Postnatal Changes in the Localization of Prostaglandin D Synthetase from Neurons to Oligodendrocytes in the Rat Brain*

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The enzymes responsible for the biosynthesis (6, 7) and degradation (8, 9) of PGD, are reported in rats. In our previous studies (16, 18), those two types of PGD synthetase were purified separately and their catalytic and immunological properties were critically examined.

In this present study, we characterized PGD synthetase in the brains of infant (2-week-old) and adult (8-week-old) rats and also examined its cellular distribution by immunohistochemical means with the specific antibodies. Our results indicate that the enzyme in the brain is the GSH-independent one and is biochemically and immunologically the same at both ages, and that the immunohistochemical localization shifts from neurons to mainly oligodendrocytes during maturation of the brain.

EXPERIMENTAL PROCEDURES

Materials—[1-14C]Arachidonic acid (52.9 mCi/mmol) was purchased from Du Pont-New England Nuclear. [1-14C]PGH, was prepared as described previously (19). PGD, PGE, and PGF, were generous gifts from Ono Central Research Institute (Osaka, Japan). Dithiothreitol and β-mercaptoethanol were obtained from Nakarai Chemicals, Kyoto, Japan. GSH was from Sigma. 1-Chloro-2,4-dinitrobenzene was from Tokyo Kasei, Tokyo, Japan. Rabbit antibodies against glial fibrillary acidic protein and carbonic anhydrase were from Immunon Inc. (Troy, MI) and Advance Co. (Tokyo, Japan), respectively. All other chemicals were of reagent grade.

Animals—Male Wistar rats were used in this study at 2 and 8 weeks after birth, when the specific activity of PGD synthetase in the brain is known to increase to its highest value and decrease to the plateau level, respectively (14).

Enzyme Purification—Rat brain PGD synthetase was purified by our previously reported method (17).

Preparation of Antibodies—Polyclonal antibodies against each GSH-independent and GSH-requiring PGD synthetase were raised in rabbits. Preparation procedures and characterization of these antibodies have been described previously (17, 18).

Two monoclonal antibodies (IgG 1) against GSH-independent PGD synthetase were obtained from BALB/c mice immunized with the purified enzyme by the cell-fusion technique with myeloma (NS-1) cells (20). In brief, the purified enzyme (30 µg) was emulsified in complete Freund's adjuvant (Difco) and injected intraperitoneally into female mice. Three weeks after the immunization, a booster was inoculated intraperitoneally. Four weeks after the booster, an additional booster with 10 µg of the enzyme in saline was injected

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1 The abbreviations used are: PG, prostaglandin; GSH, glutathione.
intravenously into mice whose serum was shown to be reactive with the enzyme. Three days after the injection, the spleen cells were fused with mouse myeloma cells.

Antibodies against rat brain PGD synthetase in the mouse serum or in the culture fluid of the hybridoma were detected by use of the dot immunoblotting test. The purified enzyme (10 ng) was spotted onto a nitrocellulose membrane (Bio-Rad) and incubated with the serum or culture fluid. The antibody bound to the enzyme was immunostained with biotinylated anti-(mouse IgG) antibody and Vectastain ABC Kit (Vector Laboratories, Inc., Burlingame, CA) reagents by the method of Hsu et al. (21).

Positive cell lines were cloned at least twice by the limiting dilution method. Ascites tumors were induced by the intraperitoneal injection of hybridoma cells into BALB/c mice. Immunoglobulin classes of the antibodies were identified by Ouchterlony double immunodiffusion with mouse and rat monoclonal typing kits (Serotec). The monoclonal antibodies were purified from ascites by use of an Affi-Gel Protein A MAPS kit (Bio-Rad). The specificity of the antibody was established on gel blots of the enzyme as described below.

Immunoblotting Analysis—Brains were homogenized with 3 and 2 volumes of distilled water in the case of 2- and 8-week-old rats, respectively. The homogenates were centrifuged at 100,000 X g for 1 h. After dialysis against distilled water at 4 °C overnight, the supernatants were subjected to two-dimensional electrophoresis by the method of O’Farrell (22). Proteins separated in the gels were electrophoretically blotted onto a hydrophobic Durapore (GVHP) membrane (Millipore Corp., Bedford, MA) and immunostained with the mouse antibody, biotinylated anti-mouse IgG antibody, and the Vectastain ABC kit.

