Lipocalin-type prostaglandin D synthase produces prostaglandin D₂ involved in regulation of physiological sleep

Wei-Min Qu*, Zhi-Li Huang*†, Xin-Hong Xu*†, Kosuke Aritake*, Naomi Eguchi*, Fumio Nambu†, Shu Narumiya§, Yoshihiro Urade*, and Osamu Hayaishi*†¶

*Department of Molecular Behavioral Biology, Osaka Bioscience Institute, Suita, Osaka 565-0874, Japan; †State Key Laboratory of Medical Neurobiology, Shanghai Medical College of Fudan University, Shanghai 200032, China; ‡Ono Pharmaceutical Co., Ltd., Osaka 618-8585, Japan; and §Department of Pharmacology, Kyoto University Faculty of Medicine, Kyoto 606-8501, Japan

Contributed by Osamu Hayaishi, September 28, 2006 (sent for review September 14, 2006)

Prostaglandin (PG) D₂ has been proposed to be essential for the initiation and maintenance of the physiological sleep of rats because intracerebroventricular administration of selenium tetrachloride (SeCl₄), a selective inhibitor of PGD synthase (PGDS), was shown to reduce promptly and effectively the amounts of sleep during the period of infusion. However, gene knockout (KO) mice of PGDS and prostaglandin D receptor (DP₁R) showed essentially the same circadian profiles and daily amounts of sleep as wild-type (WT) mice, raising questions about the involvement of PGD₂ in regulating physiological sleep. Here we examined the effect of SeCl₄ on the sleep of WT and KO mice for PGDS and DP₁R and that of a DP₁R antagonist, ONO-4127Na, on the sleep of rats. The i.p. injection of SeCl₄ into WT mice decreased the PGD₂ content in the brain without affecting the amounts of PGE₂ and PGF_{2α}. It inhibited sleep dose-dependently and immediately after the administration during the light period when mice normally sleep, increasing the wake time; and the treatment with this compound resulted in a distinct sleep rebound during the following dark period. The SeCl₄-induced insomnia was observed in hematopoietic PGDS KO mice but not at all in lipocalin-type PGDS KO, hematopoietic and lipocalin-type PGDS double KO or DP₁R KO mice. Furthermore, the DP₁R antagonist ONO-4127Na reduced sleep of rats by 30% during infusion into the subarachnoid space under the rostral basal forebrain at 200 pmol/min. These results clearly show that the lipocalin-type PGDS/PGD₂/DP₁R system plays pivotal roles in the regulation of physiological sleep.

antagonist | enzyme | knockout mice | receptor | selenium tetrachloride

The somnogenic activity of prostaglandin (PG) D₂ was originally discovered serendipitously when we microinjected nanomolar quantities of PGD₂ in the preoptic area of rats (1). Subsequent studies using a continuous-infusion circadian sleep bioassay system revealed that the effect was specific to PGD₂ because other PGs were almost totally inactive (2). Infusion with as little as picomolar quantities per minute was sufficient to induce excess amounts of both non-rapid eye movement (NREM) and rapid eye movement (REM) sleep. Sleep induced by PGD₂ was indistinguishable from physiological sleep as judged by several behavioral and electrophysiological criteria, including power spectral analysis (3). PGD₂ was not pyrogenic, and it actually caused a slight decrease in the body temperature, as is observed to occur during physiological sleep. These results suggested the possibility that PGD₂ might be a sleep hormone (4–7).

