Dominant expression of mRNA for prostaglandin D synthase in leptomeninges, choroid plexus, and oligodendrocytes of the adult rat brain

(prostaglandin D2 in situ hybridization/immunohistochemistry/arachnoid membrane/β-trace)

YOSHIRO URADE*, KUNIO KITAHAMA†, HITOSHI OHISHI‡, TAKESHI KANEKO‡, NOBORU MIZUNO‡, AND OSAMU HAYAISHIZ

*International Research Laboratories, Ciba-Geigy Japan Ltd., Takarazuka 665, Japan; †Institut National de la Santé et de la Recherche Médicale, Unité 52, Département de Médecine Expérimentale, Université Claude Bernard, 69373 Lyon Cedex 08, France; ‡Department of Morphological Brain Science, Faculty of Medicine, Kyoto University, Kyoto 606-01, Japan; and §Osaka Bioscience Institute, Suita, Osaka 565, Japan

Contributed by Osamu Hayaishi, June 28, 1993

ABSTRACT Glutathione-independent prostaglandin D synthase [prostaglandin-H2 D-isomerase; (5Z,13E)-(15S)-9α,11α-epidioxy-15-hydroxyprosta-5,13-dienoate D-isomerase, EC 5.3.99.2] is an enzyme responsible for biosynthesis of prostaglandin D2 in the central nervous system. In situ hybridization with antisense RNA for the enzyme indicated that mRNA for the enzyme was predominantly expressed in the leptomeninges, choroid plexus, and oligodendrocytes of the adult rat brain. The findings agree with those obtained by immunohistochemical staining with antibodies against the enzyme. It was further revealed that prostaglandin D synthase activity was considerably greater in the isolated leptomeninges (14.2 nmol per min per mg of protein) and choroid plexus (7.0 nmol per min per mg of protein) than the activity in the whole brain (2.0 nmol per min per mg of protein). These results, taken together, indicate that the enzyme is mainly synthesized and located in the leptomeninges, choroid plexus, and oligodendrocytes in the brain.

Prostaglandin D2 (PGD2) is a major prostaglandin in the brain of rats and humans and functions as a neuromodulator of several central actions such as sleep–wake cycles, body temperature, luteinizing hormone release, and odor responses (for review, see ref. 1). Among several enzymes catalyzing the conversion of PGG2 to PGD2, glutathione-independent PGD synthase [prostaglandin-H2 D-isomerase; (5Z,13E)-(15S)-9α,11α-epidioxy-15-hydroxyprosta-5,13-dienoate D-isomerase, EC 5.3.99.2] is responsible for biosynthesis of PGD2 in the central nervous system (3), retina (4), and cochlea (5). Immunocytochemical studies with a polyclonal and two monoclonal anti-rat brain PGD synthase antibodies revealed that the major cellular location of the enzyme in the brain shifts postnataally from neurons of 1- to 2-week-old rats to oligodendrocytes in adult animals (6), suggesting that the enzyme may have distinct functions in those cells at each specific developmental stage and thus plays important roles in both maturation and maintenance of the central nervous system.

We previously isolated cDNAs encoding this enzyme in the brain of rats (7) and humans (8). The homology search in data bases of protein primary structure (8–10) indicated that the enzyme is a member of the lipocalin superfamily consisting of a variety of secretory proteins involved in the transport of small hydrophobic molecules (11). PGD synthase is the only lipocalin known to be associated with enzyme activity. The gene structure of rat brain PGD synthase (12) is also remarkably conserved among members of this protein family in terms of the numbers and sizes of exons and phases and sites of exon/intron splicing.

Inorganic quadrivalent selenocompounds, such as SeCl4 and Na2SeO3, have recently been found to inhibit rat brain PGD synthase activity without affecting the activities of other enzymes in the arachidonate cascade (13). Those selenocompounds were subsequently shown to inhibit sleep in unrestrained rats, promoting physiological wakefulness, in a time- and dose-dependent manner, when administered into the third ventricle via a microdialysis probe (14). These results further support our hypothesis that PGD2 is involved in the promotion of sleep under physiological conditions and imply that the PGD synthase may be a key enzyme in regulation of sleep–wake activities.

We report here, by using in situ hybridization with antisense RNA for the enzyme, that mRNA for glutathione-independent PGD synthase is dominantly expressed in the leptomeninges and choroid plexus, as well as in oligodendrocytes, of the adult rat brain. These tissues and cells were also found to be enriched in both the immunoreactivity and the enzyme activity of PGD synthase, indicating that the major site of synthesis of the enzyme, and also of PGD2, is predominantly, if not exclusively, located in the leptomeninges, choroid plexus, and oligodendrocytes.

