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Microglia-specific expression of microsomal prostaglandin E_2 synthase-1 contributes to lipopolysaccharide-induced prostaglandin E_2 production

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Abstract

Microsomal prostaglandin E_2 synthase (mPGES)-1 is an inducible protein recently shown to be an important enzyme in inflammatory prostaglandin E_2 (PGE_2) production in some peripheral inflammatory lesions. However, in inflammatory sites in the brain, the induction of mPGES-1 is poorly understood. In this study, we demonstrated the expression of mPGES-1 in the brain parenchyma in a lipopolysaccharide (LPS)-induced inflammation model. A local injection of LPS into the rat substantia nigra led to the induction of mPGES-1 in activated microglia. In neuron-glial mixed cultures, mPGES-1 was co-induced with cyclooxygenase-2 (COX-2) specifically in microglia, but not in astrocytes, oligodendrocytes or neurons. In microglia-enriched cultures, the induction of mPGES-1, the activity of PGES and the production of PGE_2 were preceded by the induction of mPGES-1 mRNA and almost completely inhibited by the synthetic glucocorticoid dexamethasone. The induction of mPGES-1 and production of PGE_2 were also either attenuated or absent in microglia treated with mPGES-1 antisense oligonucleotide or microglia from mPGES-1 knockout (KO) mice, respectively, suggesting the necessity of mPGES-1 for microglial PGE_2 production. These results suggest that the activation of microglia contributes to PGE_2 production through the concerted *de novo* synthesis of mPGES-1 and COX-2 at sites of inflammation of the brain parenchyma.

Keywords: cyclooxygenase, inflammation, lipopolysaccharide, microglia, microsomal prostaglandin E_2 synthase, substantia nigra.

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Localized inflammatory responses in the brain parenchyma have been associated with the pathogenesis of a number of neurological disorders, including infection, multiple sclerosis, Alzheimer's disease and Parkinson's disease (McGeer *et al*. 1988; Sherman *et al*. 1992; Dickson *et al*. 1993). At such lesion sites, activated microglia release several types of inflammatory mediators (Liu and Hong 2003). Among these mediators, prostaglandin (PG) E_2 may be one of the most potent in terms of the initiation and propagation of brain inflammation. PGE_2 is accumulated in the cerebrospinal fluid and/or at lesion sites (Griffin *et al*. 1994; Mattammal *et al*. 1995; Montine *et al*. 1999), and agents which reduce PGE_2 synthesis exhibit neuroprotective activity in certain neuroinflammation models (Nakayama *et al*. 1998; Lim *et al*. 2000; Teismann *et al*. 2003).

PGE_2 is sequentially synthesized from arachidonic acid in two enzymatic steps: cyclooxygenase (COX) and PGE_2

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Abbreviations used: CNPase, 2′3′-cyclic nucleotide 3′-phosphodiesterase; COX, cyclooxygenase; cPGES, cytosolic PGES; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetate; EIA, enzyme immunoassay; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium-binding adapter molecule 1; KO, knockout; LPS, lipopolysaccharide; MAP-2, microtubule-associated protein-2; mPGES-1, microsomal prostaglandin E_2 synthase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Neu-N, neuron-specific nuclear protein; PBS, phosphate-buffered saline; PG, prostaglandin; PGES, PGE_2 synthase; SDS, sodium dodecyl sulfate; TBS-T, Tris-buffered saline-Tween 20; TLR4, Toll-like receptor-4; TX, thromboxane; VWF, von Willebrand factor; WT, wild-type.

synthase (PGES). Two isoforms of COX have been isolated: COX-1 is recognized as a constitutive form and COX-2 as an inducible form (Nakayama *et al*. 1998; Smith *et al*. 2000). In the normal brain, COX-2 has been immunohistochemically detected specifically in neurons (Cao *et al*. 1995) and has recently been identified in non-neuronal cells as well in some lesion sites, e.g. in microglia in the brains of patients with multiple sclerosis and chronic cerebral ischemia (Tomimoto *et al*. 2000; Rose *et al*. 2004).

Recently, three major isoforms of PGES were isolated: cytosolic PGES (cPGES), microsomal PGES (mPGES)-1, and mPGES-2. While cPGES and mPGES-2 are constitutively expressed in various cells and tissues, mPGES-1 is induced by proinflammatory stimuli and in various models of inflammation, and is functionally coupled to COX-2 (Jakobsson *et al*. 1999; Murakami *et al*. 2000, 2002; Tanikawa *et al*. 2002). The phenotypic profile of mPGES-1 knockout (KO) mice strongly supports the hypothesis that mPGES-1 plays an important role in inflammatory PGE_2 production and the subsequent propagation of inflammation (Uematsu *et al*. 2002; Engblom *et al*. 2003; Kamei *et al*. 2004). Nonetheless, our understanding of the behavior of PGES in the brain is still rudimentary compared with our knowledge of COX-2.

In the brains of animal models of burn injury, lipopolysaccharide (LPS)-induced pyresis and carrageenan-induced paw edema, mPGES-1 is induced in the vascular endothelial cells (Yamagata *et al*. 2001; Guay *et al*. 2004; Ozaki-Okayama *et al*. 2004), likely because these types of inflammation are initiated peripherally or systemically. Recently, intraparenchymal injection of interleukin-1β has been reported to induce mPGES-1 in the cerebral cortex of mice (Moore *et al*. 2004). However, the types of cells expressing mPGES-1 remain to be identified. Although LPS has been reported to induce mPGES-1 expression in cultured microglia (Ajmone-Cat *et al*. 2003; Greco *et al*. 2003), the effect of intraparenchymal injection of LPS on mPGES-1 induction in brain microglia, the cell-type specificity of LPS-induced mPGES-1 expression and the actual contribution of mPGES-1 to PGE_2 production all remain to be determined.

In order to address these issues, we performed both an *in vivo* study by intraparenchymal injection of LPS and an *in vitro* study using neuron-glia and microglial-enriched cultures from both mPGES-1 KO and wild-type (WT) mice. In the *in vivo* study, we investigated the effects of a local injection of LPS into the substantia nigra on mPGES-1 expression, because the substantia nigra is known to contain a higher density of microglia than do other brain regions (Kim *et al*. 2000). Here, we demonstrated that mPGES-1 expression is induced in microglia at the site of the localized inflammatory response *in vivo*. Furthermore, in our *in vitro* study, we identified the microglia-specific expression of mPGES-1 among brain parenchymal cells, i.e. astrocytes, neurons and oligodendrocytes, and precisely demonstrated the nature of mPGES-1 expression in microglia. Furthermore, using microglia obtained from mPGES-1 KO mice, we demonstrated the necessity of mPGES-1 induction for microglial PGE_2 production.

Materials and methods

Materials

Lipopolysaccharide of *Escherichia coli* O111:B4, dexamethasone, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), anti-rat microtubule-associated protein-2 (MAP-2), anti-pig glial fibrillary acidic protein (GFAP) and anti-human 2′3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) monoclonal antibodies were purchased from Sigma (St Louis, MO, USA). The rabbit anti-human mPGES-1, mPGES-2 and cPGES polyclonal antibodies, anti-ovine COX-1 monoclonal antibody and PGH_2 were from Cayman Chemical (Ann Arbor, MI, USA). Other materials and their sources were as follows: goat anti-human COX-2 polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-neuron-specific nuclear protein (Neu-N) monoclonal antibody (Chemicon, Temecula, CA, USA); anti-CD11b monoclonal antibody (Serotec Inc., Oxford, UK); anti-von Willebrand factor (VWF) monoclonal antibody (Dako, Carpinteria, CA, USA); anti-rat ionized calcium-binding adapter molecule 1 (Iba-1) polyclonal antibody (a gift from Dr Shinichi Kohsaka, National Institute of Neuroscience, Tokyo, Japan); multiple-labeling-grade secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA); and α-^{32}P dCTP (Amersham Biotech, Arlington Heights, IL, USA). Other reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Animals

Male 240–280 g and timed-pregnant female Wistar rats were purchased from the Shizuoka Laboratory Animal Cooperative (Shizuoka, Japan). The mPGES-1 KO mice and littermate WT mice (C57BL/6 × 129/SvJ background) were described previously (Uematsu *et al*. 2002). Rats and mice were kept on a 12 h light/dark cycle with *ad libitum* access to food and water, and all experiments were performed under approved institutional guidance.