PGD₂ is the most abundant prostanoid in the brains of rats (8) and other mammals, including humans (9); and it is produced in the brain from the substrate PGH₂, a common intermediate of various prostanoids, by the action of PGD synthase (PGDS; PGH₂ D-isomerase, EC 5.3.99.2). Two distinct types of PGDS were purified from the brain (10) and spleen (11) of rats in our and other laboratories, and their structures were determined by

x-ray crystallography (12, 13). One was the lipocalin-type PGDS (L-PGDS) (10, 14, 15), mainly expressed in the arachnoid membrane, choroids plexus, and oligodendrocytes in the brain (14, 16); and the other was hematopoietic PGDS (H-PGDS) (17, 18), localized in mast cells (19) and microglia (20, 21). Inorganic tetravalent selenium compounds were found to be potent, relatively specific, and reversible inhibitors of PGDS when tested *in vitro* (22). These inhibitors seemed to interact with the free sulfhydryl group in the active site of PGDS because the inhibition could be reversed by the addition of excess amounts of SH compounds such as glutathione or DTT. In 1991, Matsumura and coworkers (23) administered selenium tetrachloride (SeCl₄) into the third ventricle of sleeping rats, and they found that sleep was inhibited almost completely after ≈2 h from the start of the infusion. The effect was reversible because when the infusion was interrupted, sleep was restored. Furthermore, the inhibition was reversed by the simultaneous infusion of SH compounds such as DTT and glutathione, as in the case of the *in vitro* enzyme activity. These results indicated that PGDS plays an essential role in the maintenance of sleep and, therefore, that PGD₂ is involved in sleep under physiological conditions in rodents.

Our recent studies with gene knockout (KO) mice for PGDS and prostaglandin D receptors (DP₁R) specific for PGD₂ showed that mice depleted of these genes did not exhibit NREM sleep rebound after sleep deprivation, suggesting that PGD₂ is crucial for the homeostatic regulation of NREM sleep (24). However, KO mice for PGDS or DP₁R did not show any changes in the circadian profile and total time of NREM and REM sleep compared with WT mice under baseline conditions, raising questions about the involvement of PGD₂ in the regulation of physiological sleep.

In this work, we administered SeCl₄ by an i.p. bolus injection during the sleeping period into wild-type (WT) mice as well as into KO mice lacking H-PGDS, L-PGDS, H- and L-PGDS (HL-PGDS), or DP₁R, and we studied their sleep–wake behavior. The SeCl₄ administration inhibited sleep promptly and effectively in WT and H-PGDS KO mice but not at all in the other three types of KO mice, indicating that SeCl₄ inhibited sleep by blocking the endogenous production of PGD₂ by

Author contributions: W.-M.Q., Z.-L.H., Y.U., and O.H. designed research; W.-M.Q., Z.-L.H., X.-H.X., K.A., N.E., and F.N. performed research; F.N. and S.N. contributed new reagents/analytic tools; W.-M.Q., Z.-L.H., X.-H.X., N.E., Y.U., and O.H. analyzed data; and W.-M.Q., Z.-L.H., Y.U., and O.H. wrote the paper.

The authors declare no conflict of interest.

Abbreviations: DP₁R, prostaglandin D receptor; EEG, electroencephalogram; EMG, electromyogram; KO, knockout; NREM, non-rapid eye movement; PG, prostaglandin; PGDS, prostaglandin D synthase; H-PGDS, hematopoietic PGDS; L-PGDS, lipocalin-type PGDS; HL-PGDS, double H- and L-PGDS; PLSD test, probable least-squares difference test; REM, rapid eye movement; SeCl₄, selenium tetrachloride.

¶To whom correspondence should be addressed. E-mail: hayaishi@obi.or.jp.

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Fig. 1. Sleep-stage distribution produced by ONO-4127Na infusion in rats. (A) Time course changes in ONO-4127Na (200 pmol/min) treatment group. Each circle represents the hourly mean $\pm$ SEM of NREM and REM sleep and wakefulness. Open and filled circles indicate the baseline and experimental day profiles, respectively. ONO-4127Na was given between 0900 and 1500 for 6 h, as indicated by the horizontal bar, on the experimental day. The horizontal open and filled bars on the x-axes indicate the 12-h light and 12-h dark periods, respectively. (B) Total time spent in NREM and REM sleep and wakefulness for 7 h; that is, during ONO-4127Na perfusion for 6 h and 1 h postinfusion. Open and filled bars show the profiles for the respective baseline day (vehicle infusion) and experimental day (ONO-4127Na infusion). Values are means $\pm$ SEM ($n = 6$). *, $P < 0.05$; **, $P < 0.01$ by the paired *t* test.

