

Prostaglandin transporter in the rat brain: its localization and induction by lipopolysaccharide

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Abbreviations: PGE₂, prostaglandin E₂; PGT, prostaglandin transporter; COX-2, cyclooxygenase-2; LPS, lipopolysaccharide; CSF, cerebrospinal fluid; DIG, digoxigenin; PBS, phosphate buffered saline.

Prostaglandin E₂ (PGE₂) is produced in the brain during infectious/inflammatory diseases, and it mediates acute-phase responses including fever. In the recovery phase of such diseases, PGE₂ disappears from the brain through yet unidentified mechanisms. Rat prostaglandin transporter (PGT), which facilitates transmembrane transport of PGE₂, might be involved in the clearance of PGE₂ from the brain. Here, we examined the cellular localization of PGT mRNA and its protein in the brains of untreated rats and those injected intraperitoneally with a pyrogen lipopolysaccharide (LPS) or saline. PGT mRNA was weakly expressed in the arachnoid membrane of untreated rats and saline-injected ones, but was induced in blood vessels of the subarachnoid space and choroid plexus and in arachnoid membrane at 5 h and 12 h after LPS injection. In the same type of cells, PGT-like immunoreactivity was found in the cytosol and cell membrane even under nonstimulated conditions, and its level was also elevated after LPS injection. PGT-positive cells in blood vessels were identified as endothelial cells. In most cases, PGT was not colocalized with cyclooxygenase-2, a marker of prostaglandin-producing cells. The PGE₂ level in the cerebrospinal fluid reached its peak at 3 h after LPS, and then dropped over 50% by 5 h, which time point coincides with the maximum PGT mRNA expression and enhanced level of PGT protein. These results suggest that PGT is involved in the clearance of PGE₂ from the brain during the recovery phase of LPS-induced acute-phase responses.

Introduction

Prostaglandin E₂ (PGE₂) in the brain is the final mediator of fever.^{1–6} The mechanism of PGE₂ production in the brain during infection/inflammation is now well documented: upon a systemic challenge with pyrogens, the immune system releases inflammatory cytokines⁷ that are carried by the blood flow to brain endothelial cells to induce PGE₂-synthesising enzymes, including cyclooxygenase-2 (COX-2)⁸ and microsomal-type prostaglandin E synthase.^{9–12} PGE₂ is biosynthesized there and released into the brain. Then, PGE₂ acts on the EP3-positive hypothalamic neurons, by which the neural pathway of fever is activated.^{13,14}

On the other hand, what causes the termination of fever remains unknown. To cease the febrile response, the brain PGE₂ level should be lowered. However, prostaglandins, including PGE₂, are not effectively metabolized in the adult brain.¹⁵ In addition, they are charged organic anions at physiological pH, and

their membrane permeability is fairly low¹⁶; thus, it is unlikely that PGE₂ disappears from the brain by simple diffusion. Therefore, the following scenario of PGE₂ clearance has been suggested: the removal of prostaglandins from the brain is mediated by a specific transport mechanism across the blood-brain or blood-cerebrospinal fluid (CSF) barrier, and final metabolism of PGE₂ is performed by other tissues, e.g., the lung.^{15,17} Indeed, some pioneering studies showed that febrile response was enhanced when the animals were treated with organic anion transport inhibitors, such as bromocresol green and probenecid, which inhibit the transport of prostaglandins.^{18,19} As a candidate molecule of prostaglandin carriers, the prostaglandin transporter (PGT), or solute carrier organic transporter family 2A1 (SLCO2A1), and its gene were first identified in rats,²⁰ and then in humans and mice.^{21,22} PGT has high affinity for PGE₂, PGF_{2α}, PGD₂ and PGH₂; and it transports them across the plasma membrane in an energy-requiring fashion^{23,24} and bromocresol green-sensitive manner.²⁰ PGT is

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likely to mediate the uptake of PGE₂ into cells rather than the release of it from them.^{17,25}

Based on these physiological and molecular background data, we hypothesized that PGT is involved in the clearance of PGE₂ from the brain during the recovery phase of fever. In this study, as the first step to examine this hypothesis, we investigated, using *in situ* hybridization and immunohistochemical techniques, if PGT is present in the central nervous system or induced there after systemic LPS stimulation.

Results

Localization PGT in the central nervous system

PGT mRNA was induced in a certain type of blood vessels and the arachnoid membrane 5 h after intraperitoneal injection of LPS (Fig. 1B), but not after saline injection (Fig. 1A). As for blood vessels, such signals were restricted to venous-type blood vessels in the subarachnoid space and choroids plexus (Fig. 1E); PGT mRNA signals were found neither in arteries of the subarachnoid space nor in any type of blood vessels in brain parenchyma. As for the arachnoid membrane, PGT mRNA was found at the entire brain surface. Similarly, in the spinal cord, we found LPS-induced expression of PGT mRNA in the arachnoid membrane and the subarachnoidal veins or velunes (Fig. 1G). Detailed observation revealed that the arachnoid membrane, but not the blood vessels, constitutively expressed a low level of PGT mRNA under non-stimulated conditions, i.e., in saline-injected rats (Figs. 1A and 2H) and untreated rats (Fig. 2A). There were

no such signals when the sections were hybridized with the sense RNA probe (Fig. 1C).

Figure 2 shows the appearance of PGT mRNA at each time points after LPS injection. PGT mRNA signals started to increase by 1.5 h (Fig. 2B), reached their highest level at 5 h (Fig. 2D), still remained at high levels until 12 h (Fig. 2E), and then declined to the basal level by 48 h (Fig. 2G) after the LPS injection. PGT mRNA signals in the blood vessels were spot-like and countable. This uneven distribution of PGT mRNA signals within vascular cells probably represented perinuclear distribution of rough endoplasmic reticulum-bound PGT mRNA. On the other hand, PGT mRNA in the arachnoid membrane was spread along the whole cell and uncountable. Because of these differences, we semiquantified the PGT mRNA signals in blood vessels and arachnoid membrane in different ways (details were described in Materials and Methods). In blood vessels, the number of PGT mRNA-positive cells started to increase by 1.5 h, reached its peak at 5 h, and remained high until 12 h, and then declined to the basal level by 48 h after the LPS injection (Fig. 3A). In the arachnoid membrane, the signal intensity of PGT mRNA became elevated earlier than that in the blood vessels: it rose to a high level by 3 h, reached the maximum at 5 h, and then slightly declined by 12 h and returned to the basal level by 48 h after the LPS injection (Fig. 3B). In either tissue, PGT mRNA signals were most intense at 5 h after LPS injection.