LPS injection *in vivo*

Male rats were anesthetized with sodium pentobarbital (50 mg/kg) and positioned in a small-animal stereotaxic apparatus. Injection of LPS into the substantia nigra was made using the stereotaxic coordinates of 4.8 mm posterior, 2.0 mm lateral and 8.2 mm ventral from the bregma. LPS (5 μg in a volume of 2 μL of phosphate-buffered saline; PBS) was injected into the right side of the substantia nigra over a period of 2 min, and the injection needle was kept in place for 2 min after the injection. Control injections of PBS alone were made into the left side of the substantia nigra under the conditions described above. Six animals were used to evaluate the effect of nigral injection of LPS.

Cell culture

Mixed neuron-glia cultures, enriched microglial cells, astrocytes and meningeal fibroblasts were generated from the forebrains of postnatal day 1 Wistar rats or mPGES-1 KO or WT mice, and neuronal cells were taken from embryonic day 17 rats as described previously (Ikeda *et al*. 1997). Briefly, the dissected forebrains were

completely removed of meninges. Each of them was then minced with scalpels and incubated with a mixture of 0.25% trypsin and 0.01% deoxyribonuclease I at 37°C for 30 min. After termination of the reaction by addition of horse serum, the tissue fragments were centrifuged, the pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and penicillin G/streptomycin, and single cells were dissociated by gently passing through a plastic tip. The cell suspension was passed through two sheets of nylon net (25 μm mesh) and the dissociated cells were plated at the appropriate conditions. For mixed neuron-glia culture, cells were plated at an initial density of 1×10^5 cells/cm^2 onto polyethylenimine-coated glass coverslips and cultured for 7 days. For neuron-enriched culture, the dissociated cells were plated at an initial density of 1×10^5 cells/cm^2 onto poly-L-lysine-coated dishes. Twenty-four hours after plating, the medium was changed to serum-free modified Eagle's medium containing N2 supplements (100 μg/mL transferrin, 5 μg/mL insulin, 20 nm progesterone, 100 μm putrescine, and 30 nm selenium) and the neurons then grown for an additional 3 days. In this neuron-rich culture, more than 95% of cells were positively stained with antibody for MAP-2, a specific marker for neurons. For astroglial culture, the dissociated cells were plated on uncoated 75 cm^2 flasks at a density of 6×10^5 cells/cm^2, as described previously (Ikeda *et al*. 2000). A monolayer of type I astrocytes was obtained 12–14 days after the plating. Non-astrocytes such as microglia were detached from the flasks by shaking and removed by changing the medium. The primary cells were dissociated by trypsinization and re-seeded on 35 mm dishes at a density of 1×10^4 cells/cm^2. When the cells had become confluent (6–8 days after the plating) they were used for the experiments. In this astrocyte-enriched culture, more than 95% of cells were positively stained with antibody for GFAP, a specific marker for astrocytes. For enriched microglial culture, 3×10^7 cells obtained from the cerebral cortex of rats or mice were seeded on a 225 cm^2 culture flask. When a confluent monolayer of glial cells had been obtained, microglia were shaken off (100 r.p.m. for 2 h) and re-plated at 1.2×10^6 cells/35 mm dish, 3.0×10^6 cells/35 mm dish, 5.0×10^6 cells/60 mm dish, or 1.0×10^5 cells/well (96-well plate) for western blotting, northern blotting, measurement of PGES activity or MTT assay, respectively. For immunocytochemistry, cells were seeded onto polyethylenimine-coated glass coverslips at 1×10^5 cells/cm^2. One hour later, cells were washed with PBS to remove the non-adherent cells and new medium was added. Twenty-four hours later, microglial cells were used for the experiment. In this microglia-enriched culture, more than 95% of cells were positively stained with antibody for CD11b, a specific marker for microglia. For fibroblasts, the meninges were minced and dissociated with trypsin and deoxyribonuclease I at 37°C for 30 min. The dissociated cells were plated on uncoated 35 mm dishes at a density of 1×10^5 cells/cm^2. A monolayer of fibroblasts was obtained 4 days after the plating and used for the experiment. All cultures were performed at 37°C in a humidified atmosphere with 5% CO_2.

Activation of cells

Cells were activated by the addition of LPS and the reaction was terminated by washing out the LPS-containing medium with PBS at the indicated time. Cells were pre-treated with dexamethasone for 1 h before LPS application.

Immunocytochemistry

For the *in vivo* studies, animals were anesthetized with sodium pentobarbital and then perfused transcardially with saline, followed by 4% paraformaldehyde in PBS. The brains were removed, post-fixed overnight in a solution containing 4% paraformaldehyde and 4% sucrose in PBS, and then cryoprotected in solutions containing 10%, 15% and 20% sucrose in PBS for 1 day each. The brains were then frozen in dry-ice powder and coronal sections (20 μm) were cut using a cryostat. The sliced tissues and cultured brain mixed cells were fixed in a solution containing 4% paraformaldehyde and 4% sucrose in PBS for 15 min, permeabilized with 0.3% Triton-X for 10 min and then treated with 3% bovine serum albumin for 15 min in PBS to block non-specific binding. The preparations were incubated with appropriate first antibodies against mPGES-1 (1 : 250 dilution), COX-2 (1 : 250), CD11b (1 : 50), MAP-2 (1 : 100), GFAP (1 : 400), CNPase (1 : 100), VWF (1 : 25) and Neu-N (1 : 1000) in PBS containing 3% albumin at 4°C overnight, and FITC- or TRITC-conjugated second antibodies (1 : 100 dilution for each) in PBS containing 3% albumin for 1 h. The coverslips were mounted with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA). The cultured cells and sliced tissues were examined using an AxioPlan-2 fluorescence microscope connected to a Zeiss AxioCam digital camera and confocal laser scanning system (Micro Radiance, Bio-Rad, Hercules, CA, USA) equipped with an ECLIPSE TE300 (Nikon, Tokyo, Japan) inverted microscope, an argon ion laser and a host computer system, respectively. The specificity of the antibody against mPGES-1 was evaluated by an absorbance control experiment using an antigen peptide for mPGES-1 (Cayman Chemical).

Western blot analysis

The cells were lyzed in 10 mM Tris-buffered saline, pH 7.4, containing 0.1% sodium dodecyl sulfate (SDS) and 0.5 M EDTA, by sonication three times for 10 s and centrifuged at 15 000 *g* for 5 min. The protein concentration in the supernatant fluid was determined by the Bradford assay (Bio-Rad). A total of 3 μg of proteins of each sample was denatured by boiling for 5 min in sample buffer [0.05 M Tris-HCl, pH 6.8, 1% (w/v) SDS, 0.1% (v/v) glycerol, 0.02% (v/v) 2-mercaptoethanol, 0.025% (w/v) bromophenol blue], separated by electrophoresis on SDS-polyacrylamide gels (15% for COX-1, COX-2, mPGES-1, mPGES-2, cPGES, GFAP and Iba-1, and 7.5% for MAP-2) and transferred electrophoretically onto immobilon-P polyvinylidene difluoride membranes (Millipore Corporation, Bedford, MA, USA). The membranes were blocked overnight in Tris-buffered saline-Tween 20 (TBS-T) containing 5% skim milk. After washing with TBS-T, the membranes were incubated with the appropriate first antibodies against mPGES-1 (1 : 500 dilution), mPGES-2 (1 : 200), cPGES (1 : 250), COX-1 (1 : 250), COX-2 (1 : 1000), Iba-1 (1: 2000), MAP-2 (1 : 250) or GFAP (1 : 1000) in TBS-T for 1.5 h. After washing the membranes three times with TBS-T, horseradish peroxidase-conjugated secondary antibodies were added at a 1 : 10 000 dilution in TBS-T and the membranes incubated for 1 h. After three washes with TBS-T, protein bands were visualized with ECL western blot detection reagents (Amersham Biotech).