MATERIALS AND METHODS

Preparation of Complementary RNA Probe. A 352-base-pair fragment corresponding to nucleotides 122–473 of the cDNA for rat brain PGD synthase (M61900) (7) was subcloned into BlueScript II (Stratagene). After linearization of the template DNA, antisense RNA probe was transcribed by T7 RNA polymerase in the presence of CTP[35S].

In Situ Hybridization. Adult male Sprague–Dawley rats (200–300 g) were used in this study. Under deep ether anesthesia, animals were killed by decapitation. Tissues were removed immediately and flash frozen in isopentane at −70°C. Sections of 10 μm thickness were cut on a cryostat, thaw-mounted onto poly(1-lysine)-coated slides, and air-dried briefly. The sections were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate (pH 7.3) for 10 min, rinsed in phosphate buffer, and then acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine/0.9% NaCl for 10 min at room temperature. After dehydration by passage through an ethanol series, the sections were air-dried and stored at −80°C until use.

The in situ hybridization was carried out as reported elsewhere (15). In brief, the tissue sections were incubated...
with $^{35}$S-labeled RNA probe in a solution of 50% formamide, 2x standard saline citrate (SSC; 1x SSC = 0.15 M NaCl/0.015 M sodium citrate), 10 mM Tris-HCl (pH 7.5), 1x Denhardt’s solution, 10% dextran sulfate, 0.2% SDS, 100 mM dithiothreitol, 500 µg of sheared single-stranded salmon sperm DNA per ml, and 250 µg of yeast tRNA per ml. After incubation at 60°C for 5 h, the slides were washed in 2x SSC containing 10 mM 2-mercaptoethanol first at room temperature overnight and then at 60°C for 1 h. The slides were then treated with RNase A (20 µg/ml) for 30 min in 0.5 M NaCl/10 mM Tris-HCl, pH 7.5/1 mM EDTA, followed by an additional wash at a final stringency of 0.1x SSC at 60°C for 1 h. After dehydration, the slides were dipped in autoradiographic emulsion (NTB 2) diluted 1:1 with distilled water. After exposure for 2 weeks at 4°C, the dipped slides were developed in Kodak D-19, fixed, and counterstained with cresyl violet. The specificity of in situ hybridization was assessed by incubation of adjacent sections with the labeled probe in the presence of 100-fold excess unlabeled probe.

**Immunocytochemistry.** Immunoperoxidase staining was performed as reported (6) with mouse monoclonal and rabbit polyclonal antibodies against rat brain PGD synthase (2, 6) by use of the avidin–biotin–peroxidase complex method. The IgG fraction obtained from nonimmunized mice or rabbits was used for control staining.

**Enzyme Assay.** Under pentobarbital anesthesia (50 mg/kg), rats were perfused through the left ventricle of the heart with Krebs–Ringer solution (1.5 times body weight). The brain was quickly removed and the leptomeninges and choroid plexus were carefully isolated with fine forceps under a dissecting microscope. The isolated tissues were homogenized in 10 mM potassium phosphate (pH 7.0) with a Kinematica Polytron homogenizer (Lucerne, Switzerland). After centrifugation at 4°C for 1 h at 100,000 x g, the supernatant was recovered and used as the enzyme source. Enzyme activity was determined by incubation of the supernatant at 25°C for 1 min with 40 µM [1-14C]PGH$_2$ in 50 µl of 0.1 M Tris-HCl (pH 8.0) and 1 mM glutathione (reduced form) as described (2).

**RESULTS**

Figs. 1 and 2 show the autoradiograms obtained after in situ hybridization with the $^{35}$S-labeled antisense RNA for rat brain PGD synthase. The mRNA for the enzyme was intensely expressed in the choroid plexus (Figs. 1a and 2d) and the leptomeninges (Figs. 1a–c and 2a) throughout the brain and spinal cord of the adult rat. Large intracerebral vessels (Fig. 2a) as well as oligodendrocytes (Fig. 2b and c) also displayed the signals indicating mRNA for the enzyme. No signals, however, were detected in capillaries and neurons throughout the central nervous system. The signals disappeared almost completely when the labeled probe was incubated with the tissue sections in the presence of 100-fold excess of unlabeled probe (Fig. 1d).

