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RESEARCH PAPER

Microsomal prostaglandin E synthase-1 contributes to ischaemic excitotoxicity through prostaglandin E_2 EP_3 receptors

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Background and purpose: Although microsomal prostaglandin E synthase (mPGES)-1 is known to contribute to stroke injury, the underlying mechanisms remain poorly understood. This study examines the hypothesis that EP_3 receptors contribute to stroke injury as downstream effectors of mPGES-1 neurotoxicity through Rho kinase activation.
Experimental approach: We used a glutamate-induced excitotoxicity model in cultured rat and mouse hippocampal slices and a mouse middle cerebral artery occlusion–reperfusion model. Effects of an EP_3 receptor antagonist on neuronal damage in mPGES-1 knockout (KO) mice was compared with that in wild-type (WT) mice.
Key results: In cultures of rat hippocampal slices, the mRNAs of $EP_{1–4}$ receptors were constitutively expressed and only the EP_3 receptor antagonist ONO-AE3-240 attenuated and only the EP_3 receptor agonist ONO-AE-248 augmented glutamate-induced excitotoxicity in CA1 neurons. Hippocampal slices from mPGES-1 KO mice showed less excitotoxicity than those from WT mice and the EP_3 receptor antagonist did not attenuate the excitotoxicity. In transient focal ischaemia models, injection (i.p.) of an EP_3 antagonist reduced infarction, oedema and neurological dysfunction in WT mice, but not in mPGES-1 KO mice, which showed less injury than WT mice. EP_3 receptor agonist-induced augmentation of excitotoxicity *in vitro* was ameliorated by the Rho kinase inhibitor Y-27632 and *Pertussis* toxin. The Rho kinase inhibitor HA-1077 also ameliorated stroke injury *in vivo*.
Conclusion and implications: Activity of mPGES-1 exacerbated stroke injury through EP_3 receptors and activation of Rho kinase and/or G_i. Thus, mPGES-1 and EP_3 receptors may be valuable therapeutic targets for treatment of human stroke.
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Keywords: ischaemia; prostaglandin E_2; prostaglandin E synthase; mPGES-1; EP_3 receptors; Rho kinase; excitotoxicity

Abbreviations: CBF, cerebral blood flow; COX, cyclooxygenase; iNOS, inducible nitric oxide synthase; KO, knockout; MAP, mean arterial pressure; MBP, myosin-binding protein; MCA, middle cerebral artery; mPGES-1, microsomal prostaglandin E_2 synthase; OGD, oxygen–glucose deprivation; PG, prostaglandin; PGES, PGE_2 synthase; PI, propidium iodide; PTX, *Pertussis* toxin; RT-PCR, reverse transcriptase polymerase chain reaction; TTC, triphenyltetrazolium chloride; WT, wild-type

Introduction

Stroke remains a major cause of death and neuronal disability worldwide. In the early stages of cerebral ischaemia, activation of glutamate receptors initiates the ischaemic cascade that causes most of the cerebral damage (Butcher *et al.*, 1990; Lee *et al.*, 1999). At later times after ischaemia, inflammation is a major factor in the progression of the injury (Barone and Feuerstein, 1999; Dirnagl *et al.*, 1999).

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Prostaglandin E_2 (PGE_2), one of the most likely candidates for propagation of inflammation, is known to be produced and to accumulate at the lesion sites of the ischaemic brain (Kempski *et al.*, 1987; Iadecola *et al.*, 2001; Ikeda-Matsuo *et al.*, 2006). PGE_2 is sequentially synthesized from arachidonic acid in two enzymic steps: cyclooxygenase (COX) and PGE_2 synthase (PGES). Among the two COX isoforms, COX-1 and COX-2, COX-2 is the more highly inducible form (Kaufmann *et al.*, 1997) and is involved in the pathogenic events occurring in cerebral ischaemia (Nogawa *et al.*, 1997; Iadecola *et al.*, 2001; Sasaki *et al.*, 2004). Similarly, of the three PGES isozymes, cytosolic PGES, microsomal PGES (mPGES)-1 and mPGES-2, mPGES-1 is the only inducible form (Jakobsson *et al.*, 1999; Murakami *et al.*, 2002; Ikeda-Matsuo *et al.*, 2005). Recently, using a model of focal cerebral ischaemia in

mPGES-1 knockout (KO) mice, we demonstrated that induction of mPGES-1 contributed to the exacerbation of stroke injury through ischaemic PGE_2 production (Ikeda-Matsuo *et al*., 2006). More recently, we have found that mPGES-1 activity is required for COX-2 to exert neuronal damage in ischaemic injury (Ikeda-Matsuo *et al*., 2010).

How, then, is the toxicity of post-ischaemic PGE_2 mediated? PGE_2 acts on four G protein-coupled receptors (EP_1–EP_4; nomenclature follows Alexander *et al*., 2009) that have very distinct and potentially antagonistic signalling cascades. EP_1 receptors couple to G_q, and activation of this receptor results in increased intracellular Ca^{2+} concentrations (Kawano *et al*., 2006); EP_2 and EP_4 receptors couple to G_s to increase cyclic AMP (cAMP) formation, whereas most EP_3 receptor isoforms, which are generated by alternative splicing, couple to G_i to decrease cAMP (Narumiya *et al*., 1999). EP_3 receptors can also be coupled to the $G_{12/13}$, resulting in activation of the small G protein Rho followed by activation of Rho kinase, an effector of RhoA (Katoh *et al*., 1996; Hatae *et al*., 2002; Shum *et al*., 2003; Macias-Perez *et al*., 2008). Thus, there is great potential for variability in the response of target cells to PGE_2 based on the receptors activated (Narumiya *et al*., 1999). The various roles of the PGE_2 receptors in neuronal death induced by excitotoxicity and ischaemic stroke have been clarified by genetic deletion and selective inhibition of each EP receptor. Deletion and inhibition of the EP_1 receptors partially reduce the neuronal damage caused by excitotoxicity and ischaemic stroke (Ahmad *et al*., 2006; Kawano *et al*., 2006). Deletion of the EP_2 receptors increases the infarct volume in mice after ischaemic stroke (McCullough *et al*., 2004). In contrast, a selective agonist of the EP_2 receptor induces caspase-dependent apoptosis (Takadera *et al*., 2004) and a EP_4 receptor agonist has been shown to reduce excitotoxic brain injury (Ahmad *et al*., 2005). Interestingly, a recent study showed that genetic deletion of the EP_3 receptor reduces the neuronal damage caused by oxygen/glucose deprivation (OGD) or ischaemic stroke (Saleem *et al*., 2009), while the EP_3 receptor agonist exacerbates acute excitotoxic or ischaemic-induced brain injury (Ahmad *et al*., 2007). We have also confirmed the amelioration of excitotoxicity and stroke injuries by inhibition and deletion of EP_3 receptors through anti-inflammatory and anti-apoptotic mechanisms (under submission).

Thus, EP_3 receptors could be one of the important effectors for the neurotoxicity of PGE_2. However, several points remain to be elucidated. First, is the EP_3 receptor a downstream effector of mPGES-1 neurotoxicity? Second, can an EP_3 receptor antagonist protect against excitotoxicity? And finally, what is the mechanism underlying EP_3 receptor-mediated neurotoxicity? In order to address these issues, we examined the hypothesis that EP_3 receptors contribute to the exacerbation of stroke injury as downstream effectors of mPGES-1 neurotoxicity and that the activation of the EP_3 receptors exerted its neurotoxic effect through Rho kinase and/or G_i activation. We performed both an *in vitro* study using hippocampal slices exposed to glutamate and an *in vivo* study employing transient focal ischaemia models in mPGES-1 KO and wild-type (WT) mice. The results demonstrated that an EP_3 receptor antagonist conferred protection against neurotoxicity *in vitro* and *in vivo* in WT mice, but not in mPGES-1 KO mice, and that Rho kinase was involved in EP_3 receptor-mediated neurotoxicity and ischaemic stroke.

Methods

Animals

All animal care and experimental procedures complied with the guidelines given by the Japanese Pharmacological Society. mPGES-1 KO mice and WT mice (C57BL/6J × 129/SvJ background) back-crossed to C57BL/6J mice for >8 generations to avoid artefactual differences caused by genetic background were used (Uematsu *et al*., 2002). In spite of the reported gender differences in infarct size after middle cerebral artery (MCA) occlusion and reperfusion (Hayashi *et al*., 2005), our preliminary data showed no significant gender differences in not only infarct volume, but also degree of oedema, neurological score and ischaemic PGE_2 production 24 h after ischaemia (Ikeda-Matsuo *et al*., 2006). Therefore, both male and female mice were studied at a weight of 25–30 g and data from both sexes were pooled. Hayashi *et al*. (2005) used age-matched younger animals and a 90 min occlusion model, while we used adult animals weighing 24–30 g and a 120 min occlusion model to ensure adequacy of occlusion. The reason we could not observe gender differences may be connected to the severity of our protocol compared with that of Hayashi *et al*. (2005). Wistar rat pups were purchased from SLC (Shizuoka, Japan).

