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Diurnal Change of Arachidonate 12-Lipoxygenase in Rat Pineal Gland

Hiroo Kawajiri,* Na Qiao,* Da-Ming Zhuang,* Tanihiro Yoshimoto,*,[1] Hiroshi Hagiya,† Shozo Yamamoto,† Hiroyoshi Sei,‡ and Yusuke Morita‡

**Department of Pharmacology, Kanazawa University School of Medicine, Kanazawa 920, Japan; and †Department of Biochemistry and ‡Department of Physiology, Tokushima University School of Medicine, Tokushima 770, Japan*

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Rat pineal gland contains a 12-lipoxygenase as demonstrated by the enzyme activity, RNA blot analysis and *in situ* hybridization. Using rats maintained with 12-h dark and light cycles, dynamic changes of the enzyme in pineal gland were examined. When the crude extract of pineal glands was incubated with arachidonic acid and the reaction products were analyzed by reverse-phase HPLC, the glands obtained from rats in the dark showed a higher 12-lipoxygenase activity than those obtained from rats in the light. The pineal 12-lipoxygenase activity decreased after the light was on at 7 o'clock and reached the lowest level around 16 o'clock. Upon Western blot analysis the amount of 12-lipoxygenase protein in pineal glands was high in the dark and lowest around 16 o'clock. A half life of the enzyme protein was estimated to be approximately 2.8 h in organ culture of rat pineal glands. Northern blot analysis also revealed a higher 12-lipoxygenase mRNA level in pineal glands obtained in the dark than those obtained in the light. Thus, the 12-lipoxygenase of rat pineal glands shows a diurnal fluctuation that is regulated at the transcription level, and may play a certain role in the regulation of neuroendocrine processes of this gland.

Arachidonate 12-lipoxygenase introduces one molecule of oxygen into C-12 of arachidonic acid to produce 12-HPETE (1). Earlier we found a high 12-lipoxygenase activity in rat pineal glands (2), and recently cloned a cDNA of the enzyme from this gland (3). Essentially the same cDNA for 12-lipoxygenase was cloned from whole brain of rat (4). The 12-lipoxygenase mRNA was exclusively localized in pineal gland as demonstrated by RNA blot analysis and *in situ* hybridization of rat brain (3). There are two isoforms of 12-lipoxygenase (leukocyte- and platelet-types) that are distinguishable by their substrate specificity, reactivity to antibody and primary structures (1). We showed that the enzyme of rat pineal glands was of leukocyte-type based upon catalytic properties and primary structure (3). With regards to physiological roles of 12-lipoxygenase in pineal gland, the primary reaction product, 12-HPETE, was demonstrated to stimulate melatonin synthesis in rat pineals (5). 12-HPETE was transformed to hepoxilin A_3 which decreased cAMP level in cultured rat pineals (6). It is well-known that melatonin and cAMP levels show circadian rhythms, which are controlled by arylalkylamine N-acetyltransferase activity (7) and clock-driven norepinephrine release (8), respectively. Since 12-lipoxygenase products have regulatory effects on the production of these compounds as mentioned above, we investigated whether or not 12-lipoxygenase of rat pineal gland is involved in such a diurnal fluctuation.

MATERIALS AND METHODS

Materials. [1-^{14}C]Arachidonic acid, [α-^{32}P]dCTP, Megaprime DNA labeling system, nylon membrane (Hybond N+), Rapid hybridization buffer and ECL Western blotting system were purchased from Amersham International (Bucks), unlabeled arachidonic acid from Nu-Check-Prep (Elysian), precoated silica gel 60 F-254 glass plates for TLC from Merck (Darmstadt), β-actin DNA probe solution and *o*-phenylenediamine from Wako (Osaka), ISOGEN from Nippon gene (Osaka), and Freund's adjuvants from Difco (Detroit). An expression vector pKK223-3 was obtained from Pharmacia (Uppsala), pQE-32 and Ni-NTA-agarose from QIAGEN (Chatsworth), and isopropyl β-D-thiogalactoside and cycloheximide from Sigma (St. Louis). ABC kit and nitrocellulose membrane were purchased from Vector (Burlingame) and Schleicher & Schuell (Dassel), respectively. Male Wister rats aging 5 weeks were obtained from SLC (Shizuoka), and were kept in a room with light on from 7 o'clock to 19 o'clock for at least one week prior to experiments. Dulbecco's modified minimum essential medium and fetal bovine serum were purchased from Nissui (Tokyo) and CSL limited (Victoria), respectively.