Immunotitration—The brains were homogenized with 3 volumes of 10 mM potassium phosphate (pH 6.0) containing 0.5 mM dithiothreitol. The homogenate from rats of either age was centrifuged at 100,000 X g for 1 h, and the supernatant was obtained in which about 90% of the PGD synthetase activity in the brain homogenate was recovered. A given volume of the supernatant was incubated with various amounts of antibody at 4 °C for 2 h. In the case with the monoclonal antibody or nonimmunized mouse IgG, the mixture was further incubated with an excess amount (2 mg) of anti-mouse IgG antibody (Cappel) at room temperature for 30 min. The mixture was then centrifuged at 10,000 X g for 10 min to remove the immunocomplexes, and the resulting supernatant was recovered for determination of the residual PGD synthetase activity.

Immunohistochemistry—Under pentobarbital anesthesia, rats were perfused for 10 min through the left ventricle of the heart with Krebs-Ringer solution (1.5 volumes of the body weight), followed with the same volume of 2% paraformaldehyde in 0.1 M sodium phosphate (pH 7.5) at room temperature for 30 min. The brain was removed, cut into coronal slices of 2-mm thickness, and immersed in Bouin’s fixative (saturated picric acid solution: formalin: glacial acetic acid (15:5:1, v/v/v)) at 4 °C for at least 3 days. Subsequently, 40-μm-thick sections were prepared with a cryostat and incubated with an excess amount of the antibodies (10 μg/ml) at 4 °C overnight under a free-floating condition. The sections were then immunostained by the avidin-biotin-peroxidase complex method (21). Control staining was performed with the polyclonal antibody (20 μg/ml) preabsorbed with the purified enzyme and with the IgG fraction (20 μg/ml) obtained from nonimmunized rabbits or mice.

For electron microscopy, the immunoperoxidase staining procedure was performed on sections of 100-μm thickness, which were prepared with a Vibratome after fixation at 4 °C overnight in Zamboni’s solution (2% paraformaldehyde and 25% saturated picric acid in 0.1 M sodium phosphate, pH 7.2). The staining intensity under these conditions was significantly lower at both ages than that with the avidin biotin-peroxidase complex described above, such that the immunoreactive deposit was detected only in adult (8-week-old) rats.

Enzyme Assays—The PGD synthetase activity was determined with 40 μM [1-14C]PGH2 as reported previously (17, 18). In this study, the enzyme reaction was performed in 0.1 M Tris-HCl (pH 8.0) containing 1 mM GSH to determine both GSH-independent and GSH-requiring PGD synthetase activities. When the requirement of the enzyme for sulfhydryl compounds was examined, the enzyme solution was dialyzed at 4 °C overnight against 10 mM potassium phosphate buffer (pH 7.0) containing 150 mM NaCl and used for the assay. Protein concentration was determined by the method of Lowry et al. (23) using bovine serum albumin as standard.

RESULTS

Biochemical and Immunological Characterization of PGD Synthetase in the Brains of 2- and 8-Week-old Rats—After perfusion of rats at 2 and 8 weeks after birth, crude extracts of the brain were prepared and their PGD synthetase was assessed. The total and specific activities in infant (2-week-old) rats (153.4 nmol/min/brain and 2.8 nmol/min/mg of protein) were 1.2- and 1.5-fold higher, respectively, than those in adult (8-week-old) rats. When crude extracts at these ages were mixed, the PGD synthetase activity was additive; i.e. neither activation nor inhibition was found. At both ages, the crude extract showed the same PGD synthetase activity in the presence of either 0.5 mM dithiothreitol, 1 mM 3-mercaptopropanethiol, or 1 mM GSH, and no significant GSH-requiring enzyme activity was detected. However, PGD synthetase in the brain at these ages was almost inactive without those sulfhydryl compounds in the assay mixture (<0.1 nmol/min/mg of protein). The activity in crude extracts was almost completely inhibited by 1 mM 1-chloro-2,4-dinitrobenzene. The K_m value for PGH2 determined in the crude extracts was about 20 μM. These properties are the same as those of GSH-independent PGD synthetase (17) and clearly different from those of GSH-requiring enzyme (18).

