Colocalization of prostaglandin F$_{2\alpha}$ receptor FP and prostaglandin F synthase-I in the spinal cord

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Running title: Immunohistochemistry of FP in the spinal cord
**Abbreviations:** ABC, avidin-biotin complex; CLSM, confocal laser scanning light microscopy; COX, cyclooxygenase; PG, prostaglandin; PGFS, prostaglandin F synthase
ABSTRACT

Prostaglandin F$_{2\alpha}$ is synthesized by prostaglandin F synthase, which exists in two types, prostaglandin F synthase I (PGFS I) and prostaglandin F synthase II (PGFS II). Prostaglandin F$_{2\alpha}$ binds to its specific receptor, FP. Our previous immunohistochemical study showed the distinct localization of prostaglandin F synthases in rat spinal cord. PGFS I exists in neuronal somata and dendrites in the gray substance, and PGFS II exists in ependymal cells and tanycytes surrounding the central canal. Both enzymes are also present in endothelial cells of blood vessels in the white and gray substances of the spinal cord. In the present study, we found that FP localizes in neuronal somata and dendrites, but not in ependymal cells, tanycytes, or endothelial cells. Immunohistochemical analysis of serial sections showed the colocalization of FP and PGFS I. FP immunoreactivity was intense in spinal laminae I and II of the dorsal horn, a connection site of pain transmission, and was similar to that of PGFS I in neuronal elements. These findings suggest that prostaglandin F$_{2\alpha}$ synthesized in the neuronal somata and dendrites exert an autocrine action there.
INTRODUCTION

In the central and peripheral nervous systems, prostaglandin (PG) F2α plays unique roles in various physiological and pharmacological activities such as pain transmission (allodynia) in the spinal cord of conscious mice (1) and induction of depolarization in the postsynaptic actions of cerebellar Purkinje cell dendrites (2). These actions of PGF2α are controlled by its synthesis and binding site. PGF2α is synthesized by PGF synthase (PGFS) (EC 1.1.1.188) and binds to a specific receptor, FP.

PGFS exists as three isozymes: PGFS I (formerly lung-type PGFS), PGFS II (formerly liver-type PGFS) (3, 4), and prostamide/prostaglandin F synthase (5). The first two isozymes, PGFS I and PGFS II, belong to the aldo–keto reductase superfamily based on substrate specificity, molecular weight (~37 kDa), and amino acid sequences. In arachidonate metabolism, PGFS I and PGFS II catalyze two reductions of PGH2 to PGF2α and PGD2 to 9α,11β-PGF2. PGH2 is synthesized from arachidonate by cyclooxygenase (COX), and PGD2 is synthesized from PGH2 by PGD synthase. The isozymes have different Km values for PGD2, i.e., 120 µM for PGFS I and 10 µM for PGFS II. Prostamide/prostaglandin F synthase was identified recently as a new type of the enzyme that belongs to the thioredoxin-like superfamily (5). Prostamide/prostaglandin F synthase catalyzes both the reduction of prostamide H2 to prostamide F2α and that of PGH2 to PGF2α. However PGD2 does not serve as a substrate for the synthase.

PGF2α is one of the major prostanoids produced in the rat central nervous system, including the spinal cord (6), where PGF synthase activity is also detected (7). We have reported on the existence and localization of PGFS I and PGFS II in the rat...
spinal cord. Our previous immunocytochemical study of PGFS I in the rat spinal cord showed that the immunoreactivity was distributed widely in the gray substance and was especially strong in neuronal dendrites in laminae I and II of the dorsal horn, and in lamina IX of the ventral horn (8). In contrast, PGFS II was not found in the neuronal elements and but was found in ependymal cells and tanycytes surrounding the central canal (9). Both enzymes were found in endothelial cells of blood vessels in the rat spinal cord. To clarify the biological relevance of the distinct localization of PGFS I and PGFS II, the binding site of PGF$_{2\alpha}$ (i.e., localization of the PGF$_{2\alpha}$ receptor, FP) must be identified. FP transduces the PGF$_{2\alpha}$ signal by coupling with the G$_q$ protein (10). In FP-deficient mice, the targeted allele is expressed highly in the corpus luteum of the ovary and in the distal tubules of the kidney (11, 12). However, no one has reported on the morphological analysis of FP in the central nervous system. In the present study, we demonstrate the localization of FP in the rat spinal cord using a specific antibody.
MATERIALS AND METHODS

Animals and anesthesia

Twelve male and 12 female specific pathogen-free Wistar rats (6–8 weeks old, weighing 150–180 g; provided by Japan SLC Inc., Shizuoka, Japan), 2 male FP-deficient mice (11), and 2 male C57BL/6 mice (SLC, Inc.) (9 weeks old, weighing 23–27 g) were used in the present study. The animals were anesthetized deeply with sodium pentobarbital (100 mg/kg of body weight) before the subsequent procedures. All protocols were approved by the Exclusive Committee on Animal Research at The University of Tokushima and Okayama Prefectural University and the research was conducted in conformity with the Public Health Service (PHS) policy.

