reaction [25] with the BCA Protein Assay Kit from Pierce (Rockford, IL, U.S.A.). Pineal proteins (15 μ g, or as indicated otherwise) were electrophoresed on 12 % polyacrylamide gels. The proteins were transferred by electroblotting on to Immobilon-P membranes (Millipore, Bedford, MA, U.S.A.), and blocked with 5 % (w/v) non-fat dried milk in TBS buffer (pH 7.4) containing 0.1 % (v/v) Tween 20 (T-TBS buffer). The membranes were incubated with 12-LOX-specific antibody (Cayman Chemicals, Ann Arbor, MI, U.S.A.), followed by incubation with horseradish-peroxidase-conjugated anti-rabbit antibody (Sigma). The membranes were washed with T-TBS buffer, and subsequently subjected to enhanced chemiluminescence detection (Amersham, Arlington Heights, IL, U.S.A.). The protein bands were quantified by densitometry, and the statistical analysis was performed by using Student's *t* test.

Pineal gland culture

To examine whether melatonin affects directly 12-LOX protein levels, rat pineal glands were cultured according to methods described previously [26,27] with slight modifications. Each pineal gland (collected between 11:00–16:00h) was placed in a well of a 24-well culture dish and incubated with 100 μ l of BGJb medium (Gibco, Grand Island, NY, U.S.A.) at 37 °C under an air/CO₂ (19:1) atmosphere. The culture medium was supplemented with L-glutamine (0.2 mM), ascorbic acid (0.1 mg/ml), penicillin (100 units/ml) and streptomycin (100 μ g/ml). The pineal glands were conditioned for 48 h, with the medium changed after every 24 h. After the preincubation period, the glands were treated with melatonin and collected at the specified time points. Independently, the pineal glands were incubated with 100 nM luzindole (Torcris, Ballwin, MO, U.S.A.) for 1 h, and then treated with melatonin for an additional hour. Luzindole was initially dissolved in DMSO and diluted so that the final concentration of DMSO was 0.1 %. Pineal protein isolation and Western blot analysis were performed as described above.

RESULTS

Determination of LOX metabolites and melatonin levels

The major endogenous LOX metabolite detected was 12-HETE derived from AA. Using the GC/MS-NCI technique, along with d₈-12-HETE as an internal standard, it was possible to determine 12-HETE in the rat pineal gland using a portion of the lipid extract from each pineal gland (Figure 1A). Among several techniques that can be used to quantify melatonin levels, the GC/MS-NCI technique was chosen in this study because of its excellent sensitivity and specificity [21,28]. In the SIM mode, and using d₄-melatonin as the internal standard, it was possible to detect endogenous melatonin using only a portion of the lipid extract from each pineal gland (Figure 1B). This approach thus allowed the determination of both 12-HETE and melatonin levels from each single pineal gland. Metabolites from DHA, 14- and 17-HdoHEs (< 20 pg/pineal), as well as 15-HETE (< 100 pg/pineal), were also produced, but at such a low level so as not to allow consistent quantitative analyses to be made from each pineal gland. Therefore only endogenous 12-HETE was determined as an indicator of pineal lipoyxygenation.

It was observed that the endogenous levels of LOX metabolites, as well as melatonin, varied slightly from one group of animals to another, depending on factors such as age, body mass and the time of killing. However, these differences did not interfere with the overall trends observed throughout this study.

Diurnal variations in the levels of melatonin and LOX metabolites

Melatonin levels in the rat pineal gland showed a typical diurnal variation during the regular light–dark cycle (light from 07:00–19:00h) (Figure 2A). Daytime melatonin levels were 72–85 pg/pineal and did not change significantly from 10:00–17:00h. At 20:00h, only 1 h after the dark cycle had started, the melatonin level was twice that found at 17:00h, and reached a maximum between 23:00h and 02:00h the following day (1132–1232 pg/pineal). Melatonin levels had decreased significantly by 05:00h on the second day (166 pg/pineal), returning to basal levels by 10:00h.

Endogenous 12-HETE levels showed a nocturnal reduction in relation to melatonin levels (Figure 2B). The pineal 12-HETE levels were higher during the light period (260 pg/pineal at 10:00h) compared with those during the dark phase (203 pg/pineal at 02:00h). The diurnal variation in 12-HETE level correlated ($r = -0.87$, $P < 0.001$) with that of melatonin in an off-phase fashion.