When we purified PGD synthetase separately from the brains of 2- and 8-week-old rats, the enzymes at both ages were biochemically indistinguishable as judged by K_m for PGH2 (~20 μM), requirement of sulfhydryl compounds, M, (26,000), and pi values (4.35~4.75) of the final preparations. During purification, we found that the purified PGD synthetase showed microheterogeneity as evidenced by bands with slightly different pi values (4.35, 4.55, 4.60, 4.70, and 4.75). However, the specific activity of each component was almost the same even at both ages (data not shown).

In the Ouchterlony test (Fig. 1), the polyclonal antibody against the GSH-independent enzyme showed a linear immunoprecipitin line against both the purified enzyme and crude extracts of the brains at both ages. In immunoblotting tests with the polyclonal antibody after two-dimensional elec-

![Fig. 1. Ouchterlony double immunodiffusion test. Center well contained polyclonal antibody against GSH-independent PGD synthetase. Well 1, purified enzyme obtained from 4-week-old rats, as previously reported (17); well 2, crude extract of the brain of 2-week-old rats; well 3, crude extract of the brain of 8-week-old rats; well 4, IgG fraction obtained from nonimmunized rabbits.](image-url)
bands were seen at the same positions with the same profiles as those of the purified enzyme. Identical results were obtained when monoclonal antibody-1 was used. In the case of monoclonal antibody-2, in immunoblots made after isoelectric focusing of the crude extracts obtained from rats at both ages, the positive bands were seen at the same positions with the same profiles as the crude extracts obtained from rats at both ages, the positive bands were seen at the same positions with the same profiles as those of the purified enzyme (data not shown). However, the antigenic determinant of this antibody was apparently inactivated by incubation with sodium dodecyl sulfate, for no immunoreactive spot found in the immunoblot after two-dimensional electrophoresis (data not shown).

In immunotitration with the polyclonal antibody and monoclonal antibody-1 specific for GSH-independent enzyme (Fig. 3), the PGD synthetase activity in crude extracts of the brain dose-dependently decreased to about 20% of the initial activity and showed identical titration curves at these different ages. Identical titration curves at both ages were also observed by titration with monoclonal antibody-2. In this case, however, about 60% of the PGD synthetase activity could not be absorbed by the maximum amount of antibody we examined, indicating that the affinity of monoclonal antibody-2 is significantly lower than those of the polyclonal antibody and monoclonal antibody-1. On the other hand, the activity remained unchanged when titrated with antibody against GSH-requiring enzyme or the IgG fraction obtained from nonimmunized rabbits or mice.

These results both confirm the specificity of the antibodies and indicate that PGD synthetase in the brain at these different ages is biochemically and immunologically the same.

Immunohistochemical Localization of PGD Synthetase in the Brains of 2- and 8-week-old Rats—When the polyclonal and monoclonal antibodies were utilized for immunoperoxidase staining to reveal the cellular localization of PGD synthetase in the brains at both ages, intensely positive staining was observed. In contrast, no positive stain was detected with the absorbed antibody, nonimmunized IgG, and antibody against GSH-requiring PGD synthetase (data not shown).

Although the immunostaining was more intense with the polyclonal antibody and monoclonal antibody-1 than with the monoclonal antibody-2, the profile or distribution of the positive staining at each age was the same with all three antibodies specific for GSH-independent PGD synthetase. However, the localization of the immunoreactivity of this enzyme was quite different between these two ages. The results obtained with monoclonal antibody-1 are shown in Fig. 4.

In 2-week-old rats (Fig. 4a and c), intensely positive staining was seen in neural somata, dendrites, and proximal portions of axons of pyramidal and stellate cells in layers II-VI of the cerebral cortex; but no cell bodies were stained in layer I. In layers II-VI, the immunoreactive neurons constituted about 20% of the neurons viewed in an adjacent Nissl-stained section. Stained cells were widely distributed in the neocortex and in the pyriform and cingulate cortical regions. Intensely or moderately stained neurons were also found in the amygdala, hippocampus, lateral hypothalamic area, and rostral portion of the caudate putamen. Only a few immunoreactive neurons were seen in the brain stem, cerebellum, and spinal cord.