Western blot analysis

Spinal cords from male and female rats and the uteri were homogenized in a lysis buffer containing 25 mM Tris (pH 8.0), 1 mM EDTA, 0.5 mM DTT, 10 mM MgCl₂, 1% protein inhibitor cocktail (Sigma Chemical Co., St Louis, MO), and 0.25 M sucrose, and centrifuged at 800 × g for 15 min at 4°C. The homogenate was centrifuged at 100,000 × g for 1 h at 4°C, the supernatant was removed, and the expression of FP was measured in the precipitate. The protein concentration of each fraction was measured using a BCA protein assay reagent kit (Pierce, Rockford, IL). Forty micrograms of each precipitate fraction was subjected to electrophoresis in a 10–20% SDS-polyacrylamide gel (Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan). The bands were transferred electrophoretically to a Hybond-P polyvinylidene difluoride membrane (Amersham Biosciences, Buckinghamshire, UK). The membrane was blocked with nonspecific binding with Block Ace (Dainippon Seiyaku,
Osaka, Japan) and incubated at 4°C overnight with rabbit anti-FP IgG (catalogue no. 101 802, lot no. 111553-111554, Cayman Chemical Co., Ann Arbor, MI) diluted to 1:200. The anti-FP IgG is a polyclonal antibody against murine FP receptor amino acid 2-16 (SMNSSKQPVSPAAGL). The specific bands that cross-reacted with the anti-FP IgG were confirmed using the antigen-absorbed anti-FP IgG diluted to 1:200. The antigen was a purified peptide coded murine FP receptor amino acid 2-16 (catalogue no. 301 802, Cayman Chemical Co.). The immunoreactive proteins were visualized using BM chemiluminescence Western blotting kit (Roche, Penzberg, Germany).

**Immunohistochemistry**

Anesthetized male and female rats were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and the spinal cords were removed. The spinal cords were cut into 2–3 mm thick transverse slices and immersed in the same fixative solution for 6 h at 4°C. Fifty micrometer-thick sections were cut serially on a Microslicer (Dosaka Inc., Osaka, Japan), rinsed for 1–2 h in PBS, and processed for subsequent immunocytochemistry.

For single immunolabeling for PGFS I or FP, the sections were rinsed in PBS and treated with 3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. Nonspecific binding was blocked with blocking buffer containing 12.5% Block Ace in PBS at room temperature for 3 h, and the sections were incubated with rabbit anti-PGFS I antiserum provided by Dr. Kikuko Watanabe (University of East Asia, Shimonoseki, Japan) diluted to 1:5,000 at 4°C overnight or with rabbit anti-FP IgG diluted to 1:400 at 20°C for 48 h. The sections were treated with biotinylated anti-rabbit IgG diluted to 1:200 (Vector Laboratories, Burlingame, CA) at 32°C for 2
h, and then with ABC (avidin-biotin complex) Elite kit (Vector Laboratories) at 32°C for 1 h. The immunoreactivity was visualized in 50 mM Tris (pH 7.6) containing 0.01% 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.01% hydrogen peroxide at 37°C, and examined with a light microscope (Nikon Optiphoto II; Nikon Co., Tokyo, Japan). For controls, some sections were incubated with the antigen-absorbed anti-FP IgG diluted to 1:400. The specificity of anti-FP IgG was immunohistochemically confirmed using spinal cord sections (50 µm thickness) of C57BL/6 and FP-deficient mice. The sections were subjected to a single immunostaining by enzyme antibody method as described above. To confirm the co-localization of FP and PGFS I, two serial sections (10-20 µm thickness) were subjected to single immunostaining using each antibodies. The cut surface of one section was immunostained using anti-FP IgG, and that of the other was done using anti-PGFS I antiserum.