Organotypic hippocampal culture

Brains were rapidly removed from 7-day-old Wistar rat, mPGES-1 KO or WT mouse pups, and 300-μm-thick horizontal entorhino-hippocampal slices were placed on transparent membranes (Millicell-CM, Millipore, Bedford, MA, USA) in 6-well culture plate and cultured with 700 μl of culture medium consisting of 50% minimal essential medium, 25% horse serum and 25% Hanks' balanced salt solution supplemented with 3 $mg \cdot mL^{-1}$ glucose, 2 mM L-glutamine, 100 $U \cdot mL^{-1}$ penicillin G and 125 $\mu g \cdot mL^{-1}$ streptomycin in a humidified incubator at 37°C in 5% CO_2. After 7 days *in vitro*, slice cultures were exposed to 1 mM glutamate for 15 min for rats and 30 min for mice with or without EP receptor antagonists, EP receptor agonists and/or NS-398. Then medium was changed to normal culture medium containing 5 $\mu g \cdot mL^{-1}$ propidium iodide (PI) with or without kinase inhibitors, EP receptor antagonists, EP receptor agonists and/or NS-398 and cultured for 24 h. After that, all cells were killed by 24 h incubation with 0.3% Triton X-100 at a low temperature (4°C). PI fluorescence images were obtained with the confocal laser scanning system (LSM510) on an Axiovert200M inverted microscope (Carl Zeiss, Germany). The fluorescence intensity at CA1 pyramidale was obtained by measuring averaged grey-scale values of the desired area using graphics software (Photoshop ver. 7.0, Adobe Systems, San Jose, CA, USA).

mRNA analysis

RNA extraction and the semiquantitative reverse transcriptase polymerase chain reaction were performed as described (Ikeda

et al., 2000). PCR cycles were titrated to establish amplification conditions for each primer, to document linearity, and to permit quantitative analyses of signal strength; the conditions were as follows: denaturation at 94°C for 30 s, annealing at T°C, which is described below for each set of primers, for 30 s, and extension at 72°C for 45 s (X cycles). The cDNA fragments for EP_1, EP_2, EP_3 and EP_4 receptors was amplified with specific primers for mice (EP_1: sense 5′-TTAA CCTGAGCCTAGCGGATG-3′ and antisense 5′-CGCTGAG CGTATTGCACACTA-3′, 665 bp, T = 56°C, X = 30 cycles; EP_2: sense 5′-AGGACTTCGATGGCAGAGGAGAC-3′ and antisense 5′-CAGCCCCTTACACTTCTCCAATG-3′, 402 bp, T = 60°C, X = 36 cycles; $EP_{3\alpha}$: sense 5′-TGACCTTTGCCTGCAACCTG-3′ and antisense 5′-AGCTGGAAGCATAGTTGGTG-3′, 377 bp, T = 56°C, X = 33 cycles; $EP_{3\beta}$: sense 5′-CTAATTGCAGTT CGCCTGGCT-3′ and antisense 5′-CGTCTCAAGTGCAGA GTCTTC-3′, 320 bp, T = 58°C, X = 34 cycles; $EP_{3\gamma}$: sense 5′-TGACCTTTGCCTGCAACCTG-3′ and antisense 5′-AGACA ATGAGATGGCCTGCC-3′, 409 bp, T = 56°C, X = 33 cycles; EP_4: sense 5′-CATTCCGCTCGTGGTGC-3′ and antisense 5′-AGGTGGTGTCTGCTTGGGT-3′, 425 bp, T = 56°C, X = 36 cycles) and rats (EP_1: sense 5′-TTAACCTGAGCCTAGTG GATG-3′ and antisense 5′-CGCTGAGCGTATTACACACTA-3′, 665 bp, T = 56°C, X = 33 cycles; EP_2: sense 5′-AGG ACTTCTATGGCGGAGGAGAC-3′ and antisense 5′-CGGCC CTTTACGTTCCTCCAACG-3′, 402 bp, T = 60°C, X = 36 cycles; EP_3: sense 5′-CCCGGCACGTGGTGCTTCAT-3′ and antisense 5′-AGCTGGAAGCATAGTTGGTG-3′, 437 bp, T = 56°C, X = 36 cycles; EP_4: the same as mice). The quality of RNA samples was evaluated using GAPDH-specific primers (5′-AGA-CAG-CCG-CAT-CTT-CTT-GT-3′, 5′-CCA-CAG-TCT-TCT-GAG-TGG-CA-3′, T = 56°C, X = 20 cycles). PCR products were analysed on 2% agarose gels and visualized by ethidium bromide staining.

PGE_2 assay

The concentration of the PGE_2 in dissected brain tissues or culture medium was determined using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA) as described previously (Ikeda-Matsuo *et al*., 2005). The brain tissues were quickly frozen in liquid nitrogen and weighed to determine the wet weight. Prostanoids were extracted by homogenization of the tissues in 70% methanol solution containing 10 μM indomethacin and centrifugation at 15 000× *g* for 20 min at 4°C. The supernatant was evaporated and dissolved and diluted with the assay buffer. The hippocampal culture medium was also diluted with the assay buffer. The PGE_2 concentration was determined according to the instructions provided with the kit.

Induction of transient focal ischaemia

MCA occlusion was carried out under halothane anaesthesia as described previously (Ikeda-Matsuo *et al*., 2006). The right common carotid artery was exposed through a midline incision, and occlusion of the MCA was achieved by inserting a 6-0 nylon monofilament with a heat-blunted tip coated with silicon thread through the proximal external carotid artery into the internal carotid artery and up to the MCA (9 mm from the internal carotid/pterygopalatine artery bifurcation). The occlusion of the MCA was maintained for 2 h, followed by reperfusion for 24 h. The incision wound was treated with povidone iodine to avoid infection. A rectal thermometer was used to measure body temperature, which was maintained at 37°C by using a warm pad during the operation. The mortality associated with the technique was 6.4%. In sham-operated animals, an incision was made and the external carotid artery was tied off but the monofilament was not inserted, thereby avoiding MCA ischaemia. The EP_3 receptor antagonist ONO-AE3-240 was suspended in 1% sodium carboxymethylcellulose (CMC) in physiological saline, and the vehicle consisted of 1% CMC saline alone. The EP_3 receptor antagonist (3 $mg \cdot kg^{-1}$; i.p.) or vehicle was administered 2, 8 or 14 h after MCA occlusion. HA-1077 was diluted in saline and administered i.p. at 10 $mg \cdot kg^{-1}$ per day for 2 days before MCA occlusion. These mice were killed 24 h after MCA occlusion.

Quantification of infarct volume

The animals were killed and the brain tissue was removed. Brains were sectioned coronally into five 2 mm sections and incubated with 2% triphenyltetrazolium chloride (TTC) in saline for 10 min at 37°C. The area of infarct, identified by the lack of TTC staining, was measured on the rostral and caudal surfaces of each slice using Scion image software (Scion Corp., Frederick, MD, USA) and numerically integrated across the thickness of the slice to obtain an estimate of the infarct volume in each slice. The volumes from all slices were summed to calculate the total infarct volume over the entire infarcted hemisphere. The infarct volume was measured separately in the cerebral cortex, striatum and hemisphere, and corrected for swelling by comparing the volume of the neocortex in the infarcted hemisphere with that in the non-infarcted hemisphere. The infarct volumes of cortex or striatum was expressed as percentage of the whole brain volume [infarct volume = (infarct volume of cortex or striatum/volume of whole brain) × 100]. The degree of oedema was calculated as follows [oedema per cent = (volume of post-ischaemic hemisphere − volume of contralateral hemisphere)/volume of contralateral hemisphere × 100].

Behavioural experiment

All mice used for infarct volume estimation and Western blot analysis for Rho kinase activation were evaluated for post-ischaemic neurological deficits on a 5-point scale at 1 day of reperfusion by an investigator unaware of the treatment and genotypic conditions, as follows: 0, no deficit; 1, flexion of the torso and contralateral forelimb when lifted by the tail; 2, contralateral forelimb weakness upon application of pressure to the side of the body; 3, circling to the affected side; and 4, no spontaneous locomotor activity.