L-PGDS and not by some nonspecific or general toxic effects of selenium *per se*. We also examined the effect of a DP₁R antagonist ONO-4127Na on the sleep of rats, and we found that this antagonist reduced the sleep during infusion into the subarachnoid space under the rostral basal forebrain, in which DP₁R is dominantly localized (25). These results indicate again that PGD₂ plays a crucial role in the regulation of physiological sleep.

Results

Blockade of DP₁R Decreased NREM and REM Sleep in Rats. We infused the DP₁R antagonist ONO-4127Na into the subarachnoid space underlying the rostral basal forebrain of rats through a cannula for 6 h from 0900 to 1500 to investigate the changes in the sleep-stage distribution. As shown in Fig. 1A, ONO-4127Na infusion at 200 pmol/min caused a significant decrease in NREM and REM sleep: ONO-4127Na decreased NREM sleep hourly by 30–40%, and it reduced REM sleep by 60–90% commencing ≈ 2 h after the beginning of ONO-4127Na infusion compared with the baseline. This reduction in sleep time was accompanied by an increase in wakefulness. ONO-4127Na (200 pmol/min) increased the wakefulness time 2.4-fold starting ≈ 2 h after the beginning of the infusion, and this increase lasted until 1 h after the cessation of infusion. There was no further disruption of the sleep architecture during the subsequent period. Similar time course profiles were observed with a lower concentration of 100 pmol/min. However, ONO-4127Na infusion at 50 pmol/min had little effect on the sleep-stage distribution.

We calculated the total time spent in wakefulness and NREM and REM sleep for 7 h, that is, during the ONO-4127Na

Fig. 2. SeCl₄ inhibition of the enzymatic activity of both recombinant mouse L-PGDS and H-PGDS *in vitro* (A) and PGD₂, PGE₂, and PGF_{2α} contents in the brain 2 h after SeCl₄ administration in WT mice (B). Values are means ± SEM (*n* = 6). **, *P* < 0.01 by one-way ANOVA, followed by the Fisher PLSD test.

perfusion for 6 h and 1 h postinfusion (Fig. 1B). ONO-4127Na given at 100 and 200 pmol/min reduced NREM sleep by 23% and 28% and REM sleep by 49% and 63%, and it increased the total amount of wakefulness by 1.6- and 1.8-fold, respectively, during that 7-h period. There was essentially no difference between ONO-4127Na (50 pmol/min) infusion and the baseline day. These results clearly indicate that ONO-4127Na dose-dependently reduced NREM and REM sleep and concomitantly increased wakefulness.

Inhibition of Mouse Brain PGDS *In Vitro* and *In Vivo* by SeCl₄. When we examined the effect of SeCl₄ on the activities of mouse L-PGDS and H-PGDS *in vitro*, we found that it inhibited both L-PGDS and H-PGDS efficiently in a concentration-dependent manner, giving IC₅₀ values of 40 and 90 μM, respectively (Fig. 2A). To investigate whether SeCl₄ inhibits PGDS *in vivo*, we then determined the PGD₂ content in the brain of WT mice at 2 h after an i.p. injection of SeCl₄ at a dose of 1.25, 2.5, or 5 mg/kg of body weight. As shown in Fig. 2B, whereas SeCl₄ given at 1.25 mg/kg had little effect on the PGD₂ content in the brain, at 2.5 and at 5 mg/kg it reduced the PGD₂ content by 52% and 59%, respectively, without changing the PGE₂ and PGF_{2α} contents, indicating that the SeCl₄ administration selectively inhibited the production of PGD₂ in the brain *in vivo* without affecting the production of other PGs.

Inhibition of NREM and REM Sleep of WT Mice by SeCl₄ Administration.