For multiple immunostaining for confocal laser scanning light microscopy (CLSM), the sections were incubated with a mixture of the rabbit anti-FP IgG diluted to 1:200 and mouse monoclonal anti-microtubule-associated protein 2 (MAP2) (a marker of neuronal dendrites) IgG (Sigma) diluted to 1:10,000 or mouse monoclonal anti-vimentin (a marker of ependymal cells and tanyocytes in the adult rat spinal cord diluted, Zymed Laboratories, San Carlos, CA) antibody to 1:10,000 at 20°C for 48 h. For double immunostaining with anti-FP and anti-MAP2 or anti-vimentin antibodies, the sections were incubated with a mixture of Cy3-labeled donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted to 1:200 and FITC-labeled donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) diluted to 1:200 at room temperature for 2 h. For multiple fluorescein-staining with anti-FP, anti-MAP2, and Lycopersicon esculentum lectin, the sections were incubated
with a mixture of Cy3-labeled donkey anti-rabbit IgG diluted to 1:200, Cy5-labeled donkey anti-mouse IgG diluted to 1:200, and fluorescein-labeled *Lycopersicon esculentum* lectin (tomato lectin, a marker of endothelial cells, Vector Laboratories) diluted to 1:500 at room temperature for 2 h.

The sections were analyzed using CLSM, (Radiance 2000, Bio-Rad Laboratories, Inc., Hercules, CA) on the light microscope (Nikon Eclipse E800) at 488 nm and 568 nm wavelengths for excitation with the appropriate filter sets. These immunocytochemical procedures generally followed those in our previous studies (8, 9).
RESULTS

Expression of FP in rat spinal cord

Expression of FP in the rat spinal cord was investigated by Western blot analysis (Fig. 1). The single protein band of FP (~64 kDa) was detected in rat spinal cord and uterus as a positive marker. The protein band was not detected with the antigen-absorbed anti-IgG.

Localization of FP in the rat spinal cord

To examine the distribution of FP in the rat spinal cord, transverse sections were subjected to single immunostaining (Fig. 2). FP immunoreactivity was distributed diffusely in the gray substance of the rat spinal cord and was especially intense in laminae I, II, and IX. The immunoreactivity was similar to the distribution of PGFS I shown in our previous report (8).

At higher magnification (Fig. 3), immunoreactivity was found in the neuronal somata and dendrites at all segmental levels. However, it was not found in the vascular endothelium in the whole spinal cord or in the ependymal layer surrounding the central canal in lamina X. The immunoreactivity was stronger in dendrites than in the somata. The immunoreactivity of FP in the somata and dendrites was similar to that of PGFS I (8). Some colored dots in the white substance (Fig. 3A) were also observed in the section treated with antigen-absorbed anti-FP IgG (Fig. 3E), and we consider this to indicate a nonspecific reaction. No immunoreactivity was observed in the gray matter of the control sections (Fig. 3F). To confirm the specificity of anti-FP IgG, the immunohistochemistry was carried out in C57BL/6 and FP-deficient mice spinal cords (Fig. 4). The intense immunoreactivity was found in gray substance of C57BL/6 mouse spinal cord while the positive immunoreaction was disappeared in
FP-deficient mouse. The immunoreactivity in C57BL/6 mouse spinal cord was similar to that in rat spinal cord. These data indicated the specificity of the FP-immunoreactivity in the spinal cord. We also confirmed the colocalization of FP and PGFS I, as shown in each single immunostaining, in separated serial sections (Fig. 5), which showed that the same neuronal somata and dendrites were immunostained by both of the antibodies.

**Multiple immunofluorescent staining**

We performed double immunostaining with anti-FP and anti-MAP2 antibodies to confirm the immunoreactive elements in the neuronal somata and dendrites. CLSM images indicated that all FP-immunoreactive cells were also immunoreactive for MAP2 (Fig. 6). The colocalization pattern of FP and MAP2 was similar to that of PGFS I and MAP2 (8), suggesting that FP colocalizes with PGFS I in the rat spinal cord. The fluorescence intensity of MAP2 was relatively homogeneous in the whole region of the gray substance but that of FP was higher in laminae I and II of the dorsal horn. In additional multiple staining, FP colocalized with MAP2 but not with tomato lectin, a marker of endothelial cells (Fig. 6C). In the ependymal cells and tanycytes, FP did not colocalize with vimentin (Fig. 6D).
DISCUSSION