Northern blot analysis

Total RNA was extracted with TRIzol reagent (Life Technologies, Grand Island, NY, USA) from cultured brain cells. Approximately

equal amounts (about 8 μg) of the total RNAs were applied to separate lanes of 1.2 M formaldehyde-agarose gels, electrophoresed, and transferred to Immobilon-N membranes (Millipore). The resulting blots were hybridized with the respective cDNA probes. The cDNA fragments for rat PGES, COX-2 and GAPDH were generated by PCR amplification from lypopolysaccharide-stimulated rat microglia with specific primers (PGES: sense 5′-ATGACTTCCCTGGGTTTG-3′ and antisense 5′-TCACAGATGGTGGGCCAC-3′, 461 bp; COX-2: sense 5′-AGACAGATCATAAGCGAGGACC-3′ and antisense 5′-CACTTGCGTTGATGGTGGCTGT-3′, 1158 bp; GAPDH: sense 5′-AGACAGCCGCATCTTCTTGT-3′ and antisense 5′-CCACAGTCTTCTGAGTGGCA-3′, 607 bp). The PCR products were cloned using the TA cloning kit with pCR2.1 vector and DH5α (Invitrogen, Carlsbad, CA, USA). The cDNA probes of rat PGES, COX-2 and GAPDH were obtained by enzymatic digestion of recombinant clones. The probes had been labeled with ^{32}PdCTP by a Megaprime DNA labeling system (Amersham Biotech). Hybridizations were carried out at 42°C for 16 h in a solution comprising 50% (v/v) formamide, 0.75 M NaCl, 75 mM sodium citrate, 0.1% (w/v) SDS, 1 mM EDTA, 10 mM sodium phosphate, pH 6.8, 1 × Denhardt's solution, 10% (w/v) dextran sulfate and 100 μg/mL salmon sperm DNA. The membranes were washed twice at 25°C with 300 mM NaCl, 30 mM sodium citrate and 0.1% SDS for 10 min each, followed by two washes at 65°C with 150 mM NaCl, 15 mM sodium citrate and 0.1% SDS for 20 min each, followed by a wash at 65°C with 15 mM NaCl, 1.5 mM sodium citrate and 0.1% SDS for 30 min. The blots were visualized by autoradiography with Kodak X-Omat AR films (Eastman Kodak, Rochester, NY, USA) and double intensifying screens at − 80°C.

Prostanoids assay

The concentration of the PGE_2, thromboxane (TX) B_2 and 6-keto-$PGF_{1\alpha}$ in the culture medium was determined using enzyme immunoassay (EIA) kits (Cayman Chemicals). Samples (2 mL) of culture medium from each dish harvested with 1.2×10^6 cells of microglia were diluted with the assay buffer. The concentrations of each prostanoid were determined in duplicate samples according to the instructions provided with the kits and extrapolated from standard curves, and were expressed in nanograms per millilitre of culture medium.

Assay of enzymatic activity of PGES

PGES activities in cell lysates were measured by assessment of the conversion of PGH_2 to PGE_2. The cells were scraped from the dishes and disrupted by sonication (10 s, three times, at 1 min intervals) in 100 μL 0.2 M Tris-HCl, pH 8.0. After centrifugation of the sonicates at 15 000 *g* for 15 min at 4°C, the supernatant fluids were used as the enzyme source. An aliquot of each lysate (90 μg protein equivalents) was incubated with 2 μg PGH_2 for 30 s at 24°C in 100 μL 0.2 M Tris-HCl, pH 8.0, containing 2 mM glutathione and 14 μM indomethacin. After terminating the reaction by the addition of 100 mM $FeCl_2$, PGE_2 contents in the supernatant fluids were quantified using an EIA kit (Cayman Chemical). For preparation of the microsomal membrane fraction and soluble fraction, the scraped cells were sonicated in 100 μL ice-cold 1 M Tris-HCl, pH 8.0, containing protease inhibitors (Roche, Indianapolis, IN, USA) and centrifuged as described above. The supernatant fluids were further centrifuged at 100 000 *g* for 1 h at 4°C, then the soluble fractions (supernatant fluids) and microsomal membranes (pellets which were resuspended in 100 μL 1 M Tris-HCl, pH 8.0, containing protease inhibitors) were subjected to measurement of PGES activity. An aliquot of each lysate (10 μg protein equivalents) was incubated with 0.5 μg PGH_2 for 30 s at 24°C in 100 μL 1 M Tris-HCl, pH 8.0, containing 2 mM glutathione and 14 μM indomethacin. After terminating the reaction by the addition of 100 mM $FeCl_2$, PGE_2 contents were quantified as described above.

Antisense experiments

The mPGES-1 antisense and sense S-oligonucleotide (480 pM), 5′-GAGGACCACGAGGAAATGTAT-3′ and 5′-ATACATTTCCTCGTGGTCCTC-3′, respectively, were transfected into microglia with a LipofectAMINE 2000 kit (Invitrogen) according to the instructions provided with the kit. After 1 h, cells were stimulated with LPS for 12 h, and the PGE_2 released into the supernatant fluids was quantified. The cell lysates were subjected to western blotting to verify mPGES-1 expression.

MTT assay

Toxicity of the LPS was determined by MTT assay. After treatment with various concentrations of LPS for 24 and 48 h, 10 μL of a 2.5 mg/mL MTT stock solution were added to each well containing 100 μL of medium; the reaction mixture was then incubated for 2 h. Afterwards, the reaction was stopped by adding 100 μL of a solution containing 50% dimethylformamide and 20% SDS (pH 4.7). After overnight incubation at 37°C, the absorbance was measured with a microplate reader at a test wavelength of 570 nm and a reference wavelength of 655 nm.

Statistical analysis

Results are expressed as the means ± SE. Statistical significance was evaluated with one-way analysis of variance followed by Tukey's method. Values of $p < 0.05$ were considered to indicate statistical significance.

Results

Induction of mPGES-1 in microglia by LPS injection into the substantia nigra

To determine whether or not mPGES-1 expression is induced in the brain at sites of localized inflammatory response *in vivo*, we investigated the effects of a local injection of LPS into the brain on mPGES-1 expression. LPS was stereotaxically injected into the adult rat substantia nigra and 48 h later the brain was removed and further processed. The injection of LPS dramatically increased the number of mPGES-1-positive cells in the substantia nigra compared with the contralateral site, used as a control (Figs 1a, b, d and g). On the control side, the mPGES-1 immunoreactive cells were primarily localized along the needle tract (i.e. on the inside of the broken line in Fig. 1a). On the LPS-injected side, mPGES-1 immunoreactive cells were observed not only along the needle tract but also in the area far from the needle tract (Fig. 1b). The immunostaining of mPGES-1 observed

