

Cyclooxygenase-1 Inhibition Shortens the Duration of Diazepam-Induced Loss of Righting Reflex in Mice

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Cyclooxygenase-1 (COX-1) inhibition by a selective inhibitor valeryl salicylate, or nonselective inhibitors at 10 mg/kg, including aspirin, ibuprofen, indomethacin, and piroxicam, attenuated by 29%-46% the duration of loss of righting reflex induced by diazepam (20 mg/kg) in mice. On the other hand, arachidonic acid (20 mg/kg) increased the duration of diazepam-induced loss of righting reflex by 48%. This effect of arachidonic acid was abolished by aspirin. However, aspirin at 10

mg/kg also did not alter the effects of diazepam (5 mg/kg) on spontaneous activity and rotarod performance. These findings strongly suggest that one or more COX products, most likely prostaglandins, play a significant role in modulating the hypnotic effect of diazepam. Elucidating the mechanism involved may further our understanding of the pharmacology of benzodiazepines.

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Prostaglandins (PG) are members of the eicosanoid family derived mainly from arachidonic acid by the action of cyclooxygenase (COX), which produces prostaglandin H₂ (PGH₂), which is then transformed by a range of specific enzymes into the primary prostanoids such as prostaglandin D₂ (PGD₂) and E₂ (PGE₂). There are two isoforms of COX: COX-1 is constitutive and predominant in most tissues whereas COX-2 is inducible and tightly regulated at transcription/translation levels.

Ethanol-induced loss of righting reflex (LORR) in mice was significantly decreased by COX inhibitors or nonsteroidal antiinflammatory drugs (NSAIDs) such as aspirin, indomethacin, mefenamic acid, and flufenamic acid (1). In addition, prostanoids could enhance the sedative effects of ethanol (2). Endogenous PG levels were increased by ethanol to a larger extent in LS mice ("long sleep" mice sensitive to ethanol) than in SS mice ("short sleep" mice insensitive to ethanol) (3). Therefore, these observations indicate that ethanol, at least in part, mediates its central nervous system (CNS) depressant effects by increasing the production of PGs. However, similar results were not

obtained for other anesthetic drugs such as pentobarbital and chloral hydrate (1).

The benzodiazepines are the most widely used CNS depressant drugs. They act specifically on the benzodiazepine receptor of the gamma-aminobutyric acid (GABA)_A receptor complex and thus modify the GABA-induced chloride flux. However, it has been demonstrated that muscimol, a selective GABA_A receptor agonist, stimulates glioma C₆ cells to release arachidonic acid, which is converted to PGD₂ in the presence of diazepam (4). PGD₂ is an endogenous sleep promoting substance (5). It is interesting, therefore, to investigate whether PGs play a role in the CNS depressant effects of diazepam. We report here that COX-1 inhibitors decreased whereas arachidonic acid increased diazepam-induced LORR in mice.

Methods

This work was approved by the Animal Ethics Committee of National University of Singapore and all efforts were made to minimize the number of mice used. Male Swiss-Albino mice (25 to 30 g) were obtained from the Laboratory Animal Center and housed in groups of 5 with food and water available *ad libitum* on a 12-h light-dark cycle. Mice were assigned to experimental groups at random and each was used in only one experiment.

All drugs were administered by intraperitoneal (IP) injection with an injection volume of 5 mL/kg. Aspirin, arachidonic acid and small-dose diazepam were

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dissolved in 30% dimethyl sulfoxide (DMSO). All other drugs were dissolved in DMSO. Control mice received the appropriate vehicle only. Diazepam was administered 30 min after pretreatment.

After the administration of diazepam (20 mg/kg), mice were put on their backs to test for LORR. The righting reflex was considered lost if the mice could not right themselves within 1 min, and regained when they managed to right themselves 3 times within 1 min. The duration of LORR was recorded for each mouse. All experiments were conducted between 1000 and 1200 h.