We examined PGT protein localization as well by immunohistochemistry. Western blotting revealed that the antibody used for the immunohistochemistry properly recognized a

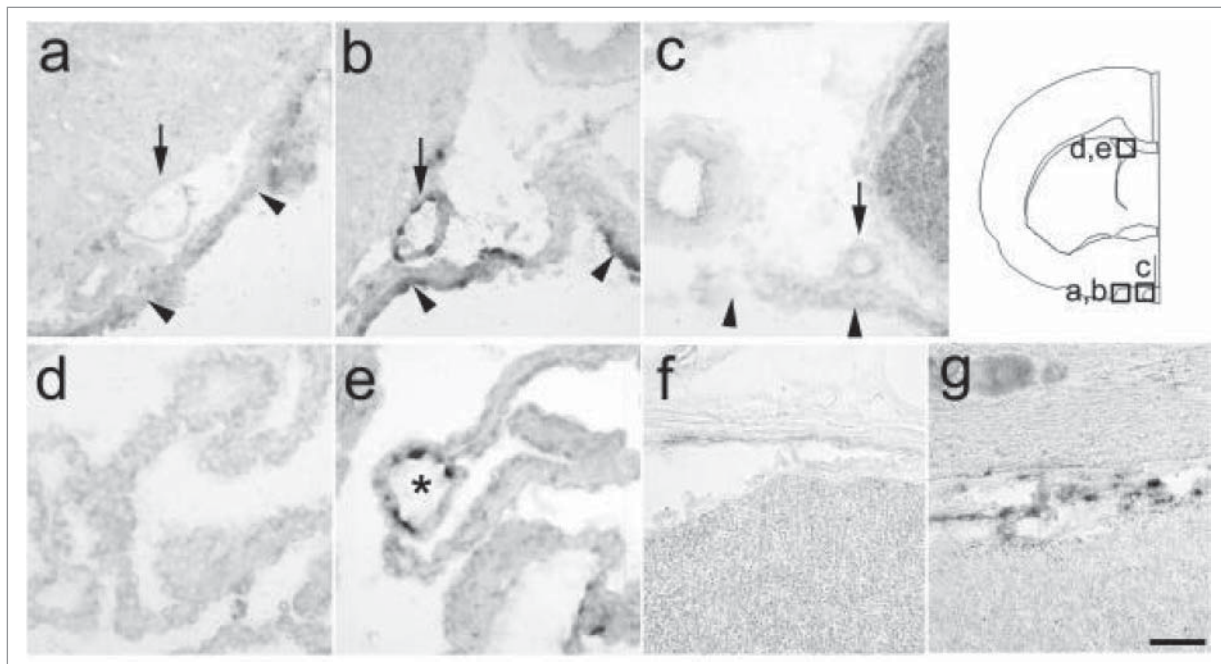


Figure 1. Expression of PGT mRNA in the subarachnoidal space (a, b, c) and choroid plexus (d, e) of the brain and in the subarachnoidal space of the spinal cord (f, g). (A, D, F) 5 h after saline injection, (B, E, G) 5 h after LPS injection, (C) hybridization with sense probe (5 h after LPS injection). PGT mRNA was expressed in the arachnoid membrane (arrowheads), subarachnoidal blood vessels (arrows), and blood vessels in the choroid plexus (asterisk) after the LPS injection. A line drawing at the upper right corner indicates brain regions corresponding to a–e. Scale bar: 50 μ m.

single protein band (Fig. 4A), which is close to the deduced molecular weight of rat PGT (70.5 kD).²⁶ To find the best conditions for tissue fixation and antibody dilution, we immunostained the lung and kidney tissues, both of which are known to constitutively express high levels of PGT. The antibody was applicable to immunohistochemistry in acetone-fixed tissues with a dilution of 1:100. In the lung, alveolar cells, but not bronchial cells, constitutively expressed a high level of PGT protein (Fig. 4B). In a magnified view, the PGT signals were expressed in a vesicular pattern (Fig. 4C). Preabsorption of the primary antibody with PGT antigen peptide eliminated the staining (Fig. 4D). In the brain, the arachnoid membrane (Fig. 4E) and subarachnoid blood vessels (Fig. 4G) of LPS-injected rats expressed PGT protein in a vesicular pattern. Preabsorbed antibody did not yield such staining (Fig. 4F, H).

Figure 5 shows the time-dependent changes in the level of PGT protein after the LPS injection. The obtained PGT expression pattern was similar to that found in the *in situ* hybridization study: PGT was induced in the arachnoid membrane and the blood vessels, mainly those in the subarachnoid space, after the LPS injection (Fig. 5B–E) but not after saline injection (Fig. 5A). The intensity of PGT protein signals was highest at 12 h (Fig. 5C) and 24 h (Fig. 5D) after the LPS injection, at which time the elevation in the signal density was statistically