Next we examined the sleep–wake pattern of WT mice by measuring their electroencephalogram (EEG) and electromyogram (EMG) before and after an i.p. bolus injection of SeCl_4 at doses of 1.25, 2.5, 4, and 5 mg/kg at 1100 during the sleep period. The typical time courses of the hourly amounts of NREM and REM sleep and wakefulness and their cumulative amounts for 5 h after injection are summarized in Figs. 3 and 4, respectively. In the case of 1.25 mg/kg SeCl_4 , the sleep–wake pattern was almost identical before and after the injection, similar to the pattern of PGD_2 content in the brain. SeCl_4 at 2.5 mg/kg slightly reduced both NREM and REM sleep for 2–3 h after the injection (Fig. 3A). At this dose, the cumulative amounts of NREM and REM sleep for 5 h postinjection significantly decreased by 23% and 44%, respectively, and the amount of

Fig. 3. Time courses of the sleep–wake profiles after i.p. administration of SeCl_4 (2.5 and 5 mg/kg) to WT mice (A and B) and SeCl_4 (5 mg/kg) to H-PGDS (C), L-PGDS (D), HL-PGDS double (E), and DP_1R (F) KO mice. Each circle represents the hourly mean $\pm$ SEM of NREM and REM sleep and wakefulness. Open and filled circles stand for the baseline- and experimental-day profiles, respectively. SeCl_4 was given at 1100, as indicated by the arrow, on the experimental day; and the vehicle was used for the baseline day. The horizontal open and filled bars on the x-axes indicate the 12-h light and 12-h dark periods, respectively. Values are means $\pm$ SEM ($n = 6$ or 7). *, $P < 0.05$; **, $P < 0.01$ by the paired t test.

wakefulness increased 1.5-fold (Fig. 4). At doses of 4 and 5 mg/kg SeCl_4 , both NREM and REM sleep decreased promptly after the injection; and SeCl_4 -injected mice displayed almost complete insomnia within 1 h. The sleep suppression gradually decreased thereafter, lasting ≈ 3 and 5 h after the injection of 4 and 5 mg/kg SeCl_4 , respectively; and both NREM and REM sleep increased remarkably during the following nighttime (Fig. 3B). SeCl_4 given at 4 and 5 mg/kg reduced the 5-h cumulative amount of NREM sleep during the daytime by 31% and 45% and that of REM sleep by 63% and 81%, respectively, and it increased that of wakefulness time by 1.7- and 2.1-fold, respectively (Fig. 4). It also induced a strong rebound-like reaction during the following nighttime to increase the 6 h-cumulative amount of NREM sleep 2.2- and 2.4-fold, REM sleep 5.5- and 3.9-fold, and to decrease the amount of wakefulness time by 36% and 41%, respectively. These results revealed that SeCl_4 given

during the light period dose dependently inhibited NREM and REM sleep of WT mice and increased both during the following dark period.

Effect of SeCl_4 on NREM and REM Sleep of KO Mice Lacking H-PGDS, L-PGDS, HL-PGDS, or DP_1R . We then administered SeCl_4 at 5 mg/kg to WT or various KO mice lacking H-PGDS, L-PGDS, HL-PGDS, or DP_1R , and we compared the effect on the sleep–wake cycle between the WT and these KO mice. In H-PGDS KO mice, SeCl_4 also inhibited both NREM and REM sleep by 80% and almost completely, respectively, and it increased the wake time ≈ 2 -fold for 1 h after the injection (Fig. 3C). The hourly amounts of NREM sleep and wakefulness returned to the vehicle-injected level within 2–3 h after the administration, but the REM sleep suppression was prolonged for 6 h. H-PGDS KO mice showed strong NREM and REM sleep induction and suppression of

