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## Full Length Article

# Methylmercury promotes prostacyclin release from cultured human brain microvascular endothelial cells via induction of cyclooxygenase-2 through activation of the EGFR-p38 MAPK pathway by inhibiting protein tyrosine phosphatase 1B activity



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## ABSTRACT

Methylmercury is an environmental pollutant that exhibits neurotoxicity when ingested, primarily in the form of neuropathological lesions that localize along deep sulci and fissures, in addition to edematous and inflammatory changes in patient cerebrums. These conditions have been known to give rise to a variety of ailments that have come to be collectively termed Minamata disease. Since prostaglandins I<sub>2</sub> and E<sub>2</sub> (PGI<sub>2</sub> and PGE<sub>2</sub>) increase vascular permeability and contribute to the progression of inflammatory changes, we hypothesize that methylmercury induces the synthesis of these prostaglandins in brain microvascular endothelial cells and pericytes. To test this theory, human brain microvascular endothelial cells and pericytes were cultured and treated with methylmercury, after which the PGI<sub>2</sub> and PGE<sub>2</sub> released from endothelial cells and/or pericytes were quantified by enzyme-linked immunosorbent assay while protein and mRNA expressions in endothelial cells were analyzed by western blot analysis and real-time reverse transcription polymerase chain reaction, respectively. Experimental results indicate that methylmercury inhibits the activity of protein tyrosine phosphatase 1B, which in turn activates the epidermal growth factor receptor–p38 mitogen-activated protein kinase pathway that induces cyclooxygenase-2 expression. It was also found that the cyclic adenosine 3',5'-monophosphate pathway, which can be activated by PGI<sub>2</sub> and PGE<sub>2</sub>, is involved in methylmercury-induced cyclooxygenase-2 expression. Since it appears that protein tyrosine phosphatase 1B serves as a sensor protein for methylmercury in these mechanisms, it is our belief that the results of the present study may provide additional insights into the molecular mechanisms responsible for edematous and inflammatory changes in the cerebrum of patients with Minamata disease.

## 1. Introduction

Methylmercury is an organometallic compound that is well-known for its neurotoxic properties (Sanfeliu et al., 2003). However, while such organometallic compounds are often toxic, their toxicity characteristics cannot be assumed from those of either the inorganic metal or the organic structure of the organometallic compounds (Kohri et al., 2015). In fact, the toxicity of methylmercury is completely different from its inorganic mercury and methane components. In human beings, methylmercury toxicity is characterized by neuropathological lesions localized along deep sulci and fissures in the cerebral cortex, such as carcalin fissures, and the granular cell layer in patient cerebellums (Eto, 1997). The resulting neuropathological affects, which has come to be known as Minamata disease, include concentric visual field contraction,

articulation disorder, hearing impairment, and ataxia (Chang et al., 1973; Uchino et al., 2005). However, while the molecular mechanisms underlying the abovementioned localization have not yet been clarified, Eto et al. (2001) hypothesized that methylmercury induces edematous changes that cause a circulation disorder; after which nerve cells, especially along deep sulci and fissures, are secondarily damaged in the cerebrum. This “edema hypothesis” appears to rationally explain why methylmercury cerebral damage tends to localize along deep sulci and fissures in the cerebral cortex. Additionally, it is likely that inflammatory changes in the neurovascular unit (NVU) contribute to the progression of the edematous changes (Stokum et al., 2016).

Vascular endothelial cells and pericytes are constituent cell types of the NVU, which regulates the functions of brain microvessels such as the blood-brain barrier (BBB) function, glucose and amino acid

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transport, and vascular tonus (Abbott et al., 2006; Bechmann et al., 2007; Orledge and D'Amore, 1987; Kutcher and Herman, 2009). Our previous studies of methylmercury-induced functional cell damage have shown that the compound also inhibits the repair of wounded monolayers in endothelial cells by suppressing the expression of fibroblast growth factor-2 (Hirooka et al., 2009). Additionally, it has been shown that methylmercury activates the vascular endothelial growth factor (VEGF) paracrine system among endothelial cells and pericytes by inducing the expression of VEGF and VEGF receptor-2 in pericytes and endothelial cells, respectively (Hirooka et al., 2013). These results suggest that the activation of VEGF system suppresses the expression of tight junction proteins (Wang et al., 2001; Fischer et al., 2002), increases hydraulic permeability of vessels (Bates and Curry, 1996), and promotes edema formation (van Bruggen et al., 1999). Recently, we reported that methylmercury induces the expression of hyaluronan in endothelial cells and pericytes (Hirooka et al., 2017), which can contribute to the progression of edematous change by retaining water in the extracellular matrix. Such methylmercury-induced edematous changes may – at least in part – result from functional abnormalities in brain microvascular endothelial cells and pericytes.

Prostaglandins (PGs) are arachidonic acid metabolites that are involved in the inflammatory and subsequent edematous changes in brain microvascular endothelial cells and pericytes. Cyclooxygenases (COXs) metabolize arachidonic acid to prostaglandin  $H_2$  (Miyamoto et al., 1976; Van der Ouderaa et al., 1977), which is subsequently processed by downstream enzymes to the various prostanoids such as prostaglandins  $I_2$  and  $E_2$  ( $PGI_2$  and  $PGE_2$ ) that cause edematous and inflammatory changes by increasing vascular permeability and vasodilation (Williams, 1979). There are two COX isoforms: COX-1 and COX-2. The expression of COX-1 is constitutive, whereas COX-2 is inducible and involved in the inflammatory response (Seibert et al., 1994; Dubois et al., 1998). The induction of COX-2 results in a shift in arachidonic acid metabolism from the production of several prostanoids to the preferential synthesis of  $PGI_2$  and  $PGE_2$  (Brock et al., 1999). The mitogen-activated protein kinase (MAPK) and cyclic adenosine 3',5'-monophosphate (cAMP) pathways are involved in the induction of COX-2 expression (Tsatsanis et al., 2006).

We hypothesize that methylmercury influences PG synthesis in brain microvascular endothelial cells and pericytes. We examined this hypothesis using a cell culture system of these cell types with the goal of revealing the intracellular signaling by which methylmercury induces COX-2 to promote  $PGI_2$  synthesis in endothelial cells.

## 2. Materials and methods

### 2.1. Materials

Human brain microvascular endothelial cells and pericytes were purchased from DS Pharma Biomedical (Osaka, Japan). Collagen-coated tissue culture dishes and plates were obtained from AGC Technoglass (Chiba, Japan). HuMedia-EG2 and HuMedia-SG2 were purchased from Kurabo (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM) and  $Ca^{2+}$ ,  $Mg^{2+}$ -free phosphate-buffered saline (CMF-PBS) were obtained from Nissui Pharmaceutical (Tokyo, Japan). Methylmercury chloride was purchased from Tokyo Kasei Kogyo (Tokyo, Japan), while bovine serum albumin (BSA), Tris base, and anti- $\beta$ -actin antibodies (20–33) were obtained from Sigma-Aldrich Chemical (St. Louis, MO, USA).