[1] To whom correspondence should be addressed. Fax: +81-76-234-4227. E-mail: yoshimot@med.kanazawa-u.ac.jp.

Abbreviations: 12-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; 12-HPETE, 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid; PG, prostaglandin.

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Locomotor activity. To evaluate the feature of diurnal rhythm in rats, locomotor activity was measured using animal movement analyzing system (Scanet MV-10LD, MATYS, Toyama, Japan). The system consisted of a rectangular enclosure (48 cm width × 48 cm depth × 6 cm height), the side walls of which were equipped with 144 pairs of photosensors. Each pair of photosensors was scanned every 0.1 s to detect animal movements. Locomotor activity was calculated from the scanning data and accumulated every 10 min. Each rat was individually placed in a transparent Plexiglas cage (28 × 44 × 18 cm) which was fixed at the center of the apparatus under a controlled light and dark condition and a constant ambient temperature (23 ± 2 °C). After an adaptation period of about 24 h, locomotor activity was measured for 30 h.

12-Lipoxygenase assay. Pineal glands were obtained from rats anesthetized with pentobarbital (50 mg/kg), and homogenized in 100 volumes of 20 mM Tris-HCl buffer at pH 7.4 containing 1 mM EDTA and 1 mM dithiothreitol by a Potter-Elvehjem's homogenizer. The homogenate was centrifuged at 10,000 × g for 10 min, and the supernatant solution was used as the crude extract. Protein concentration was determined by the method of Lowry *et al.* using bovine serum albumin as a standard (9). For the 12-lipoxygenase assay the crude extract was incubated with 25 μM [1-^{14}C] arachidonic acid (50,000 cpm in 5 μl of ethanol) in 50 mM Tris-HCl buffer at pH 7.4 at 30 °C for 10 min. The products extracted with diethyl ether were separated by silica gel TLC plate using a solvent mixture of diethyl ether/petroleum ether/acetic acid (85/15/0.1, by volume). Radioactivity on the plate was monitored, and the enzyme activity was determined as described previously (10).

HPLC analysis. Reverse-phase HPLC analysis was carried out on a Waters system with a TSK ODS-120T column (5 μm particle, 4.6 × 250 mm) using a solvent mixture of methanol/water/acetic acid (80/20/0.01, by volume) at a flow rate of 1 ml/min. Absorbance at 240 nm due to a conjugated diene was monitored by a Waters dual wavelength detector model 490.

Western blotting. An expression vector for rat pineal 12-lipoxygenase cDNA was constructed using pKK223-3 with *tac* promoter. cDNA fragments of Eco RI-Kpn I (nucleotides 142-689) and Kpn I-Hin cII (nucleotides 689-2222) were inserted into pKK223-3 which had been digested with Eco RI and Sma I. N-Terminal region was amplified by PCR using primers and pQE-32 containing a DNA sequence encoding 6 histidine residues onto the 5′ end of the 12-lipoxygenase cDNA as a template. The sequences of the primers were 5′-GGAATTCATGAGAGGATCGCAGCACCA-3′ (upstream primer containing overhang Eco RI site as underlined) and 5′-GCATTTGGCTAGGAGCCAG-3′ (downstream primer complementary to nucleotides 1119-1138). The amplified DNA was digested with Eco RI, and a fragment (nucleotides −35-142) was inserted into an Eco RI site of the above recombinant pKK223-3. *E. coli* was transformed with the expression vector, and expression of the 12-lipoxygenase was induced by the addition of 0.1 mM isopropyl β-D-thiogalactoside. *E. coli* was harvested by centrifugation and disrupted by sonication. A high-speed supernatant was fractionated with ammonium sulfate (40% saturation), and subjected to Ni-NTA-agarose chromatography under manufacturer's instruction. Approximately 15 mg of the purified 12-lipoxygenase was obtained from 12-liter culture. The enzyme could be stored at −70°C without appreciable loss of activity for at least 6 months. Polyclonal antibody against the purified 12-lipoxygenase was raised in New Zealand white female rabbits as described elsewhere (11). For Western blot analysis the crude extracts of pineal glands were separated in 8.5% polyacrylamide gel containing 0.1% SDS. Proteins were then electrophoretically transferred to a nitrocellulose membrane. The membrane was incubated with polyclonal anti-rat-12-lipoxygenase antiserum (2,500-fold dilution), biotinylated anti-rabbit-IgG, and ABC kit. 12-Lipoxygenase protein was visualized with ECL Western blotting system using a Fuji X-ray film.