Fig. 4. SeCl₄ inhibition of sleep in WT and H-PGDS KO mice, but not in L-PGDS, HL-PGDS double, and DP₁R KO mice. Total time spent in NREM and REM sleep and in wakefulness for 5 h after SeCl₄ administration into WT and KO mice is shown. Open and filled bars show the profiles for the baseline and experimental days, when the mice were treated with vehicle and SeCl₄, respectively. Values are means $\pm$ SEM ($n = 6$ or 7). *, $P < 0.05$; **, $P < 0.01$ compared with its own control; †, $P < 0.05$; ††, $P < 0.01$ compared with WT mice administered SeCl₄ at 5 mg/kg; ‡, $P < 0.05$; ‡‡, $P < 0.01$ compared with H-PGDS KO mice i.p. injected with SeCl₄ at 5 mg/kg, by two-way ANOVA, followed by the Fisher PLSD test.

wakefulness during the following dark period, similar to the WT mice. In the comparison of the cumulative amounts, NREM and REM sleep for 5 h after the injection decreased by 27% and 78%, respectively, and the wakefulness increased by 1.7-fold (Fig. 4). The cumulative amounts of NREM and REM sleep for 6 h during the following dark period increased 2.4- and 4.1-fold, respectively, and that of wakefulness decreased by 47%. Between the WT and H-PGDS KO mice, there was no statistically significant difference in the responsiveness of NREM and REM sleep and wakefulness to the SeCl₄ administration, suggesting that H-PGDS was most likely not involved in the SeCl₄-induced insomnia.

In contrast, when L-PGDS KO mice were examined, SeCl₄ did not change the amounts of NREM and REM sleep or the time spent in wakefulness at all during the daytime (Figs. 3D and 4), clearly indicating that the sleep inhibition immediately after the administration of SeCl₄ depended on L-PGDS. However, interestingly, the SeCl₄ administration to L-PGDS KO mice increased both NREM and REM sleep and decreased wakefulness time during the following nighttime (Fig. 3D). The 6-h cumulative amounts of the NREM and REM sleep during the dark period increased almost 2-fold, and that of wakefulness time decreased $\approx 25\%$. Between the WT and L-PGDS KO mice, there was no statistically significant difference in the increase in NREM or REM sleep or in the decrease in wakefulness during the nighttime after the SeCl₄ administration, indicating that L-PGDS was not involved in the delayed sleep induction by SeCl₄. The delayed sleep induction was considered to be mediated by PGD₂ produced by H-PGDS and recognized by DP₁R because it was not observed in HL-PGDS double KO mice or in DP₁R KO mice, as described below.

When SeCl₄ was administered to HL-PGDS double KO mice (Fig. 3E) or to DP₁R KO mice (Fig. 3F) under the same experimental conditions, neither the inhibition of sleep immediately after administration of SeCl₄ nor the delayed increase in sleep during the night was observed. These results clearly show that SeCl₄ was not toxic to inhibit sleep *per se*, but it suppressed sleep by inhibiting the endogenous production of PGD₂, whose information is transferred by DP₁R to induce sleep. These results also suggest that sleep in WT mice is controlled by PGD₂ produced by L-PGDS, rather than H-PGDS, and it is recognized by DP₁R under physiological conditions.

Discussion

The present study showed that SeCl₄ inhibited sleep almost immediately after the administration of it to WT mice and H-PGDS KO mice, but it failed to do so in L-PGDS KO, HL-PGDS double KO, and DP₁R KO mice, indicating that L-PGDS is the enzyme responsible for the synthesis of PGD₂ involved in sleep regulation and that the selenium compound inhibited sleep through the L-PGDS/PGD₂/DP₁R system (7).