Mice were screened 24 h before experimentation for their ability to stay on a rotarod (Acceler Rota-rod, Ugo Basile, Comerio, Italy), which accelerated from 2 to 20 revolutions/min in 5 min. Only mice that could stay on for the full 5 min were used in the experiment. After the administration of diazepam (5 mg/kg), mice were put into an activity cage. Spontaneous ambulation was monitored for 3 min after a delay of 1 min. The activity cage (Columbus Instruments, Columbus, OH) consisted of a transparent polypropylene box of dimension 40 (l) × 20 (w) × 20 (h) cm. The movement of a mouse inside the box was monitored by 15 infrared beams placed equidistant, and the distance between the first and the last beam was 38 cm. Beam interceptions were recorded automatically by a computer-based system. Ambulatory activities were recorded at 30-s time blocks. Three min after the completion of the activity cage measurements, the mouse was then placed onto the rotarod and the fall latency was recorded; otherwise, the mouse was given a latency of 5 min. All experiments were conducted between 1900 and 2300 h.

Mice were given an IP injection (4 mL/kg) of either saline (control) or diazepam (20 mg/kg). They were killed by cervical dislocation 30 min after the injection. Cerebral cortex and hypothalamus/thalamus were collected and homogenized (Heidolph DIAX 900 Homogenizer, Heidolph Instruments LLC, Cinnaminson, NJ) in ice-cold phosphate buffer containing 10 μ M indomethacin. The homogenates were then centrifuged at 15,000 rpm and the supernatant was removed and stored at -20°C until the PG assay. Indomethacin inhibits both COX enzymes and 15-hydroxyprostaglandin dehydrogenase that catalyzes the catabolism of PG and was added to minimize errors resulting from the possible synthesis and degradation of PG in the homogenate. The concentration of PGD₂ was determined using commercial kits according to manufacturer protocol (Cayman Chemical, Ann Arbor, MI). The PGE₂ kits commercially available use anti-mouse IgG. To minimize possible cross-reaction, a modified PGE₂ methoxime enzyme immunoassay kit was used with reagents purchased from Cayman.

Mice were treated as described in the previous section. Total RNA was extracted from the cerebral cortex using Trizol reagent. RNA concentration and purity were assessed by measuring the absorbance at 260 and 280 nm wavelength (Shimadzu UV-1601 UV-visible Spectrophotometer Shimadzu Corp., Tokyo, Japan). Only RNA with absorbance ratio between 1.8 and 2.0 was used for further reverse transcriptase-polymerase chain reaction (RT-PCR) analysis.

Aliquots of total RNA (5 μ g) were used in the RT reaction mixed with 0.5 μ g oligo(dT) primer. First-strand cDNA synthesis was then performed with the use of Avian Myeloblastosis Virus RT (Invitrogen, Carlsbad, CA). cDNA was then amplified by PCR using primers specific to the key enzymes in PG synthesis pathway. β -actin, a ubiquitously expressed gene, was used as an internal control. The specificity of each pair of primers was tested by blasting their sequences to the entire mice genome. The quality of the primers was tested by the software, NetPrimer (Shimadzu).

β -actin primers were as follows: forward, 5'-ATC-TGGCACCACACCTTCTACAATGAGCTGCG-3'; reverse: 5'-TACTCCTGCTTGCTGATCCACATCTGC-3', which yield a PCR product of 832bp. Cox-1 primers were as follows: forward, 5'-AGGAGATGGCTGCT-GAGTTGG-3'; reverse: 5'-AATCTGACTTTCTGAGT-TGCC-3', which result in a PCR product of 601bp.