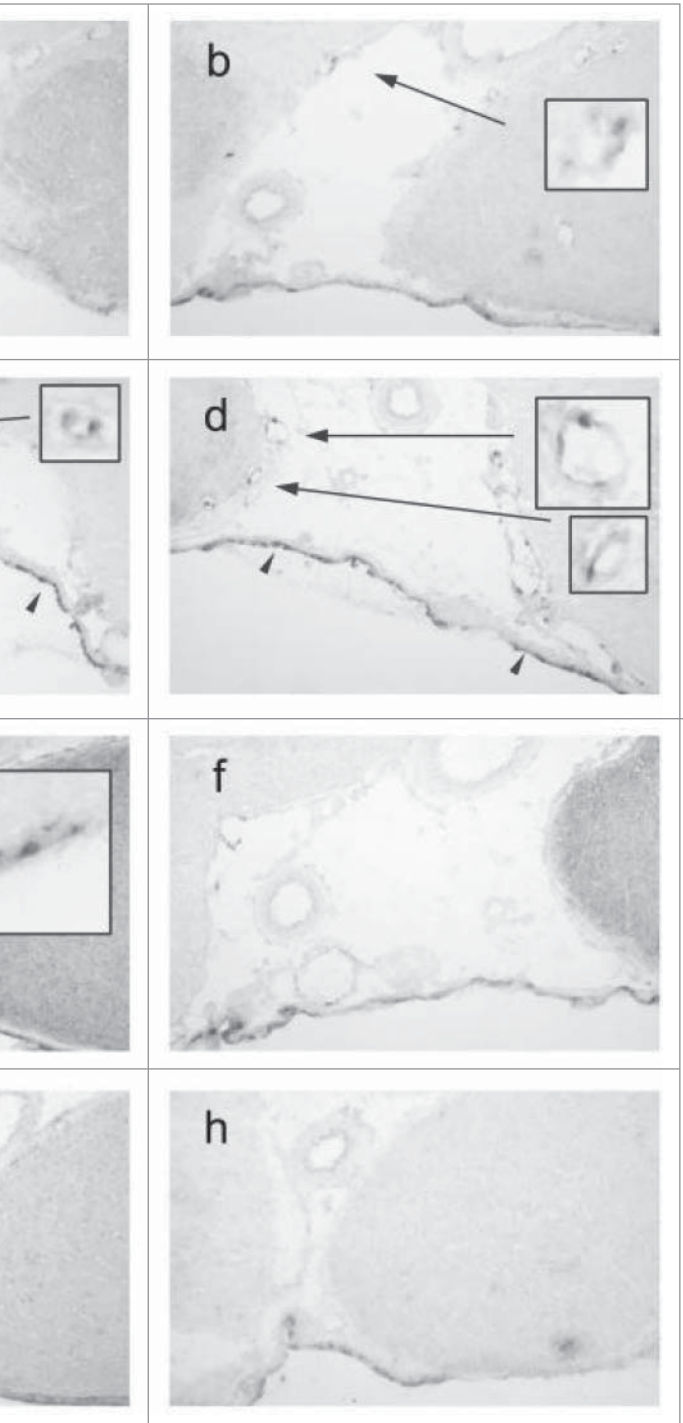


Figure 2. Time-dependent expression of PGT mRNA in the brain subarachnoid space. (A) untreated rat, (B) 1.5 h, (C) 3 h, (D) 5 h, (E) 12 h, (F) 24 h, (G) 48 h after the LPS injection. (H) 5 h after the saline injection. Levels of PGT mRNA in the arachnoid membrane (arrowheads) and subarachnoid blood vessels (arrows) reached their peak at 5 h after the LPS injection. Inlets show 3-times magnified views of blood vessels. Scale bar: 100 μ m.

significant compared to that of saline-injected one (Fig. 5F). To determine the type of cells that expressed PGT in the blood vessels, we incubated some brain sections with antibody against von Willebrand factor, an endothelial cell marker, after they had been stained with PGT antibody (Fig. 6B). PGT-positive

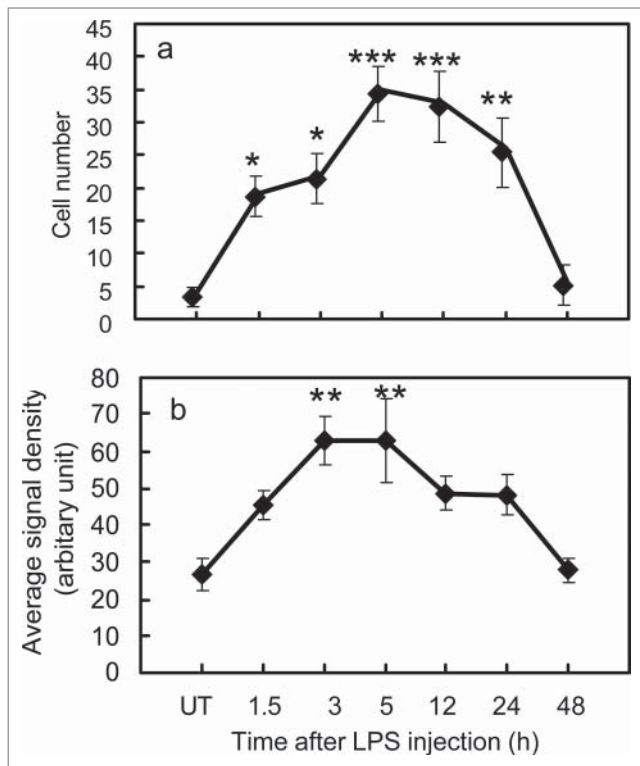


Figure 3. Semiquantitative analysis of PGT mRNA. (A) changes in the number of PGT mRNA-positive cells in blood vessels in the subarachnoidal space after LPS injection, (B) changes in signal intensity of PGT mRNA in the arachnoid membrane after LPS injection. The values denoted by asterisks were significantly different from that of UT (untreated) group (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

cells in the blood vessels (Fig. 6A) were identified as endothelial cells (Fig. 6C).

Colocalization of PGT mRNA and COX 2-positive cells

To examine whether PGT mRNA is expressed in the same blood vessels as COX-2, we studied the COX-2 expression by using consecutive brain sections. In the subarachnoidal space, we found that PGT mRNA was expressed in the same blood vessels as COX-2 (Fig. 6D, E). However, in the brain parenchyma, there were few PGT-positive blood vessels; whereas COX-2 positive blood vessels were found abundantly throughout the brain (Fig. 6F, G). On the other hand, PGT mRNA-positive cells in arachnoid membrane were completely negative for COX-2. Thus, PGT and COX-2 were not colocalized in most cases with an exception of subarachnoidal veins.

PGE₂ level in the CSF after LPS injection

To obtain further insight into the relation between PGT and PGE₂ in the brain, we also measured PGE₂ concentration in the CSF, which was sampled just before decapitation. The PGE₂ level in the CSF started to increase at 1.5 h, reached its maximum at 3 h, and then declined to less than half of the peak level at 5 h and returned to the basal level by 12 h after the LPS injection. Figure 7 shows the temporal relationship between the

PGE₂ concentration in the CSF (indicated by columns) and PGT mRNA expression in the blood vessels (solid line) and the arachnoid membrane (dotted line). In this figure, values of PGT mRNA signals were converted to a percentage of the maximum level obtained at 5 h after the injection. PGT mRNA signals reached the maximum level at 5 h after the LPS injection, at which time the PGE₂ level was already declining, being in line with the hypothesis that PGT is involved in the clearance of PGE₂ from the brain during the recovery phase of fever. Interestingly, PGT mRNA signals stayed relatively high for a while after the PGE₂ level had returned to the basal level.