In 8-week-old rats (Fig. 4b, d, and e), the immunoreactivity was scarcely detected in neural cells except in some stellate neurons in layer I (Fig. 4d) and in the superficial part of layer II of the neo- and mesocortices. Several clusters of immunoreactive dendrites were also seen in the pyriform cortex. At this age, the immunoreactivity was mainly seen in small non-neuronal cells having a few fine processes; their soma diameter was about 10 μm (Fig. 4e). These small immunoreactive cells were distributed throughout the brain and spinal cord, more densely in the white matter than in the gray. In sections doubly stained with antibodies against PGD synthetase and glial fibrillary acidic protein (a marker of astrocytes), no cells showed both immunoreactivities simultaneously. However, in sections stained with antibodies against PGD synthetase and carbonic anhydrase (a marker of oligodendrocytes), these two immunoreactivities were observed with a high frequency (>90%) in the same glial cells. From the morphological characteristics, distribution profiles, and coexistence of a marker-enzyme, the immunoreactive cells were considered to be oligodendrocytes.

**Fig. 2.** Immunoblotting with polyclonal antibody against GSH-independent PGD synthetase after two-dimensional electrophoresis of the purified enzyme (a) and crude extracts of the brain of 2 (b) and 8 (c)-week-old rats. First dimension, isoelectric focusing (IEF) in 5% polyacrylamide gel containing 2% Ampholine, pH 3.5-10.0, and 1% Ampholine, pH 4.0-6.0; second dimension, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (12.5% gel).

**Fig. 3.** Immunotitration test of the PGD synthetase activity in the brains of 2 (open symbols)- and 8 (closed symbols)-week-old rats. Symbols represent the polyclonal antibody (O, ◦), monoclonal antibody-1 (△, □), and monoclonal antibody-2 (V, ▼) specific for GSH-independent PGD synthetase, polyclonal antibody against GSH-requiring PGD synthetase (□, ◦), nonimmunized rabbit IgG (●, ○), and nonimmunized mouse IgG (●, ○). The residual enzyme activities are expressed as percentages of those before incubation (2.0 nmol/min).
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FIG. 4. Immunoperoxidase staining with monoclonal antibody for GSH-independent PGD synthetase in coronal sections (40 μm) of the parietal cortex of 2 (a and c) and 8 (b, d, and e)-week-old rats. a and b, low-power views; c, immunoreactive pyramidal and stellate neurons in layer V of 2-week-old rat; d, immunoreactive stellate neurons in layer I of adult rat; e, immunoreactive oligodendrocytes in layer III of adult rat. At higher magnifications (c–e), positively stained cells are not in the same focal plane. Bars represent 100 μm.

Electron microscopy (Fig. 5) revealed that the immunoreactive cells had scanty cytoplasm and contained a small oval nucleus without indentation and that no synaptic terminals were associated with these cells. The immunoreactivity appeared to be localized in the rough-surfaced endoplasmic reticulum and outer nuclear membrane of the glial cells. However, no electron-dense staining was detected on the plasma membrane or mitochondria.

DISCUSSION

In this study, by critical examination of catalytic (—SH requirement, $K_a$ for PGH$_2$, and inhibition by 1-chloro-2,4-dinitrobenzene), molecular ($M$ and pl values), and immunological properties (Figs. 1–3) of PGD synthetase in the brains of infant (2-week-old) and adult (8-week-old) rats, we demonstrated that the enzyme at both ages was the same as the GSH-independent enzyme which we previously purified and characterized (17). On the other hand, no significant activity of GSH-requiring enzyme was detected in crude extracts of the brain-independent enzyme activity at each age was found to be independent and neither activation nor inhibition was observed. Furthermore, the activity at both ages showed identical immunotitration curves (Fig. 3). Therefore, it appears that the difference noted in the enzyme activity in the brain between both ages is due to that of the content of GSH-independent enzyme.