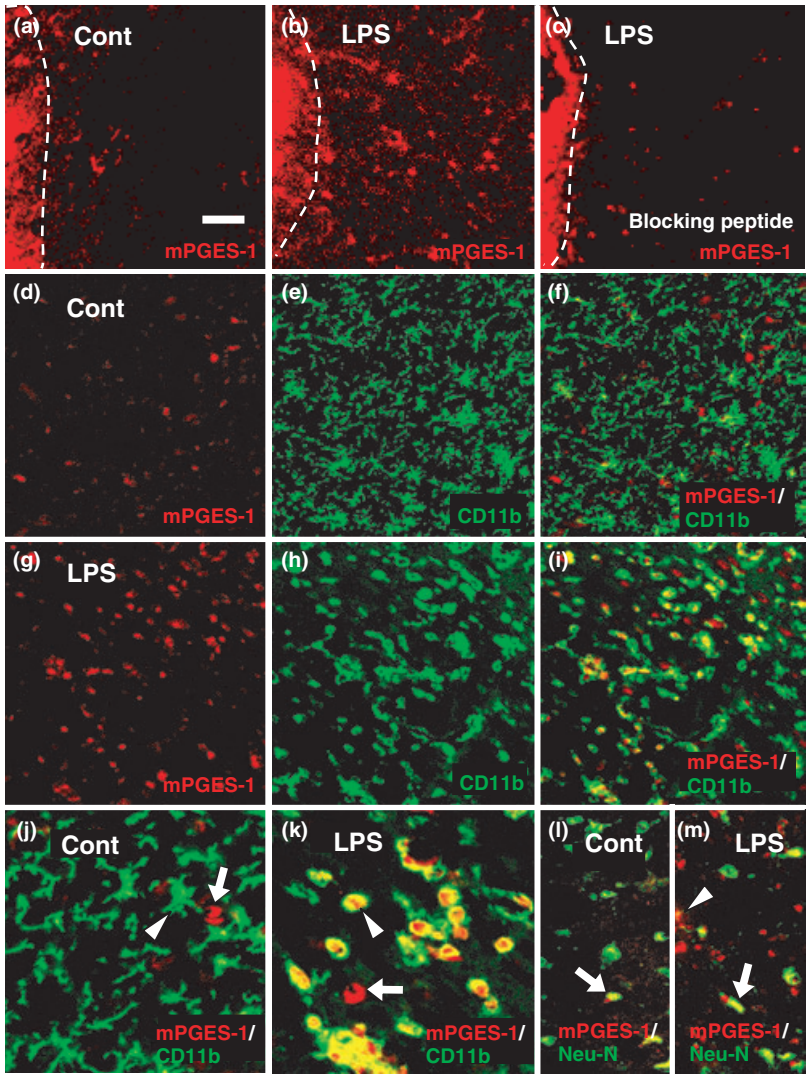

Fig. 1 Immunostaining of mPGES-1 and cell type-specific proteins (Neu-N for neurons and CD-11b for microglia) in the rat substantia nigra 48 h after injection of LPS. LPS was injected into the right side of the substantia nigra (b, c, g–i, k, m). Control injections of vehicle were made into the left side of the substantia nigra (a, d–f, j, l). In all panels, red and green indicate mPGES-1 and cell type-specific protein immunoreactivity, respectively. The staining of mPGES-1 around the needle tract of the control side is shown in (a), and that of the LPS-injected side in (b). The staining of mPGES-1 around the needle tract of the LPS-injected side using pre-absorbed mPGES-1 antibody is shown in (c). The inside of the broken lines in (a), (b) and (c) indicates the area of the needle tract. (d–k) Double immunostaining of mPGES-1 (d, g) and CD11b (e, h) with overlay (f, I–k). (j), (k) High magnification of (f) and (i), respectively. The arrowhead and arrow in (j) indicate the ramified microglia and basal mPGES-1 expression, respectively, in the vehicle-injected substantia nigra. The arrowhead and arrow in (k) indicate the ameboid (activated) microglia and non-microglial cell, respectively, expressing mPGES-1 protein in the LPS-injected substantia nigra. (l, m) Double immunostaining of mPGES-1 and Neu-N. The arrows in (l) and (m) indicate mPGES-1 expression in neurons. The arrowhead in (m) indicates a significant induction of mPGES-1 in non-neuronal cells of the LPS-injected substantia nigra. Scale bar, 50 μm for (a–i, l, m); 20 μm for (j, k).

on the LPS-injected side was similar to that on the control side when a pre-absorbed antibody with an antigen peptide for mPGES-1 was used (Fig. 1c). These results indicated that the signals seen along the needle tract were the non-specific type of signals that are occasionally seen at the edges of tissue sections. However, we cannot exclude the possibility that mPGES-1 was induced along the needle tract due to mechanical injury-induced activation, since such activation has previously been shown to lead to the accumulation and activation of microglia (Kim *et al*. 2000). To avoid confusing the effects of mechanical injury and the effects induced by LPS, we investigated mPGES-1 induction in an area that was approximately 300–500 μm away from the needle tract (Fig. 1d–m).

On the control side, the majority of CD11b-immunopositive cells exhibited a resting or ramified state (Fig. 1e), whereas at the LPS-injected site, CD11b-immunopositive cells exhibited the characteristics of activated microglia, i.e. an increase in cell volume and a morphological change into rounder somata (Fig. 1h). These data indicate that we successfully induced an inflammatory response at the LPS-injected site.

Double immunostaining of mPGES-1 and CD11b showed that mPGES-1 was induced in activated microglia (Figs 1i and k). mPGES-1-positive cells were occasionally observed on the vehicle-treated side, but they were negative for CD11b, indicating that the cells constitutively expressing mPGES-1 were not microglia (Figs 1f and j). mPGES-1-positive cells on the vehicle-treated side were positive for the neuronal marker protein, Neu-N (Fig. 1l), whereas on the LPS-injected side, mPGES-1-positive cells were largely negative for Neu-N (Fig. 1m). Although there is increasing evidence that mPGES-1 is induced in vascular endothelial cells in the brain in some models, including the intraperitoneal injection model of LPS-induced fever (Yamagata *et al*. 2001), the kainate-induced seizure model (Ciceri *et al*. 2002) and the burn injury model (Ozaki-Okayama *et al*. 2004), we did not observe mPGES-1 induction in endothelial cells within the substantia nigra in our LPS injection model (data not shown), indicating that a difference in the LPS injection route results in a different profile of mPGES-1 expression in the brain.

Expression of PGES and COX protein in cell-enriched cultures stimulated with LPS

Although the morphological change into rounder somata from the ramified form observed in CD11b-immunoreactive cells in the substantia nigra was typical of microglia (Figs 1j and k), we cannot exclude the possibility that those round cells may have been infiltrating monocytes, which were also immunoreactive for anti-CD11b antibody. To determine the cell type expressing the LPS-induced mPGES-1 more precisely, we prepared separate microglia-, astrocyte- and neuron-enriched cultures that contained more than 95% positive cells stained by each cell-specific marker (data not shown). Each type of cultured cell was stimulated with 1 μg/mL LPS for 24 h. Western blot analysis demonstrated that mPGES-1 protein as well as COX-2 protein, but not mPGES-2, cPGES or COX-1 protein, was potently induced by LPS in the microglia-enriched culture (Fig. 2). mPGES-1 and COX-2 proteins were also slightly induced in the astrocyte-enriched culture, but not in the neuron-enriched culture. Although the level of cPGES protein was previously reported to increase in brain tissue after intravenous injection of LPS (Tanioka *et al*. 2000), the expression of cPGES was virtually unaltered by LPS in all of the cell types tested here. The changes in mPGES-2 and COX-1 expression were also minimal. The cell purity of each sample was confirmed by marker proteins, i.e. Iba-1 for microglia, GFAP for astrocytes and MAP-2 for neurons. Because slight expression of Iba-1 was detected in the astrocyte-enriched preparations, the band of mPGES-1 obtained from the astrocyte-enriched cultures may have resulted from a contamination of the microglia. mPGES-1 protein is known to be induced by inflammatory stimuli in some fibroblasts, such as synovial or orbital fibroblasts (Han *et al*. 2002; Kojima *et al*. 2002). In order to estimate the degree to which contamination of meningeal fibroblasts contributed to the expression of mPGES-1 in the microglia-enriched culture, we also tested meningeal fibroblasts for their ability to induce mPGES-1 protein and found that it was lower than that in microglia stimulated with LPS (Fig. 2). Conversely, the band of mPGES-1 obtained from fibroblasts may also have been the result of contamination of the microglia, because slight Iba-1 expression was observed in the fibroblast preparations.

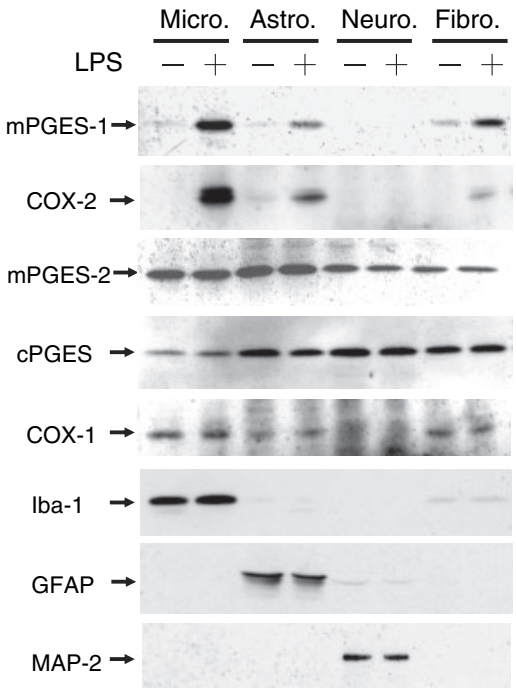

Fig. 2 mPGES-1 expression induced by LPS in microglia, astrocytes, neurons and meningeal fibroblasts. Enriched cultures of microglia (Micro.), astrocytes (Astro.) and neurons (Neuro.), and meningeal fibroblasts (Fibro.), were obtained as described in the Materials and methods. The cells were incubated for 12 h with or without 1 μg/mL LPS, and lysates were subjected to western blot analysis for mPGES-1, COX-2, mPGES-2, cPGES, COX-1 and cell type-specific protein (Iba-1 for microglia, GFAP for astrocytes and MAP-2 for neurons). Representative immunoblots from three separate experiments are presented.

