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Activation of interleukin-6 and -8 expressions by methylmercury in human U937 macrophages involves RelA and p50

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ABSTRACT: The accumulation of macrophages has been observed around lesions of the brain in patients with Minamata disease. In this condition, mercury has been detected histochemically in macrophages throughout the brain. However, the role of macrophages in the neurotoxicity of methylmercury (MeHg) and the molecular mechanisms of their response to MeHg exposure remain to be elucidated. Here, we investigated how MeHg affects the expression of proinflammatory cytokines such as interleukin (IL)-6 and IL-8 in cultured human U937 macrophages. Compared with controls, IL-6 and IL-8 mRNA expression was maximally induced in U937 macrophages after treatment with 10 μM MeHg for 6 h. The protein secretion of IL-6 and IL-8 was significantly stimulated by MeHg in U937 macrophages. Results from luciferase reporter assay indicated functional activation of nuclear factor kappa B and the involvement of subunit RelA and p50 in MeHg-induced IL-6 and IL-8 activation, which was confirmed by siRNA knockdown experiments. MeHg exposure at 4 μM also significantly induced IL-8 expression in U-87 MG cells at mRNA and protein level, indicating that IL-8 induction might be a general mode of action of MeHg treatment among different cell types. These results indicate a possible involvement of an early inflammatory response, including IL-6 and IL-8 expression in the pathogenesis of MeHg. *N*-acetyl-L-cysteine suppressed MeHg-induced activation of IL-6 and IL-8 mRNA expression in U937 macrophages, indicating the effectiveness of *N*-acetyl-L-cysteine as a therapeutic drug in MeHg-induced inflammation. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: methylmercury; macrophage; interleukin-6; interleukin-8; NF-κB; RelA; p50; inflammation; *N*-acetyl-L-cysteine

Introduction

In patients with Minamata disease, accumulation of macrophages was observed around lesions in the brain. Histochemistry shows mercury to be widely distributed in the cerebrum and cerebellum, particularly in macrophages and glial cells (Okabe & Takeuchi, 1980; Takeuchi *et al*., 1989). These observations indicate the involvement of macrophages responding to the exposure of methylmercury (MeHg) in the pathophysiology of MeHg exposure in the brain.

Macrophages are one of the potent modulators of central nervous system (CNS) repair and regeneration (Hu *et al*., 2015; Murray & Wynn, 2011; Prinz & Priller, 2014). Depending on the degree of homeostatic disturbances, leukocytes will be recruited from the bloodstream. Peripherally derived macrophages and perivascular macrophages will also participate in the inflammatory response. After brain injury has occurred, microglia and/or peripherally derived monocytes and macrophages may acquire an anti-inflammatory phenotype, which causes them to remove cell debris and promote regeneration.

Brain injury and neurodegenerative disorders are associated with acute and chronic brain inflammation (Whitney *et al*., 2009). Inflammation is a complex cellular and molecular response to stress or injury that attempts to defend against insults, to clear dead and damaged cells and to return the affected area to a normal state. Therefore, clarification of the role of inflammation due to mercury compounds is important for understanding the pathophysiology of acute and chronic exposure to MeHg. Upregulation of certain inflammatory chemokines (e.g. CCL2, CCL4 and CCL7) was detected in MeHg-exposed

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mouse cerebellum using DNA microarray analysis (Hwang *et al.*, 2011). The role of MCP-1 (also known as CCL2) as a possible neuroprotective alert system in brain deficits due to MeHg intoxication has been reported (Godefroy *et al.*, 2012). A previous report showed that treatment with inorganic mercury (InHg) induced proinflammatory cytokines such as tumor necrosis factor α and interleukin (IL)-1β in the murine macrophage cell line J774A.1 (Kim *et al.*, 2002). Moreover, exposure to MeHg, InHg or ethylmercury increased the expression of tumor necrosis factor α and IL-1β in lipopolysaccharide-activated peripheral blood mononuclear cells (Gardner *et al.*, 2009) and decreased expression of IL-6 in liver of lipopolysaccharide-exposed mice (Kim & Sharma, 2005). However, only a few studies reported the direct effect of MeHg on inflammatory signaling in phagocytes, and particularly in infiltrated macrophages.