Additionally, a mitogen-activated protein kinase (MEK1/2) inhibitor PD98059, a p38 MAPK inhibitor SB203580, a c-Jun N-terminal kinase (JNK) inhibitor SP600125, a 6-keto  $PGF_{1\alpha}$  enzyme-linked immunosorbent assay (EIA) kit, and a  $PGE_2$  EIA kit were all purchased from Cayman Chemical (Ann Arbor, MI, USA). The adenylate cyclase inhibitor SQ22536 and human active protein tyrosine phosphatase (PTP1B) EIA kit used in this study were obtained from R & D Systems (Minneapolis, MN, USA). We purchased epidermal growth factor

(EGFR) inhibitor PD153035 from Calbiochem (Boston, MA, USA), a BCA protein assay reagent kit from Pierce (Rockford, IL, USA), Immobilon-P transfer membrane (pore size 0.45  $\mu m$ ) from Millipore (Billerica, MA, USA), anti-COX-2 antibody (sc-19999) from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

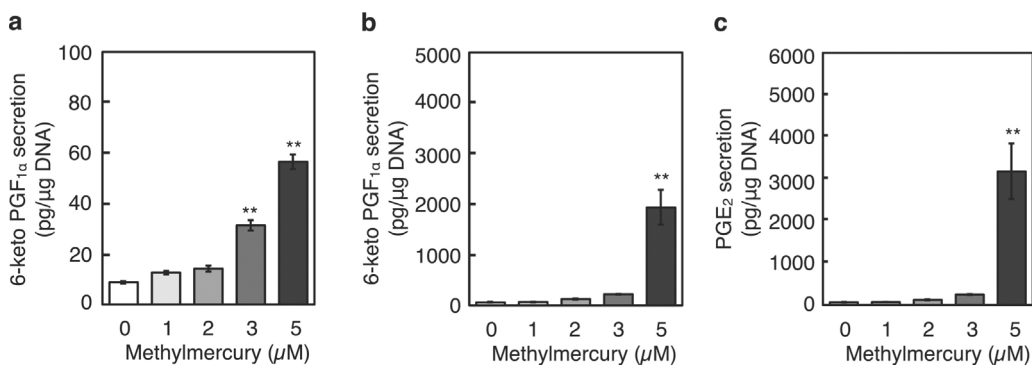
Anti-phosphorylated EGFR antibody (3777S), anti-phosphorylated ERK1/2 antibody (9101S), anti-phosphorylated p38 MAPK antibody (9211S), anti-phosphorylated JNK antibody (9255S), and horseradish peroxidase-labeled anti-mouse and anti-rabbit antibodies were purchased from Cell Signaling (Beverly, MA, USA). Fetal bovine serum (FBS), MagicMark™ XP western protein standard, and RNase DNase free water were obtained from Invitrogen (Carlsbad, CA, USA). QIAzol lysis reagent was purchased from Qiagen (Hilden, Germany), while a High-Capacity cDNA archive kit, TaqMan Universal PCR Master Mix II, TaqMan Gene Expression Assay (20  $\times$ ), and the primer and probe sets used for real-time reverse transcription polymerase chain reaction (RT-PCR) were purchased from Applied Biosystems (Foster, CA, USA). Other reagents were purchased from Wako Purechemical (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan). Methylmercury at 10 mM was stored at  $-80^\circ C$  in CMF-PBS containing L-cysteine at 10 mM until use.

### 2.2. Cell culture

Human brain microvascular endothelial cells and pericytes were cultured in HuMedia-EG2 and HuMedia-SG2 medium, respectively, in 100 mm collagen-coated dishes at  $37^\circ C$  in a humid atmosphere of 5%  $CO_2$  until confluent. The cells were then transferred into other 100 mm collagen-coated dishes, 60 mm collagen-coated dishes, or 6-well collagen-coated plates and cultured until confluent. Next, the cells were treated with methylmercury in fresh HuMedia EG-2 medium (endothelial cells) and DMEM supplemented with 1% BSA (pericytes). In a separate experiment, confluent cell cultures were treated with SB203580 (10, 20, 50 or 100  $\mu M$ ), PD153035 (2, 5, 10 or 20  $\mu M$ ), or SQ22536 (10, 20, 50 or 100  $\mu M$ ) for 3 h, and then treated with methylmercury (5  $\mu M$ ) for 6 or 24 h. The expression levels of COX-2, ERK1/2, JNK, p38 MAPK, and EGFR were determined by western blot analysis as described below. We postulated that the PTP1B is a sensor protein for methylmercury to activate the EGFR-MAPK pathway that induces COX-2 expression. It was considered that it takes a time to stimulate the PG production by activation of the EGFR-p38 MAPK-COX-2 pathway. Therefore, we designed experiments to determine PTP1B activity and EGFR phosphorylation after 20 min, MAPK phosphorylation after 1 h, and COX-2 activation/PG accumulation after 24 h of the methylmercury treatment. Additionally, we do not show the data about the cell viability, since we have already reported that endothelial cells and pericytes in our system are resistant to the cytotoxicity of methylmercury (Hirooka et al., 2007, 2010a,b).

### 2.3. Determination of $PGI_2$ and $PGE_2$

Human brain microvascular endothelial cells and pericytes in 6-well culture plates were treated with methylmercury (1–3, and 5  $\mu M$ ) for 24 h. After treatment, the conditioned medium was harvested and used for the determination of  $PGI_2$  measured as 6-keto  $PGF_{1\alpha}$  and  $PGE_2$  by the EIA kits. The cell layer was washed twice with CMF-PBS and then scraped off with a rubber policeman in the presence of 1.5 mL CMF-PBS. The well was then washed with 1.5 mL CMF-PBS and the wash was combined with the corresponding harvested cell suspension. This combined cell suspension was then sonicated and the cell homogenate was used for the determination of DNA content by the fluorometric assay. The 6-keto  $PGF_{1\alpha}$  and  $PGE_2$  accumulated in the conditioned medium were expressed as  $pg/\mu g$  DNA.



**Fig. 1.** The release of PGI<sub>2</sub> and PGE<sub>2</sub> from human brain microvascular endothelial cells and pericytes after exposure to methylmercury. The cells were treated with methylmercury (1–3, and 5 μM) for 24 h. The released PGI<sub>2</sub> was detected as 6-keto PGF<sub>1α</sub>. a) The effect of methylmercury on the release of PGI<sub>2</sub> from endothelial cells. b) The effect of methylmercury on the release of PGI<sub>2</sub> from pericytes. c) The effect of methylmercury on the release of PGE<sub>2</sub> from pericytes. PGE<sub>2</sub> release from endothelial cells was not detected. Values are means ± S.E. of four samples. \*\*Significantly different from the corresponding control,  $p < 0.01$ .