Microglia-specific expression of mPGES-1 protein in a mixed culture of brain cells stimulated with LPS

To confirm further the microglia-specific induction of mPGES-1 seen in Fig. 2 by excluding the possibility of cell

contamination, we performed immunostaining for mPGES-1 in a mixed culture of neonatal rat brain cells, primarily neurons, astrocytes, oligodendrocytes and microglial cells. Immunostaining for mPGES-1 and COX-2 revealed co-induction and co-localization of these proteins by LPS stimulation (Figs 3b and c). Not all of the mixed brain cells expressed mPGES-1 after LPS stimulation (Figs 3a and d), suggesting the heterogenous induction of mPGES-1 protein. To determine whether or not mPGES-1 induction is cell-specific, LPS-treated cells were double-stained with anti-mPGES-1 antibody and one of the following: anti-CD11b (Fig. 3d), anti-GFAP (Fig. 3e), anti-MAP-2 (Fig. 3f) or anti-CNPase antibody (Fig. 3g), the antigens of which are specific markers for microglia, astrocytes, neurons or oligodendrocytes, respectively. As shown in Fig. 3(d), the mPGES-1-positive cells were also positive for CD-11b. In contrast, none of the mPGES-1-positive cells were positive for GFAP, MAP-2 or CNPase (Fig. 3e–g). These results once again demonstrate that the induction of mPGES-1 proteins was specific to microglia, and that mPGES-1 and COX-2 were co-induced by LPS.

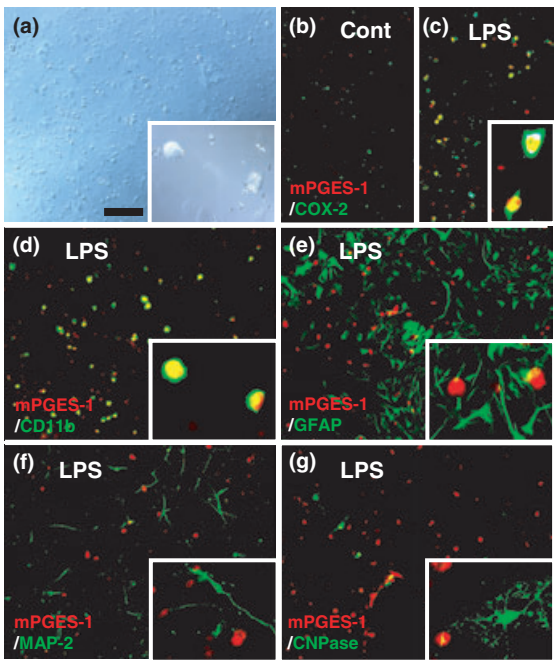

Fig. 3 Microglia-specific induction of mPGES-1 with COX-2 by LPS. Neuron-glia mixed culture (a) was incubated for 24 h with (c–g) or without (b) 1 μg/mL LPS and double-stained with the anti-mPGES-1 (red: b–g), COX-2 (green: b, c) and anti-cell type-specific antibodies (green: d–g). Microglia, astrocytes, neurons and oligodendrocytes were recognized by anti-CD11b (d), anti-GFAP (e), anti-MAP-2 (f) and anti-CNPase (g) antibodies, respectively. The insets show high magnification of each staining. The bright field photomicrography of the visual field equal to (d) is shown in (a). Representative photographs from three separate experiments are presented. Scale bar, 125 μm; insets, 20 μm.

Time course of the induction of protein and mRNA of mPGES-1 and COX-2 in microglia

To elucidate the nature of the mPGES-1 induction, we used microglia-enriched cultures. The treatment of LPS (1–1000 ng/mL) for 24 h induced mPGES-1 protein expression in a concentration-dependent manner (Fig. 4a). As was the case with COX-2 protein induction, a lower concentration of LPS (1–10 ng/mL) also had a tendency to induce mPGES-1 expression, and a higher concentration of LPS (i.e. more than 100 ng/mL) significantly induced mPGES-1 protein expression when the bands were analyzed by densitometer. Because we observed the most prominent induction of mPGES-1 at a concentration of 1 μg/mL LPS, which had no toxic effect on the microglia (Fig. 4b), we used this concentration of LPS in all subsequent experiments. We next examined the time course of LPS-elicited up-regulation of mPGES-1 and COX-2 protein in a microglia-enriched culture (Figs 5a and b). mPGES-1 and COX-2 were both expressed at almost undetectable levels under basal conditions. mPGES-1 protein was detectable after 6 h and by 24–48 h, expression

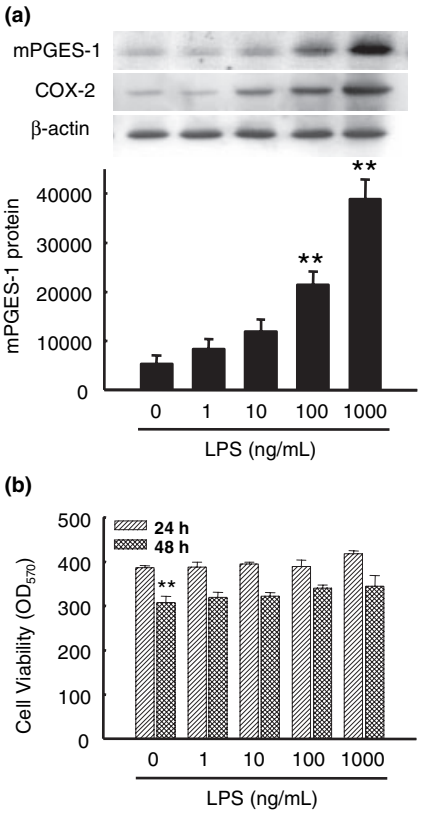

Fig. 4 Effect of LPS on mPGES-1 expression and viability of microglia. Microglia-enriched cultures were incubated with the indicated concentrations of LPS for 24 h (a, b) and 48 h (b). (a) The cell lysates were subjected to western blot analysis for mPGES-1, COX-2 and β-actin. Representative immunoblots are presented on top of bar graph. Densities of immunoblots with mPGES-1 antibody were measured ($n = 4$). (b) The cell viabilities were measured by MTT assay ($n = 4$).

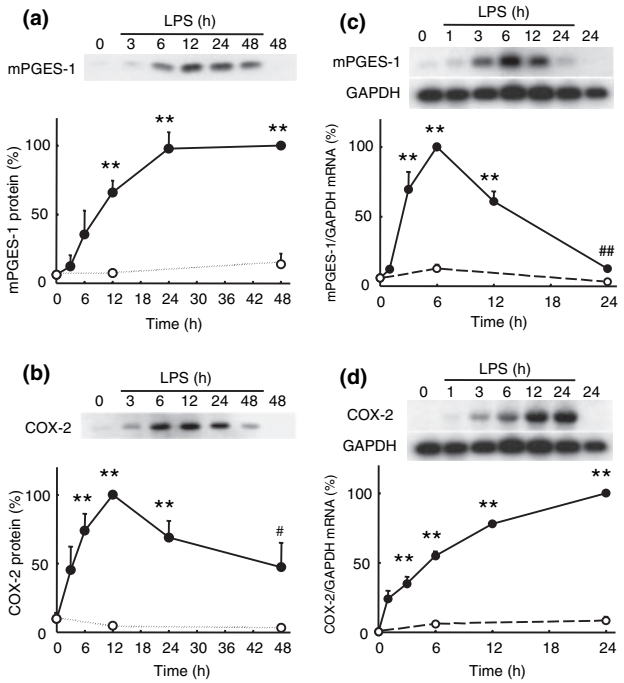

Fig. 5 Time course of LPS-induced mPGES-1 and COX-2 expression in microglia. Microglia-enriched culture was incubated with (●) or without (○) 1 μg/mL LPS for the indicated times. Lysates were subjected to western blot analysis for mPGES-1 (a) and COX-2 (b). Representative immunoblots are presented on top of each bar graph. Quantitated data ($n = 4$) were normalized and scaled to a percentage of the maximal values: 48 h for mPGES-1 and 12 h for COX-2. Total RNA from these cells was subjected to northern blot analysis for mPGES-1 (c), COX-2 (d) and GAPDH (c, d). Representative blots are presented on top of each bar graph. Quantitated data ($n = 4$) were normalized to GAPDH and scaled to a percentage of the maximal values: 6 h for mPGES-1 and 24 h for COX-2; $^{**}p < 0.01$, significant induction versus basal expression (0 h); $\#\#p < 0.01$ and $\#p < 0.05$, significant reduction versus LPS-induced maximal expression.