Organ culture of pineal glands. Organ culture was performed by the essentially same method as described elsewhere (5). Briefly, rat pineal glands were washed twice with ice-cold phosphate-buffered saline, and placed in Dulbecco's modified minimum essential medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 100 μg/ml cycloheximide. A gland was put into a well of 24-well plate containing 500 μl of the same medium, cut into several pieces, and cultured in a CO_2 (5%) incubator at 37°C. The tissues were removed at 1-h intervals, and washed with phosphate-buffered saline by centrifugation. The crude extract for Western blotting was prepared as described above.

Northern blot analysis. Total RNA from rat pineal gland was extracted with ISOGEN. Total RNAs (1.5 μg) were separated by electrophoresis on 1% agarose and transferred to a nylon membrane (Hybond N+) (12). The membrane was hybridized in a Rapid hybridization buffer. A cDNA probe (nucleotides 1162-1993 of rat pineal 12-lipoxygenase) was prepared as described previously (3) and labeled with [α-^{32}P]dCTP using the Megaprime DNA labeling system. The membrane was washed in 2 × SSPE (1 × SSPE = 0.15 M NaCl/10 mM NaH_2PO_4/1 mM EDTA) at 25°C twice for 5 min and finally in 0.7 × SSPE at 65°C for 15 min. The radioactivity on the membrane was measured by a Fujix bioimaging analyzer BAS 2000.

RESULTS AND DISCUSSION

We have previously shown that rat pineal gland had a high 12-lipoxygenase activity (2). Subsequent study revealed that the enzyme was exclusively expressed in the pineal gland among various parts of rat brain as assessed by Northern blot analysis and *in situ* hybridization (3). Since pineal gland showed circadian rhythms of melatonin and cAMP levels as described above, we examined whether or not 12-lipoxygenase of rat pineal gland showed a diurnal fluctuation. Rats were maintained in a room of the animal facility with 12-h light and dark cycles; light on from 7 o'clock to 19 o'clock. The crude extracts of rat pineals obtained at either noon or midnight were incubated with arachidonic acid, and the reaction products were analyzed by reverse-phase HPLC monitoring absorbance at 240 nm due to a conjugated diene as shown in Fig. 1. Pineal glands obtained at midnight produced larger amounts of 12-HETE than those at noon as compared with PGB_2 as an internal standard. Fig. 2A shows 12-lipoxygenase activity of pineal glands obtained at the various times of day. The enzyme activity at 16 o'clock was significantly lower than that at midnight. Locomotor activity of rats was monitored by Scanet MV-10LD, and lateral and up-and-down movements were integrated by a computer. The amount of movement was larger in the dark than in the daytime (Fig. 2B), confirming that rat is a nocturnal animal.

In order to examine the quantitative change of 12-lipoxygenase protein, we carried out Western blot analysis. Polyclonal antibody raised in rabbit against a purified recombinant 12-lipoxygenase detected a single band with a molecular weight of about 75 kDa. The antibody crossreacted equally with an enzyme of porcine leukocytes, but not with an enzyme of rat platelets as assessed by Western blot analysis (data not shown). As shown in Fig. 3, the 12-lipoxygenase protein level

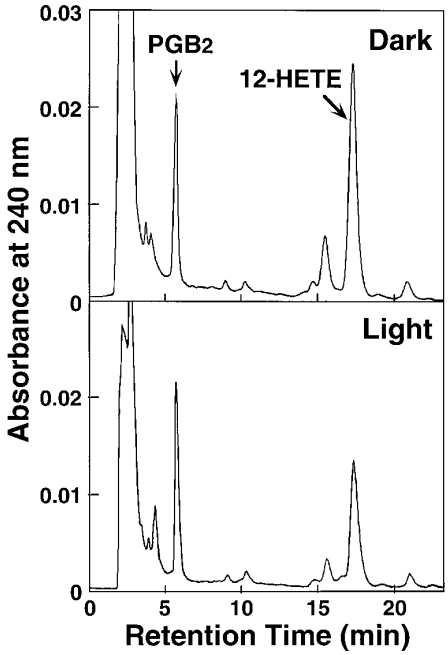

FIG. 1. 12-Lipoxygenase activity of rat pineal glands obtained at either midnight (upper) or noon (lower). Crude extracts of the glands (10,000 × g supernatant of a homogenate) were incubated with 25 μM arachidonic acid at 30°C for 10 min, and the $NaBH_4$-reduced products were analyzed on reverse-phase HPLC with PGB_2 as an internal standard. Absorbance at 240 nm of a conjugated diene was monitored. Retention times of 12-HETE and PGB_2 are shown by arrows.