Discussion

Little is known about what is happening to PGE₂ in the brain during the recovery phase of fever. Removal of excess amount of potent PGE₂ from the brain is apparently necessary to terminate the febrile response and other acute phase responses involving PGE₂. However, PGE₂ can neither be metabolized in the brain nor traverse the plasma membrane effectively. This situation suggests the existence of a transport mechanism that is involved in the clearance of PGE₂. In 1995, PGT, the first known specific transporter of prostaglandins, was molecularly identified in rats.²⁰ Rat PGT is widely expressed in the body and transports PGE₂, PGF_{2 α} , and PGD₂ with high affinity, thromboxane with intermediate affinity, and prostacyclin analogs with low affinity in an energy-dependent manner²⁰. Here, we hypothesized that PGT is involved in the clearance of PGE₂ from the brain during the recovery phase of fever. In the present study, for the first time, we demonstrated that both PGT mRNA and protein were expressed in venous blood vessels of subarachnoidal space and choroid plexus, and in the arachnoid membrane after systemic LPS injection. Ivanov et al.²⁷ reported that they could not find any significant change in the level of PGT mRNA in the brain after the intravenous injection of LPS. To examine the PGT mRNA, they used quantitative RT-PCR with homogenized hypothalamus, but we used the *in situ* hybridization technique, which has high spatial resolution. Our method probably allowed us to detect the local change in PGT in the brain more effectively. In fact, in the study of Ivanov et al.,²⁷ the PGT mRNA level in the hypothalamus increased slightly 5 h after the intravenous injection of LPS, though the increase was not statistically significant.

As discussed below, our present results and the available literature suggest that PGT is involved in the removal of PGE₂ during the recovery phase of fever.

Possible role of PGT: removal of PGE₂ from the brain or release of PGE₂ into the brain?

There are 3 possible roles of PGT: release of newly synthesized PGE₂ from endothelial cells, trans-endothelial transport of blood-borne PGE₂ into the brain or removal of PGE₂ from the brain. The first 2 may initiate PGE₂ signaling, whereas the third one may terminate PGE₂ signaling. However, the first one is unlikely for the following reasons: We found that COX-2-

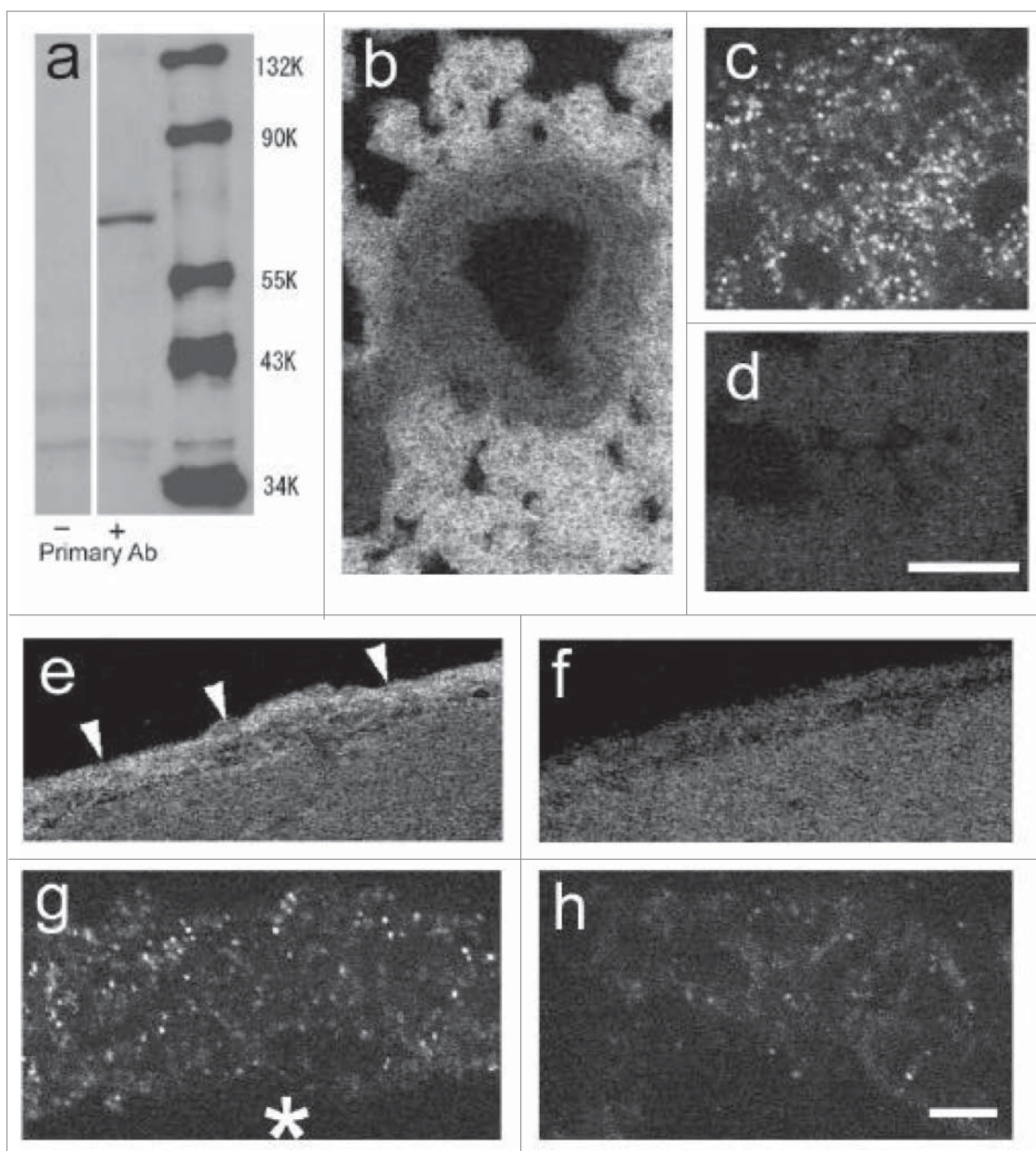


Figure 4. Expression of PGT protein. (A) Western blot of the rat brain (5 h after the LPS injection) with (+) or without (–) PGT antibody. (B–D) confocal microscopic views of the lung of saline-injected rat. (B) expression of PGT protein in the lung. (C) a magnified view of “b.” PGT is expressed in a vesicular pattern. (D) PGT antibody was incubated with the antigen peptide prior to assay. (E–H) confocal microscopic views of the brain sampled at 12 h after the LPS injection. (E) expression of PGT protein in the arachnoid membrane. (F) arachnoid membrane when incubated with the preabsorbed PGT antibody. (G) a magnified view of a blood vessel, which expressed PGT in vesicular pattern. The asterisk indicates the lumen of a blood vessel. (H) a magnified view of a blood vessel when incubated with the preabsorbed PGT antibody. Scale bars: 100 μm (b,d), 50 μm (e,f), 5 μm (c,g,h).