Lipocalin type-PGD synthase (L-PGDS) primers were as follows: forward, 5'-CAGGAAAACCAGT-GTGAGACC-3'; reverse, 5'-AGAGGGTGGCCATG-CGGAAG-3', which yield a PCR product of 194bp. Membrane-associated PGE synthase-1 (mPGES-1) primers were as follows: forward, 5'-TGTACGCGGT-GGCTGTCATC-3'; reverse, 5'-GCCAGGACAT-AGGCCCCGG-3', which result in a PCR product of 319bp. 2 μ L from each RT reaction mixture was mixed with 1 μ L of each primer (20 mM), 25 μ L PCR reaction mix and the final volume was topped up to 50 μ L. To remain in the linear range during amplification, the mixture was heated at 95°C for 5 min and the reaction was performed respectively as follows: 29 cycles (95°C for 30 s; 60°C for 30 secs and 72°C for 1 min; 10 min for the last extension) for β -actin; 30 cycles (95°C for 30 s; 55°C for 30 s; and 72°C for 1 min; 10 min for the last extension) for COX-1, 29 cycles (95°C for 30 s; 60°C for 30 s; and 72°C for 1 min; 10 min for the last extension) for L-PGDS and 39 cycles (95°C for 30 s; 60°C for 30 s; and 72°C for 1 min; 10 min for the last extension) for mPGES-1. The amplified PCR products were then separated by electrophoresis (Bio-Rad laboratories, Hercules, CA) on 1% agarose gel. A Gene Ruler 1000-bp DNA Ladder was used to determine the size of the PCR products. Densitometry of the bands was performed (Syngene Multi Genius Bioimaging System, Frederick, MD) to give rough quantifications. The expression level of each gene was estimated as its ratio to

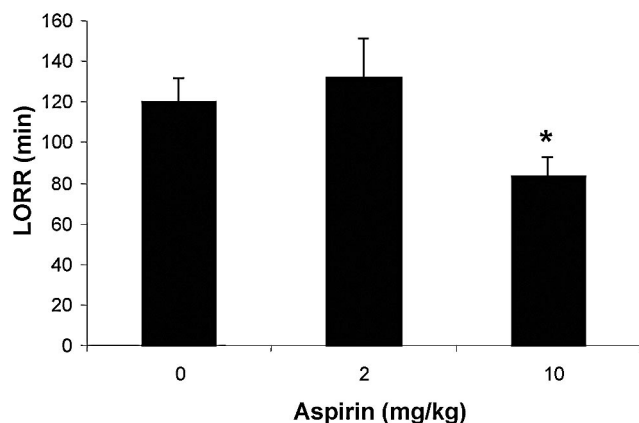


Figure 1. Aspirin (10 mg/kg) inhibited diazepam-induced loss of righting reflex. Mice were pretreated with aspirin dissolved in 30% dimethyl sulfoxide, control received only the vehicle. Diazepam (20 mg/kg) was injected 30 min after pretreatment. Data are presented as mean ± SD, $n = 6$. One-way analysis of variance: $F(2,15) = 18.542$; $P < 0.001$. * $P < 0.002$ by *post hoc* analysis with Bonferroni correction.

β -actin. Each gene in each sample was amplified twice by PCR and the average of the two ratios was taken and used for statistical analysis. To minimize possible influence of natural circadian cycle to the gene expression, all individual experiments were started at between 1400 and 1500 h.

Data are expressed as mean ± SD. All statistical analyses were performed by SPSS for Windows, Version 12 (SPSS, Chicago, IL). One-way analysis of variance or 2-way analysis of variance with repeated measures was followed by *post hoc* analysis with Bonferroni correction. In all cases, $P < 0.05$ was taken as the level of significance.

Results

Diazepam (20 mg/kg) caused a LORR for 120 ± 12 min in control mice pretreated with vehicle (30% DMSO). Pretreatment with aspirin (10 mg/kg but not 2 mg/kg) significantly reduced the duration of LORR by 31% to 83 ± 10 min ($P < 0.002$) (Fig. 1). Pretreatment with arachidonic acid (20 mg/kg), however, significantly increased the duration of LORR by 48% from 111 ± 19 min to 164 ± 35 min ($P < 0.001$) (Fig. 2). As expected, coadministration of aspirin abolished the effect of arachidonic acid.