#### 2.4. Determination of COX-2, p38 MAPK, ERK1/2, JNK, and EGFR proteins

The human brain microvascular endothelial cells and pericytes treated with methylmercury (1–3, and 5 μM) for 24 h in 6-well culture plates were washed twice with ice-cold CMF-PBS and 2 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid-containing CMF-PBS. After washing, the cells were lysed with 80 μL of 50 mM Tris-HCl buffer solution (pH 6.8) containing 10% glycerol and 2% sodium dodecyl sulfate. The ten μg proteins of the lysate were separated by SDS-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel, and then transferred onto an Immobilon-P membrane. Next, 5% polyacrylamide gel was used for EGFR separation. Membranes were treated with 5% skim milk in 20 mM Tris-HCl buffer solution (pH 7.5) containing 150 mM NaCl and 0.1% Tween 20, and then incubated overnight at 4 °C with anti-COX-2 antibody (1:200), anti-phosphorylated EGFR antibody (1:1000), anti-phosphorylated ERK antibody (1:1000), anti-phosphorylated p38 MAPK antibody (1:1000), anti-phosphorylated JNK antibody (1:1000), anti-EGFR antibody (1:1000), or anti-β-actin antibody (1:200). The membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Immunoreactive bands were visualized using enhanced chemiluminescence western blot detection reagents (Chemilumi One L; Nacalai, Kyoto, Japan) and scanned using a LAS 3000 Imager (Fujifilm, Tokyo, Japan).

#### 2.5. Real-time RT-PCR

Total RNA was extracted from human brain microvascular endothelial cells and pericytes treated with methylmercury (1–3, and 5 μM) for 12 h in 60 mm dishes. The complementary DNA (cDNA) was synthesized using the High-Capacity cDNA Reverse Transcription kit. Real-time PCR was performed using Gene Ace SYBR qPCR Mixα with 10 ng cDNA and 100 nM primers on a StepOnePlus RT-PCR system (Applied Biosystems). The thermal cycling parameters were 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. COX-2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels were quantified using the relative standard curve method. The primer and probe sets for COX-2 (Hs00153133-m1) and GAPDH (Hs99999905-m1) were obtained using the TaqMan Gene Expression Assay.

#### 2.6. PTP1B activity

Confluent cultures of human brain microvascular endothelial cells in 60 mm dishes were treated with methylmercury (3 or 5 μM) for 20 min, after which the medium was discarded and the cell layer was harvested by scraping with a rubber policeman in the presence of 200 μL 50 mM Tris-HCl buffer solution (pH 7.5) containing 150 mM NaCl, 0.1% sodium deoxycholate, 1% 3-[(3-cholamidopropyl)dimethylammonio]propane-sulfonate, 1 mM *O,O'*-bis(2-aminoethyl)ethyleneglycol-*N,N,N',N'*-

tetraacetic acid, and 0.4 mM ethylenediaminetetraacetic acid (cell lysis buffer). The cell suspension was then lysed on ice for 30 min, after which the cell lysate was centrifuged at  $2,000 \times g$  for 5 min at 4 °C. The resulting supernatant was used for the determination of active PTP1B protein and total protein content by the human active PTP1B Duoset IC kit and BCA protein assay reagent kit, respectively. The PTP1B activity was defined as the ratio of active PTP1B protein content to the corresponding total protein content.

#### 2.7. Statistical analysis

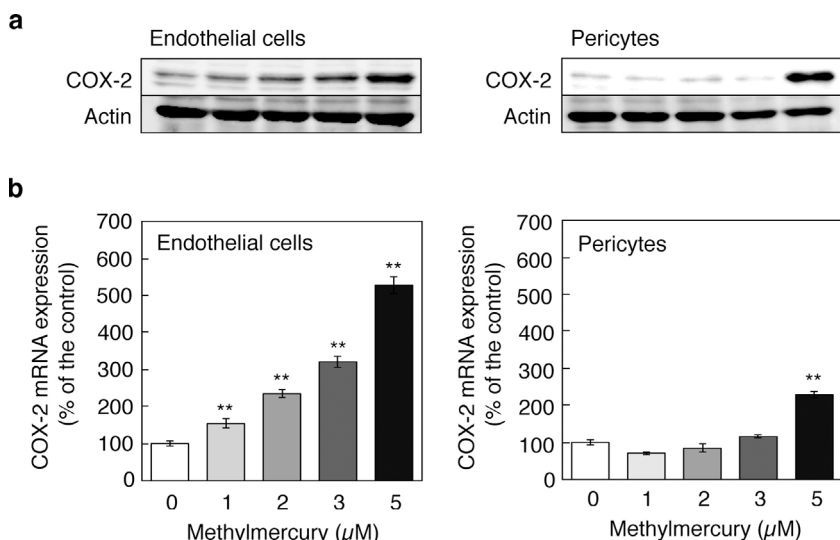
Results were analyzed for statistical significance by analysis of variance (ANOVA) and Bonferroni's multiple *t*-test, when possible. *P* values less than 0.05 were considered statistically significant.

### 3. Results

Fig. 1 shows the effects of methylmercury on the accumulation of PGI<sub>2</sub> and PGE<sub>2</sub> in the conditioned medium of human brain microvascular endothelial cells and pericytes. In endothelial cells, methylmercury levels at 5 μM and less were found to have significantly increased the accumulation of PGI<sub>2</sub> detected as 6-keto PGF<sub>1α</sub> in a concentration-dependent manner after a 24-h incubation period (Fig. 1a); however, PGE<sub>2</sub> was not detected. It was reported that vascular endothelial cells have an ability to synthesize PGE<sub>2</sub> but the major product of prostaglandins in the cells is PGI<sub>2</sub> (Chesterman et al., 1983). PGI<sub>2</sub> accumulation in the conditioned pericyte medium was found to have increased after treatment with methylmercury at 5 μM for 24 h (Fig. 1b). Additionally, methylmercury at 5 μM significantly increased the PGE<sub>2</sub> accumulation after a 24-h incubation period (Fig. 1c).

Fig. 2 shows the expression of COX-2 in human brain microvascular endothelial cells and pericytes before and after treatment with methylmercury. As can be seen in the figure, methylmercury at 5 μM and less increased the expression of COX-2 protein in endothelial cells in a concentration-dependent manner (Fig. 2a, left panel) and at 5 μM in pericytes (Fig. 2a, right panel) after a 24-h incubation period. The expression level of COX-2 mRNA in endothelial cells was significantly elevated by methylmercury treatment at concentrations of 5 μM or less in a concentration-dependent manner (Fig. 2b, left panel), and at 5 μM in pericytes (Fig. 2b, right panel) after a 24-h incubation period.