positive endothelial cells, which produce PGE_2 , did not always express PGT after systemic injection of LPS. In the brains of LPS-injected rats, PGT was well expressed in the blood vessels in the subarachnoid space but not in the blood vessels in the brain parenchyma; whereas COX-2 was expressed in blood vessels throughout the brain. Conversely, the arachnoid membrane

was negative for COX-2, but expressed PGT abundantly. If PGT had been involved in release of newly synthesized PGE_2 , we should have found colocalization of COX-2 and PGT in blood vessels throughout the brain. Our failure to do so implies that PGT is not releasing newly synthesized PGE_2 from endothelial cells.

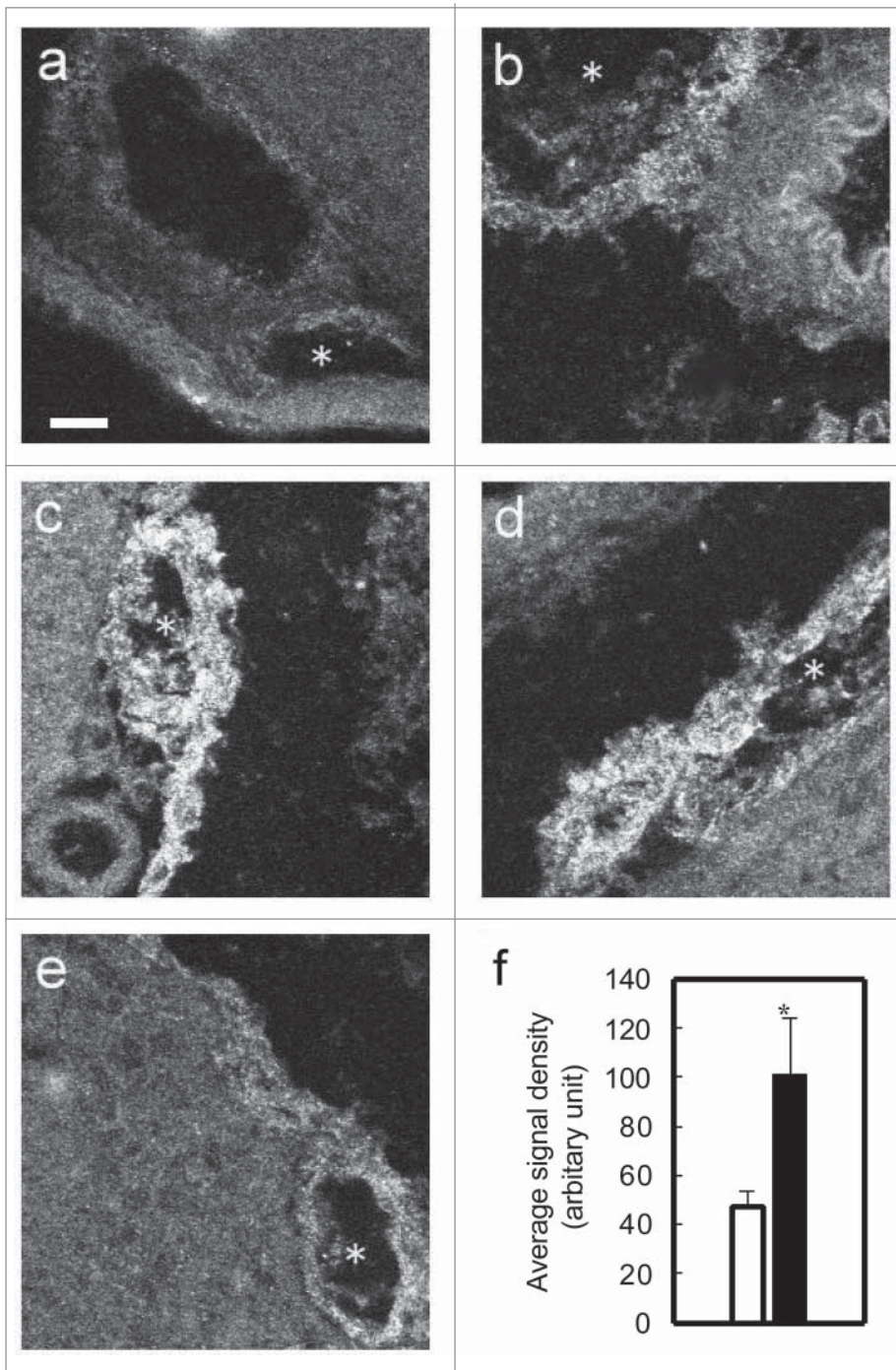


Figure 5. Expression of PGT protein in brain subarachnoid space at each time point after LPS or saline injection. (A) 5 h after the saline injection (B) 5 h, (C) 12 h, (D) 24 h, (E) 48 h after the LPS injection. PGT was induced in venous blood vessels (asterisks) after the LPS injection. Scale bar: 20 μ m (F) semiquantitative analysis of the signal intensity of PGT protein at 24 h after the LPS injection (closed bar) and 5 h after the saline injection (open bar). * $P < 0.05$.