Several studies indicated that IL-6 expression was activated by MeHg exposure. For example, microglial reactions induced by non-cytotoxic MeHg have been reported to have possible neuroprotective effects via interactions with astrocytes (Eskes *et al.*, 2002). Challenge of rat C6 glioma cells, human U251HF glioma cells or human retina pigment epithelial (ARPE-19) cells with MeHg, led to increased release of IL-6 (Chang, 2007; Chang & Tsai, 2009). In the current study, we investigated whether IL-6 expression was stimulated by MeHg in macrophages and if the activation of IL-6 expression by MeHg occurs as a general phenomenon.

The chemokine IL-8 (also known as CXCL8) is reported to have various roles in the health and pathology of the CNS (Semple *et al.*, 2010), and one important function of IL-8 is the induction of chemotaxis in its target cells (e.g. monocytes and neutrophils). In addition, Purkinje neurons in mouse cerebellar slices were reported to respond to CXCL8 and CXCL1 treatment with a transient increase in calcium, neurotransmitter release and impaired long-term depression (Giovannelli *et al.*, 1998). Puma *et al.* (2001) reported that CXCL8 modulates calcium channel excitability through CXCR2 on rat septal neurons, indicating that CXCL8/CXCR2 signaling has a critical role beyond neutrophil chemoattraction throughout the adult brain. Regarding the relationship between IL-8 expression and mercury compound, Migdal and co-authors (2010a,b) reported that the induction of IL-8 by thimerosal and ethylmercury was mediated via reactive oxygen species and calcium signaling in U937 dendritic cells, but this activation was not observed in the presence of MeHg and InHg. Accordingly, these observations indicate a need to study the effects of MeHg on inflammatory responses such as IL-6 and IL-8 expressions in macrophages and their molecular mechanisms.

Nuclear factor kappa B (NF-κB) is a highly regulated transcription factor that controls the expression of many genes involved in inflammation, including IL-6 (Murakami & Hirano, 2012) and IL-8 (Yamamoto *et al.*, 2008). In addition, activation of the MCP-1 by MeHg through RelA was reported in human 1321N1 astrocytoma cells (Kim *et al.*, 2012).

Nuclear factor-erythroid 2-related factor 2 (Nrf2) is a transcription factor that functions as the key of the redox homeostatic gene regulation. Certain Nrf2-regulated genes were reported to be involved in controlling inflammation via NF-κB (Li *et al.*, 2008). Zhang *et al.* (2005) indicated that Nrf2 caused only weak induction of IL-8 transcription, but significantly increased the half-life of IL-8 mRNA in human mesangial cells and aortic endothelial cells. From the viewpoint of MeHg toxicity, Nrf2 expression was reported to be activated by MeHg and had a protective role against MeHg toxicity (Ni *et al.*, 2010; Toyama *et al.*, 2007, 2011).

N-acetyl-L-cysteine (NAC), the acetylated precursor of L-cysteine, is a sulfhydryl-containing antioxidant, which has been used for the treatment of heavy metal toxicity and can act as an anti- inflammatory agent (Samuni *et al.*, 2013). It reduces reactive oxygen species level by raising intracellular glutathione concentrations and/or playing directly as a free radical scavenger, and inhibits activation of transcription factors in upstream signaling, which is important for inflammatory and oxidative stress responses. NAC was used to modulate peripheral and CNS inflammatory pathways and cytokine levels in neuropsychiatric disorders (Samuni *et al.*, 2013). Furthermore, NAC was reported to act as a chelating agent for mercury and accelerates urinary excretion of MeHg in mice (Aremu *et al.*, 2008), and suppressed the MeHg-activated MCP-1 and IL-6 expressions in U-87 MG cells (Muniroh *et al.*, 2015).