Other COX inhibitors were administered in DMSO due to their insolubility in aqueous solvents. Control mice pretreated with DMSO showed markedly enhanced diazepam-induced LORR (275 ± 48 min or 2.3-fold) compared with mice pretreated with 30% DMSO (Figs. 1 and 2). This is consistent with the finding that DMSO enhanced the effects of barbiturates (6). However, despite the differences in control value, ibuprofen, indomethacin, and piroxicam at the

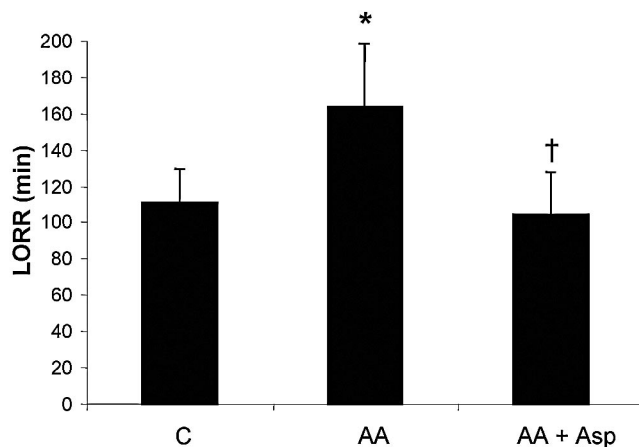


Figure 2. Arachidonic acid (AA, 20 mg/kg) enhanced diazepam-induced loss of righting reflex. Aspirin (Asp, 10 mg/kg) abolished this enhancement. Mice were pretreated with AA or AA + asp dissolved in 30% dimethyl sulfoxide, control received only the vehicle. Diazepam (20 mg/kg) was injected 30 min after pretreatment. Data are presented as mean ± SD, $n = 10-13$. One-way analysis of variance: $F(2,31) = 16.071$; $P < 0.001$. * $P < 0.001$ against control and † $P < 0.001$ against AA group by *post hoc* analysis with Bonferroni correction.

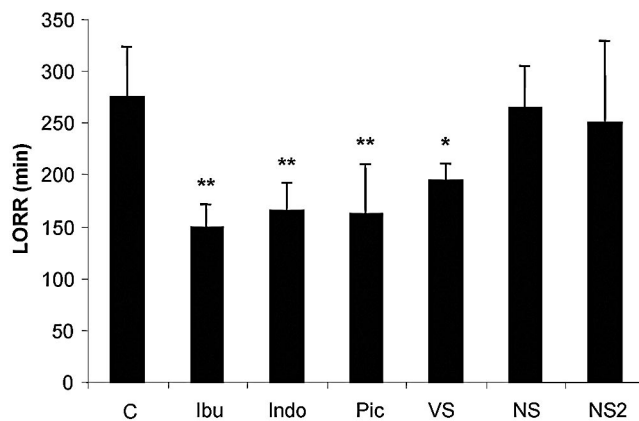


Figure 3. Effects of various selective and nonselective COX inhibitors on diazepam-induced loss of righting reflex. Mice were pretreated with ibuprofen (Ibu), indomethacin (Indo), piroxicam (Pic), valeryl salicylate (VS), or NS398 (NS) dissolved in dimethyl sulfoxide; control received only the vehicle. All drugs were administered at 10 mg/kg except NS, which was also administered at 20 mg/kg (NS2). Diazepam (20 mg/kg) was injected 30 min after pretreatment. Data are presented as mean ± SD, $n = 4-11$. One-way analysis of variance: $F(6,43) = 7.607$; $P < 0.001$. * $P < 0.05$ and ** $P < 0.005$ compared with control group by *post hoc* analysis with Bonferroni correction.

same dose of 10 mg/kg, gave similar results by reducing the diazepam-induced LORR by 46%, 40%, and 41%, respectively ($P < 0.005$, Fig. 3).

Valeryl salicylate, a selective COX-1 inhibitor at 10 mg/kg, also reduced diazepam-induced LORR by 29% to 195 ± 14 min ($P < 0.05$). In contrast, NS398, a selective COX-2 inhibitor, failed to produce any effects even when the dose was doubled to 20 mg/kg (Fig. 3).