Western blotting

For measurement of Rho kinase activity, Western blot analysis was performed as previously described (Ikeda-Matsuo *et al*., 2005; Yamaguchi *et al*., 2009) with slight modifications. The cultured slices were treated with drug(s), and the reactions

were terminated with ice-cold 5% trichloroacetic acid. The slices were sonicated and precipitated by centrifugation, and then solubilized in SDS sample buffer. Proteins were separated on SDS-polyacrylamide gels, and then transferred to PVDF membranes (Immobilon-P; Millipore). After blocking with 5% skim milk, the membranes were incubated with the appropriate primary antibody solution against anti-pT850 myosin-binding subunit (MBS) (1:1000) and anti-β-actin (1:20 000) in Solution 1 of the Can-get-Signal enhancer solutions for 1.5 h. Horseradish peroxidase (HRP)-conjugated anti-rabbit or mouse IgG antibody (1:20 000) was used as a secondary antibody and incubated in Solution 2 of the Can-get-Signal enhancer solutions for 1.5 h. Immunoreactive proteins were detected using LumiGLO, and the images were captured using a CCD camera (Light Capture; ATTO, Tokyo, Japan) and then analysed using 'CS analyzer' software (ATTO).

Statistical analysis

Results are expressed as the mean ± standard error. Statistical significance was evaluated with one-way analysis of variance followed by Tukey's method. For behavioural experiment, Steel-Dwass' method was used to analyse the data of neurological score. Values of $P < 0.05$ were considered to indicate statistical significance.

Materials

Selective agonists for EP_1 (ONO-DI-004), EP_2 (ONO-AE1-259), EP_3 (ONO-AE-248) and EP_4 (ONO-AE1-329) receptors, and selective antagonists for EP_1 (ONO-8713), EP_3 (ONO-AE3-240) and EP_4 (ONO-AE3-208) receptors were gifts from Ono Pharmaceutical (Osaka, Japan). Each agonist and antagonist is highly selective for each receptor, and the K_i values were as follows: ONO-DI-004 (150 nM for the EP_1 receptor and more than 10 μM for the other receptors); ONO-AE1-259 (3 nM for the EP_2 receptor and more than 6 μM for the other receptors); ONO-AE-248 (7.5 nM for the EP_3 receptor and more than 3 μM for the other receptors); ONO-AE1-329 (9.7 nM for the EP_4 receptor and more than 1 μM for the other receptors); ONO-8713 (0.3 nM for the EP_1 receptor and more than 1 μM for the other receptors); ONO-AE3-240 (590, 0.23 and 58 nM for the EP_1, EP_3 and EP_4 receptors respectively, and more than 10 mM for the EP_2 receptor); ONO-AE3-208 (30 and 1.3 nM for the EP_3 and EP_4 receptors respectively, and more than 10 μM for the other receptors) (Suzawa *et al*., 2000; Watanabe *et al*., 2000; Kabashima *et al*., 2002; Amano *et al*., 2003). NS-398, TTC and anti-β-actin monoclonal antibody were from Sigma (St Louis, MO, USA). H-89, Ro-31-8220 and *Pertussis* toxin (PTX) were from Calbiochem (Darmstadt, Germany). Other materials and their sources were as follows: anti-pT805 myosin-binding protein (MBP; Upstate, Charlottesville, VA, USA); HRP-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA); Y-27632 (Tocris, Ellisville, MO, USA); fasudil (HA-1077; Asahi Chemical Ind, Tokyo, Japan); LumiGLO Western blot detection reagent (Cell Signalling, Danvers, MA); Can-Get-Signal enhancer solution (Toyobo, Osaka, Japan). Other reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan).

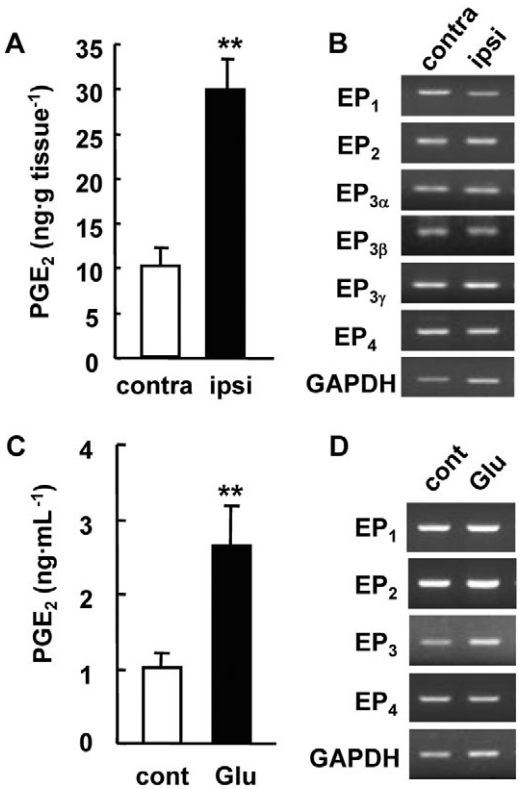

Figure 1 Production of prostaglandin E_2 (PGE_2) and the expression of EP receptors in the ischaemic cortex of the mice or in cultured rat hippocampal slices exposed to glutamate. (A) The production of PGE_2 in the ipsilateral (ipsi) and contralateral (contra) cortex of mice 24 h after middle cerebral artery occlusion. $n = 8$ mice; $^{**}P < 0.01$. (B) Reverse transcriptase polymerase chain reaction (RT-PCR) analysis for EP receptor mRNAs in the cortex 24 h after ischaemia. Representative data from three separate experiments are presented. GAPDH signals were used as loading controls. (C) The accumulation of PGE_2 in the culture medium 24 h after 1 mM glutamate exposure for 15 min. $n = 4$; $^{**}P < 0.01$. (D) RT-PCR analysis for EP receptor mRNAs in the cultured hippocampal slices 24 h after glutamate exposure. Representative data from three separate experiments are presented.

Results

Involvement of EP receptors in neuronal damage after transient ischaemic and excitotoxic injury

Before starting the *in vitro* study of ischaemic neurotoxicity, we first examined whether or not the production of PGE_2 and the expression of EP receptors in cultured hippocampal slices exposed to glutamate showed tendencies similar to those in ischaemic cortices *in vivo*. We investigated the PGE_2 content and EP mRNA levels in the mouse brain after 2 h of focal ischaemia followed by 24 h of reperfusion. In our experimental stroke model, the PGE_2 production in the ipsilateral cortex of MCA-occluded mice was about threefold higher than that in the contralateral cortex (Figure 1A). Expression of the mRNAs of each of the EP_1, EP_2, $EP_{3\alpha}$, $EP_{3\beta}$, $EP_{3\gamma}$ and EP_4 receptors was observed in both the contralateral and ipsilateral cortex, and the mRNA levels of these receptors in the ipsilateral cortex were almost the same as those in the contralateral cortex (Figure 1B). We next investigated the production of PGE_2 and expression of EP receptors in an *in vitro* ischaemia model. Rat hippocampal slices were stimulated with 1 mM