Effect of isoproterenol on endogenous melatonin and LOX metabolites

It is well established that stimulation of the β -adrenergic receptor increases melatonin production in the pineal gland [16,17]. To examine whether such an increase affects pineal lipoyxygenation, rats were injected with isoproterenol intravenously and the levels of melatonin and LOX metabolites were determined 3 h after the injection. As shown in Figure 3, isoproterenol elevated melatonin levels by more than 14-fold over the basal level. On the other hand, endogenous 12-HETE levels were reduced by more than 20 % (Figure 3). This reduction in endogenous 12-HETE levels after isoproterenol injection was negatively correlated with melatonin levels ($r = -0.91$, $P < 0.001$), which provided a strong agreement with the off-phase correlation observed during the light–dark cycle (Figure 2).

Figure 1 Detection of endogenous HETEs (A) and melatonin (B) in the rat pineal gland

(A) A typical ion chromatogram of HETEs was obtained from one-third of the total extract from one single pineal gland. The $(M-181)^{-}$ ions of the derivatized HETEs and added internal standards, d₈-12-HETE and d₈-15-HETE, were monitored at m/z 399 and 407 respectively. (B) A typical ion chromatogram of melatonin was obtained from one-fourth of the total extract from one single pineal gland. The $(M-2HF)^{-}$ and $(M-HD-HF)^{-}$ ions of the derivatized melatonin and d₈-melatonin were recorded at m/z 320 and 323 respectively.

Effect of melatonin on *in vitro* lipoygenation

To determine if melatonin had any effect on 12-lipoxygenation by inhibiting LOX activity, pineal homogenates were incubated with [³H]AA in the presence of exogenous melatonin. Exogenous melatonin levels up to 20 nM did not have any noticeable effect on LOX activity in the pineal homogenate. At 200 nM, melatonin led to a slight inhibition ($12.3 \pm 3.8\%$ reduction, $P < 0.05$) of the conversion of [³H]AA into [³H]12-HETE. Further inhibition ($21.3 \pm 7.1\%$, $P < 0.01$) of 12-lipoxygenation by melatonin was achieved at 10 μ M. Since injection of isoproterenol has been shown to elevate pineal cAMP levels [16], the direct effects of cAMP and isoproterenol on pineal lipoygenation were also

examined. Neither cAMP nor isoproterenol affected the conversion of [³H]AA into [³H]12-HETE in the pineal homogenates (results not shown).

High melatonin levels are associated with decreased 12-LOX expression *in vivo*

To examine if 12-lipoxygenation in the rat pineal gland is regulated by gene expression, 12-LOX mRNA and protein levels were determined during the light (10:00–12:00h) and dark (00:00–02:00h) phases (Figure 4A), and after isoproterenol administration (Figure 4B). When melatonin levels were high during the dark phase and after isoproterenol injection, 12-LOX

Figure 2 Diurnal variations of endogenous pineal melatonin (A) and 12-HETE (B) levels

Data are expressed as means $\pm$ S.D. values from six different animals for each time point. Statistically significant differences were found among values labelled with different letters ($P < 0.05$, Bonferroni–Dunn *post-hoc* analysis).

Figure 3 Effect of isoproterenol on endogenous levels of melatonin and 12-HETE in the rat pineal gland

Isoproterenol (5 mg/kg body mass) was injected into the tail vein of the rats, and pineal glands were processed 3 h after the injection. Data are expressed as means $\pm$ S.D. values. ***, $P < 0.001$ versus control values (unpaired Student's *t* test).

mRNA levels were significantly decreased. Similarly, 12-LOX protein levels were also reduced under these conditions. The reduction in 12-LOX protein was $22 \pm 1\%$ ($P < 0.01$) during the dark phase, as determined by densitometry measurements. Time-

A. Effect of Light and Dark Cycle on 12-LOX Expression

B. Effect of Isoproterenol Injection on 12-LOX Expression

Figure 4 Effect of light and dark cycles (A) and isoproterenol injection (B) on 12-LOX expression

12-LOX mRNA and 12-LOX protein levels were determined for the pineal glands collected during the light (10:00–12:00h) and dark (00:00–02:00h) phases (A), and after isoproterenol (Iso) injection (B) (3 or 6 h after the administration at 10:00–12:00h). Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as an internal control for mRNA determination. Each lane of the Western blot analysis represents a separate pineal gland treated individually. The data presented are representative of experiments, each of which was performed using four to six animals per group and repeated three times.

dependent reduction in 12-LOX protein levels was observed after isoproterenol injection. While the plasma melatonin levels rose progressively during the first 6 h, 12-LOX protein levels decreased by $28 \pm 1\%$ ($P < 0.01$) and $44 \pm 5\%$ ($P < 0.001$) at 3 and 6 h after isoproterenol administration respectively. The reduced 12-LOX mRNA/protein levels were consistent with the decrease found in 12-HETE production under the same conditions.