The immunohistochemical studies with the antibody against the enzyme revealed the immunoreactivity in the leptomeninges (Fig. 2e) and choroid plexus (Fig. 2h). The immunoreactivity for the enzyme was also found in oligodendrocytes (Fig. 2f and g), confirming our data reported previously (6), but these positive cells were almost ubiquitously distributed in various parts of the brain and were not

**Fig. 1.** Distribution of mRNA for PGD synthase in the adult rat brain and spinal cord. Negative film images of in situ hybridization of a parasagittal section (a) and coronal sections of the spinal cord (b) and the brain (c and d) are shown. Sections were hybridized with an antisense RNA probe for the enzyme in the presence (d) or absence (a–c) of 100-fold excess unlabeled probe. ac, Anterior commissure; Cb, cerebellum; cc, corpus callosum; CP, choroid plexus; Cx, cerebral cortex; M, midbrain; MO, medulla oblongata; OB, olfactory bulb; P, pons; T, thalamus. (Bar = 500 µm.)
FIG. 2. Bright-field photomicrographs show location of mRNA (a–d) and immunoreactivity (e–h) of PGD synthase in adult rat brain. Leptomeninges of the surface of the neocortex and along intracortical blood vessels (arrows) (a, e), choroid plexus (d, h), and oligodendrocytes in layer V of the frontoparietal neocortex (b, f) and in the corpus callosum (c, g) are labeled by in situ hybridization with the RNA probe (10-μm sections) and immunoperoxidase staining with the anti-PGD synthase antibody (40-μm sections). Nonpyramidal cells in layers I and II of the neocortex show the immunoreactivity (e) but no signals for the transcript (a). (a, e, bar = 100 μm; b–d, f–h, bar = 20 μm.)
confined to any specific area. The immunoreactivity was occasionally observed in somata and dendrites of nonpyramidal neurons (Fig. 2e) and apical dendrites of pyramidal neurons in the superficial part of the cortex. No signals for the mRNA of the enzyme were, however, detected in these cells (Fig. 2a). Essentially the same pattern of immunostaining was observed with either the rabbit polyclonal or mouse monoclonal antibody for the enzyme. No immunoreactivity was observed with nonimmunized mouse IgG, nonimmunized rabbit IgG, or with the polyclonal antibodies preabsorbed with excess amounts of the purified enzyme.

PGD synthase activity was remarkably enriched in the isolated leptomeninges (14.2 nmol per min per mg of protein) and choroid plexus (7.0 nmol per min per mg of protein) of the adult rat brain, as compared with the activity in the whole brain (2.0 nmol per min per mg of protein) (Table 1). Furthermore, enzyme activity in the cerebral cortex (1.1 nmol per min per mg of protein) decreased to \( \approx 50\% \) after careful removal of the leptomeninges, becoming lower than the activity in the white matter (0.8 nmol per min per mg of protein).

These results, taken together, clearly indicate that PGD synthase is predominantly present in the leptomeninges and choroid plexus, as well as in oligodendrocytes, in the adult rat brain.

**DISCUSSION**

During the past 10 years, we have shown that PGD\(_2\) is the major endogenous sleep-regulating substance in the brain of rats, monkeys, and probably humans, and that its site of action is near or in the preoptic area (1). However, the major site of synthesis of PGD\(_2\) has not yet been clearly demonstrated. In this study, mRNA for PGD synthase was shown to be predominantly expressed in the leptomeninges, choroid plexus, and oligodendrocytes in the adult rat brain. PGD synthase-like immunoreactivity was also demonstrated mainly in these tissues and cells. Furthermore, enzyme activity was enriched in these tissues. The experimental evidence, therefore, strongly indicates that PGD synthase is present mainly, if not exclusively, in choroid plexus, leptomeninges, and oligodendrocytes, and that PGD\(_2\) is produced mostly in these tissues and cells.

We demonstrated previously that PGD synthase is a member of the lipocalin superfamily (8–10). Most lipocalins are soluble secretory proteins, and bind and transport small lipophilic molecules (11). PGD synthase is the only known exception, being an enzyme rather than a lipophilic ligand-carrier protein. Earlier we characterized PGD synthase as a membrane-associated enzyme, because the enzyme is N-glycosylated, has a putative signal sequence (7), and is immunoelectron microscopically located on the rough-surfaced endoplasmatic reticulum and outer nuclear membrane of oligodendrocytes (6). These characteristics are also shared by various secretory proteins. Human cerebrospinal fluid contains a significant PGD synthase activity (10–80 nmol per min per mg of protein; T. Suzuki, K. Watanabe, S. Ito, and O.H., unpublished observation). Zahn et al. (16) recently demonstrated that the protein termed \( \beta \)-trace, a major constituent of human cerebrospinal fluid, shows a high degree of homology in its N-terminal 28 amino acids with the corresponding sequence of the rat and human PGD synthase (68% and 93% identity, respectively). Together with two previous papers (17, 18), imply that \( \beta \)-trace may possibly be closely related, if not exactly identical, to PGD synthase. The \( \beta \)-trace has already been shown immunohistochemically to be localized in oligodendrocytes in various species (19), which is consistent with our immunohistochemical studies with antibodies against PGD synthase in the rat brain (6). Although final identification of \( \beta \)-trace awaits the results of further experiments, we may conjecture that the major sites of expression and synthesis of \( \beta \)-trace are the leptomeninges and choroid plexus of the human brain, in addition to oligodendrocytes. Furthermore, Achen et al. (20) recently demonstrated that the major protein secreted by amphibian choroid plexus has the highest homology (41% identity and 86% similarity) with the rat and human PGD synthase among lipocalins so far identified. Thus, the enzyme may be secreted from those original sites of synthesis into the cerebrospinal fluid.