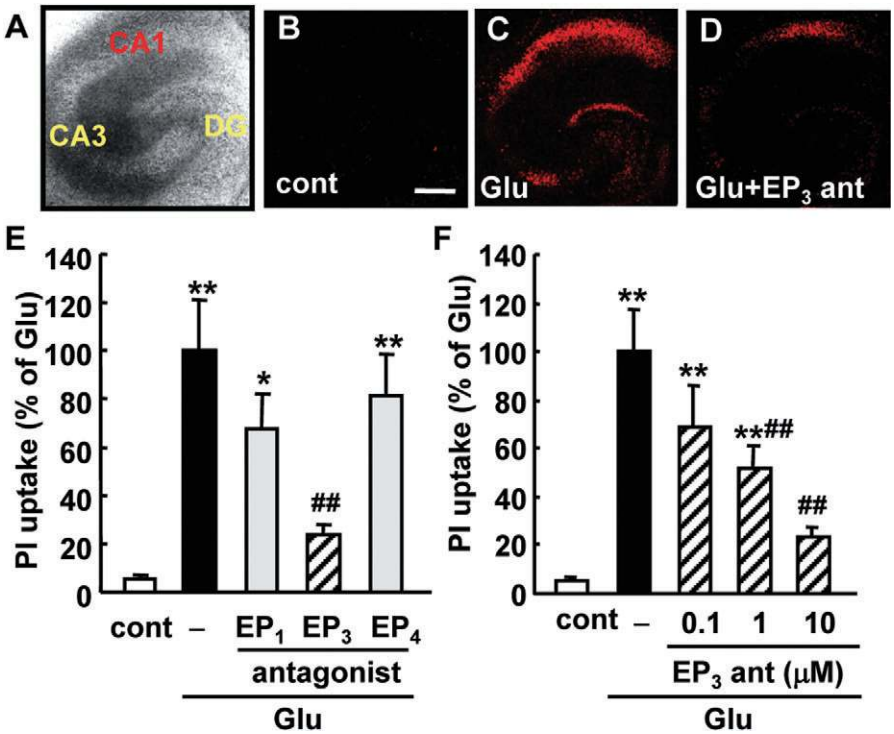

Figure 2 Effects of EP receptor antagonists on the glutamate-induced excitotoxicity in a rat hippocampal slice culture. EP receptor antagonists were added during and after the 1 mM glutamate exposure for 15 min. Twenty-four hours later, propidium iodide (PI) uptake was analysed. A representative differential interference microscopic image of a cultured hippocampal slice (A) and confocal images of PI fluorescence of a control slice (cont, B), a slice that received glutamate exposure (Glu, C) and a slice that received glutamate exposure with 10 μM of an EP_3 receptor antagonist, ONO-AE3-240 (Glu + EP_3 ant, D) are shown (scale bar: 400 μm). (E) Quantitative data from PI uptake analysis in the CA1 region with or without glutamate (Glu) and/or the EP_1 receptor antagonist ONO-8713 (10 μM), EP_3 receptor antagonist ONO-AE3-240 (10 μM) or EP_4 receptor antagonist ONO-AE3-208 (10 μM) were scaled to a percentage of the response of glutamate alone. $n = 5$–14 slices per group. (F) Concentration-dependent protective effect of ONO-AE3-240 on glutamate-induced PI uptake in CA1. $n = 5$–12 slices per group. $^{**}P < 0.01$, $^{*}P < 0.05$ versus control, $\#\#P < 0.05$ versus glutamate alone, $^{\$\$}P < 0.01$ versus glutamate with NS-398.

glutamate for 15 min and then cultured with normal medium for 24 h. In hippocampal slice cultures, glutamate increased the PGE_2 levels up to 2.5-fold higher than the control level (Figure 1C). All of the EP receptors were constitutively expressed in the hippocampal slices with or without glutamate exposure (Figure 1D).

To elucidate the roles of EP receptors in the excitotoxicity induced by glutamate, cellular damage in the hippocampal slices was assessed by fluorescent image analysis of PI uptake (Figure 2A–D). The exposure of slices to glutamate resulted in neuronal death, which was detected as an increase in the uptake of PI in the CA1 region of the hippocampus (Figure 2C). The increase in PI uptake induced by glutamate exposure was inhibited significantly by the EP_3 receptor antagonist ONO-AE3-240 in a concentration-dependent manner (0.1–10 μM), but not by other EP receptor antagonists even at a concentration of 10 μM (Figure 2E and F). Thus, we next investigated the effect of the EP_3 receptor agonist ONO-AE-248 on glutamate-induced excitotoxicity. However, we could not detect any enhancement of glutamate-induced excitotoxicity even at the concentration of 1 μM (data not shown). Because we observed significant PGE_2 production after glutamate exposure (Figure 1A) and a protective effect of the EP_3 receptor antagonist on glutamate-induced excitotoxicity (Figure 2F), it was suggested that the extent of the contribution by endogenous PGE_2 does not leave room for an additive effect on glutamate-induced excitotoxicity by EP receptor activation in our model. Therefore, to investigate the effect of EP receptor agonists, endogenous PGE_2 production was inhibited by the COX-2 inhibitor NS-398 (Table 1). As expected, NS-398 attenuated approximately 50% of the glutamate-induced excitotoxicity (Figure 3A and B). The EP_3 receptor agonist ONO-AE-248 exaggerated the excitotoxicity, while agonists for EP_2 (ONO-AE1-259) and EP_4 receptors (ONO-AE1-329) had no effects at the concentration of 1 μM (Figure 3D and E). Because the K_i values of ONO-AE-248, ONO-AE1-259 and ONO-AE1-329 for the EP_2, EP_3 and EP_4 receptors, respectively, are similar, while that of the EP_1 agonist (ONO-DI-004) for the EP_1 receptor is 10–100 times lower (see the *Methods* section), we examined the effect of ONO-DI-004 at higher concentration. ONO-DI-004 did not affect glutamate-induced excitotoxicity even at the concentration of 10 μM (Figure 3C and E). ONO-AE-248 exacerbated the glutamate-induced excitotoxicity in a concentration-dependent manner (0.01–1 μM, Figure 3F), while it alone had no effect on the survival of CA1 neurons even at the concentration of 1 μM (data not shown).

EP_3 receptors as downstream effectors of mPGES-1 neurotoxicity

We have previously reported that mPGES-1 enhanced neuronal death through massive PGE_2 production in the

Table 1 Production of PGE_2 in the culture medium of hippocampal slices

	Rat	*mPGES-1$^{+/+}$ mice*	*mPGES-1$^{-/-}$ mice*
CTL	1.01 ± 0.19	0.182 ± 0.020	0.183 ± 0.031
GLU	2.66 ± 0.52**	0.273 ± 0.024*	0.188 ± 0.028#
GLU + NS-398	0.29 ± 0.03##	–	–

The amount of PGE_2 (ng·mL^{-1}) in the culture medium of slices from rats, mPGES-1 knockout (–/–) and wild-type (+/+) mice was measured 24 h after 1 mM glutamate (GLU) exposure and in control hippocampal slices (CTL). NS-398 (1 μM) were applied during and after the glutamate exposure (n = 3).
**P < 0.01, *P < 0.05 versus control, ##P < 0.01, #P < 0.05 versus glutamate.
mPGES, microsomal prostaglandin E synthase; PGE_2, prostaglandin E_2.

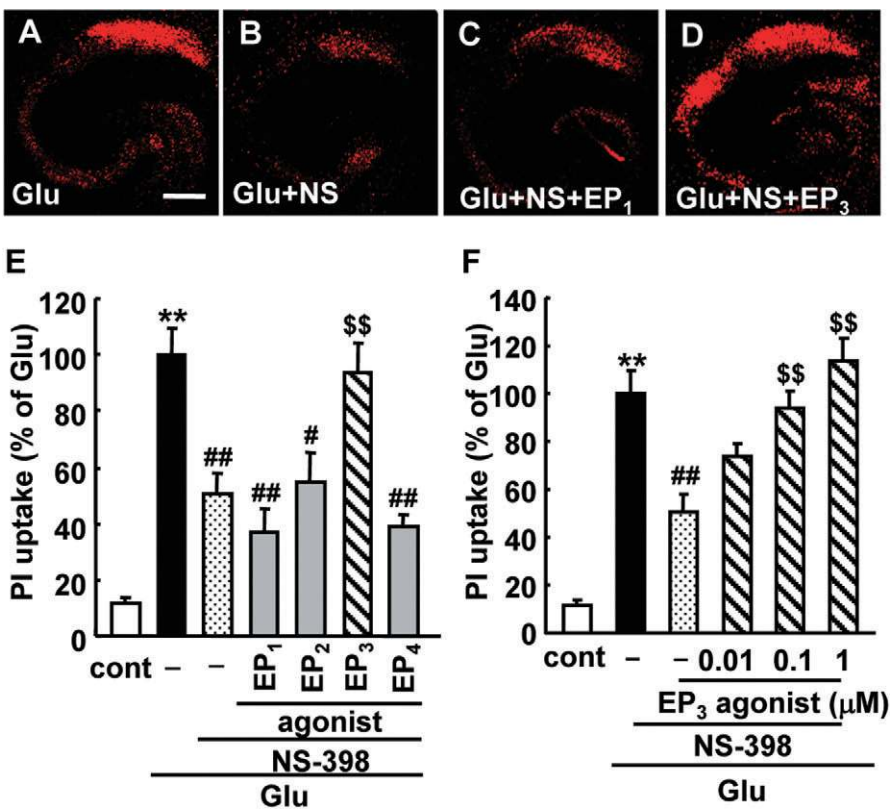

Figure 3 Effects of EP receptor agonists on the glutamate-induced excitotoxicity in a rat hippocampal slice culture. Representative confocal images of propidium iodide (PI) fluorescence of a slice that received 1 mM glutamate exposure for 15 min (Glu, A), a slice that received glutamate exposure with 1 μM NS-398 (Glu + NS, B), a slice that received glutamate exposure with NS-398 and 10 μM EP_1 agonist ONO-DI-004 (Glu + NS + EP_1, C) and a slice that received glutamate exposure with NS-398 and 0.1 μM EP_3 agonist ONO-AE-248 (Glu + NS + EP_3, D) are shown (scale bar: 400 μm). (E) Quantitated data from PI uptake analysis in the CA1 region with or without glutamate (Glu) and/or the EP_1 agonist ONO-DI-004 (10 μM), EP_2 agonist ONO-AE1-259 (1 μM), EP_3 agonist ONO-AE-248 (0.1 μM), EP_4 agonist ONO-AE1-329 (1 μM) and NS-398 (1 μM) were scaled to a percentage of the response of glutamate alone. n = 8–19 slices per group. (F) Concentration-dependent toxic effect of ONO-AE-248 on glutamate-induced PI uptake in CA1 in the presence of NS-398 (1 μM). n = 9–19 slices per group. **P < 0.01 versus control, ##P < 0.01, #P < 0.05 versus glutamate alone, $$$P$ < 0.01 versus glutamate with NS-398.