Western blot analysis of the rat spinal cord showed FP expression and a specificity of the antibody detecting the single band at ~64 kDa. Immunohistochemical analysis showed intense FP-immunoreactivity in the dorsal horn, which was similar to PGFS I-immunoreactivity. The dorsal horn is a site of a pain transmission, and our result supports other reports on the physiological role of PGF$_{2\alpha}$ as a pain modulator (1). Muratani et al. (13) reported that FP-expressing cells appear in the deeper layer (lamina III and deeper) of the dorsal horn of the spinal cord in conscious mice. Their findings differ slightly from our immunohistochemical data. Using naive rats, we found intense FP expression in the superficial layer of the dorsal horn (laminae I and II). The difference between their physiological data and our morphological data might reflect differences in the experimental approaches and animals. Muratani et al. characterized the distribution of FP-expressing cells according to their sensitivity to PGF$_{2\alpha}$ by measuring intracellular free calcium concentration in spinal cord slices of the mouse after induction of allodynia. The FP-expressing cells were characterized functionally as those cells responsive to both PGF$_{2\alpha}$ and N-methyl-D-aspartate. Their results may reflect the particular pharmacological condition, and maybe suggesting that the localization or the expression level of FP is changed in this kind of pathological condition.

A new type of prostamide/prostaglandin F synthase has been identified recently that catalyzes the conversion of prostamide H$_2$ to prostamide F$_{2\alpha}$, and PGH$_2$ to PGF$_{2\alpha}$ but does not use PGD$_2$ as a substrate (5). The prostamide/prostaglandin F synthase is highly expressed in mouse brain and spinal cord, and immunohistochemical study showed that the enzyme localizes to the superficial layer of the dorsal horn and in the...
motor neurons of the ventral horn (5). The immunoreactivity of prostamide/prostaglandin F synthase seems to be intense in the somata of the motor neurons. Additionally, the enzyme also localizes in glia of the white substance differing from the localization of PGFS I and FP. It is possible that prostamide/prostaglandin F synthase also acts with PGFS I to synthesize PGF$_{2\alpha}$ in the neurons of the gray substance, although its intracellular localization might differ from that of PGFS I in the neurons of the spinal cord. Under basal conditions, COX-2 exists in the neurons of all spinal laminae, particularly laminae I, II, in laminae III–VI and X, and in motor neurons but not in glial cells in the gray substance where neurons contain some COX-1 (14, 15). Together with our previous and present data indicate that the complete synthesis pathway from arachidonic acid to PGF$_{2\alpha}$ and binding sites are present morphologically in the neurons including motor neurons and interneurons in the spinal cord. PGFS I and FP also colocalize in neurons in other brain regions such as the hippocampus and cerebellum (unpublished data). COX-2 is expressed constitutively in the rat hippocampus, and its expression is upregulated by kainic acid, which induces seizure. Kainic acid treatment stimulates the production of large amounts of PGF$_{2\alpha}$, and this production is inhibited by the selective COX-2 inhibitor, NS398 (16). This suggests that the PGF$_{2\alpha}$ synthetic pathway through COX-2 and PGFS I is present and that PGF$_{2\alpha}$ acts in an autocrine fashion in some region of brain.

Previous studies demonstrate that specific inhibitors of COX-2, SC236, and celecoxib decrease the production of prostaglandins, proinflammatory cytokines, reactive oxygen species, and free radicals, and so significant protection against the loss of spinal motor neurons in amyotrophic lateral sclerosis (ALS) (17, 18). These studies implicate the prostaglandins produced by COX-2 as a mediator of both excitotoxic and inflammatory processes, which cause motor neuron death. COX-2
activity contributes to neuronal toxicity in hippocampal and cerebral ischemia (19, 20). Increased COX-2 activity may also contribute to neuronal death in the neuronal degeneration of Parkinson’s (21, 22) and Alzheimer’s (23) diseases. It is unclear which prostaglandin produced by COX-2 is a trigger of neuronal death. Administration of PGF$_{2\alpha}$ into the spinal cord causes significant cell loss and increases the extracellular levels of hydroxyl radicals and malondialdehyde, an end product of membrane lipid peroxidation (24). The concentration of PGF$_{2\alpha}$ measured by microdialysis sampling increases immediately in experimental impact injury to the rat spinal cord. On the other hand, another major prostaglandin, PGE$_2$, paradoxically protects motor neurons in the model of ALS (25) and cerebral ischemia (26, 27). Activation of the isoforms of the PGE$_2$-specific receptors EP1-EP4, EP2, and EP3 protect motor neurons in the spinal cord, and EP2 does the same in hippocampal neurons. Thus, the balance between PGE$_2$ and PGF$_{2\alpha}$ production and activation of the specific receptors might be involved in neuronal plasticity.