It is known that selenium compounds are toxic to several species of mammals (26), despite being an essential trace element and the fact that selenocysteine is present in the active site of glutathione peroxidase (27) and a component of other proteins such as selenoprotein P (26). If the observed reduction in sleep had been caused by some toxic effect of selenium, similar changes in the sleep–wake profiles would have been expected in WT, L-, H-PGDS, HL-PGDS double and DP₁R KO mice, let alone the overt behavioral changes that are common consequences of toxicity. Nevertheless, SeCl₄ merely caused a significant reduction in the amount of sleep of the WT and H-PGDS KO mice, and it had no effect on the other KO mice, thereby eliminating the possibility of some toxicological effect of SeCl₄. In addition, when SeCl₄ was infused into the third ventricle of rats during the light period, sleep was inhibited both time- and dose-dependently, and the inhibition was reversed when the infusion was interrupted. Likewise, the effect was reversed by the simultaneous infusion of sulfhydryl compounds such as DTT and reduced glutathione. Furthermore, throughout the sleep-inhibition period caused by selenium administration, all rats displayed normal behaviors similar to those observed during the natural wake phase (23). Recently, Lee *et al.* (28) reported that intracerebroventricular infusion of SeCl₄ increased the arousal-like behavior in unanesthetized fetal sheep and that the effect could be abolished by subsequent administration of PGD₂. These findings further indicate that SeCl₄-induced sleep inhibition may indeed be attributed to L-PGDS inhibition and not to selenium intoxication.

It is noteworthy that the amount of sleep in L-PGDS KO mice increased during the dark period when SeCl₄ was given during the light period, whereas HL-PGDS double KO and DP₁R KO mice were not affected in this way, suggesting that H-PGDS may be involved in the increase in sleep during the dark period. The physiological meaning of this “pseudo-rebound” phenomenon is unknown at present, and further experiments are required to clarify this issue.

Here we showed L-PGDS and DP₁R to be important in sleep induced by PGD₂. When the DP₁R antagonist ONO-4127Na was infused into the subarachnoid space in the rostral basal forebrain of a sleeping rat, where DP₁R are densely localized, sleep was inhibited dose- and time-dependently and reversibly during the period of infusion. However, L-PGDS, DP₁R, and HL-PGDS double KO mice displayed NREM and REM sleep circadian profiles identical to those of WT mice under baseline conditions. Similar phenomena were also found in mice with knockout of other sleep–wake-related genes, such as those for the histamine H₁ receptor (29, 30), orexin (31), and the adenosine A₁ and A_{2A} receptors (32, 33). Considering that sleep is essential for life, it

is not surprising that the sleep-regulatory system would be composed of a complicated network of systems in which the deficiency of one system may be effectively compensated by the other systems during embryonic development (24). However, when mice were subjected to sleep deprivation, L-PGDS KO mice exhibited little NREM sleep rebound, without any increase in PGD₂ content in the brain, whereas WT mice displayed a pronounced sleep rebound especially in the NREM sleep, with concomitant increase in PGD₂ content in the brain after sleep deprivation (24), indicating that endogenous PGD₂ is involved in the homeostatic regulation of NREM sleep.

In conclusion, SeCl₄ inhibited sleep in WT mice, concomitant with a reduction in the PGD₂ content in the brain. The sleep inhibition was also observed in H-PGDS KO mice, but not in L-PGDS, HL-PGDS double or DP₁R KO mice. We conclude that PGD₂ is not only involved in the homeostatic regulation of sleep but also essential for the initiation and maintenance of normal circadian sleep under physiological conditions.

Materials and Methods

Animals. L-PGDS KO and H-PGDS KO mice were generated at Osaka Bioscience Institute (Osaka, Japan) and DP₁R KO mice, at Kyoto University (Kyoto, Japan), as reported in detail by Eguchi *et al.* (34), Trivedi *et al.* (35), and Matsuoka *et al.* (36), respectively. All of these KO mice were back-crossed to the C57BL/6 strain for >20 generations. Heterozygous mice for either the H- or L-PGDS gene mutation were cross-bred to generate double heterozygous mice. These double heterozygous mice were then cross-bred to produce the WT, H-PGDS KO, L-PGDS KO, and HL-PGDS double KO mice used in this work. Male WT, L-, H-, and HL-PGDS double and DP₁R KO mice of the inbred C57BL/6 strain, weighing 25–28 g (11–13 weeks), were maintained at Oriental Bioservice (Kyoto, Japan).

H- and L-PGDS deficiencies were confirmed by Southern blotting and PCR analysis of DNA prepared from clipped tails and by Western blotting with antibodies against H- and L-PGDS proteins, as described in refs. 34 and 35. Physical attributes of the HL-PGDS double KO mice were basically the same as those of the WT, H-PGDS KO, and L-PGDS KO mice as judged by the growth curves of body weight covering up to 40 weeks of age and visual observation of their behaviors.