ischaemic cortex (Ikeda-Matsuo *et al*., 2006). To address the question of whether or not the activation of EP_3 receptors is involved in the mPGES-1-dependent neurotoxicity resulting from massive PGE_2 production, we compared the effect of an EP_3 receptor antagonist on the excitotoxicity in mPGES-1 KO and WT slices. ONO-AE3-240 significantly attenuated the glutamate-induced excitotoxicity in the slices obtained from WT mice (Figure 4A). In the mPGES-1 KO slices, the glutamate-induced PGE_2 production observed in WT slices was almost completely abolished (Table 1). The glutamate-induced excitotoxicity was reduced, but not completely abolished, in the mPGES-1 KO slices; it was at almost the same level as observed in ONO-AE3-240-treated WT slices (Figure 4A and B). However, ONO-AE3-240 did not attenuate the excitotoxicity in mPGES-1 KO slices, while the EP_3 receptor agonist ONO-AE-248 exacerbated it (Figure 4B and C).

To extend these observations of slice culture to models of ischaemia *in vivo*, we used a mouse MCA occlusion–reperfusion model which showed an increase in PGE_2 production in the post-ischaemic cortex (Figure 1A). ONO-AE3-240 (3 mg·kg^{-1}) was injected i.p. three times, once each at 2, 8 and 14 h after MCA occlusion. ONO-AE3-240 reduced the infarct volume in the cortex of WT mice (Figure 5A); the infarct volume in the cortex of mice injected with ONO-AE3-240 was less than 40% that of vehicle-injected mice, while that in the striatum was not affected by the injection of ONO-AE3-240

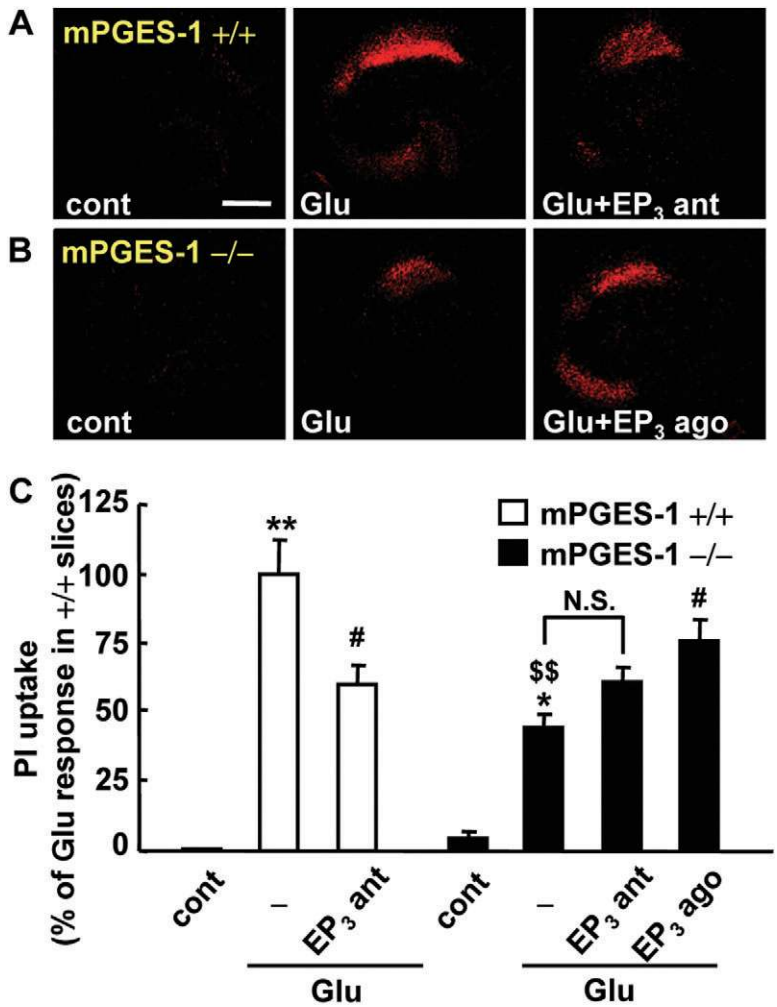

Figure 4 Effect of an EP_3 receptor antagonist and agonist on the glutamate-induced excitotoxicity in a microsomal prostaglandin E synthase (mPGES-1) knockout (KO) (–/–) and a wild-type (WT) (+/+) mouse hippocampal slice culture. The EP_3 receptor antagonist (ONO-AE3-240) and agonist (ONO-AE-248) were added during and after the 1 mM glutamate exposure for 30 min. Twenty-four hours later, propidium iodide (PI) uptake was analysed. Representative confocal images of PI fluorescence of a control slice (cont), a slice that received glutamate exposure (Glu) and slices that received glutamate exposure with 10 μM of an EP_3 receptor antagonist (Glu + EP_3 ant) or 0.1 μM of an EP_3 receptor agonist (Glu + EP_3 ago) in WT (+/+) mice (A) and mPGES-1 KO (–/–) mice (B) are shown (scale bar: 400 μm). (C) Quantitative data from PI uptake analysis in the CA1 region with or without glutamate (Glu) and EP_3 receptor antagonist (EP_3 ant) or agonist (EP_3 ago) were scaled to a percentage of the glutamate response in slices from WT mice. $n = 6$–11 slices per group. $^{**}P < 0.01$, $^{*}P < 0.05$ versus the control slice from WT mice, $^{\#}P < 0.05$ versus glutamate alone in each genotype, $^{\$\$}P < 0.01$ versus the control slice from mPGES-1 KO mice, N.S. (not significant).

(Figure 5B). As seen in our previous study (Ikeda-Matsuo *et al*., 2006), mPGES-1 KO mice in the present study were partially resistant to transient ischaemic injury; the infarct volume in the cortex of mPGES-1 KO mice was almost the same as that of ONO-AE3-240-injected WT mice. Interestingly, ONO-AE3-240 did not cause additional reduction of infarction in mPGES-1 KO mice. The degree of oedema was also reduced by ONO-AE3-240 in WT mice; the reduced level was almost identical to the level in mPGES-1 KO mice (Figure 5C). ONO-AE3-240 had no further protective effect on oedema in mPGES-1 KO mice. To determine the functional role of EP_3 receptors in behavioural symptoms, we investigated the neurological dysfunction observed after transient ischaemia. ONO-AE3-240 ameliorated neurological deficits in WT mice to almost the same level as in mPGES-1 KO mice, while it did not ameliorate the neurological deficits of mPGES-1 KO mice (Figure 5D). ONO-AE3-240 did not alter mean arterial pressure (MAP), changes in cerebral blood flow (CBF) after reperfusion, or rectal temperature (data not shown). As we have reported previously, there were no significant differences between mPGES-1 KO and WT mice in MAP, changes in CBF before, during or after MCA occlusion, the anatomy of the circle of Willis or the origins of the cerebral arteries (Ikeda-Matsuo *et al*., 2006). Expression of the mRNAs of the EP_1, EP_2, $EP_{3\alpha}$, $EP_{3\beta}$, $EP_{3\gamma}$ and EP_4 receptors was observed in both the contralateral and ipsilateral cortex of mPGES-1 KO mice, and the mRNA levels of these receptors in the bilateral cortex of mPGES-1 KO mice were almost the same as those of WT mice (Figure 1B and other data not shown).