Because a concentration-dependent response to methylmercury was observed in endothelial cells but not in pericytes, the following experiments were performed using endothelial cells. Since the expression of COX-2 is upregulated by activation of MAPKs (Lin et al., 2009), both the phosphorylation of MAPKs (p38 MAPK, ERK1/2, and JNK) and the involvement of MAPKs in methylmercury-induced COX-2 expression were determined (Fig. 3). The phosphorylation of ERK1/2 and JNK was unaffected, whereas that of p38 MAPK was increased by methylmercury in a concentration-dependent manner (Fig. 3a). Methylmercury-induced expression of COX-2 protein was suppressed by SB203580, a p38



**Fig. 2.** The expression of COX-2 in from human brain microvascular endothelial cells and pericytes after exposure to methylmercury. a) The effect of methylmercury on the expression of COX-2 protein. The cells were treated with methylmercury (1–3, and 5 μM) for 24 h b) The effect of methylmercury on the expression of COX-2 mRNA. The cells were treated with methylmercury (1–3, and 5 μM) for 12 h. Values are means ± S.E. of four samples. \*\*Significantly different from the corresponding control,  $p < 0.01$ .

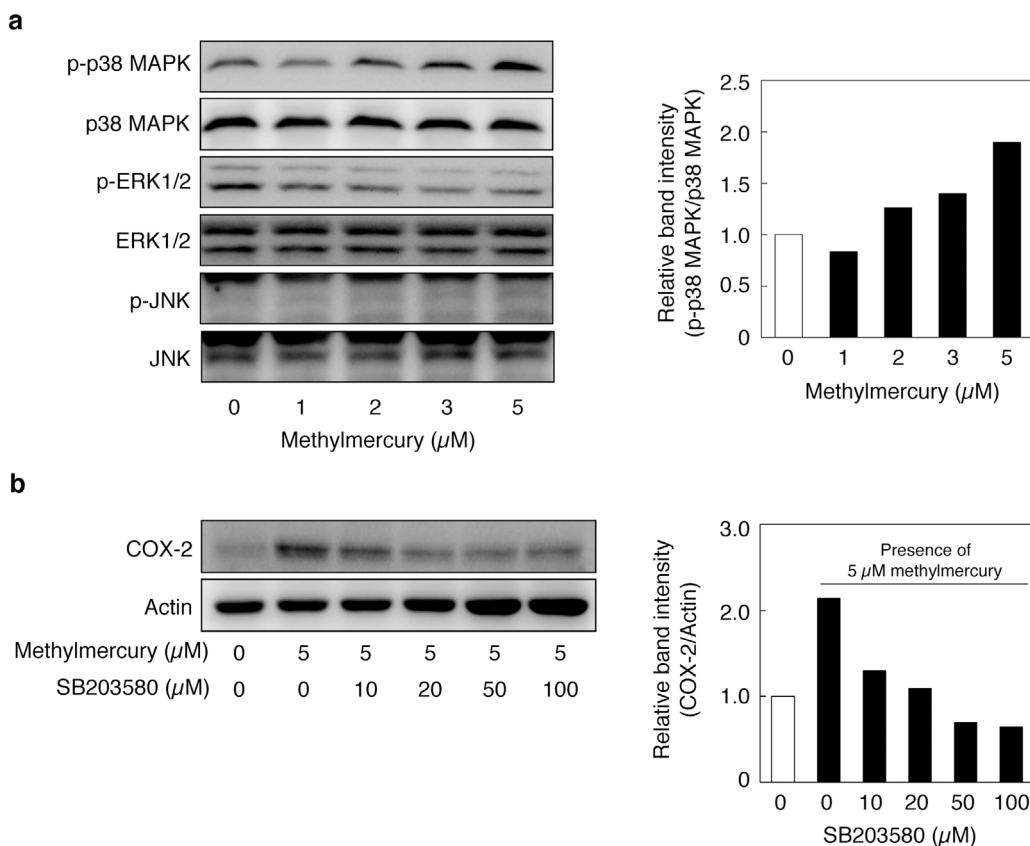
MAPK inhibitor, thereby suggesting that the activation of the p38 MAPK signal is involved in methylmercury-induced COX-2 expression in human brain microvascular endothelial cells (Fig. 3b).

Although the mechanism by which methylmercury activates p38 MAPK has previously been unclear, we paid specific attention to EGFR due to the following reasons:

- (1) Auto-phosphorylation occurs in EGFR, and this activation is negatively regulated by the de-phosphorylating enzyme, PTP1B (Barford et al., 1995).
- (2) Electrophiles including zinc ion can inhibit the activity of PTP1B and then activate EGFR (Bellomo et al., 2014; Iwamoto et al., 2007).
- (3) Methylmercury has an electrophile characteristic.

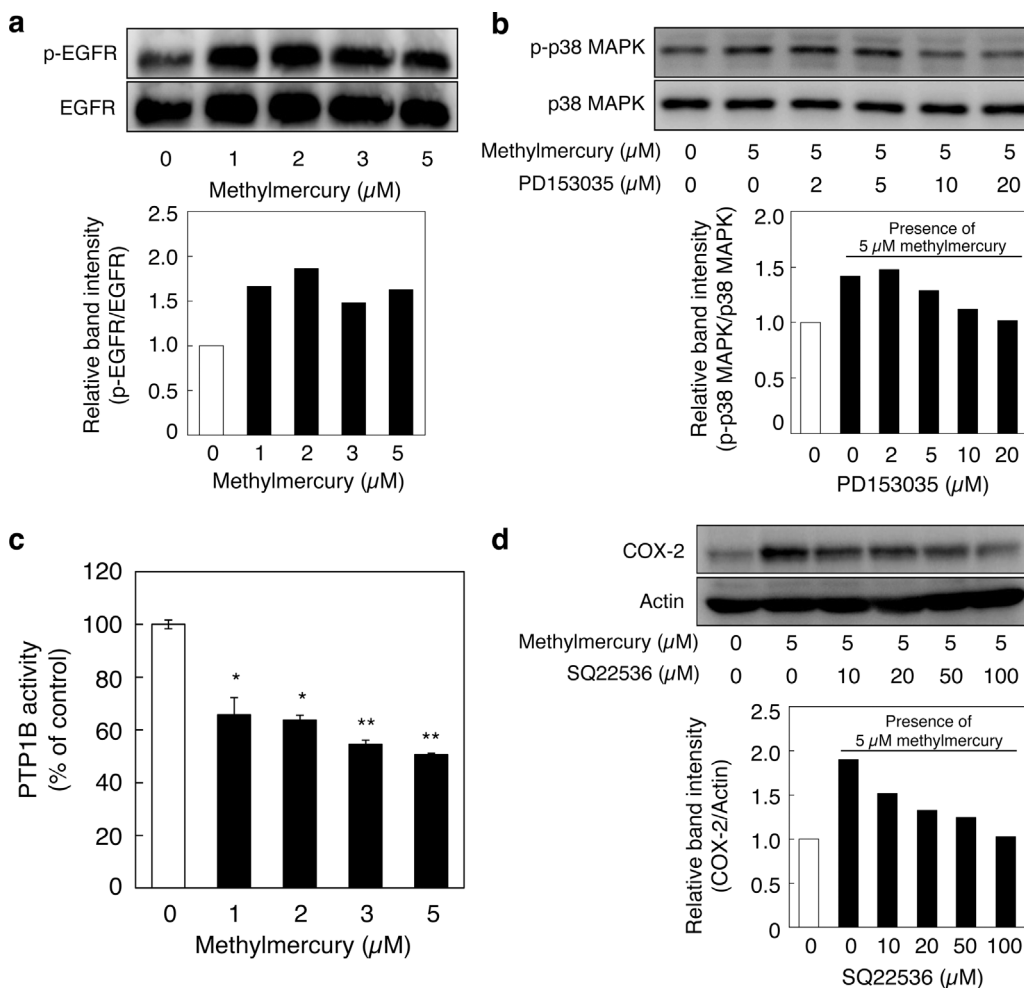
It was found that EGFR phosphorylation was increased by treatment with methylmercury for 20 min at levels of 1 μM and more (Fig. 4a), and that PD153035, an EGFR inhibitor, diminished methylmercury-induced p38 MAPK activation in a concentration-dependent manner (Fig. 4b). This suggests that the methylmercury-induced p38 MAPK activation is mediated by the EGFR phosphorylation. Furthermore, methylmercury at 1 μM and more significantly decreased the activity of PTP1B (Fig. 4c), thus supporting the hypothesis that methylmercury inhibits this phosphatase, although other mechanisms cannot be excluded. Taken together, these results suggest that methylmercury-induced COX-2 expression is mediated by the EGFR-p38 MAPK pathway, which is activated by PTP1B inhibition.