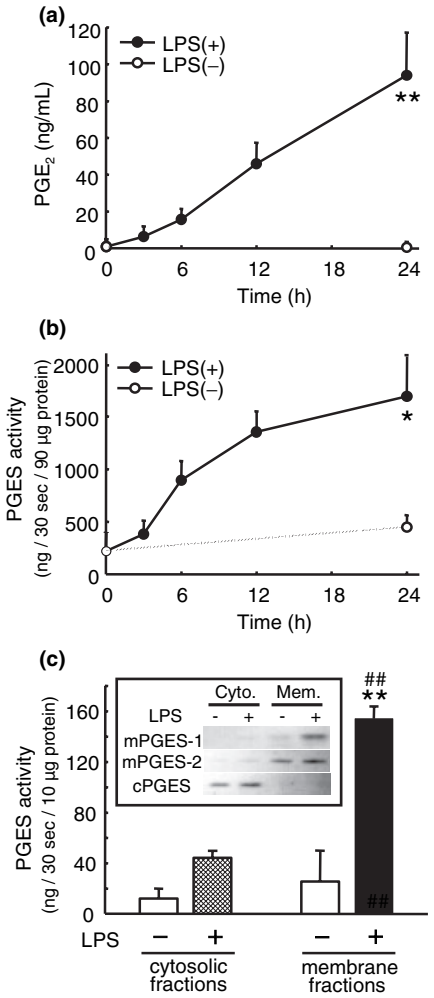

Fig. 6 Effect of LPS on PGE_2 synthesis and PGES activity in microglia. Microglia-enriched culture was incubated with (●) or without (○) 1 μg/mL LPS for the indicated times. (a) The amount of PGE_2 in the culture medium was measured by immunoassay ($n = 4$). (b, c) PGES activity was measured as the conversion of exogenous PGH_2 to PGE_2 for 30 s by the cell lysate (b) and cytosolic (Cyto.) and membrane (Mem.) fractions (c). The amount of PGE_2 was measured by immunoassay (b, $n = 4$; c, $n = 3$). Insets of (c) membrane and cytosolic fractions of microglia stimulated with or without LPS for 24 h were subjected to western blot analysis for mPGES-1, mPGES-2 and cPGES. Representative immunoblots from three separate experiments are presented; $^{*}p < 0.05$, $^{**}p < 0.01$ and $\#\#p < 0.01$, significant induction versus the vehicle-treated control culture and significant difference versus the cytosolic fraction of LPS-treated culture, respectively.

had reached a maximal level that was at least 10-fold higher than the control level (Fig. 5a). Importantly, this time course was in good correspondence with the LPS-induced increases in PGES activity and PGE_2 levels (Fig. 6). The induction of COX-2 protein was more rapid and transient than that of mPGES-1. The level peaked after 12 h, at a value of at least 10-fold the baseline value, and then decreased gradually (Fig. 5b). A significant reduction in COX-2 expression was observed at 48 h versus maximal induction at 12 h.

To determine whether or not the induction of mPGES-1 and COX-2 is regulated at the pre-translational level, we carried out a northern blot analysis. The transcript encoding mPGES-1 was slightly detectable under basal culture conditions and was substantially increased 3 h after LPS treatment (Fig. 5c). The mRNA levels of mPGES-1 peaked after 6 h at a value at least 10-fold above the initial levels. After 12 h, levels began to decrease and had returned to the basal levels by 24 h. COX-2 mRNA was almost undetectable under the control conditions and was up-regulated by LPS treatment; levels of COX-2 mRNA increased gradually up to 24 h (Fig. 5d).

LPS up-regulates PGE_2 production and PGES activity in microglia

To investigate the roles of mPGES-1, we first investigated changes in PGE_2 production and PGES enzymatic activity

induced by applying LPS to the culture for up to 24 h. In our microglia-enriched cultures, LPS elicited a dramatic increase in the level of PGE_2, as reported previously (Minghetti and Levi 1995). The PGE_2 level began to increase after 6 h but did not reach an apparent steady state, even at 24 h (Fig. 6a). At 24 h, the PGE_2 level was nearly 100-fold that of the control value.

For the PGES activity assays, we measured the conversion of exogenous PGH_2 to PGE_2 using cell lysates of LPS-stimulated cultures at various times. As shown in Fig. 6(b), the PGES activity was also increased beginning at 6 h of the LPS challenge, and reached a maximal level of approximately 800% of the initial levels at 24 h. To determine which type of PGES, i.e. membrane-associated PGES or cytosolic PGES, contributes to the up-regulation of PGES activity, the cytosolic and membrane fractions of microglia were separated and subjected to PGES assay. The membrane fractions exhibited elevated PGES activity when stimulated with LPS for 24 h (Fig. 6c). The PGES activity of the LPS-stimulated membrane fractions was significantly higher than that of the cytosolic fractions. In the cytosolic fractions, PGES activity was slightly up-regulated by LPS stimulation, suggesting that cytosolic PGES also contributes to LPS-induced PGE_2 production. However, the PGES activity observed in the cytosolic fractions may have resulted from contamination by membrane-associated PGES, because mPGES-1 was slightly expressed in the cytosolic fraction (Fig. 6c, inset). These data suggest that most of the increased PGES activity in LPS-stimulated microglia is membrane-associated. The observation of simultaneous increases in mPGES-1 expression (Fig. 5a), PGES activity and PGE_2 levels suggests that LPS-stimulated PGE_2 production may be mediated, at least in part, by an increase in PGES activity through the induction of mPGES-1 protein.

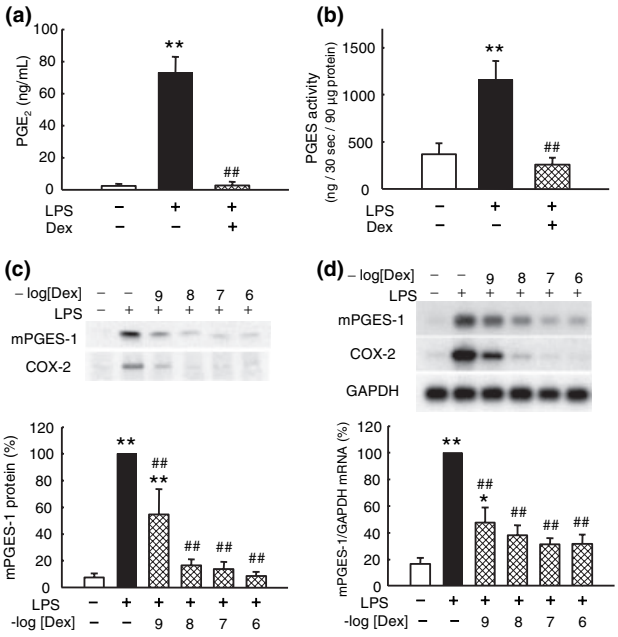

Fig. 7 Effect of dexamethasone (Dex) on LPS-induced PGE_2 synthesis, PGES activity and mPGES-1 expression in microglia. (a, b) Microglia-enriched culture was pre-incubated for 1 h in the presence or absence of 1 μM Dex and was then incubated for 24 h with or without 1 μg/mL LPS. (a) The amount of PGE_2 in the culture medium was measured by immunoassay ($n = 4$). (b) PGES activity was measured as the conversion of exogenous PGH_2 to PGE_2 for 30 s by the cell lysates ($n = 4$). (c, d) Microglia-enriched culture was pre-incubated for 1 h in the presence or absence of the indicated concentration of Dex, and then incubated for 12 or 6 h with or without 1 μg/mL LPS for the western blot or the northern blot analysis, respectively. (c) Representative immunoblots for mPGES-1 and COX-2 are presented on top of the bar graph. Quantitated data ($n = 4$) from immunoblotting with mPGES-1 antibody were normalized and scaled to a percentage of the response of LPS alone. (d) Representative blots of northern blot analysis for mPGES-1, COX-2 and GAPDH are presented on top of the bar graph. Quantitated data ($n = 4$) from blotting of mPGES-1 probe were normalized to GAPDH and scaled to a percentage of the response of LPS alone; $^*p < 0.05$, $^{**}p < 0.01$ and $\#\#p < 0.01$, significant induction versus the vehicle-treated control culture and significant inhibition versus the LPS-treated culture, respectively.