Male Sprague–Dawley rats weighing 250–320 g (8–10 weeks) were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). Both mice and rats were housed in an insulated sound-proofed recording room maintained at an ambient temperature of 24 ± 0.5°C with a relative humidity of 60 ± 2% on an automatically controlled 12-h light/dark cycle (light on at 0800), and they had free access to food and water. The experimental protocols were approved by the Animal Care Committee of Osaka Bioscience Institute, and every effort was made to minimize the number of animals used as well as any pain and discomfort.

Chemicals. ONO-4127Na was produced and provided by Ono Pharmaceutical Co., Ltd. (Osaka, Japan). This compound exhibited a high specific binding affinity for the DP₁R ($K_i = 0.0025 \mu\text{M}$) compared with other prostanoid receptors (Table 1, which is published as supporting information on the PNAS web site) and antagonism toward DP₁R ($pA_2 = 9.73$; see Table 2, which is published as supporting information on the PNAS web site), based on the results of competitive binding assays and agonist-stimulated cAMP accumulation assays with mouse DP₁R-expressing stable transformants of Chinese hamster ovary cells (37–40). ONO-4127Na is almost insoluble in physiological saline or artificial cerebrospinal fluid, but it is soluble in 5% sucrose with a solubility limit at 5×10^{-4} M. Therefore, the maximum dosage of the drug allowed for infusion into the rat brain was 200 pmol at a rate of 0.4 $\mu\text{l/min}$. SeCl₄ (Wako Pure Chemicals,

Osaka, Japan) was dissolved in sterile saline. Aliquots were prepared and stored frozen at –20°C. For each experiment, the aliquots were thawed, and the pH was adjusted to 7.2 by the addition of 0.2 M NaOH immediately before use.

Enzyme Assay. The effect of SeCl₄ on the enzymatic activity of L- and H-PGDS was measured by incubating either enzyme at 25°C for 1 min with [1-¹⁴C]PGH₂ (final concentration of 40 μM) in 50 μl of 0.1 M Tris·HCl (pH 8.0) containing different concentrations of SeCl₄ and 1 mM glutathione (41). [1-¹⁴C]PGH₂ was prepared from [1-¹⁴C]arachidonic acid (2.20 GBq/mmol; PerkinElmer, Wellesley, MA) as described in ref. 10.

PGD₂, PGE₂, and PGF_{2 α} Enzyme Immunoassays. Two hours after SeCl₄ had been injected by the i.p. route to WT mice at a dose of 1.25, 2.5, or 5 mg/kg, the brains were harvested and immediately frozen in liquid nitrogen. They were then homogenized in ethanol containing 0.02% HCl at pH 2.0 and centrifuged at $500 \times g$ for 20 min. ³H-labeled PGD₂, PGE₂, and PGF_{2 α} (60 Bq for each per assay; PerkinElmer) were added to the supernatant as tracers for estimation of recovery. The recovery values were ≈60%. PGD₂, PGE₂, and PGF_{2 α} were extracted with ethyl acetate, which was evaporated under nitrogen; and the samples were then separated by a HPLC (Gilson, Middleton, WI) (42). The quantification of PGD₂, PGE₂, and PGF_{2 α} was performed with a PGD₂, PGE₂, and PGF_{2 α} enzyme immunoassay kit for these prostanoids (Cayman Chemicals, Ann Arbor, MI).

Polygraphic Recordings in Rats and Mice. Under pentobarbital anesthesia (50 mg/kg, i.p.), rats underwent surgery for implantation of electrodes for EEG and EMG recordings and placement of a stainless-steel cannula (0.35-mm diameter) for infusion of ONO-4127Na as described earlier (43). The cannulae were inserted in a midline position, 1.1 mm anterior to bregma, to a depth of 7.8–8.0 mm below the dura. The cannulae thus inserted were directed at the subarachnoid space under the rostral basal forebrain, the area defined as a PGD₂-sensitive sleep-promoting zone (43). Postoperatively, the rats were housed individually for 8–10 days.