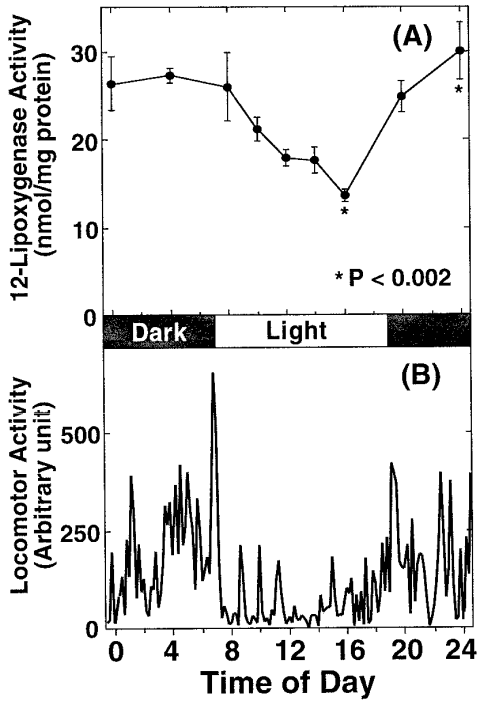

FIG. 2. Diurnal change of 12-lipoxygenase activity of rat pineal gland (A) and rat locomotor activity (B). (A) 12-Lipoxygenase activity of pineal glands obtained at indicated times was determined by the incubation with ^{14}C-arachidonic acid. Data are means ± SD of triplicate experiments. (B) Locomotor activity of rats was examined as described under Materials and Methods. Presented are average activity values of three rats with 10-min integrations.

was significantly lower at 16 o'clock than at 8 o'clock and 24 o'clock.

The stability of intracellular 12-lipoxygenase protein has not yet been examined in any tissue or cell. We wondered if rat pineal 12-lipoxygenase protein had a reasonably short half life for a day-night fluctuation. Pineal glands were cultured in the medium containing cycloheximide to inhibit translation of the enzyme protein. We monitored the degradation of 12-lipoxygenase protein in a course of pineal culture by Western blotting. Fig. 4 shows a gradual decrease of enzyme protein during the tissue culture of four independent experiments. A half life of the enzyme was estimated to be approximately 2.8 h. This short half life of the pineal 12-lipoxygenase can account for a diurnal fluctuation of the enzyme protein.

Since 12-lipoxygenase protein showed a day-night fluctuation, we examined whether mRNA of the enzyme also changed by the exposure of rats to light. As shown in Fig. 5, RNAs were extracted from pineal glands obtained at 8, 16 and 24 o'clock, and subjected to Northern blot analysis using the 12-lipoxygenase cDNA as a probe. 12-Lipoxygenase mRNA bands at 16 o'clock was lighter than those at 8 and 24 o'clock as examined by a BAS-2000 bioimaging analyzer. β-Actin signals as a control did not change significantly.

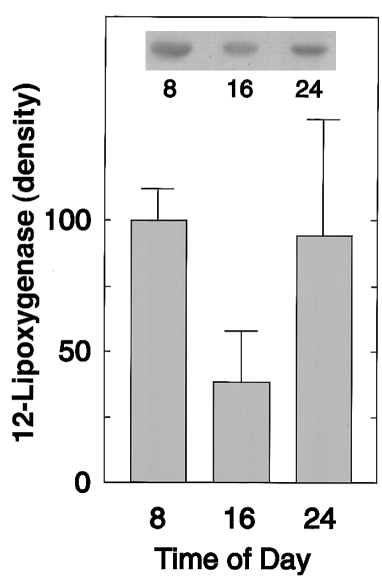

FIG. 3. Diurnal change of 12-lipoxygenase protein of rat pineals as examined by Western blotting. The crude extracts (8 μg protein) of rat pineal glands obtained at the indicated times were subjected to Western blot analysis as described under Materials and Methods. The density of bands on the blot was analyzed using NIH image software (version 1.6). Data are expressed as means ± SD using 3 pineals. Inset shows a representative blot.