The immunoreactivity is often observed in somata and dendrites of stellate neurons and apical dendrites of pyramidal neurons in the superficial part of the cerebral cortex, although the mRNA for the enzyme was not detected in these cells (Fig. 2a and e). This difference might be due to the fact that the lower limit of detection of immunoreactivity is higher than that of the mRNA, although we used a highly sensitive method with the RNA probe for *in situ* hybridization. Alternatively, these results may be interpreted to mean that the enzyme was originally produced in the cells of leptomeninges, choroid plexus, and oligodendrocytes and that it may be secreted and transported through the cerebrospinal fluid to other parts of the brain, including certain neurons, where the supply of its substrate, PGH\(_2\), is presumably more abundant.

Several lines of experimental evidence presented in this paper and elsewhere, therefore, pose a number of intriguing and challenging hypotheses that should be critically evaluated. Is PGD\(_2\), a sleep regulator, produced mainly in the leptomeninges, choroid plexus, and oligodendrocytes? Leptomeninges and choroid plexus have long been believed to play a role in production of cerebrospinal fluid for the mechanical support and chemical homeostasis of the brain. However, a more positive function to provide a neuroendocrine pathway for communication through the cerebrospinal fluid within the central nervous system has also been suggested recently (21). Glucose utilization reportedly increases in the choroid plexus during slow-wave sleep (22). The presence of the sleep-promoting substance(s) in the cerebrospinal fluid has been shown by a number of previous investigators and suggests that a humoral mechanism is critical in sleep regulation (23–25). PGD\(_2\) concentrations were selectively and markedly elevated in the cerebrospinal fluid of the advanced-stage patients of African sleeping sickness (26). Thus, PGD\(_2\) may act not as a classical or typical neurotransmitter, but rather as a "neurohormone," circulating in the cerebrospinal fluid throughout the ventricles, extracellular space of the brain, subarachnoid space, and superior sagittal sinus. Secretion of PGD\(_2\) (or PGD synthase) into the cerebrospinal fluid from choroid plexus and leptomeninges may provide an efficient means for communicating its signal to target cells in the central nervous system. Alternatively, PGD

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Enzyme activity (nmol per min per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptomeninges</td>
<td>14.2 ± 2.0</td>
</tr>
<tr>
<td>Choroid plexus*</td>
<td>7.4 ± 6.5</td>
</tr>
<tr>
<td>Whole brain (with leptomeninges)</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>Cerebral cortex (with leptomeninges)</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Superficial part with leptomeninges</td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td>Deep part</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>White matter</td>
<td>0.8 ± 0.2</td>
</tr>
</tbody>
</table>

Values are shown as means ± SE of at least five determinations. *For each experiment, samples were pooled from 10 rats.
synthase, being a member of the lipocalin family, may serve not only to produce but also to transport PGD₂ and/or other lipophilic molecules.

Note Added in Proof. After submission of this manuscript, a full account of ref. 18 appeared (27).

We are grateful to Prof. Michel Jouvet (Université Claude Bernard), Prof. S. Inoue (Tokyo Medical Dental University), and Drs. F. I. Tsuji and K. Mori (Osaka Bioscience Institute) for critical reading of the manuscript and useful discussion. We also thank Mr. A. Uesugi (Kyoto University) for photographic help and Dr. A. Nagata (Tokyo University) and Dr. M. Igarashi (Osaka University) for technical assistance. O.H. is grateful to Prof. S. Nakanishi and Dr. R. Shigemoto (Kyoto University) for helpful suggestions, and to Dr. M. Mäder (Göttingen University) and Dr. C. Murphy (University of Tennessee Medical Center, Knoxville) for making unpublished data available and for many helpful discussions. This work was supported in part by research grants from the Ministry of Education, Science and Culture of Japan; the Yamanouchi Foundation for Research on Metabolic Disorders; and the Japan Academy.