Effects of dexamethasone on LPS-induced PGE_2 production, PGES activity and mPGES-1 expression in microglia

To determine the importance of the concerted induction of mPGES-1 and COX-2 to PGE_2 production in activated microglia, we examined the effects of synthetic glucocorticoid dexamethasone, which is known to inhibit eicosanoid production via the down-regulation of COX-2 expression (Weidenfeld *et al*. 1993; Pistritto *et al*. 1999), on LPS-induced responses. The microglia-enriched cultures were stimulated with LPS in the presence or absence of dexamethasone. Consistent with previous reports (Minghetti *et al*. 1999), LPS-induced PGE_2 production was suppressed almost completely by dexamethasone under our experimental conditions (Fig. 7a). Furthermore, the up-regulation of PGES activity by LPS was also inhibited completely by pre-treatment with dexamethasone (Fig. 7b). Western and northern blot analyses were performed to evaluate the inhibitory effects of dexamethasone on mPGES-1 expression. The microglia-enriched cultures were pre-treated with various concentrations of dexamethasone (0.001–1 μM), followed by exposure to LPS. The LPS-induced expression of mPGES-1 protein, as well as that of COX-2 protein, was inhibited by dexamethasone in a concentration-dependent manner and was almost completely suppressed at 10 nM or more (Fig. 7c). The induction of mPGES-1 mRNA was also attenuated by dexamethasone in a concentration-dependent manner (Fig. 7d). Taken together, these results indicate that dexamethasone inhibits PGE_2 production not only by decreasing the COX-2 levels but also, by reducing PGES activity via the inhibition of mPGES-1 expression at the pre-translational level.

Role of mPGES-1 in PGE_2 production by LPS-stimulated microglia

To evaluate the contribution of mPGES-1 to PGE_2 production by LPS-stimulated microglia, we first took advantage of an antisense approach. As compared with the microglia transfected with mPGES-1 sense oligonucleotide, LPS-induced PGE_2 production was significantly reduced in microglia transfected with mPGES-1 antisense oligonucleotide, in which mPGES-1 expression was also decreased, without a reduction in either COX-2 or mPGES-2 expression (Fig. 8). A partial change in the amount of mPGES-1 could affect PGE_2 production, suggesting that the expression level of mPGES-1 regulates the rate of PGE_2 production.

To confirm further the contribution of mPGES-1 to PGE_2 production by LPS-stimulated microglia, we carried out studies in mPGES-1 KO mice. LPS-stimulated PGE_2 production was completely abolished in microglia derived from mPGES-1 KO mice (Fig. 9a). Constitutive expression of mPGES-2, cPGES and COX-1, and inducible expression of COX-2, were similar between WT- and KO-derived microglia (Fig. 9b), indicating that a mPGES-1 deficiency

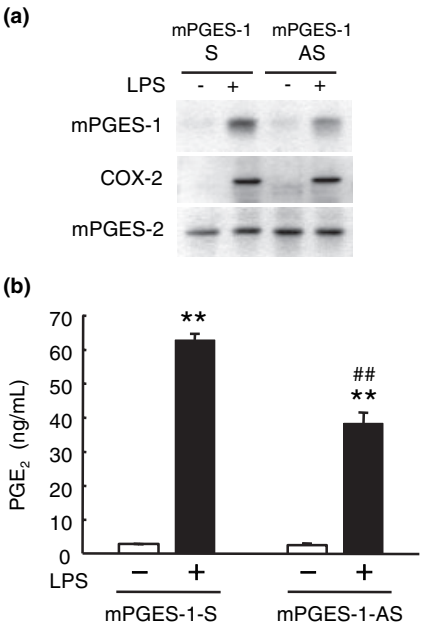

Fig. 8 Inhibitory effects of an mPGES-1 antisense oligonucleotide on mPGES-1 expression and PGE_2 production. (a) Effects of the antisense and the control sense oligonucleotides (mPGES-1-AS and mPGES-1-S, respectively) on mPGES-1, COX-2 and mPGES-2 protein levels determined by western blotting in the microglial culture with (+) or without (–) LPS (1 μg/mL) stimulation for 12 h. Representative immunoblots from three separate experiments are presented. (b) Effects of the antisense and the sense oligonucleotides on medium PGE_2 level in the microglial culture with (closed column) and without (open column) LPS stimulation ($n = 4$); $^{**}p < 0.01$ and $\#\#p < 0.01$, significant induction versus the vehicle-treated control culture and significant inhibition versus the sense-oligonucleotide-treated culture, respectively.

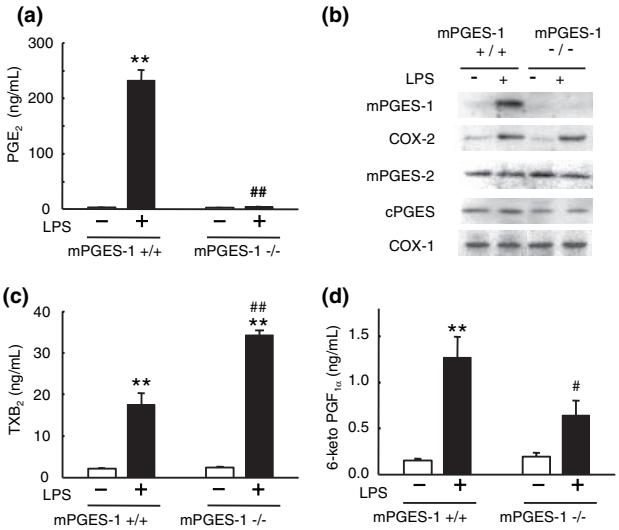

Fig. 9 mPGES-1 is an essential component for PGE_2 production by LPS-stimulated microglia. Microglia obtained from WT and mPGES-1 KO mice (+/+ and –/–, respectively) were incubated with (+) or without (–) 1 μg/mL LPS for 24 h. (a) The production of PGE_2 by WT and KO microglia treated with (closed column) or without (open column) LPS was measured ($n = 4$). (b) The cell lysates from microglia obtained from WT and mPGES-1 KO mice were subjected to western blotting for the PGE_2 biosynthetic enzymes. Representative immunoblots from three separate experiments are presented. (c, d) Production of other prostanoids by WT and KO microglia after 24 h of culture with (+) or without (–) LPS. The amount of TXB_2 (c) and 6-keto $PGF_{1\alpha}$ (d) was measured ($n = 4$); $^{**}p < 0.01$, significant induction versus the vehicle-treated control culture; $\#p < 0.05$ and $\#\#p < 0.01$, significant changes from the LPS-treated microglial culture obtained from WT mice.

does not affect the expression of other enzymes implicated in PGE_2 synthesis in these cells. We also determined the levels of other prostanoids, TXB_2 (a stable end product of TXA_2) and 6-keto-$PGF_{1\alpha}$ (a stable end product of PGI_2), in the culture medium. A significant increase in TXB_2 (1.74-fold, $p < 0.01$) was observed in LPS-treated mPGES-1 KO microglia compared with that of WT cells (Fig. 9c), which may reflect a shunting effect due to a defect in the metabolic flow from PGH_2 to PGE_2. On the other hand, LPS-treated mPGES-1 KO microglia released 50% less 6-keto-$PGF_{1\alpha}$ ($p < 0.05$) than did WT cells (Fig. 9d). Although the clarification of the role of mPGES-1 in PGI_2 synthesis will require further study, the reduction in 6-keto-$PGF_{1\alpha}$ observed in the LPS-treated mPGES-1 KO microglia suggests that PGI_2 synthesis is at least partially dependent on mPGES-1-derived PGE_2 in these cells.