The synthesis and secretion of pineal melatonin shows the day-night fluctuation as a result of the change of its biosynthetic enzyme, arylalkylamine N-acetyltransferase (7). Present work showed that the 12-lipoxygenase activity, protein, and mRNA level in rat pineal gland were high at night and low during the day, indicating a diurnal rhythm of this enzyme. It has been reported that melatonin is a natural ligand for retinoid Z receptor (13). The nuclear retinoid receptor is a superfamily which comprises retinoid Z receptors (RZRα and RZRβ) and three splicing variants of orphan retinoid receptors (RORα_1, α_2, and α_3). A responsive element in the promoter of human 5-lipoxygenase binds specifically RZRα and RORα_1, and melatonin down-regulated the 5-lipoxygenase expression about 5-fold in B lymphocytes (13). The fact that 12-lipoxygenase is higher at night where melatonin level is high raises a question whether melatonin upregulates pineal 12-lipoxygenase. Gene structures of human 12-lipoxygenase (14,15) and 5-lipoxygenase (16) are very different in size (8 kb and >82 kb, respectively). Moreover, their deduced amino acid sequences show only about 40% homology (1). Therefore, the transcription of human 5-lipoxygenase and 12-lipoxygenase genes may be differently regulated. Alternatively, the changes of melatonin and 12-lipoxygenase level may be two independent phenomena. It is well known that distribution of lipoxygenases shows species difference (1). Namely, although mouse (17) and rat pineal glands (2,3) have 12-lipoxygenase, it is uncertain whether human pineal gland has any lipoxygenase, the gene of which responds to melatonin and retinoid receptor. Physiological roles of diurnal change of 12-lipoxygenase in rat pineal gland have to be delineated by further investigations.

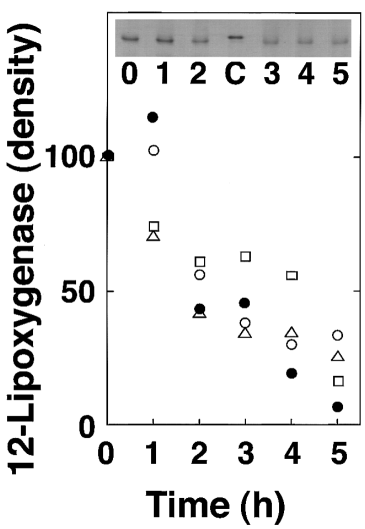

FIG. 4. Half life of pineal 12-lipoxygenase protein as examined by Western blot analysis. Pineals were cultured in the medium containing cycloheximide as described under Materials and Methods. The 12-lipoxygenase protein at 1-h intervals was estimated by comparing with an amount at time 0. Four serial experiments were performed, and data were shown by different marks. Inset shows a representative blot at various times, and lane C contained 10 ng of recombinant 12-lipoxygenase. The recombinant 12-lipoxygenase contained 6 histidine residues at the N-terminal which caused a slight retardation upon SDS polyacrylamide gel electrophoresis.

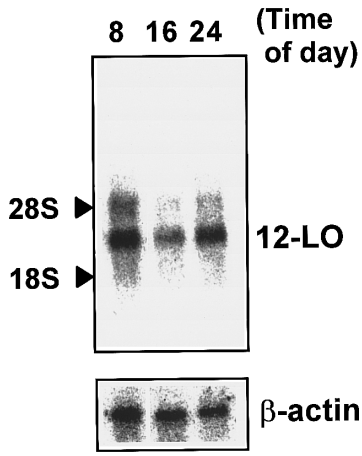

FIG. 5. RNA blot analysis of 12-lipoxygenase mRNA in pineal gland. Total RNA (1.5 μg) of pineal glands obtained at the indicated times was subjected to Northern blotting as described under Materials and Methods. Hybridization with a β-actin probe was also carried out as a control. Blots were analyzed with a Fujix BAS-2000 bioimaging analyzer. Ribosomal RNAs (28S and 18S) are shown by arrows. 12-LO denotes 12-lipoxygenase.

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