Both PGI<sub>2</sub> and PGE<sub>2</sub> can increase the intracellular level of cAMP by



**Fig. 3.** Involvement of the phosphorylation of MAPKs (p38 MAPK, ERK1/2, and JNK) in the induction of COX-2 protein by methylmercury in human brain microvascular endothelial cells. a) The effect of methylmercury on the phosphorylation of MAPKs. The cells were treated with methylmercury (1–3, and 5 μM) for 1 h. Western blot analysis (left panel) and its quantitative analysis (right panel). b) The effect of p38 MAPK inhibitor SB203580 on the expression of COX-2 protein. The cells were treated with methylmercury (1–3, and 5 μM) for 24 h after pretreatment with SB203580 (10, 20, 50, and 100 μM) for 3 h. Western blot analysis (left panel) and its quantitative analysis (right panel).





**Fig. 4.** Involvement of the activation of EGFR in the phosphorylation of p38 MAPK and induction of COX-2 protein by methylmercury in human brain microvascular endothelial cells. **a)** The effect of methylmercury on the phosphorylation of EGFR. The cells were treated with methylmercury (1–3, and 5  $\mu\text{M}$ ) for 20 min. Western blot analysis (upper panel) and its quantitative analysis (lower panel). **b)** The effect of EGFR inhibitor PD153035 on methylmercury-induced phosphorylation of p38 MAPK. The cells were treated with methylmercury (1–3, and 5  $\mu\text{M}$ ) for 1 h after pretreatment with PD153035 (2, 5, 10, and 20  $\mu\text{M}$ ) for 3 h. Western blot analysis (upper panel) and its quantitative analysis (lower panel). **c)** The effect of methylmercury on the activity of PTP1B. The cells were treated with methylmercury (3 and 5  $\mu\text{M}$ ) for 20 min. Values are means  $\pm$  S.E. of three samples. Significantly different from the corresponding control, \* $p < 0.05$ ; \*\* $p < 0.01$ . **d)** The effect of adenylate cyclase inhibitor SQ22536 on methylmercury-induced expression of COX-2 protein. The cells were treated with methylmercury (1–3, and 5  $\mu\text{M}$ ) for 24 h after pretreatment with SQ22536 (10, 20, 50, and 100  $\mu\text{M}$ ) for 3 h. Western blot analysis (upper panel) and its quantitative analysis (lower panel).

activation of adenylate cyclase through the activation of relaxant prostaglandin receptors – EP2, EP4, or IP (Narumiya and Furuyashiki, 2011). Additionally, COX-2 expression can be upregulated by the cAMP-protein kinase A signaling pathway (Debey et al., 2003; Steinert et al., 2009; Díaz-Muñoz et al., 2012). Based on these results, involvement of the cAMP pathway in methylmercury-induced COX-2 expression was determined (Fig. 4d). More specifically, after a 24-h incubation period, it was shown that SQ22536, an adenylate cyclase inhibitor, diminished the induction of COX-2 expression by methylmercury in a concentration-dependent manner. This suggests that there is a positive feedback loop among methylmercury, COX-2, and cAMP that induces COX-2 expression in endothelial cells.

#### 4. Discussion

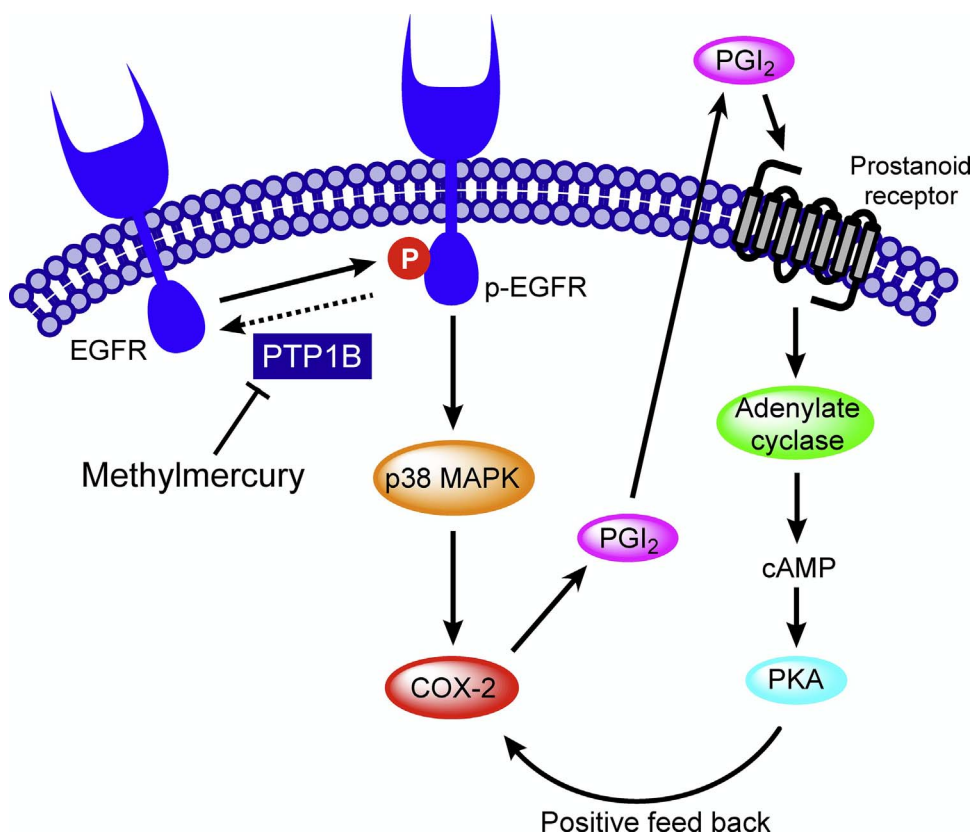
It is important to clarify the effects of methylmercury on the synthesis of PGI<sub>2</sub> and PGE<sub>2</sub> in brain vascular cells in order to understand the inflammatory changes that accompany the progression of edematous variations in the brain after methylmercury exposure. The following results were obtained from our experiments using a cell culture system:

- (1) Methylmercury increased the release of PGI<sub>2</sub> from human brain microvascular endothelial cells and both PGI<sub>2</sub> and PGE<sub>2</sub> from human brain microvascular pericytes.
- (2) Methylmercury induced the expression of COX-2 in both endothelial cells and pericytes.
- (3) Inhibition of methylmercury-activated p38 MAPK suppressed methylmercury-induced COX-2 expression.

- (4) Methylmercury activated EGFR and inhibited PTP1B activity. In addition, the inhibition of EGFR suppressed the activation of p38 MAPK by methylmercury.
- (5) Inhibition of adenylate cyclase suppressed the expression of COX-2 induced by methylmercury.

When these results are examined together, it can be postulated that methylmercury activates EGFR by inhibition of PTP1B, and that this activation consequently induces the activation of p38 MAPK that increases the expression of COX-2. The induced COX-2 then increases the synthesis of PGI<sub>2</sub> in endothelial cells and both PGI<sub>2</sub> and PGE<sub>2</sub> in brain microvessel pericytes. Additionally, it is suggested that PGI<sub>2</sub>- and PGE<sub>2</sub>-induces the activation of adenylate cyclase (Coleman et al., 1994; Narumiya et al., 1999) and increases the intracellular cAMP that induces the COX-2 expression (Debey et al., 2003; Steinert et al., 2009; Díaz-Muñoz et al., 2012). In other words, the EGFR–p38 MAPK pathway mediates the methylmercury-induced expression of COX-2 that synthesizes PGI<sub>2</sub> and PGE<sub>2</sub> in vascular endothelial cells and pericytes. In addition to this intracellular signaling cascade, the PGI<sub>2</sub>/PGE<sub>2</sub>–adenylate cyclase–cAMP–protein kinase A pathway may serve as a positive feedback loop that enhances COX-2 induction by methylmercury, since protein kinase A is a cAMP-dependent protein kinase (Shabb, 2001) and induce COX-2 expression (Debey et al., 2003). Furthermore, PTP1B appears to be a sensor protein for methylmercury, which initiates the consequent intracellular signaling. These biological chain reactions are summarized in Fig. 5.

Although PGI<sub>2</sub> and PGE<sub>2</sub> have multiple physiological roles, one of their common functions is involvement with the inflammatory changes caused by an increase in vascular relaxation and permeability



**Fig. 5.** The present data suggest the intracellular signal transduction that mediates methylmercury-induced synthesis of PGI<sub>2</sub> in human brain microvascular endothelial cells. Methylmercury inhibits the activity of PTP1 B and increases the phosphorylation of EGFR. The activated EGFR increases the phosphorylation of p38 MAPK, and the EGFR–p38 MAPK pathway mediates the methylmercury-induced expression of COX-2 that synthesizes PGI<sub>2</sub>. PGI<sub>2</sub> stimulates the prostanoid receptor(s) such as EP2, EP4, and IP that can activate the cAMP pathway. The cAMP pathway may serve as a positive feedback loop that enhances PGI<sub>2</sub> synthesis via COX-2 induction.

(Williams, 1979). Accordingly, the induction of PGI<sub>2</sub> and PGE<sub>2</sub> release may be a part of the mechanisms underlying the edematous changes in brains exposed to methylmercury. Note that the PGI<sub>2</sub> and PGE<sub>2</sub> receptors are IP and EP, respectively (Ushikubi et al., 1995; Narumiya et al., 1999), both of which are G protein-coupled. Activation of the IP receptor increases the intracellular level of cAMP via the activation of adenylate cyclase. On the other hand, the EP receptor has four subtypes: EP1, EP2, EP3, and EP4. Among these, EP2 and EP4 increase the intracellular cAMP level by coupling with the G<sub>s</sub> protein (Coleman et al., 1994; Narumiya et al., 1999). Both PGI<sub>2</sub> and PGE<sub>2</sub> can elevate the cAMP level via their receptors. Additionally, increased cAMP leads to activation of protein kinase A (Steinert et al., 2009; El-Haroun et al., 2008). We reported that methylmercury can activate the paracrine VEGF system between endothelial cells and pericytes, which can then enhance vascular permeability via induction of the VEGFR1 expression in endothelial cells and VEGF-A expression in pericytes (Hirooka et al., 2013). Since the cAMP level, which can be increased by either PGI<sub>2</sub> or PGE<sub>2</sub>, increases the expression of VEGF (Höper et al., 1997; Bradbury et al., 2005), it is suggested that VEGF-A system-induced vascular permeability is potentiated by cAMP levels in the progression of edematous changes in brains exposed to methylmercury. Additionally, cAMP mediates the decrease in the anticoagulant heparan sulfate proteoglycan synthesis (Kaji et al., 1996) and fibrinolytic activity (Francis and Neely, 1989) in vascular endothelial cells, thereby suggesting that methylmercury can reduce the anti-coagulant and anti-fibrinolytic activities that can contribute to the microangiopathy that secondarily induces damage to nerve cells around the microvessels. Therefore, it is suggested that increases in PGI<sub>2</sub>/PGE<sub>2</sub> synthesis in vascular endothelial cells, and that pericytes are significantly involved in methylmercury-induced edematous and inflammatory changes in the brain.

Our previous and present studies have worked to clarify the molecular mechanisms underlying the edematous changes that accompany the inflammatory changes leading to secondary nerve cell damage in brains exposed to methylmercury. Specifically, it has and continues to

be speculated that the vasogenic edema caused by methylmercury is initiated by the activation of the VEGF system between the endothelial cells and pericytes in brain microvessels (Hirooka et al., 2013). When that occurs, hyaluronan levels increase in the extracellular matrix of endothelial cells and pericytes (Hirooka et al., 2017). Hyaluronan then absorbs water seepage from blood vessels whose permeability has been enhanced by the activated VEGF system following methylmercury exposure. PGI<sub>2</sub> and PGE<sub>2</sub> would also increase vascular permeability further, and thus contribute to the inflammatory changes in the progression of edematous changes caused by methylmercury. Thus, our experimental results presented in this study provide clear insights into part of the molecular basis in the “edema hypothesis”, and our obtained data highlights the importance of vascular toxicity in understanding the methylmercury neurotoxicity.