Discussion

Two major conclusions can be derived from the present experiments. First, by employing a cell type-specific staining technique and cell type-specific culture preparations, we have

shown that mPGES-1 is induced specifically in microglia *in vivo* and *in vitro* after LPS treatment. Second, we have demonstrated the necessity of mPGES-1 for PGE_2 production in brain parenchymal cells. Using mPGES-1 antisense oligonucleotide and a cell type-specific culture of mPGES-1 KO mouse cells, we were able to provide unequivocal evidence that mPGES-1 contributes to the formation of PGE_2 in microglia stimulated with LPS.

Microglial activation observed in a localized area in the brain parenchyma is a histopathological hallmark in several neurodegenerative brain diseases, including Parkinson's disease, Alzheimer's disease, multiple sclerosis and the AIDS dementia complex (McGeer *et al*. 1988; Dickson *et al*. 1993; Raine 1994). In this study, we were able to detect such activated microglial morphology in an LPS-injected area of the rat substantia nigra. Activated microglia produce various types of cytokines and inflammatory mediators, including PGE_2 (Minghetti and Levi 1995; Elmquist *et al*. 1997). It is well known that COX-2 induction may be predominant in the course of inflammatory brain PGE_2 production (Nogawa *et al*. 1997; Lipsky 1999; Tomimoto *et al*. 2000; Hoozemans *et al*. 2001; Iadecola *et al*. 2001; Teismann *et al*. 2003). However, the role of PGES, the terminal enzyme for PGE_2 synthesis, in brain PGE_2 production has remained unclear. Recently, increasing evidence has been reported suggesting that vascular endothelial cells are one of the major sources of PGE_2 in the brain; for example, endothelial mPGES-1 induction has been observed in animal models of burn injury, LPS-induced pyresis and carrageenan-induced paw edema (Yamagata *et al*. 2001; Guay *et al*. 2004; Ozaki-Okayama *et al*. 2004). Because these types of inflammation were initiated peripherally or systemically, the site of initial exposure to pathological signals in the brain might have been the vascular endothelial cells of the brain. On the other hand, in our LPS-induced localized inflammation model in the brain parenchyma, activated microglia-specific induction of mPGES-1 was observed at the site of LPS injection. The same cell-type specificity of mPGES-1 induction was observed in cultured brain cells stimulated with LPS. Therefore, these results suggest that the microglia-specific induction of mPGES-1 and the production of PGE_2 may occur at the localized inflammation site in the brain parenchyma, in which microglial activation is known as a histopathological hallmark. However, we cannot exclude the possibility that the mPGES-1 induction observed in our *in vivo* study was due to the infiltrating monocytes. The reasons are that we cannot distinguish microglia from infiltrating monocytes by immunostaining of brain slices since CD11b, a marker for microglia, is also expressed in infiltrating monocytes, and LPS induces expression of mPGES-1 in cultured macrophages (Murakami *et al*. 2000; Kamei *et al*. 2004); also, the endothelial mPGES-1 induction may occur at an earlier time point following intraparenchymal injection of LPS, as reported in a systemic LPS injection model (Yamagata *et al*. 2001). In neurological disorders such as infection, ischemic brain injury, multiple sclerosis, Alzheimer's disease and Parkinson's disease, COX-2 is known to be induced in both neurons and microglia (Nogawa *et al*. 1997; Lipsky 1999; Tomimoto *et al*. 2000; Hoozemans *et al*. 2001; Teismann *et al*. 2003; Rose *et al*. 2004). Moreover, amyloid β has been shown to induce mPGES-1 mRNA in cultured rat astrocytes (Satoh *et al*. 2000). Although we were unable to detect mPGES-1 induction in either neurons or astrocytes stimulated with LPS, the neuronal and/or astroglial induction of mPGES-1 may occur in the brains of patients with various neurological disorders. Additional studies will be needed to determine the cellular source of PGE_2 in the brain in patients with these diseases.

In cultured microglia, the co-expression of mPGES-1 and COX-2 protein was preceded by mRNA induction. Therefore, the induction of mPGES-1, as well as that of COX-2, is regulated, at least in part, at the pre-translational level. The mechanisms underlying mPGES-1 induction have been reported to involve the activation of Toll-like receptor-4 (TLR4), MyD88 and NF-IL6, and the binding of Egr-1 to the proximal GC box in the *mPGES-1* promoter region (Naraba *et al*. 2002; Uematsu *et al*. 2002). Although the mechanism of the LPS-induced expression of microglial mPGES-1 requires further study, the cell-specific induction of mPGES-1 observed here suggests that there may be some differences between microglia and other brain cells with respect to the activation of these proteins. Indeed, the mRNA of TLR4 exists in microglia, but not in astrocytes or oligodendrocytes, as determined by RT-PCR methods (Lehnardt *et al*. 2002).

LPS-treated microglia exhibited the co-expression of mPGES-1 and COX-2. However, the time courses of induction of these proteins differed from each other. mPGES-1 expression, PGES activity and PGE_2 production all increased gradually until 24 h after LPS stimulation, whereas the enhanced expression of COX-2 began to decline as early as 12 h. The production of both PGD_2 and TXB_2 (products of the same substrate as PGE_2) was reported to increase rapidly between 4 and 8 h, and then remain stable for up to 24 h in LPS-stimulated microglia (Minghetti and Levi 1995). The differences between PGE_2 and other products, as observed in terms of the kinetics of accumulation, are thought to depend on the nature of each prostanoid terminal synthase, because all of these prostanoids are synthesized from a single common substrate, PGH_2. Thus, the continued increase in mPGES-1 expression observed here indicates that microglial mPGES-1 plays an important role in the regulation of the amount of PGE_2, particularly during the later phases of LPS-activated PGE_2 production. The importance of the regulation of mPGES-1 expression for PGE_2 production in microglia was also suggested by the inhibitory effects of dexamethasone on the LPS-activated induction of PGES activity and mPGES-1 expression, as well as on the LPS-activated production of PGE_2. We observed a marked

down-regulation of the expression of the mRNA and protein of mPGES-1 at physiologically relevant concentrations of dexamethasone. The data therefore imply that mPGES-1, as well as COX-2, is a biochemical target of glucocorticoid regulation.

Although we found no apparent changes in the expression of cPGES or mPGES-2 after the application of LPS, the possibility that other types of PGES contribute to elevated PGES activity could not be excluded. However, the finding that the induction of mPGES-1 protein was coincident with both the up-regulation of PGES activity and the production of PGE_2 strongly suggests the importance of mPGES-1 in microglial PGE_2 production. Indeed, the reduction in the level of endogenous mPGES-1 by antisense oligonucleotide resulted in a reduction in PGE_2 production. Furthermore, mPGES-1-deficient microglia were unable to produce PGE_2, even when they were stimulated with LPS, thus indicating the predominant role of mPGES-1 in PGE_2 formation in microglia. A significant increase and decrease in TXB_2 and 6-keto $PGF_{1\alpha}$, respectively, were observed in LPS-treated mPGES-1-deficient microglia compared with normal cells. Thus, the absence of mPGES-1 slightly altered the production profiles of other prostanoids in microglia, although in a qualitatively different manner.

In summary, we have shown that LPS induces PGE_2 production through the microglia-specific induction of both mPGES-1 and COX-2. Our results demonstrated that the elevation in PGE_2 production after an LPS challenge was correlated with mPGES-1 protein levels rather than with COX-2 protein levels, and that increases in PGE_2 production and PGES activity occurred simultaneously. Furthermore, the critical involvement of mPGES-1 in LPS-induced PGE_2 production in microglia was confirmed using mPGES-1 KO mice. Thus, microglial mPGES-1 was shown to play a pivotal role, particularly during the later phases of LPS-activated PGE_2 production. Considering that COX inhibitors may non-selectively suppress many types of prostanoids that are essential for normal physiological function of the brain (Kaufmann *et al*. 1997), our results suggest that mPGES-1 is a promising novel target for therapeutic strategies aimed at modulating brain PGE_2 synthesis, and for protection against certain types of inflammatory diseases in the brain. Our interpretation of the data obtained here may cautiously be applied to account for the real events that occur during central inflammation, although the unequivocal elucidation of the roles of microglial mPGES-1 will still require chronic experiments using pathological animal models and human patients. The results presented here will hopefully provide a useful framework for further investigations.

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