Effect of Rho kinase inhibitor on glutamate-induced excitotoxicity and ischaemic injury

To further investigate the underlying mechanisms of EP_3 receptor-mediated neurotoxicity, we examined the effects of

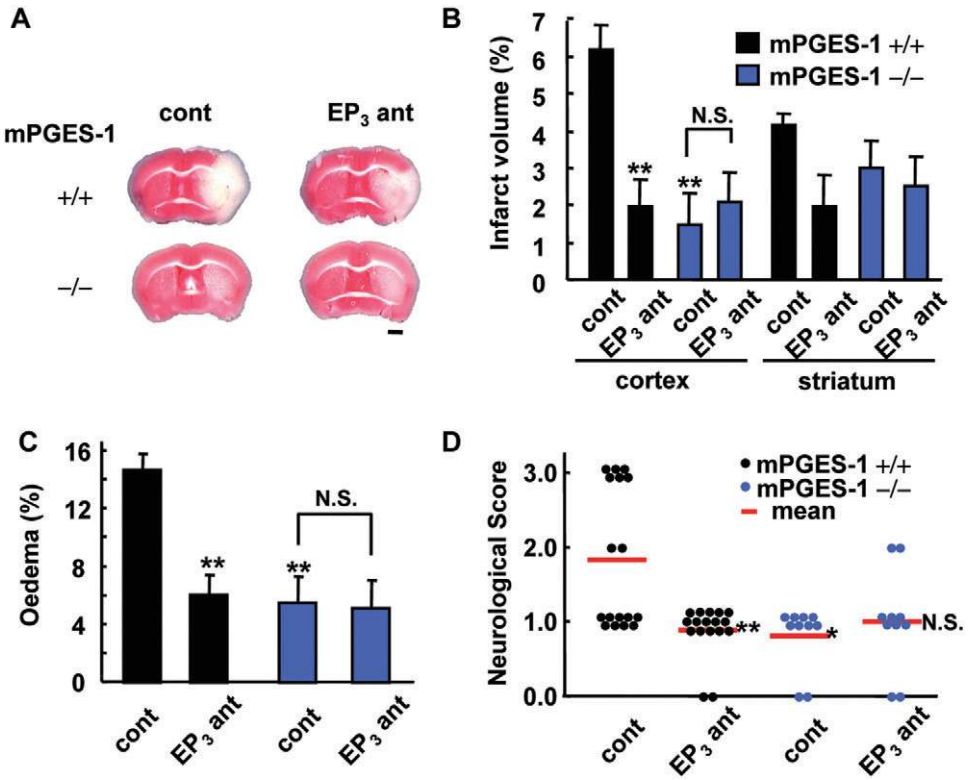

Figure 5 Protective effect of an EP_3 receptor antagonist on post-ischaemic symptoms in wild-type (WT) mice but not in microsomal prostaglandin E synthase (mPGES-1) knockout (KO) mice. ONO-AE3-240 (3 mg·kg^{-1}, i.p.) was injected three times at 2, 8 and 14 h after middle cerebral artery occlusion. (A) Representative triphenyltetrazolium chloride-stained coronal sections of the WT (+/+) mice and mPGES-1 KO (–/–) mice injected with vehicle (cont) or ONO-AE3-240 (EP_3 ant) (scale bar: 1 mm). (B) The volume of the infarcted cortex and striatum 24 h after ischaemia was estimated and expressed as a percentage of the corrected tissue volume. $n = 9$ mice per group. (C) The corrected oedema percentage in the EP_3 antagonist-injected WT and mPGES-1 KO mice. $n = 9$ mice per group. (D) Neurological dysfunction in the EP_3 antagonist-injected WT and mPGES-1 KO mice 24 h after ischaemia. $n = 10$–17 mice per group, $^{**}P < 0.01$ versus vehicle-treated WT mice (cont), N.S. (not significant) versus vehicle-treated mPGES-1 KO mice (cont).

protein kinase inhibitors on glutamate-induced excitotoxicity using rat hippocampal slices. EP_3 receptors have been found to exert their effects through the small G protein Rho-involved pathway (Hatae *et al.*, 2002; Macias-Perez *et al.*, 2008). The glutamate-induced excitotoxicity was significantly inhibited by PTX, an inhibitor of G_i protein, and Y-27632, an inhibitor of Rho kinase, while H-89, a PKA inhibitor, and Ro-31-8220, a PKC inhibitor, had no protective effect (Figure 6A). In the presence of NS-398, in which the endogenous PGE_2 production induced by glutamate was completely abolished (Table 1), the attenuated glutamate-induced excitotoxicity was not further inhibited by PTX and Y-27632, or by the EP_3 receptor antagonist ONO-AE3-240 (Figure 6B). On the other hand, the attenuated glutamate-induced excitotoxicity in the presence of NS-398 was exacerbated by H-89 and Ro-31-8220. Exacerbation of glutamate-induced excitotoxicity induced by the EP_3 receptor agonist ONO-AE-248, in the presence of NS-398 was completely inhibited by an EP_3 receptor antagonist, ONO-AE3-240 (Figure 6C). PTX and Y-27632 significantly inhibited the ONO-AE-248-induced enhancement of the excitotoxicity, while H-89 and Ro-31-8220 had no protective effect (Figure 6C). Thus, PTX and Y-27632 completely inhibited the excitotoxicity related to EP_3 receptor activation.

We next investigated the activation of Rho kinase by the EP_3 receptor agonist ONO-AE-248 in rat hippocampal slices. Rho kinase activity, as measured by the Thr^{850} phosphorylation of MBS, was increased by more than twofold at 1 min after treatment of 0.1 μM ONO-AE-248 (Figure 7). This activation of Rho kinase was completely inhibited by treatment with 10 μM Y-27632. We also investigated the activation of Rho kinase 24 h after treatment with ONO-AE-248; however, we could not detect significant changes in Rho kinase activation at this time point (data not shown). In another experiment, we confirmed that the expression level of not only β-actin, but also pan-MBP, was not changed by treatment with ONO-AE-248 for 1 min (data not shown). In an *in vivo* ischaemic model, we also investigated the activation of Rho kinase in the mouse brain 24 h after transient ischaemia, but we could not detect the activation of Rho kinase in the ischaemic cortex (data not shown).

To further elucidate the role of Rho kinase in the ischaemic brain in morbidity *in vivo*, we investigated the effect of HA-1077, another Rho kinase inhibitor clinically used for cerebrovascular disorder, on ischaemic brain injury. HA-1077 almost completely protected against infarction in both the cortex and striatum and against oedema formation after transient ischaemia (Figure 8A–C). Furthermore, HA-1077 significantly ameliorated the neurological dysfunction (Figure 8D).