The following reports also ensure that PGT is not involved in the release of newly synthesized PGE₂ from the endothelial cells: Although the intrinsic permeability of the plasma membrane to PGE₂ is low, PGE₂ can efflux from cells by simple diffusion.²³

uptake is dependent on the lactate gradient created by intracellular glycolysis.³⁵

Through time course analysis, we found that the level of PGE₂ in the brain reached its peak at as early as 3 h, and then declined

The electrochemical driving force in a cell is considered far more likely to facilitate diffusive efflux of PGE₂ than influx on the grounds that the inside-negative voltage of cells preferentially drives efflux of PGE₂, which exists as a charged anion within the cells, and that accumulated protons in the cells lead to protonation of PGE₂, which form also tends to efflux from the cells.¹⁷ Endo et al.²⁸ compared bradykinin-induced PGE₂ release between normal Madin-Darby canine kidney cells and PGT-transfected ones. There was no difference in PGE₂ release between these 2 cell groups, indicating that PGT is not involved in the release of PGE₂ from cells. On the other hand, several studies have demonstrated that multidrug resistance protein 4 (MRP4) is responsible for the release of PGE₂ from cells.²⁹⁻³²

It is demonstrated that the first phase of fever, which starts at approximately 30 min after systemic injection of LPS, is supported by trans-endothelial transport of blood-borne PGE₂ into the brain.^{33,34} PGT in endothelial cells might be involved in this process either by uptaking PGE₂ from the blood or by releasing PGE₂ into the brain. Indeed, we found that PGT protein is constitutively expressed in subarachnoidal endothelial cells. However, the level of constitutive PGT is low compared to that after LPS injection. PGT protein in endothelial cells reached the highest level between 12 h and 24 h after LPS injection, at which time PGE₂ levels in the CSF has decreased almost to the basal level. Thus, the role of PGT induced in the later time is unlikely involved in the elevation of PGE₂ in the brain.

The third possible role of PGT during fever is to remove PGE₂ from the brain to terminate the febrile response, as we hypothesized. In fact, it was demonstrated that PGT mediates uptake of PGE₂ into cultured cells against its concentration gradient.²³ It was also reported that PGT seemed to mediate uptake of prostaglandins in exchange for lactate, and that the degree of its PGE₂

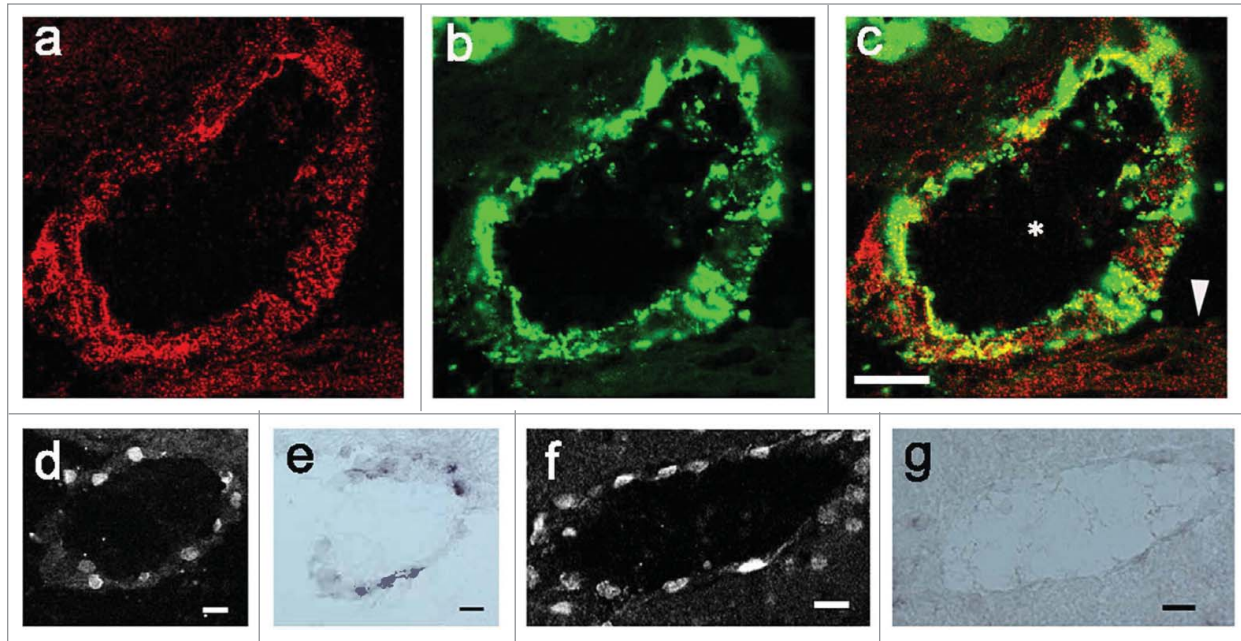


Figure 6. PGT protein localization in blood vessels. (A–C) confocal microscopic views of a venous blood vessel. (A) expression of PGT protein (B) expression of von Willbrand Factor, an endothelial marker. (C) double-stained image of PGT and von Willbrand Factor. PGT was expressed in endothelial cells of the venous blood vessels (whose lumen is indicated by the asterisk) and arachnoid membrane (arrowhead). (D, F) COX-2 protein expression in a sub-arachnoidal blood vessel (d) and a parenchymal blood vessel (f). (E, G) PGT mRNA expression in subarachnoidal blood vessel (e) in an adjacent section to (D) and in a parenchymal blood vessel (g) in an adjacent section to (F). PGT mRNA and COX-2 were coexpressed in subarachnoidal venous blood vessels. In parenchymal blood vessels, little PGT mRNA was expressed, whereas, COX-2 was expressed well. Scale bar: 20 μm .

to less than half of the peak level at 5 h after the LPS injection. On the other hand, around 5 h after the LPS injection, the expression of PGT mRNA reached its highest level and its protein level was also elevated. This observation implies that the

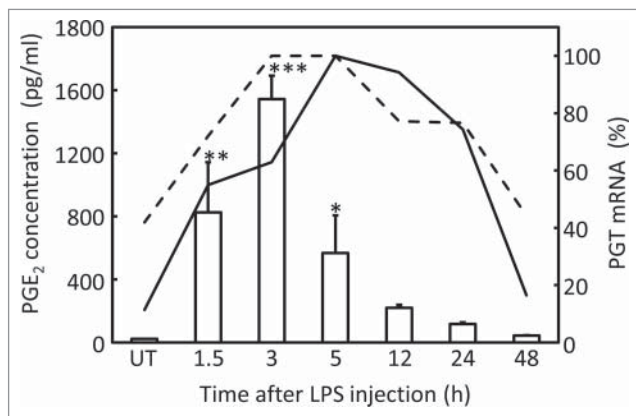


Figure 7. Time-course analysis of PGE₂ concentration in the CSF (open columns), and PGT mRNA in the brain (subarachnoidal blood vessels: solid line, arachnoid membrane: dotted line) after LPS injection. Values of PGT mRNA signals were converted to a percentage of the maximum level obtained at 5 h after the injection. The original values and SE were shown in **Figure 3**. PGE₂ concentration denoted by asterisks were significantly different from that of UT (untreated) group (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