It should also be noted that the present study revealed, for the first time, that the presence of methylmercury induced the release of PGI<sub>2</sub> in endothelial cells and pericytes, and in both PGI<sub>2</sub> and PGE<sub>2</sub> in pericytes. This was traced to the induction of COX-2 expression mediated by the EGFR–p38 MAPK pathway. Additionally, we found that PTP1B served as a sensor protein for methylmercury, and that EGFR was activated by the inhibition of this enzyme in endothelial cells after methylmercury exposure. Furthermore, our results suggest that the PGI<sub>2</sub>/PGE<sub>2</sub>–adenylate cyclase–cAMP–protein kinase A pathway provides a positive feedback loop that increases the COX-2 induction caused by methylmercury. Since other experimental results show that the activated cAMP pathway appears to be secondarily and extensively involved in methylmercury neurotoxicity, further studies should be undertaken to clarify the functional damage to brain microvascular cells as part of efforts aimed at understanding the edematous and inflammatory changes caused by methylmercury. However, studies on the mechanisms underlying not only vasogenic edema, but also cytotoxic edema, will be necessary from the viewpoint of signal toxicology (Kanno, 2016) because activation of the PTP1B–EGFR–p38 MAPK–COX-2–PGs–adenylate cyclase–cAMP–protein kinase A signaling

cascade may be one of the most important methylmercury signal toxicities.

### Conflict of interest

None.