Human U937 cell line is an established cell model to differentiate into macrophage cell types by treatment with phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and the resulting U937-derived macrophages are a well characterized model of the mammalian cellular response to various inflammatory stimuli (Vogel *et al.*, 2007; Yamamoto *et al.*, 2008). Astrocytes play an important role in the neurotoxicity of MeHg (Farina *et al.*, 2011; Noguchi *et al.*, 2013), and are known to produce a wide variety of cytokines and chemokines during the process of CNS inflammation (Whitney *et al.*, 2009). The U-87 MG glioblastoma/astrocytoma cell line is widely used as an *in vitro* model of astrocytes (Maresca *et al.*, 2015; Muniroh *et al.*, 2015). Therefore, we tested whether MeHg-activated IL-8 expression is a general effect of MeHg exposure by using U-87 MG cells next to U937 macrophages.

MeHg is well known to cause neuronal cell death and there are many studies concerning the mechanisms of cell death caused by MeHg (Farina *et al.*, 2011). On the other hand, the molecular responses and the effects of MeHg at low concentrations where no obvious cell death occurred are not well understood. Based on the above, we investigated the inflammatory responses to the non-cytotoxic concentration of MeHg by examining the effects on IL-6 and IL-8 expressions in U937 macrophages. In addition, we also examined the involvement of NF-κB and Nrf2 during transcriptional activation of the cytokines to elucidate the underlying signaling pathways.

Materials and methods

Cell culture and methylmercury treatment

The human U937 and U-87 MG cell lines were purchased from Sumitomo Dainippon Pharma (Osaka, Japan). The U937 cells was grown in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; SAFC Biosciences, Lenexa, KS, USA), and were maintained at 37 °C in a humidified 5% CO_2 atmosphere. Differentiation of U937 monocytes (initial concentration: 2×10^5 cells ml^{-1}) to macrophages was initiated by the addition of TPA (Wako Pure Chemical Industries, Osaka, Japan) into the regular medium at a final concentration of 10 nM and allowed to proceed for 48 h. TPA stock solution (10 mM) was dissolved in dimethyl sulfoxide and stored at −80 °C. Further dilutions of TPA were made in cell culture medium immediately before use. The final dimethyl sulfoxide concentration did not exceed 0.1% (v/v). The U-87 MG cells were grown in Eagle's minimum essential medium supplemented with penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}) and 10% heat-inactivated FBS. An initial concentration of 1×10^5 cells ml^{-1} was used for each experiment.

Cells were incubated with MeHg for the periods indicated. MeHg chloride was obtained from Tokyo Chemical Industry Co. Ltd (Tokyo, Japan). MeHg stock solution (10 mM) was dissolved in Dulbecco's phosphate-buffered saline (Sigma-Aldrich, St. Louis, MO, USA) with L-cysteine (Cys; Hg/Cys = 1:1) and kept at −80 °C. The solution was diluted with regular medium immediately before use. NAC (Wako Pure Chemical Industries) was diluted with RPMI 1640 medium without FBS immediately before use. U937 macrophages were exposed to MeHg in the presence of 5 mM NAC.

Cytotoxicity

Experiments were performed at an initial concentration of 2×10^5 cells ml^{-1}. U937 macrophages were treated with MeHg (0–100 μM) for 24 h. Cell proliferation was determined using a WST-8 Cell Counting kit (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's instructions. Values represent the mean ± SEM of four experiments.

Analysis of mRNA expression

mRNA level was analyzed as previously described protocol (Yamamoto *et al*., 2012). Total RNA from U937 macrophages and U-87 MG cells was isolated using an RNeasy Plus Mini kit (Qiagen, Tokyo, Japan), which includes removal of genomic DNA contamination before cDNA synthesis. Samples were collected from three separate culture experiments. cDNAs were synthesized from total RNA (1 μg) using QuantiTect Reverse Transcription (Qiagen).