decrease in the PGE₂ level in the brain is linked to the removal of PGE₂ by PGT. Interestingly, Inoue et al.¹⁰ pointed out previously that levels of PGE₂-synthesising enzymes in the brain and level of PGE₂ in the CSF were correlated well in the early phase of fever. However, in the late phase of fever, when the level of PGE₂ started to decrease, the levels of those enzymes were still increasing. This dissociation between the enzyme levels and PGE₂ level was most remarkable at 5 h after the LPS injection, being in line with the time point at which the PGT mRNA level was highest. During the later phase of fever, newly expressed PGT may be effectively removing the excess amount of PGE₂ from the brain while PGE₂-synthesising enzymes are still producing PGE₂ for a while. Thus, our time course data are in line with the hypothesis that PGT is involved in the removal of PGE₂ during the recovery phase of fever. PGT protein was expressed well at 5 h: but, in fact, the peak time point of it was around 12 h and 24 h after the LPS injection, being somewhat later than the period when there might be the highest demand for the removal of PGE₂. This later accumulation of PGT protein might be in preparation for the possible next attack of pathogens.

In our immunohistochemical study, PGT was expressed in a vesicular pattern and was localized along the plasma membrane and throughout the cytoplasm in the endothelial cells and arachnoid membrane. In line with this result, Bao et al.³⁶ also reported the vesicle-like expression of PGT in the rat kidney, and assumed that PGT is recruited to the plasma membrane by exocytosis, as is the case with many transporters. We assume that, during the

recovery phase of fever, PGT is recruited to the plasma membrane of endothelial cells to mediate uptake of PGE₂ from the CSF. The efflux of PGE₂ across the plasma membrane of endothelial cells might occur by simple diffusion, or through MRP4. Then PGE₂ enters the blood flow to be metabolized by other organs, such as the lung.

We have no idea about the destination of PGE₂ after it is taken up by the arachnoid membrane. The arachnoid membrane is not vascularized very well. PGE₂ might be carried to blood vessels in the dura mater, which is juxtaposed to arachnoid membrane and well vascularized. Another possibility is that there are PGE₂-catabolizing enzymes in the arachnoid membrane itself. This latter possibility may sound unlikely according to the literature so far, but these available literature denies PGE₂ metabolism only in the brain parenchyma. As far as we know, there is no report of any study that examined PGE₂ metabolism in the arachnoid membrane. In our preliminary RT-PCR experiment, mRNA of 15-hydroxy-prostaglandin dehydrogenase, a PGE₂-metabolizing enzyme, was detected in a mixture of arachnoid membrane and subarachnoidal blood vessels. In fact, some studies demonstrated that the arachnoid membrane is involved in drug metabolism.³⁷

Mechanism of PGT induction after the LPS stimulation

What triggers PGT expression in the brain after the LPS stimulation remains unknown. As inflammatory cytokines activate the arachnoid acid cascade and initiate PGE₂ production, we first speculated that some products of the arachnoid acid cascade might induce PGT. However, PGE₂ itself is not likely to cause PGT induction because coinjection of LPS with dicrofenac, a potent inhibitor of COX-1 and COX-2, did not suppress PGT mRNA expression in the brain (our unpublished observation). Since almost all LPS actions are mediated by inflammatory cytokines, we currently assume that some of the inflammatory cytokines are responsible for PGT induction by LPS. On the other hand, Topper et al.³⁸ reported that inflammatory stimuli (LPS, recombinant human interleukin-1 β , tumor necrosis factor- α) did not induce PGT in cultured human umbilical vein endothelial cells. They also reported that human PGT was induced in cultured vascular endothelial cells in response to a physiological fluid mechanical stimulus, suggesting that blood flow as a stimulus may trigger the PGT induction. Since PGE₂ has various physiological roles in different tissues, it may not be surprising that PGT has tissue-specific behavior depending on the role of local PGE₂.

In addition to the work of Topper et al.,³⁸ there is another intriguing report in terms of the difference in PGT regulation between the brain and other tissues. Ivanov et al.²⁷ demonstrated that after the intravenous injection of LPS, PGT and another PGE₂ carrier, i.e., moat1 (multispecific organic anion transporter 1), and 2 PGE₂-metabolizing enzymes were downregulated in the lung and the liver, but not in the brain. We also observed down regulation of PGT in the kidney after LPS injection, in that PGT mRNA was strongly expressed in the kidney medulla of the control rat, while those mRNA signals disappeared after

LPS injection (unpublished observation). We need to conduct further studies on the regulation of PGT induction by the LPS stimulus.

Limitations of this study

PGT is the carrier with the highest affinity for PGE₂ but there are several prostaglandin carriers identified other than PGT, such as moat1,³⁹ and OAT3.⁴⁰ Thus, we cannot exclude the possibility that those carriers also take part in the removal of PGE₂ from the brain during the recovery phase of fever. To establish the physiological role of PGT in the brain, we need specific inhibitors for PGT such as the one recently reported by Chi et al.⁴¹

Materials and Methods

Materials

All experiments were carried out under the Guideline for Animal Experiments of Kyoto University and Osaka Institute of Technology. Male Wistar rats (8 weeks old) were purchased from Japan SLC (Shizuoka, Japan). They were housed 5 or 6 to a cage in a room at 26 \pm 2°C with a standard 12:12 h light:dark cycle, and were given free access to food and water. Other materials and their sources were as follow: LPS of *Escherichia coli* 026:B6 (Sigma, St. Louis, MO); digoxigenin (DIG) RNA labeling mix (Roche Diagnostics Corporation, Indianapolis, IN); NBT/BCIP (Roche Diagnostics Corporation); alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics Corporation); rabbit polyclonal antibody against rat PGT (PGT11-A, Alpha Diagnostic International Inc., San Antonio, TX), which was raised against a 20 aa peptide (SWRMKKNREYSLQENTSGLI) of rat PGT conjugated to KLH and affinity purified; rat PGT control peptide (Alpha Diagnostic International Inc.); Cy3-labeled anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA); sheep polyclonal antibody against rat von Willbrand factor (Affinity Biologicals, Ont., Canada); goat polyclonal antibody against rat COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA); PGE₂ monoclonal enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI).