Until now, the morphological localization of the specific receptor of PGF$_{2\alpha}$, FP, has not been analyzed accurately in the central nervous system. Our experiments show that FP localizes in the rat spinal cord and that FP colocalizes with one of the PGF$_{2\alpha}$ synthases, PGFS I. These results suggest that FP or PGFS I play a role in controlling neuronal homeostasis. Other approaches, such as electrophysiology, are needed to confirm the physiological roles of PGFS I and FP in the neurons of the spinal cord.
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REFERENCES


FIGURE LEGENDS

Figure 1. Western blot analysis of FP in rat spinal cord. Rat uterus (lane 1) and spinal cord (lane 2) microsomal fractions centrifuged at 100,000 × g (40 µg of protein) were used as the protein source and applied on 10–20% SDS-polyacrylamide gel. These were analyzed using anti-FP IgG (A) or antigen-absorbed antibody (B). The analyses were performed under the same condition as described in the text. The positive band in the spinal cord using anti-FP IgG migrated to a position of ~60 kDa, which is visible in a same position in the uterus, used as a positive control.

Figure 2. Immunohistochemistry of FP and PGFS I in a transverse section of rat spinal cord. In the observation with Nomarski differential interference microscope of single immunostaining, the coloration of the DAB reaction indicate the localization of FP and PGFS I. The scale bar represents 500 µm.

Figure 3. Immunohistochemistry of FP in rat spinal cord. A: Transverse section immunostained for FP. B: Lamina X surrounding the central canal. C: The dorsal gray column at higher magnification. D: Immunoreactivity in lamina IX of the ventral gray column. E: A section treated with antigen-absorbed anti-FP IgG as a negative control. F: Higher magnification of lamina IX of a part of the image shown in E. The immunoreactive neuronal somata (large arrowheads) and dendrites (arrows) appear. Small arrowheads and asterisk indicate blood vessels and the central canal, respectively. The scale bars represent 500 µm in A and E, 200 µm in C, and 100 µm in B, D, and F.

Figure 4. Immunohistochemistry of FP in C57BL/6 mouse and FP-deficient mouse spinal cords. The specificity of the anti-FP IgG was confirmed using
C57BL/6 mouse (A-C) and FP-deficient mouse (D-F). A and D: Transverse section immunostained for FP. B and E: Immunoreactivity in lamina IX of the ventral gray column. C and F: The dorsal gray column at higher magnification. The scale bars represent 100 µm in A and D, 20 µm in B and E, and 50 µm in C and F.

**Figure 5. Immunohistochemistry of FP and PGFS I in serial sections of lamina IX.** Single immunostaining of FP or PGFS I in serial sections developed an image after the DAB reaction. The same neuronal somata (arrows) and dendrites with transverse and vertical sections (arrowheads) were immunoreactive for FP and PGFS I. The scale bar represents 50 µm.

**Figure 6. Multiple immunofluorescent labeling of FP, MAP2, tomato lectin, and vimentin.** A and C: Lamina IX of the ventral gray column. B: The dorsal gray column. D: Lamina X surrounding the central canal. An optical section using CLSM shows that FP (red) and MAP2 (green in A and B) colocalized in the neuronal somata and dendrites (yellow in A and B). The merged image shows that FP colocalized with MAP2 (violet) but not with tomato lectin (green) in C. In lamina X, FP (red) did not colocalize with vimentin (green in D). The scale bars represent 100 µm in A, B, and D, and 20 µm in C.
Supplemental Figure 1. Immunohistochemistry of FP in the dorsal gray column of rat spinal cord. In the high magnification of the dorsal gray column, the strong immunoreactivity was observed in many dendrites. Some somata were also immunoreactive (arrows), and the immunoreactivity was weaker than that of dendrites. The scale bars represent 50 µm.

Supplemental Figure 2. Double immunofluorescent labeling of FP and MAP2 in the gray substance of rat spinal cord. A: The half transverse section immunostained for FP (red) and MAP2 (green). B: The dorsal gray column at higher magnification. C: The ventral gray column at higher magnification. MAP2-immunoreactivity was relatively homogeneous in the whole region of the gray substance. In comparison, FP-immunoreactivity was higher in laminae I and II of the dorsal horn. The scale bars represent 500 µm in A, 50 µm in B and C.