Expressed genes were detected quantitatively using a LightCycler instrument (Roche Diagnostics Japan, Tokyo, Japan) with LightCycler FastStart DNA Master^{PLUS} SYBR Green I (Roche Diagnostics) according to the manufacturer's instructions. The primers for each gene were designed and synthesized on the basis of data from the National Center for Biotechnology Information, using Premier Biosoft software (Palo Alto, CA, USA) or Primer3 (http://frodo.wi.mit.edu/primer3/), such that the targets were 80–300 bp in length (Sigma-Aldrich Japan, Hokkaido, Japan). The primer sequences used were: β-actin, forward (5′-3′), ACC CCG TGC TGC TGA CC, reverse (5′-3′), CCA GAG GCG TAC AGG GAT AGC; IL-6, forward (5′-3′), GAA CTC CTT CTC CAC AAG CG, reverse (5′-3′), TTT TCT GCC AGT GCC TCT TT; IL-8, forward (5′-3′), GAC ATA CTC CAA ACC TTT CC, reverse (5′-3′), CTT CTC CAC AAC CCT CTG.

Polymerase chain reaction (PCR) amplification was performed in a total volume of 20 μl containing cDNA and each primer (0.5 μM). The PCR cycling conditions were 95 °C for 10 min followed by 45 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 15 s. The fluorescent product at the end of the 72 °C extension period was determined. All PCR assays were performed at least three times. The data obtained were analyzed using the LightCycler analysis software. To confirm the amplification specificity, we subjected the PCR products to melting curve analysis. Threshold cycle values of the target genes were normalized to those of the internal control genes. The relative expression in each sample to that of the control sample was calculated according to the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001).

Cytokine protein quantification

IL-6 and IL-8 protein levels were measured in cell supernatants collected at 12 h of incubation from MeHg-treated U937 macrophages and U-87 MG cells. Cytokine concentrations were assessed by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The minimum detectable concentrations of IL-6 and IL-8 are typically less than 0.7 and 3.5 pg ml^{-1}, respectively.

Luciferase reporter assay and knockdown of nuclear factor-κB and nuclear factor-erythroid 2-related factor 2 with siRNA

The luciferase reporter assay was performed as described previously (Vogel *et al*., 2014). For transient transfection of U937 macrophages, cells were plated in RPMI 1640 with 10% FBS and TPA for 2 days. NF-κB luciferase reporter was from Clontech Laboratories (Mountain View, CA, USA). Transfection of plasmid DNA into U937 cells was performed via Nucleofector technology. Briefly, 10^6 U937 cells were resuspended in Nucleofector Solution V (100 μl; Lonza, Basel, Switzerland) and nucleofected with plasmid DNA (1 μg) or siRNA (3 μg) using program V-001, which is preprogrammed into the Nucleofector device. After nucleofection, the cells were immediately mixed with pre-warmed RPMI 1640 medium (500 μl) and transferred into six-well plates containing RPMI 1640 medium (1.5 ml $well^{-1}$). Twenty-four hours after transfection, cells were treated with MeHg for 6 h. To control the transfection efficiency, the cells were co-transfected with β-galactosidase reporter construct (0.1 μg $well^{-1}$). Luciferase activity was measured using the Luciferase Reporter Assay System (Promega, Madison, WI, USA) with a luminometer (Lumit LB 9501/16; Berthold Technologies, Pittsburgh, PA, USA). Relative light units were normalized to β-galactosidase activity.

Nrf2 reporter assay was conducted according to the protocol for NF-κB. U937 macrophages were treated with 10 μM MeHg or 10 μM *tert*-Butylhydroquinone (EMD Millipore, Billerica, MA, USA) as a positive control for Nrf2 activation (Li *et al*., 2005).

siRNA to target human RelA, RelB, p50 and a negative control siRNA were synthesized by Qiagen (RelA: 5′-AAGATCAATGGCTACACAGGA-3′, RelB: 5′-GGAUUUGCCGAAUUA ACAA-3′, p50: 5′-GACGCCATCTATGACAGTAAA-3′ and a negative control siRNA [catalog no. 10272280]). The Nrf2 siRNA was purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA).