Spontaneous ambulatory activity was significantly decreased by diazepam (5 mg/kg) over the entire observation period of 3 min ($P < 0.05$). Pretreatment with aspirin

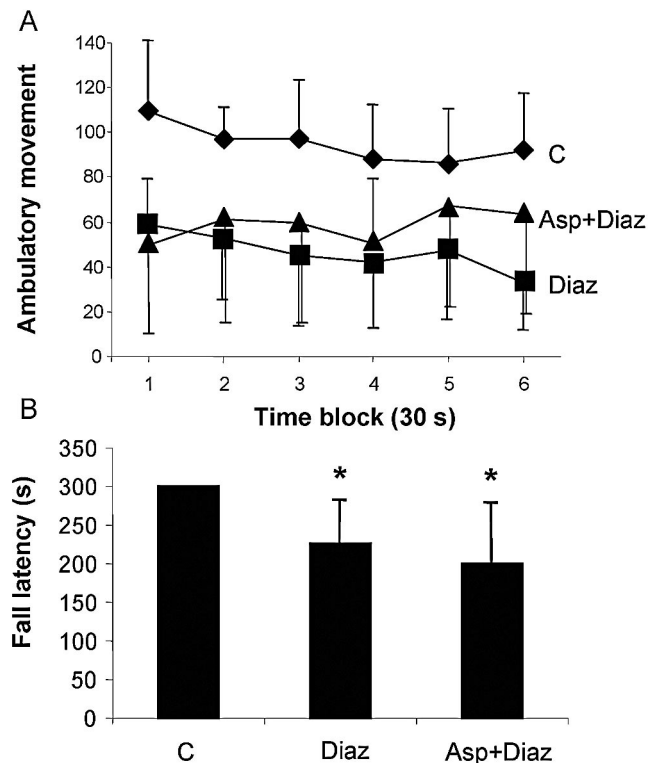


Figure 4. A. Spontaneous activities of mice at a sedative dose of diazepam. Mice (Asp + Diaz) were injected diazepam (5 mg/kg) dissolved in 30% dimethyl sulfoxide 30 min after pretreatment with aspirin (10 mg/kg). Diaz group received diazepam after pretreatment with vehicle whereas control (C) received only the vehicle. Data are presented as mean \pm SD, $n = 5 - 7$. Two-way analysis of variance (General Linear Model) with repeated measures: $F(2,16) = 4.294$; $P < 0.05$ for the main effect of treatment on ambulatory movement. Post hoc analysis with Bonferroni correction showed significant difference between control (C) and diazepam (Diaz) group ($P < 0.05$) but no difference between Diaz and Asp + Diaz group. B. Rotarod performance of mice at a sedative dose of diazepam. Rotarod test was performed immediately after the spontaneous ambulation as shown in Fig. 4A. Data are presented as mean \pm SD, $n = 5 - 7$. One-way analysis of variance: $F(2,16) = 4.357$; $P < 0.05$. * $P < 0.05$ compared to controls by *post hoc* analysis with Bonferroni correction.

did not significantly alter the effect of diazepam on spontaneous ambulation ($P > 0.7$) (Fig. 4A).

Consistent with reduced spontaneous motor activity, diazepam-injected mice showed significantly impaired performance on the rotarod, with a 25% decrease in the fall latency ($P < 0.05$). Again, aspirin pretreatment did not significantly alter the diazepam effect on the rotarod performance (Fig. 4B).

The cerebral cortex and hypothalamus/thalamus are responsible for the regulation of consciousness and sleep/wake cycle, respectively (7). They are thus selected for the measurement of PGD_2 and PGE_2 and the expression of related enzymes. Endogenous PGD_2 and PGE_2 levels in the cerebral cortex and hypothalamus/thalamus homogenates were determined at 30 min after injection. PGD_2 was found to be 31% less ($P < 0.05$) in the cortex but not in the hypothalamus/thalamus. In contrast, PGE_2 remained unchanged in