Sample preparation

To study the time course of PGT expression during fever, we intraperitoneally injected rats with LPS (100 μ g/kg in 0.5 ml of saline) or saline (0.5 ml) between 9:00 and 11:00 am, then obtained samples at the following time points: 1.5, 3, 5, 12, 24, and 48 h after the injection. At each time point, 4 rats were anesthetized with halothane, and their heads were fixed in a stereotaxic apparatus; and then the CSF was sampled from the cisterna magna with a 26-gauge needle connected to a microsyringe (100 μ l) via PE20 tubing. Immediately after CSF sampling, they were killed by decapitation, and brains and spinal cords were removed. The CSF samples were quickly frozen in liquid nitrogen, and brains and spinal cords were frozen in isopentane that had been cooled with liquid nitrogen. The CSF samples were stored at -30°C, and the brains and the spinal cords were stored at -80°C until the day of further processing.

***In situ* hybridization**

All brain samples were processed for PGT mRNA *in situ* hybridization. Frozen brain sections (14- μ m thickness) were made in a cryostat so that the sections contained the rostral part of the preoptic area, which is the putative fever center. The sections were thaw mounted on silane-coated glass slides and then fixed with 4% paraformaldehyde for 10 minutes at 4°C. The following protocol was carried out at room temperature otherwise stated. They were further treated with proteinase K (1 μ g/ml in phosphate buffered saline (PBS)) for 10 minutes, glycine buffer (2 mg/ml in PBS) for 10 minutes, and then prehybridized for 1 h with hybridization buffer containing 50% formamide, 5 X saline sodium citrate (SSC: 1 X SSC = 0.15 M NaCl, 0.015 M sodium citrate), 5 X Denhardt's solution, 0.25 mg/ml yeast tRNA, and 0.5 mg/ml herring sperm DNA. The sections were hybridized overnight at 55°C with the DIG-labeled antisense or sense PGT riboprobe dissolved in the hybridization buffer at a concentration of approx. 0.25 μ g/ml. These DIG-labeled cRNA probes were prepared from a 1.6 kb-fragment of the cDNA sequence of rat PGT. The following day, the sections were washed at 65°C with 0.1 X SSC several times, and processed for immunodetection of DIG. After having been treated with 10% normal goat serum, the sections were incubated with alkaline phosphatase-conjugated anti-DIG antibody at a dilution of 1:1000, and then the signals were detected with NBT/BCIP solution. Some spinal cord samples were examined for PGT mRNA at 5 h and 12 h after LPS injection, at 5 h after saline injection, and at 0 h (untreated).

Semiquantitative analysis of PGT mRNA signals

All brain sections examined by *in situ* hybridization were semiquantitatively analyzed for signal intensity. Since the appearance of PGT mRNA signals was different between blood vessels and arachnoid membrane, we quantified them in different ways. In blood vessels, we counted the number of PGT mRNA-positive cells within a fixed area, both right and left side of the optic chiasma, through a microscope. The signal intensity in the arachnoid membrane was measured with the aid of an image analysis program, Scion Image for Windows (Scion corp., Frederick, Maryland). In brief, using a PC-driven CCD camera, we captured microscopic views of both sides of the optic nerve, converted them into 8-bit gray scale images, and then quantified the optical density within a given area in the arachnoid membrane. We also measured the optical density of a certain area in the parenchyma in each image as background signals. The signal intensity in the arachnoid membrane was calculated by subtracting the background value from the mean optical density of the arachnoid membrane in each image.

Immunohistochemistry

Brain sections (14- μ m thickness) were fixed with acetone for 10 minutes at 4°C, washed with 0.1 M PBS (pH 7.4), treated with 10% normal donkey serum for 1 h, and then incubated overnight with rabbit anti-rat PGT antibody at a dilution of 1:100. After removal of the primary antibody, the

sections were washed with PBS and then incubated for 2 h with Cy3-labeled anti-rabbit IgG at a concentration of 1:1000. Fluorescent images of PGT were captured with a laser confocal microscope (Radiance 2000, BioRad), and were converted into 8-bit gray scale images. The mean intensity of fluorescent signals was quantified with the image analysis software. To confirm the specificity of the PGT antibody, we preincubated a solution of PGT antibody with PGT antigen peptide (5 μ g/ml) at 37°C for 2 hours with gentle shaking, and then applied it to the sections. For confirmation that PGT was expressed in endothelial cells, some sections, which had been stained for PGT, were further incubated with sheep anti-rat von Willebrand factor IgG at a dilution of 1:3000, followed by FITC-labeled anti-sheep IgG at a dilution of 1:500. To examine whether or not PGT and COX-2 are expressed in the same type of cells, we immunostained COX-2 in brain sections that were adjacent to those processed for *in situ* hybridization of PGT mRNA according to the protocol described previously.¹⁰ Immunohistochemistry was carried out at room temperature otherwise stated.

Enzyme immunoassay for PGE₂ in CSF

Three CSF samples from each time point were thawed on ice. PGE₂ was extracted from 70 μ l of CSF with an organic solvent, ethylacetate, and then assayed with an enzyme immunoassay kit according to the manufacturer's instructions.

Western blot analysis of PGT

Freshly frozen brains were homogenized in 5 vol. of 20 mM Tris-HCl, pH 7.4, containing 0.25 mM sucrose, 1 mM EDTA, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 0.5 mM DTT, 10 μ g/ml leupeptin, and 1% Triton X-100. The samples were centrifuged, and the supernatants were mixed with SDS sample buffer and heated for 2 minutes, after which they were applied to 10% polyacrylamide gel, electrophoresed, and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with nonfat milk solution for 1 h, and then incubated at 4°C overnight with anti-PGT antibody at a dilution of 1:300. After having been washed with Tris-HCl buffered saline containing 0.3% Tween 20, the membrane was incubated for 2 h with horseradish peroxidase-conjugated anti-rabbit antibody at a dilution of 1:1500. Immunoreactive protein was detected with an ECL kit.

Statistics

Data were expressed as the means \pm SE. Statistical significance of the difference among multiple groups was examined by the analysis of variance followed by post hoc multiple comparison (Dunnett test). Student's t-test was used for comparison between 2 groups. A $P < 0.05$ level of significance was set for all statistical analyses.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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