Melatonin attenuates 12-LOX expression

To investigate further whether melatonin directly affects 12-LOX expression, rats were injected with melatonin. As shown in Figure 5, melatonin injection significantly decreased the endogenous level of 12-LOX protein (by $55 \pm 11\%$; $P < 0.01$). This reduction coincided with an increase in serum melatonin levels from 7.5 ± 2.7 to 104.0 ± 23.0 pg/ml ($P < 0.001$) after the injection. Similar results were obtained when rat pineal glands were cultured and treated with exogenous melatonin. As shown

Figure 5 Effect of melatonin injection on endogenous 12-LOX protein levels

Rats were injected intravenously with melatonin (0.5 mg/kg) between 10:00h and 12:00h, and pineal glands were collected 1 h after the injection. Melatonin was dissolved in saline solution containing 2% (v/v) DMSO, and 12-LOX protein levels were determined by Western blotting. Each lane represents a separate pineal gland treated individually. The data presented are representative of experiments that were performed using six animals in each group and repeated twice.

Figure 6 Effect of melatonin (A) and melatonin receptor antagonist (B) on 12-LOX protein levels in the cultured pineal glands

12-LOX protein levels were examined after the pineal glands were incubated with 100 nM exogenous melatonin for 1 or 3 h (A). Separately, the pineal glands were incubated first with 100 nM luzindole for 1 h, and then with 100 nM melatonin for an additional hour (B). Each lane represents a separate pineal gland treated individually. The data presented are representative of experiments that were performed using four pineal glands in each group and repeated three times.

In Figure 6(A), the 12-LOX protein levels decreased by $38 \pm 5\%$ ($P < 0.01$) and $73 \pm 4\%$ ($P < 0.001$) respectively after 1 and 3 h of incubation with melatonin. In the presence of the melatonin receptor antagonist luzindole, the melatonin-induced reduction of 12-LOX protein was abolished (Figure 6B). The 12-LOX level in the pineal glands preincubated with luzindole was maintained at $95 \pm 6\%$ of the control value, while melatonin alone decreased the 12-LOX protein by $30 \pm 2\%$ ($P < 0.01$), suggesting that the effect of melatonin on 12-LOX expression might be mediated through melatonin receptors.

DISCUSSION

Simultaneous analysis of endogenous levels of melatonin and LOX metabolites in the rat pineal gland has previously been a challenge due to difficulties in sample preparation and lack of specific analytical techniques. In the present study, GC/MS-NCI

procedures made it possible to detect both melatonin and LOX metabolites at low picogram levels, allowing determination of these pineal substances from each rat pineal gland under different experimental conditions. In conjunction with the use of molecular biology techniques, we were able to demonstrate for the first time that melatonin regulates 12-lipoxygenation in the rat pineal gland, and that its level correlates negatively *in vivo* with the 12-LOX metabolite (12-HETE), 12-LOX mRNA and 12-LOX protein levels.

Melatonin has been suggested to play a significant role in a variety of physiological processes [1,2,6,8]. As a neurohormone, melatonin has been shown to suppress the expression of a number of genes in various tissues and cells [12,13,29–31]. In particular, melatonin represses 5-LOX gene expression in human B lymphocytes [12]. The involvement of melatonin in 5-LOX gene expression was confirmed further in melatonin-deficient pinealectomized rats, where increased levels of hippocampal 5-LOX mRNA were observed [13]. Recently, reduced 5-LOX mRNA levels were also observed in the rat pineal gland during the dark phase [14]. In the present study, we observed that high melatonin was not only associated with reduced 12-LOX mRNA/protein levels, but also correlated negatively with the endogenous levels of 12-LOX metabolite, 12-HETE (Figures 2B, 3, 4A and 4B). Furthermore, exogenous melatonin administration significantly reduced the 12-LOX protein levels in rat pineal glands *in vivo* and in cultured pineal glands (Figures 5 and 6). These data indicate that the reduction of 12-HETE observed with increased melatonin levels is due to the suppression of 12-LOX gene expression by melatonin in the rat pineal gland, suggesting that melatonin might act as an endogenous modulator. Our present findings, and previous reports by others [12–14], share a common notion that melatonin might be an important negative modulator of LOXs in general. It should be noted, however, that one report has shown that rat pineal glands obtained at 08:00h or 00:00h showed a higher 12-LOX activity, as well as 12-LOX mRNA/protein levels, in comparison with those obtained at 16:00h [32]. This inconsistency might be attributed to the differences in experimental designs. In our study, endogenous 12-HETE and melatonin levels were monitored simultaneously at various time points, and 12-LOX mRNA and protein data were obtained from pineal glands at 2:00h for the dark phase. In addition, 12-LOX protein levels were monitored in conjunction with serum melatonin levels. In contrast, the previous report [32] examined 12-HETE production from exogenous AA (25 μ M) using 10000 g supernatant of a pineal homogenate as an enzyme source. The production *in vitro* of 12-HETE may be altered by the free AA that is present in the pineal preparation. Therefore 12-LOX activity determined by the conversion of exogenous substrate might not represent the endogenous variations of 12-HETE levels. In addition, the difference might also be due to an anaesthetic agent, phenobarbital, which was employed in the previous study [32].