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### References

- Abbott, N.J., Rönnbäck, L., Hansson, E., 2006. Astrocyte-endothelial interactions at the blood-brain barrier. *Nat. Rev. Neurosci.* 7, 41–53.
- Barford, D., Jia, Z., Tonks, N.K., 1995. Protein tyrosine phosphatases take off. *Nat. Struct. Biol.* 2, 1043–1053.
- Bates, D.O., Curry, F.T., 1996. Vascular endothelial growth factor increases hydraulic conductivity of isolated perfused microvessels. *Am. J. Physiol.* 271, H2520–H2528.
- Bechmann, I., Galea, I., Perry, V.H., 2007. What is the blood-brain barrier (not)? *Trends Immunol.* 28, 5–11.
- Bellomo, E., Massarotti, A., Hogstranda, C., Maret, W., 2014. Zinc ions modulate protein tyrosine phosphatase 1B activity. *Metallomics* 6, 1229–1239.
- Bradbury, D., Clarke, D., Seedhouse, C., Corbett, L., Stocks, J., Knox, A., 2005. Vascular endothelial growth factor induction by prostaglandin E<sub>2</sub> in human airway smooth muscle cells is mediated by E prostanoic EP2/EP4 receptors and SP-1 transcription factor binding sites. *J. Biol. Chem.* 280, 29993–30000.
- Brock, T.G., McNish, R.W., Peters-Golden, M., 1999. Arachidonic acid is preferentially metabolized by cyclooxygenase-2 to prostacyclin and Prostaglandin E<sub>2</sub>. *J. Biol. Chem.* 274, 11660–11666.
- Chang, L.W., Opitz, J.M., Pallister, P.D., Gilbert, E.F., Viseskul, C., 1973. Minamata disease: a case report and a comparative study. *Acta Neuropathol.* 26, 275–284.
- Chesterman, C.N., Ager, A., Gordon, J.L., 1983. Regulation of prostaglandin production and ectoenzyme activities in cultured aortic endothelial cells. *J. Cell. Physiol.* 116, 45–50.
- Coleman, R.A., Grix, S.P., Head, S.A., Louttit, J.B., Mallett, A., Sheldrick, R.L., 1994. A novel inhibitory prostanoid receptor in piglet saphenous vein. *Prostaglandins* 47, 151–168.
- Díaz-Muñoz, M.D., Osmá-García, I.C., Fresno, M., Iñiguez, M.A., 2012. Involvement of PGE<sub>2</sub> and the cAMP signalling pathway in the up-regulation of COX-2 and mPGES-1 expression in LPS-activated macrophages. *Biochem. J.* 443, 451–461.
- Debey, S., Meyer-Kirchraht, J., Schrör, K., 2003. Regulation of cyclooxygenase-2 expression by iloprost in human vascular smooth muscle cells. role of transcription factors CREB and ICER. *Biochem. Pharmacol.* 65, 979–988.
- Dubois, R.N., Abramson, S.B., Crofford, L., Gupta, R.A., Simon, L.S., Van De Putte, L.B., Lipsky, P.E., 1998. Cyclooxygenase in biology and disease. *FASEB J.* 12, 1063–1073.
- El-Haroun, H., Clarke, D.L., Deacon, K., Bradbury, D., Clayton, A., Sutcliffe, A., Knox, A.J., 2008. IL-1 $\beta$ , BK, and TGF- $\beta$ 1 attenuate PGI<sub>2</sub>-mediated cAMP formation in human pulmonary artery smooth muscle cells by multiple mechanisms involving p38 MAP kinase and PKA. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 294, 553–562.
- Eto, K., 1997. Pathology of Minamata disease. *Toxicol. Pathol.* 25, 614–623.
- Eto, K., Yasutake, A., Kuwana, T., Korogi, Y., Akima, M., Shimozeiki, T., Tokunaga, H., Kaneko, Y., 2001. Methylmercury poisoning in common marmosets—a study of selective vulnerability within the cerebral cortex. *Toxicol. Pathol.* 29, 565–573.
- Fischer, S., Wobben, M., Marti, H.H., Renz, D., Schaper, W., 2002. Hypoxia-induced hyperpermeability in brain microvessel endothelial cells involves VEGF-mediated changes in the expression of zonula occludens-1. *Microvasc. Res.* 63, 70–80.
- Francis Jr., R.B., Neely, S., 1989. Inhibition of endothelial secretion of tissue-type plasminogen activator and its rapid inhibitor by agents which increase intracellular cyclic AMP. *Biochim. Biophys. Acta* 1012, 207–213.
- Höper, M.M., Voelkel, N.F., Bates, T.O., Allard, J.D., Horan, M., Shepherd, D., Tuder, R.M., 1997. Prostaglandins induce vascular endothelial growth factor in a human monocytic cell line and rat lungs via cAMP. *Am. J. Respir. Cell Mol. Biol.* 17, 748–756.
- Hirooka, T., Fujiwara, Y., Yamamoto, C., Yasutake, A., Kaji, T., 2007. Methylmercury retards the repair of wounded monolayer of human brain microvascular endothelial cells by inhibiting their proliferation without nonspecific cell damage. *J. Health Sci.* 53, 450–456.
- Hirooka, T., Fujiwara, Y., Inoue, S., Shnkai, Y., Yamamoto, C., Satoh, M., Yasutake, A., Eto, K., Kaji, T., 2009. Suppression of fibroblast growth factor-2 expression: possible mechanism underlying methylmercury-induced inhibition of repair of wounded monolayers of cultured human brain microvascular endothelial cells. *J. Toxicol. Sci.* 34, 433–439.
- Hirooka, T., Fujiwara, Y., Shinkai, Y., Yamamoto, C., Yasutake, A., Satoh, M., Eto, K., Kaji, T., 2010a. Resistance of human brain microvascular endothelial cells in culture to methylmercury: cell-density-dependent defense mechanisms. *J. Toxicol. Sci.* 35, 287–294.
- Hirooka, T., Fujiwara, Y., Yamamoto, C., Yasutake, A., Kaji, T., 2010b. Cell-density-dependent methylmercury susceptibility of cultured human brain microvascular pericytes. *Toxicol. In Vitro* 24, 835–841.
- Hirooka, T., Yamamoto, C., Yasutake, A., Eto, K., Kaji, T., 2013. Expression of VEGF-related proteins in cultured human brain microvascular endothelial cells and pericytes after exposure to methylmercury. *J. Toxicol. Sci.* 38, 837–845.
- Hirooka, T., Yoshida, E., Eto, K., Kaji, T., 2017. Methylmercury induces hyaluronan synthesis in cultured human brain microvascular endothelial cells and pericytes via different mechanisms. *J. Toxicol. Sci.* 42 (3), 329–333.
- Iwamoto, N., Sumi, D., Ishii, T., Uchida, K., Cho, A.K., Froines, J.R., Kumagai, Y., 2007. Chemical knockdown of protein-tyrosine phosphatase 1B by 1,2-naphthoquinone through covalent modification causes persistent transactivation of epidermal growth factor receptor. *J. Biol. Chem.* 282, 33396–33404.
- Kaji, T., Inada, M., Yamamoto, C., Fujiwara, Y., Koizumi, F., 1996. Cyclic AMP-dependent pathway that mediates suppressive regulation of glycosaminoglycan production in cultured vascular endothelial cells. *Thromb. Res.* 82, 389–397.
- Kanno, J., 2016. Introduction to the concept of signal toxicity. *J. Toxicol. Sci.* 41, SP105–SP109.
- Kohri, K., Yoshida, E., Yasuie, S., Fujie, T., Yamamoto, C., Kaji, T., 2015. The cytotoxicity of organobismuth compounds with certain molecular structures can be diminished by replacing the bismuth atom with an antimony atom in the molecules. *J. Toxicol. Sci.* 40, 321–327.
- Kutcher, M.E., Herman, I.M., 2009. The pericyte: cellular regulator of microvascular blood flow. *Microvasc. Res.* 77, 235–246.
- Lin, C.C., Lin, W.N., Wang, W.J., Sun, C.C., Tung, W.H., Wang, H.H., Yang, C.M., 2009. Functional coupling expression of COX-2 and cPLA2 induced by ATP in rat vascular smooth muscle cells: role of ERK1/2, p38 MAPK, and NF-kappaB. *Cardiovasc. Res.* 82, 522–531.
- Miyamoto, T., Ogino, N., Yamamoto, S., Hayaishi, O., 1976. Purification of prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes. *J. Biol. Chem.* 251, 2629–2636.
- Narumiya, S., Furiyashiki, T., 2011. Fever, inflammation, pain and beyond: prostanoid receptor research during these 25 years. *FASEB J.* 25, 813–818.
- Narumiya, S., Sugimoto, Y., Ushikubi, F., 1999. Prostanoid receptors: structures, properties, and functions. *Physiol. Rev.* 79, 1193–1226.
- Orlidge, A., D'Amore, P.A., 1987. Inhibition of capillary endothelial cell growth by pericytes and smooth muscle cells. *J. Cell Biol.* 105, 1455–1462.
- Sanfelici, C., Sebastia, J., Cristófol, R., Rodríguez-Farré, E., 2003. Neurotoxicity of organomercurial compounds. *Neurotox. Res.* 5, 283–305.
- Seibert, K., Zhang, Y., Leahy, K., Hauser, S., Masferrer, J., Perkins, W., PLee, L., Isakson, P., 1994. Pharmacological and biochemical demonstration of the role of cyclooxygenase-2 in inflammation and pain. *Proc. Natl. Acad. Sci. U. S. A.* 91, 12013–12017.
- Shabb, J.B., 2001. Physiological substrates of cAMP-dependent protein kinase. *Chem. Rev.* 101, 2381–2411.
- Steinert, D., Küper, C., Bartels, H., Beck, F.X., Neuhofer, W., 2009. PGE<sub>2</sub> potentiates tonicity-induced COX-2 expression in renal medullary cells in a positive feedback loop involving EP<sub>2</sub>-cAMP-PKA signaling. *Am. J. Physiol. Cell Physiol.* 296, 75–87.
- Stokum, J.A., Gerzanich, V., Simard, J.M., 2016. Molecular pathophysiology of cerebral edema. *J. Cereb. Blood Flow Metab.* 36, 513–538.
- Tsatsanis, C., Androulidaki, A., Avenihaki, M., Margioris, A.N., 2006. Signalling networks regulating cyclooxygenase-2. *Int. J. Biochem. Cell Biol.* 38, 1654–1661.
- Uchino, M., Hirano, T., Satoh, H., Arimura, K., Nakagawa, M., Wakamiya, J., 2005. The severity of Minamata disease declined in 25 years: temporal profile of the neurological findings analyzed by multiple logistic regression model. *Tohoku J. Exp. Med.* 205, 53–63.
- Ushikubi, F., Hirata, M., Narumiya, S., 1995. Molecular biology of prostanoid receptors; an overview. *J. Lipid Mediat. Cell Signal.* 12, 343–359.
- Van der Ouderaa, F.J., Buytenhek, M., Nugteren, D.H., Van Dorp, D.A., 1977. Purification and characterisation of prostaglandin endoperoxide synthetase from sheep vesicular glands. *Biochim. Biophys. Acta* 487, 315–331.
- Wang, W., Dentler, W.L., Borchardt, R.T., 2001. VEGF increases BMEC monolayer permeability by affecting occludin expression and tight junction assembly. *Am. J. Physiol. Heart Circ. Physiol.* 280, H434–H440.
- Williams, T.J., 1979. Prostaglandin E<sub>2</sub>, prostaglandin I<sub>2</sub> and the vascular changes of inflammation. *Br. J. Pharmacol.* 65, 517–524.
- van Bruggen, N., Thibodeaux, H., Palmer, J.T., Lee, W.P., Fu, L., Cairns, B., Tumas, D., Gerlai, R., Williams, S.P., van Lookeren Campagne, M., Ferrara, N., 1999. VEGF antagonism reduces edema formation and tissue damage after ischemia/reperfusion injury in the mouse brain. *J. Clin. Invest.* 104, 1613–1620.