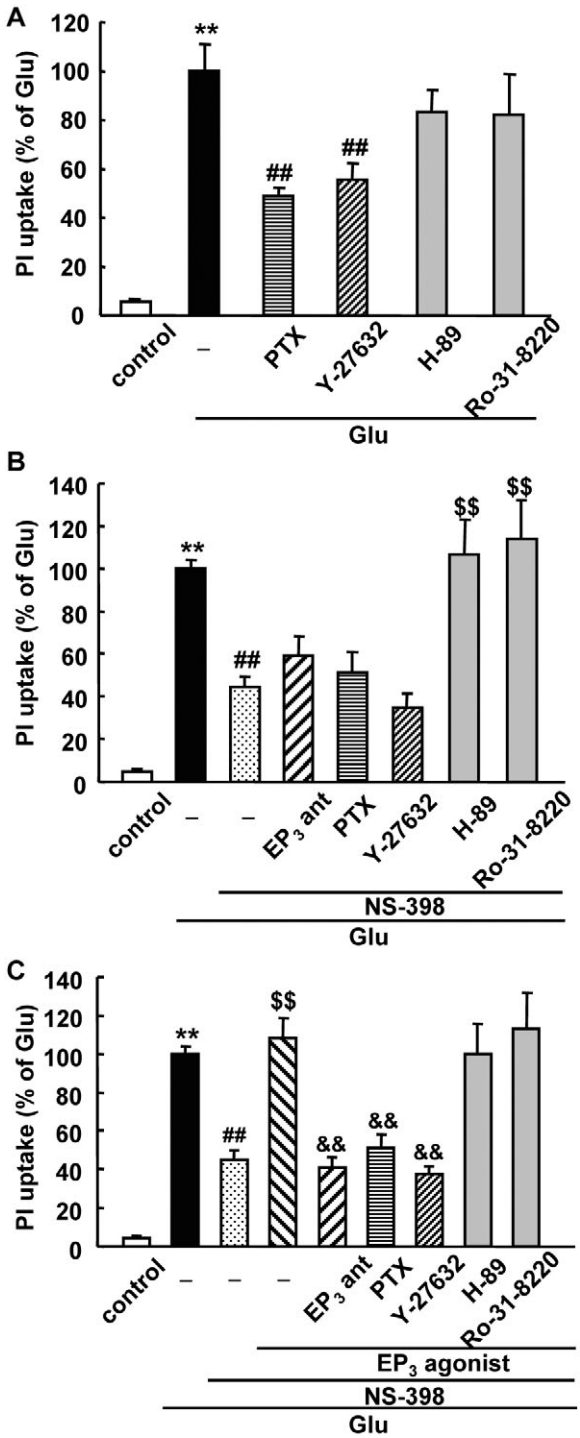

Figure 6 Effect of a Rho kinase inhibitor on glutamate-induced excitotoxicity and EP_3-induced enhancement of excitotoxicity in a rat hippocampal slice culture. Quantitative data from propidium iodide (PI) uptake analysis in the CA1 region with or without glutamate and *Pertussis* toxin (PTX) and kinase inhibitors in the absence of NS-398 (A), in the presence of NS-398 (B) or in the presence of NS-398 and EP_3 receptor agonist (C). PTX (1 $\mu g \cdot mL^{-1}$) was added from 2.5 h before 1 mM glutamate (Glu) exposure for 15 min. The EP_3 receptor agonist (ONO-AE-248, 0.1 μM), EP_3 receptor antagonist (EP_3 ant, ONO-AE-240, 10 μM) and NS-398 (1 μM) were added during and after glutamate exposure. Y-27632 (10 μM), H-89 (1 μM) and Ro-31-8220 (1 μM) were added immediately after glutamate exposure. Twenty-four hours later, PI uptake was analysed. The data were scaled to a percentage of the glutamate response. $n = 7$–41 slices per group, $^{**}P < 0.01$ versus control, $^{\#\#}P < 0.01$ versus glutamate alone, $^{\$\$}P < 0.01$ versus glutamate with NS-398, $^{\&\&}P < 0.01$ versus glutamate with ONO-AE-248 and NS-398.

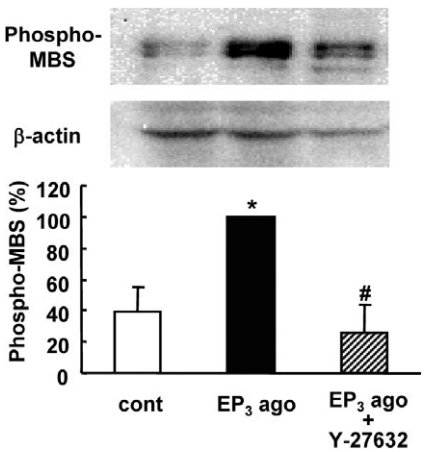

Figure 7 Effect of an EP_3 receptor agonist on Rho kinase activity in rat hippocampal slices. The EP_3 receptor agonist (EP_3 ago; ONO-AE-248, 0.1 μM) was administered for 1 min with or without Y-27632 (10 μM). Rho kinase activity was determined by immunoblotting of slice lysates with antibodies against phosphorylated myosin-binding subunit (phospho-MBS) and β-actin. Rho kinase activity was normalized to β-actin and the data were scaled to a percentage of the EP_3 agonist response. $n = 3$. $^{*}P < 0.05$ versus control (cont), $^{\#}P < 0.05$ versus EP_3 agonist alone.

Discussion and conclusions

Here we have shown that mPGES-1 exacerbated ischaemic injury and glutamate-induced excitotoxicity through activation of EP_3 receptors. Using mPGES-1 KO mice and antagonists of EP_3 receptors, we provided unequivocal evidence that activity of mPGES-1 enhances the excitotoxicity and ischaemic insult through EP_3 receptor activation both *in vitro* and *in vivo*. We have also shown that Rho kinase activation contributes to the neurotoxicity mediated by EP_3 receptors.

We have previously demonstrated that up-regulation of mPGES-1 exacerbates the stroke injury observed after ischaemia and the excitotoxicity induced by glutamate in hippocampal slices, through extensive PGE_2 production (Ikeda-Matsuo *et al*., 2006; 2010). In this study, we show that EP_3 receptors contribute to the excitotoxicity as downstream effectors of mPGES-1. This result is consistent with a recent report showing that OGD-induced cell death in hippocampal slice cultures of EP_3 receptor KO mice is lower than in those of WT mice (Saleem *et al*., 2009). While we did not detect any effects of the EP_1 receptor antagonist or agonist on glutamate-induced excitotoxicity, Kawano *et al*. (2006) showed that the EP_1 receptor antagonist SC51089 attenuated the COX-2-dependent component of the injury produced by OGD in a hippocampal slice culture. The mechanisms of neuronal death induced by glutamate-induced excitotoxicity may differ from those of neuronal cell death induced by OGD; OGD-induced PGE_2 may stimulate both EP_1- and EP_3 receptor-related pathways. Considering that an excess amount of

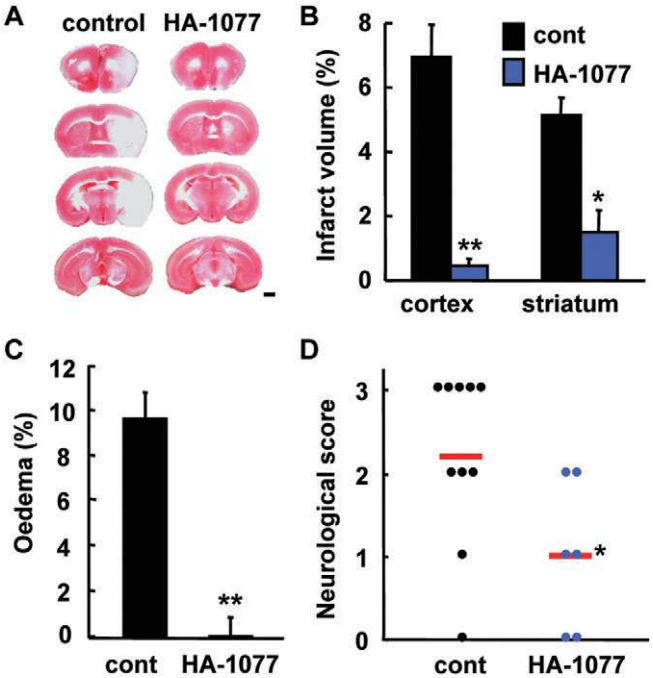

Figure 8 Effect of a Rho kinase inhibitor on stroke-reperfusion injury. HA-1077 (10 mg·kg^{-1}, i.p.) or vehicle was injected twice at 24 and 48 h before middle cerebral artery occlusion. (A) Representative triphenyltetrazolium chloride-stained coronal sections of the vehicle (control) or HA-1077-injected mice (scale bar: 1 mm). (B) Volume of the infarcted cortex and striatum 24 h after ischaemia in the vehicle (cont)- or HA-1077-injected mice was estimated and expressed as a percentage of the corrected tissue volume. (C) The corrected oedema percentage in the vehicle- or HA-1077-injected mice. (D) Neurological dysfunction in the vehicle- or HA-1077-injected mice 24 h after ischaemia. n = 6–10 mice per group, $^{**}P < 0.01$, $^{*}P < 0.05$ versus control.

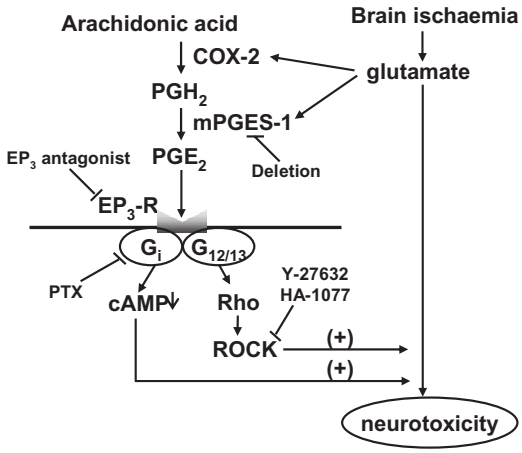

Figure 9 Pathway for EP_3 receptor activation-induced exacerbation of stroke injury. The prostaglandin E_2 (PGE_2) produced by induction of COX-2 and microsomal prostaglandin E synthase (mPGES-1) during ischaemia exacerbates glutamate-induced neurotoxicity through activation of EP_3 receptors (EP_3-R) followed by activation of Rho kinase (Rho kinase) and/or G_i.