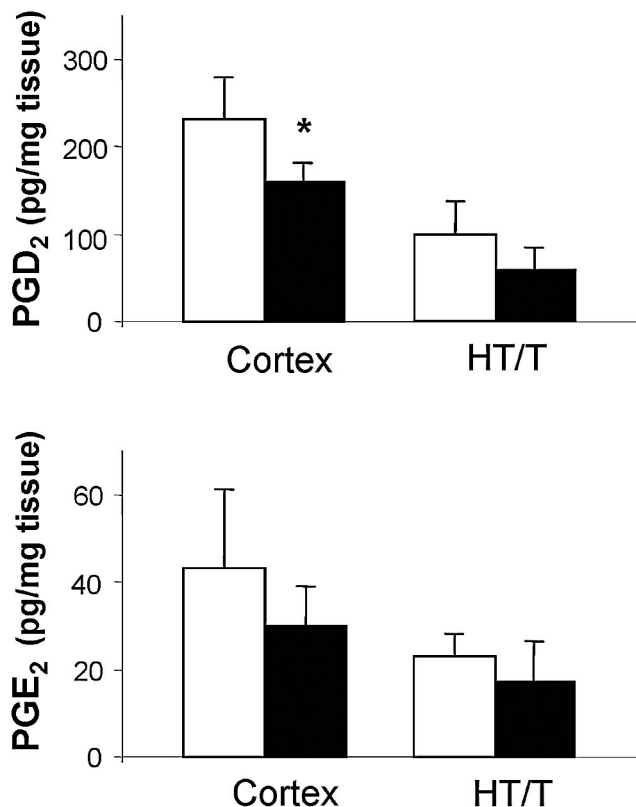


Figure 5. Effects of diazepam on endogenous levels of prostaglandin (PG) D_2 and E_2 in the cerebral cortex and hypothalamus/thalamus (HT/T). Mice were killed 30 min after administration of diazepam (20 mg/kg, solid bar), controls (open bar) received saline. Data are presented as mean \pm SD, $n = 4 - 5$. *Statistically significant by independent sample *t*-test, $t = 2.732$, $df = 7$, $P < 0.05$.

both brain regions (Fig. 5). RT-PCR analysis revealed that the expression of COX-1, L-PGDS and m-PGES-1 were not affected by diazepam (Fig. 6).

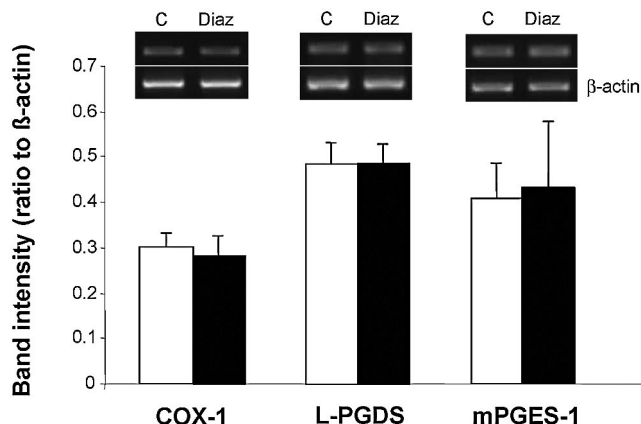


Figure 6. Expression of cyclooxygenase-1 (COX-1), lipocalin type-PGD synthase (L-PGDS) and membrane-associated PGE synthase-1 (mPGES-1) genes in the cerebral cortex by RT-PCR. Mice were killed 30 min after administration of diazepam (20 mg/kg, solid bar), controls (open bar) received saline. Band intensity is presented as a ratio to the β -actin band, mean \pm SD, $n = 6$. No statistically significant differences were observed.