Under pentobarbital anesthesia (50 mg/kg, i.p.), mice were chronically implanted with EEG and EMG electrodes for polysomnographic recordings. The implant consisted of two stainless-steel screws (1-mm diameter) serving as EEG electrodes, inserted, according to the atlas of Franklin and Paxinos (44), through the skull into the cortex (anteroposterior, +1.0 mm and +left–right, –1.5 mm from bregma or lambda); and two insulated, stainless-steel, Teflon-coated wires, serving as EMG electrodes, which were bilaterally placed into both trapezius muscles. All electrodes were attached to a microconnector and fixed to the skull with dental cement (29, 33). Postoperatively, the animals were housed individually for 8–10 days.

The recordings of EEG and EMG were carried out by means of a slip ring, designed so that behavioral movement of the animal would not be restricted. After a 10-day recovery period, the mice or rats were housed individually in transparent barrels and habituated to the recording cable for 3–4 days before starting the polygraphic recordings. The rats were infused continuously with artificial cerebrospinal fluid into the brain at a speed of 0.4 $\mu\text{l/min}$. Sleep–wakefulness states were monitored for a period of 48 h, which comprised baseline and experimental days. Baseline recordings were taken in each animal for 24 h, beginning at 0800, which served as the control for the same animal. On the next day, ONO-4127Na (50, 100, or 200 pmol/min) was infused into the rat brain between 0900 and 1500 for 6 h. Each mouse was i.p. injected with saline (20 ml/kg of body weight) at 1100 on the 1st day. The next day, again at 1100, SeCl₄ was injected i.p. at a dose of 1.25, 2.5, 4, or 5 mg/kg; and EEG/EMG activity was collected for another 24 h.

Cortical EEG and EMG signals were amplified and filtered (EEG, 0.5–30 Hz; EMG, 20–200 Hz), then digitized at a sampling rate of 128 Hz and recorded by using SLEEP-SIGN (Kissei Comtec, Nagano, Japan) as described in refs. 29 and 33. The total amounts of wakefulness and NREM and REM sleep were calculated for 7 h after beginning ONO-4127Na infusion and for 5 h after SeCl₄ administration. The amounts of the various sleep–wake states were expressed in minutes.

Vigilance State Analysis. Polygraphic recordings were automatically scored offline by 10-s epochs as wakefulness or NREM or REM sleep by SLEEP-SIGN according to standard criteria (29). As a final step, defined sleep–wake stages were examined visually, and corrected, if necessary.

Statistical Analysis. All data were expressed as the mean $\pm$ SEM ($n = 6$ or 7). For time course data, amounts of the different sleep–wake states were analyzed by the paired t test, with each

animal serving as its own control. For PGD₂, PGE₂, and PGF_{2 α} contents in the brain of WT mice and the total amounts of each vigilance stage for 5 h or 7 h after drug treatment, one- or two-way ANOVA followed by the Fisher probable least-squares difference (PLSD) test was used to determine whether the difference among groups and genotypes was statistically significant. In all cases, $P < 0.05$ was taken as the level of significance.

We thank Drs. A. A. Borbély, C. B. Saper, R. B. Wickner, and L. Frye for critical reading of the manuscript and valuable comments; and Mr. M. Wada, Ms. N. Matsumoto, N. Nagata, and Y. Hoshikawa for excellent technical assistance. This work was supported in part by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (18300129 to O.H.) and (18603011 to Z.-L.H.); the Program for Promotion of Basic Research Activities for Innovative Biosciences (to Y.U.); the Genome Network Project from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (to Y.U.); the Sankyo Foundation, Tokyo, Japan (to Z.-L.H.); Takeda Pharmaceutical Co., Ltd., Japan (to O.H.); and Ono Pharmaceutical Co., Ltd., Japan (to O.H.).

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