Western blot analysis

Western blot analysis of Nrf2 expression in the nuclear was conducted as previously described (Yamamoto *et al*., 2012). Briefly, nuclear proteins were extracted using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL, USA). Cells were harvested by scraping after washing with phosphate-buffered saline. For nuclear protein extraction, 0.1 mM EDTA with Protease Inhibitor Cocktail (Pierce) was added to the extraction solution. Nuclear protein extracts were stored at −80 °C until use. The protein concentration was determined with a BCA Protein Assay Kit (Pierce).

The protein sample solution (final concentration 0.6–1.2 mg ml^{-1}) was mixed with Tris-glycine sodium dodecyl sulfate sample buffer (Invitrogen, Carlsbad, CA, USA) and 5 mM DTT, left to stand for 30 min at room temperature, then resolved on a 4–12% Tris-glycine gel in Tris-glycine sodium dodecyl sulfate running buffer (Invitrogen). After gel electrophoresis, the protein was transferred on to PVDF membranes (Invitrogen) and immunolabeled with Nrf2 antibody (sc722; Santa Cruz Biotechnology, Dallas, TX, USA) at 4 °C for overnight with gently shaking. The specific reaction was detected using a Western Breeze Immunodetection Kit (Invitrogen). Alkaline phosphatase was used

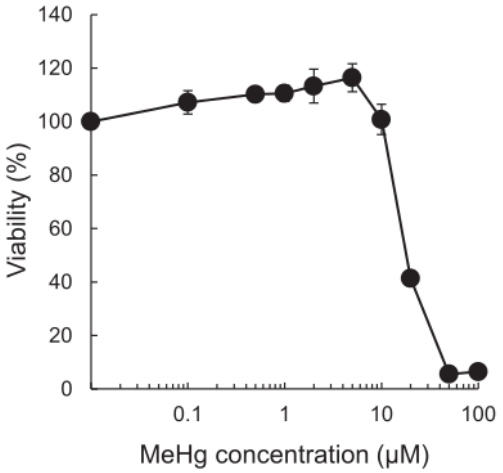

Figure 1. Cytotoxicity of MeHg in U937 macrophages. U937 macrophages were treated with MeHg (0–100 μM) for 24 h. Values represent the mean ± SEM of four experiments. MeHg, methylmercury.

as a secondary antibody and immunoreactive bands were visualized by Light Capture (AE-6971; Atto, Tokyo, Japan) with an enhanced chemiluminescent substrate, CDP-star. All western blottings were conducted using four samples of cells to confirm the reproducibility of the results. Western blot images were analyzed using computerized densitometry software (Atto).

Statistical analysis

All values are expressed as the mean ± SEM. Statistical analyses were conducted using the Mann–Whitney *U*-test. The level of significance is indicated by $*P < 0.05$.

Results

Cytotoxicity

The relative ratios of dead cells following exposure to various concentrations of MeHg (0–100 μM) are shown in Fig. 1. Cell viability declined less than 100% after exposure to more than 10 μM MeHg for 24 h. In the presence of 10 μM MeHg for 6 h, cell viability was almost 100% (data not shown).