Discussion

In preliminary dose-finding experiments, it was found that diazepam at 20 mg/kg gave consistent mean duration of LORR (90–120 min). This is appropriate for studying a potential reduction of effect by COX inhibitors. With smaller doses, there were a significant number of mice that failed to show LORR. This would cause large variations in the duration of LORR making statistical comparison between groups difficult. The human equivalent dose (HED) of 20 mg/kg in mice is 1.62 mg/kg calculated on the basis of body surface area, or approximately 113 mg for a 70-kg person. This is thus a very large dose compared with the usual clinical doses (2–10 mg) being used in patients. However, this is consistent with the reported 95% effective dose of propofol at 200 mg/kg (HED 16.2 mg/kg) for inducing LORR in mice (8), whereas its clinical dose range is 1–2.5 mg/kg in humans. At 5 mg/kg, diazepam gave a suitable reduction in locomotion and rotarod performance for the purpose of this study. Doses of 1–10 mg/kg are commonly used as motor impairing or sedative doses in mice (9).

It is well established that the benzodiazepines cause CNS depression by acting on the benzodiazepine receptors that are regulatory sites on the GABA_A receptors. When diazepam binds to this site, it enhances GABA action on this receptor, leading to increased opening frequency of the receptor chloride channel and thus hyperpolarization of the neuronal membrane. The effects of the benzodiazepines are thus limited by the action of endogenous GABA, and they are not known to produce nonspecific effects related to its lipid solubility even at very large doses (10). It is therefore intriguing that arachidonic acid could enhance, while COX inhibitors could attenuate, the hypnotic effect of diazepam. This is consistent with previous findings that PG synthesis inhibitors attenuated ethanol-induced LORR (11) whereas PGD₂ enhanced pentobarbital-induced LORR (12).

Although these findings clearly suggest that the arachidonic acid cascade can modulate the hypnotic effect of diazepam, the mechanism is unclear. Our present findings strongly suggest that one or more COX products, most likely PG, play a significant role in modulating the duration of LORR. The fact that the selective COX-2 inhibitor NS398 was ineffective, while the COX-1 selective inhibitor, valeryl salicylate, and all the nonselective inhibitors tested were effective, demonstrated the exclusive involvement of the constitutive COX-1 and not the inducible COX-2 enzyme. It is also interesting to note that aspirin did not alter the effect of small-dose diazepam on spontaneous ambulation and rotarod performance. This argues against any direct antagonistic action between aspirin and diazepam because the same aspirin dose attenuated the LORR induced by diazepam at a 20 mg/kg dose

but not the spontaneous activity and rotarod performance at a 5 mg/kg dose.

PGD₂ and PGE₂ are major PGs in the mammalian brain (13,14). PGD₂ was first reported to be a sleep-inducing substance in the rat brain (15) and then, subsequently, in the monkey (16), rabbit (17), and cat (5) brains. Infusion of PGD₂ into the preoptic area of the hypothalamus induced sleep during the day or night, which is indistinguishable from natural sleep (5,18). PGD₂ produced by brain-specific L-PGDS circulates in the cerebrospinal fluid and exhibits circadian fluctuation in parallel with the sleep-wake cycle (19). In this connection, there have been reports of decreased or disrupted sleep after administration of NSAIDs in both humans and animals during the normal sleep period (20,21).

Conversely, infusion of PGE₂ into the cerebroventricle (22) increased wakefulness. Moreover, AH6809 (a PGE₂ antagonist) increased sleep in rats (23) and PGE₂ inhibited sleep in narcoleptic dogs (24). At the molecular level, PGE₂ may cause wakefulness by activating arousal neurons in the tuberomammillary nucleus located at posterior hypothalamus via AMPA-type excitatory amino acid receptors (25). Therefore, it appears that these two PGs with opposing effects on sleep may be intimately involved in the regulation of the sleep-wake cycle (26).

Based on the known actions of PGD₂ and PGE₂, they are two likely candidates among COX-1 products that could be involved in the modulation of diazepam-induced LORR, perhaps an increase in PGD₂ and/or a decrease in PGE₂ levels. On the contrary, we observed only a small but significant decrease in PGD₂ levels in the cerebral cortex 30 min after the administration of diazepam. The exact mechanism by which diazepam interacts with the arachidonic acid cascade remains to be elucidated. This would further enhance our understanding of the pharmacology of benzodiazepines.

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