Our result showing that luzindole, a melatonin receptor antagonist, inhibited the melatonin effect on 12-LOX expression suggests the involvement of melatonin receptors in this process. Although to our knowledge there is no concrete evidence for the presence of melatonin receptors in the rat pineal gland, binding of melatonin to rat and bovine pineal glands has been observed previously [33–35], suggesting that melatonin receptors might exist in pineal glands. In order to clarify whether specific receptor subtypes are indeed involved in the regulation of 12-LOX expression, further investigation will be necessary.

It has also been suggested that melatonin is a potent antioxidant that protects cells or tissues from oxidative damage by reactive free radicals [6,7]. Considering the fact that most LOX

inhibitors have antioxidant properties [36], one can postulate that melatonin is a LOX inhibitor. The data *in vitro* obtained in the present study show that melatonin partially inhibits 12-lipoxygenation in pineal homogenates only at concentrations higher than those that are physiologically relevant. These data indicate that melatonin is not an effective 12-LOX inhibitor compared with compounds such as eicosatetraynoic acid and nordihydroguaiaretic acid [9]. However, it cannot be ruled out that melatonin might be a localized LOX inhibitor in the pineal gland, where its concentrations can reach the micromolar range during peak hours of melatonin synthesis (Figure 2A; approx. 1.2 ng/pineal).

The pineal gland has been shown to be a highly vascularized organ with a high blood flow [37]. Therefore the contribution of vascular or blood cell 12-LOX to the observed melatonin effect may be speculated upon. Such a possibility, however, is at least unlikely, if not excluded, on the basis of previous reports [9,24,38,39]. It has been demonstrated that the 12-lipoxygenation that occurs in pineal glands is clearly distinguished from that of blood cell origin [9,24]. In addition, in the brain, only the pineal gland shows any significant 12-LOX activity in conjunction with a strong 12-LOX mRNA signal, although microvessels are distributed throughout the brain regions [9,24]. Furthermore, microvessel endothelia produce prostaglandin E₂ as the major metabolite of 20:4n-6 [38], whereas the pineal gland produces mainly 12-HETE. All of these observations indicate that microvessels are not the major source of 12-LOX observed in this study.

The production of certain LOX metabolites is elevated most commonly during disease-related processes. Under normal physiological conditions, however, levels of LOX metabolites do not vary significantly. Therefore it is not surprising that modest diurnal variations in endogenous pineal 12-HETE levels (20–30%) were observed in the present study. 12-LOX and its metabolites have been suggested to play key roles in a number of neurobiological processes, including intracellular calcium release [10], modulation of ion-channel activities in *Aplysia* sensory neurons [39], neuronal degeneration [40] and neurotransmission [41]. However, the biological functions of 12-LOX in the rat pineal gland still remain unclear. It has been reported that 12-HETE can be incorporated into cerebral endothelial membranes, where it might alter cerebrovascular functions [42]. Since the pineal gland is an endocrine organ and occupies a central location in the brain, albeit outside of the blood–brain barrier, it is tempting to speculate that the pineal gland might participate in certain neurophysiological processes via the secretion of 12-LOX metabolites, which can be made available to microvessels. Obviously, further investigation is necessary to answer the question of whether changes in endogenous 12-HETE production by melatonin can lead to significant alterations in certain brain functions.

In summary, our present study provides the first evidence that melatonin is involved in the down-regulation of 12-LOX gene/protein expression, as well as the production of 12-LOX metabolites in the rat pineal gland. If a similar regulation should be found to occur in other tissues, as has been suggested by other researchers [12,13], it follows that melatonin and its synthetic analogues can be explored as pharmaceutical agents to combat 12-LOX-related pathophysiological conditions.

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