extracellular glutamate is observed in the post-ischaemic cortex (Dávalos *et al*., 2000), EP_3 receptors may contribute at least in part to post-ischaemic injury. In fact, in a recent study we demonstrated that pharmacological and genomic inhibition of EP_3 receptors attenuated ischaemic injuries 24 h after transient focal ischaemia, suggesting that EP_3 receptors have a toxic effect on the post-ischaemic brain (under submission). However, using similar methods, Li *et al*. (2008) showed that there were no differences in infarct size between WT and EP_3 receptor KO mice. The differences in results between our study and that of Li *et al*. may be due to various technical factors. We used a siliconized flexible filament to minimize blood vessel damage such as endothelial injury and to reduce intracerebral haemorrhage and mortality (Shah *et al*., 2006). We also used halothane anaesthesia to minimize the pre- and post-conditioning effects derived from other anaesthetics, such as isoflurane (Lee *et al*., 2008). In fact, using similar methods (e.g. using a siliconized flexible filament and halothane anaesthesia), Saleem *et al*. (2009) also showed reduced infarct volume and neurological dysfunctions in EP_3 receptor KO mice compared with WT mice. We used a glutamate-induced neurotoxicity model in rat and mouse hippocampal slice cultures and a mouse ischaemia-induced cerebral cortex and striatum infarction model. There might be region- and species-specific differences in the susceptibility to excitotoxicity and ischaemia and the role of EP_3 receptors. In the present study, however, the protective effects of the EP_3 receptor antagonist were observed in both rat and mouse hippocampal slices and also in the cerebral infarction model, suggesting that EP_3 receptors play a role in common mechanisms of excitotoxic and ischaemic neuronal death in both species. Furthermore, the protective effects of the EP_3 receptor antagonist observed in WT mice were completely abolished in mPGES-1 KO mice in both hippocampal slices exposed to glutamate and the focal cerebral ischaemia (Figures 4 and 5), suggesting that EP_3 receptors are the most important effectors for the mPGES-1 neurotoxicity in these ischaemic models (Figure 9). In our transient ischaemia model, although significant ischaemic injury was observed at 24 h after ischaemia, it might not have reached the maximal level at this time point, and thus further experiments will be needed to elucidate the effect of EP_3 receptor antagonists at later time points of re-perfusion and the effective time-window of EP_3 receptor antagonist treatment.

How, then, is the toxicity by EP_3 receptor activation mediated? The EP_3 receptor has been shown to play a role in the regulation of body temperature (Ushikubi *et al*., 1998; Oka *et al*., 2003). It has also been shown that the heightened temperatures induced by i.c.v. injection of PGE_2 play a significant role in escalating the neural damage caused by global ischaemia (Thornhill and Asselin, 1999). Therefore, we measured rectal temperature before and after injection of a EP_3 receptor antagonist in mice with MCA occlusion, and also in mPGES-1 KO mice. However, as we have reported recently, the rectal temperature in mPGES-1 KO mice did not differ from that of WT mice (Ikeda-Matsuo *et al*., 2006). Also, the rectal temperature after treatment with a EP_3 receptor antagonist did not differ from those of mice given vehicle (unpublished experiments). With respect to vasoconstriction, it has been reported that stimulation of EP_3 triggers vasoconstriction in porcine middle cerebral arteries (Hatae *et al*., 2002; Jadhav *et al*., 2004) and in the guinea-pig aorta (Shum *et al*., 2003).

EP_3 receptors also seem to contribute significantly to the PGE_2-dependent regulation of arterial blood pressure in male mice (Audoly *et al*., 1999). However, under our experimental conditions, no significant difference was observed in CBF or MAP between the WT and mPGES-1 KO mice before, during or after reperfusion (Ikeda-Matsuo *et al*., 2006). Also, the EP_3 receptor antagonist had no effect on MAP and the recovery of CBF after reperfusion (under submission). Therefore, the effect of EP_3 receptors on the exacerbation of stroke injury may not be attributable either to the thermoregulatory effect or to the vasoregulatory and cerebral circulatory effect of EP_3 receptors. As we are able to detect neuronal excitotoxicity after EP_3 receptor activation even in the cultured hippocampal slice system, we speculate that some or all of the stroke injury observed in our *in vivo* system was the result of direct damage through the activation of EP_3 receptors in neuronal cells at the parenchymal lesion site.

In fact, it has been demonstrated that EP_3 receptors are constitutively and abundantly expressed throughout the brain (Nakamura *et al*., 2000). In particular, EP_3 receptors are expressed exclusively in neurons and induced in glial cells after excitotoxic lesions by quinolinic acid (Slawik *et al*., 2004). In our mouse brain ischaemia models, all of the mouse EP_3 receptor subtypes were constitutively expressed, and the expression level was not changed after transient ischaemia. In cultured hippocampal slices from rats, EP_3 receptors were also constitutively expressed with or without glutamate stimulation. The enhancement of glutamate-induced excitotoxicity by an EP_3 receptor agonist in the presence of NS-398, which inhibits endogenous PGE_2 synthesis, was completely abolished by Rho kinase and G_i inhibitors, as well as by an EP_3 receptor antagonist, while glutamate-induced toxicity in the presence of NS-398 but the absence of an EP_3 receptor agonist was not attenuated by any of these inhibitors. This suggests that the neurotoxic effect of EP_3 receptor activation is mediated through activation of Rho kinase and/or G_i (Figure 9). In an EP_2 receptor-deficient mouse model, it has been shown that the neuroprotective function of the EP_2 receptor in cerebral ischaemia is dependent on cAMP signalling (McCullough *et al*., 2004). Therefore, an EP_3 receptor-related cAMP reduction through G_i activation may mediate the effects in a manner opposite to the mediation by EP_2 receptors. EP_3 receptors have also been shown to be linked to phospholipase C activation via G_i, and this activation leads to Ca^{2+} mobilization from internal stores and influx from the extracellular medium (Irie *et al*., 1994). Thus, much as in the case of the toxic effect of EP_1 receptors (Kawano *et al*., 2006), EP_3 receptors may contribute to neurotoxicity by augmenting the Ca^{2+} dysregulation underlying excitotoxic neuronal death.

We detected activation of Rho kinase by the EP_3 receptor agonist in rat hippocampal slices, but not by focal ischaemia in the mouse brain. Because phosphorylation of MBS in hippocampal slices was observed at 1 min, but not 24 h, after treatment with the EP_3 receptor agonist, the activation of Rho kinase in the ischaemic brain might have been detectable at a time point earlier than 24 h (Yamashita *et al*., 2007). In fact, we confirmed the involvement of Rho kinase not only in the glutamate-induced neurotoxicity *in vitro*, but also in the ischaemic infarction in mouse transient ischaemia models using the Rho kinase inhibitor. We used two types of Rho kinase inhibitors, Y-27632 for *in vitro* study and HA-1077 for *in vivo* study, because Y-27632 has been the most commonly used inhibitor for *in vitro* experimental studies for a long time, while HA-1077 has been clinically used for cerebrovascular disorders. The protective effect of HA-1077 was consistent with previous findings using both permanent ischaemia and transient focal ischaemia models in mice (Yamashita *et al*. 2007 and Rikitake *et al*., 2005 respectively). In addition to its protective effect on infarction, HA-1077 significantly attenuated the oedema formation and behavioural dysfunction observed after ischaemia. The protective effect of HA-1077 was more potent than that of EP_3 receptor antagonist, possibly because HA-1077 protects the neurons from ischaemic injury not only through a reduction of inducible nitric oxide synthase (iNOS) expression (Li *et al*., 2009), but also through an increase in CBF by vasodilation through endothelial nitric oxide synthase expression (Rikitake *et al*., 2005). Therefore, EP_3 may mediate nitric oxide production by induction of iNOS through activation of Rho kinase, and thereby induce apoptotic cell death (Nomura, 1998). As we were able to detect neuronal excitotoxicity by Rho kinase activation even in the cultured hippocampal slice system (Figure 6), we suggest that some of the stroke injury observed in our *in vivo* system was the result of direct damage through the activation of Rho kinase by the activation of EP_3 receptors in the parenchymal lesion site. Actually, in the spinal cord, Rho kinase has been shown to mediate nitric oxide formation by EP_3 receptor activation (Matsumura *et al*., 2005). However, the mechanisms by which EP_3 receptors/Rho kinase affects inflammation and neuronal apoptosis remain to be identified.

In summary, we have shown that EP_3 receptors, as effectors of mPGES-1 neurotoxicity, play a critical role in the infarction, oedema and behavioural dysfunctions observed after ischaemia through the activation of Rho kinase and/or G_i (Figure 9). Thus, our results suggest that the EP_3 receptor, as well as mPGES-1, is one of the most promising novel targets for treatment of human stroke.

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Conflict of interest

None.

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