Although PGD synthetase in the brain at both ages is the same, the immunohistochemical distribution, as revealed by the use of three kinds of antibody specific for this enzyme, was markedly different between the two ages: the enzyme was detected in many neurons in 2-week-old rats, but was mainly localized in oligodendrocytes in adult animals (Fig. 4). Previously, several groups have investigated the cellular localization of PGD synthetase activity in the brain. Gerritsen and Printz (24) reported that the enzyme activity was specifically localized in the capillary microvessels obtained from the cerebral cortex of adult rats. However, no significant activity of the enzyme was detected in the capillary microvessels obtained from the cerebral cortex of adult rats. However, no significant activity of the enzyme was detected in the same preparation by Reinhard et al. (25) and by Goehlert et al. (26). On the other hand, by Seregi et al. (27) and Keller et al. (28), PGD$_2$ was shown to be actively synthesized in astrocytes in the primary culture prepared from the cerebral hemisphere of newborn rats. However, we could not detect PGD synthetase immunohistochemically in the microvessels and astrocytes of either 2- or 8-week-old rats (Fig. 4).

For light microscopic immunohistochemistry, the brain was thoroughly perfused with 2% paraformaldehyde, cut into 2-mm-thick slices, and then immersed in a relatively strong fixative, Bouin’s solution. After fixation for over 3 days in this solution, positively stained cells were evenly distributed in transverse sections of the slices at both ages, indicating that the fixative penetrates into the tissue enough to immobilize the enzyme in such relatively thick slices. The profiles of the immunostaining were unchanged even when the tissues were fixed for a longer period, even up to 10 days. Further-
more, in immunoblotting analysis after incubation of the blot of crude extracts of the brain with the fixative, the antigenicity of the enzyme remained almost the same with extracts made at both ages. Therefore, the immunohistochemical distribution of the enzyme at both ages is thought to reflect the true distribution of the enzyme in the tissue and not an artifact by fixation.

By electron microscopic immunocytochemistry, the immunoactivity was observed to be associated with rough-surfaced endoplasmic reticulum and outer nuclear membrane of the glial cells in the adult rats (Fig. 5), indicated that PGD synthetase is, at least in part, a membrane-associated enzyme. This conclusion is also supported by the subcellular distribution of the PGD synthetase activity reported previously (15), in which about 30% of the total activity was recovered in the membrane (P2 + P3) fractions. The immunocytochemical localization of this enzyme is very similar to that of cyclooxygenase reported by Rollins and Smith (29). Although further studies on the localization of cyclooxygenase in oligodendroglia is necessary, the identical distribution of these two enzymes suggests a functional coupling of cyclooxygenase and PGD synthetase. Since the active site of cyclooxygenase is reportedly oriented toward the cytoplasmic surface of the endoplasmic reticulum (30), the same location of these two enzymes has advantages for immediate catalysis of the unstable endoperoxide, \( \text{PGH}_2 \), to PGD in the cell.

Although the physiological function of PGD in the brain is still unclear, PGD may play different roles in these cells at each stage of their development. In 2-week-old rats, the immunoactivity of this enzyme was transiently concentrated in many neurons of the cortex (Fig. 4, a and c). At this age, the cortical neurons actively develop their dendrites and axons to form the neural network. Interestingly, neuroblastoma (N1E-115) or hybridoma (NG-108) cells develop neurite-like structures by the addition of PGD into the culture medium (31). Thus, PGD might function in the growth of neuronal processes. In the adult rats, the immunoactivity was not detected in neurons in most regions of the brain; however, a few positive neurons and dendrites were seen in certain regions, such as layers I–II of the cerebral cortex (Fig. 4d) and the pyriform cortex, in which enrichment of PGD-binding activity has been observed (13). PGD, therefore, might participate in central nervous functions located in these regions in the adult rat. However, the immunoactivity of PGD synthetase in the brain and spinal cord of adult rats was mainly localized in oligodendrocytes (Fig. 4b and e). Therefore, PGD may play important roles in neuron-glia interactions or in several glial functions such as myelin maintenance and its metabolism.

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