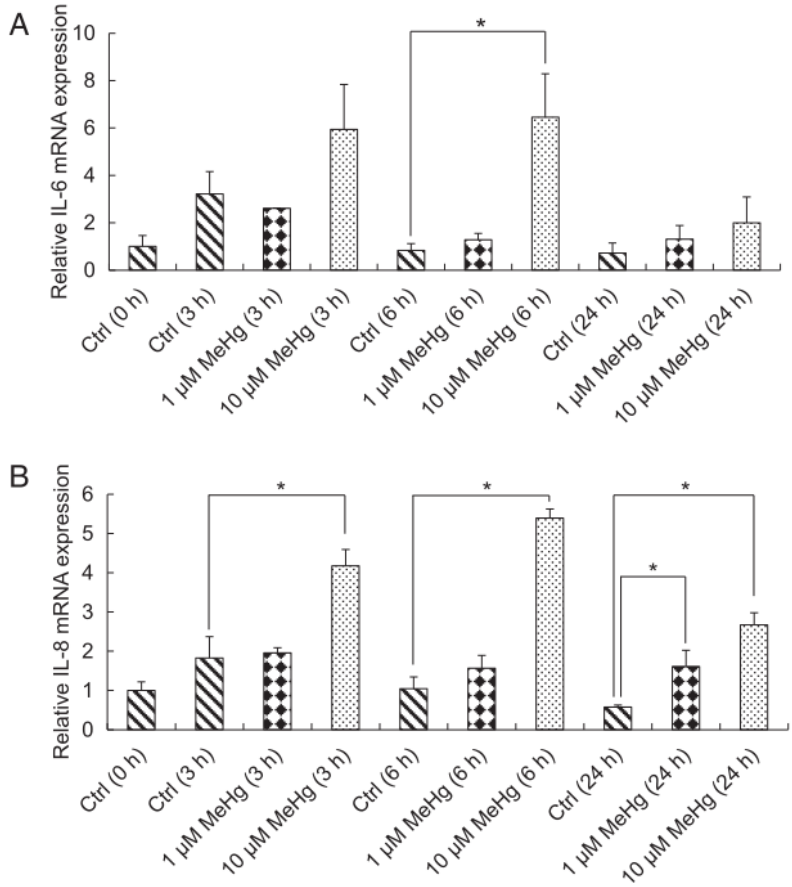

Figure 2. Effects of MeHg on IL-6 and IL-8 mRNA expression. U937 macrophages were treated for 3, 6 and 24 h with 1 or 10 μM MeHg. mRNA expression of IL-6 (A), IL-8 (B) of U937 macrophages were analyzed by real-time polymerase chain reaction. Values represent the mean ± SEM of three experiments. Level of significance is indicated by $*P < 0.05$. Ctrl, control; IL, interleukin; MeHg, methylmercury.

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Effect of methylmercury on interleukin-6 and -8 mRNA expression in U937 macrophages

We tested whether MeHg-activated IL-6 expression in U937 macrophages was the same as those in other cells. Significant activation of IL-6 mRNA expression (8.1-fold) was observed after 6 h of treatment in U937 macrophages (Fig. 2A). IL-8 mRNA expression was also significantly upregulated 2.3, 5.4 and 4.5-fold at 3, 6 and 24 h of treatment in the presence of 10 μM MeHg, and 2.7-fold at 24 h of treatment with 1 μM MeHg in U937 macrophages, respectively (Fig. 2B).

Effect of methylmercury on interleukin-6 and -8 protein secretion in U937 macrophages

To confirm the activation of IL-6 and IL-8 expression at the protein level, we conducted ELISA on supernatants from 10 μM MeHg-treated U937 macrophages collected after 12 h incubation. The result showed that protein secretions of IL-6 and IL-8 were significantly (1.5- and 2.6-fold) stimulated by MeHg, respectively (Fig. 3A,B).

Effect of methylmercury on interleukin-8 mRNA and protein expressions in U-87 MG cells

At non-cytotoxic concentration of 4 μM MeHg, we observed a significant induction of IL-6 and MCP-1 expressions in U-87 MG cells as a model of astrocytes (Muniroh *et al.*, 2015). Astrocytes are also known to secrete IL-8 and have various physiological roles in the brain (Hesselgesser & Horuk, 1999). Therefore, we tested whether the IL-8 expression was activated in the presence of MeHg in U-87 MG cells. The IL-8 mRNA expression in U-87 MG cells was significantly upregulated 11.0-, 86.6- and 3.6-fold at 3, 6 and 24 h of treatment in the presence of 4 μM MeHg, and 3.2-fold at 24 h of treatment with 1 μM MeHg in U-87 MG cells, respectively (Fig. 4A; Muniroh *et al.*, 2015). IL-8 mRNA expressions peaked at 6 h in the presence of MeHg in U-87 MG cells similar to findings in U937 macrophages.

We conducted ELISA using supernatants from 4 μM MeHg-treated U-87 MG cells collected after 12 h incubation. The result shows that the protein secretion of IL-8 is significantly (9.7-fold) stimulated by MeHg (Fig. 4B).

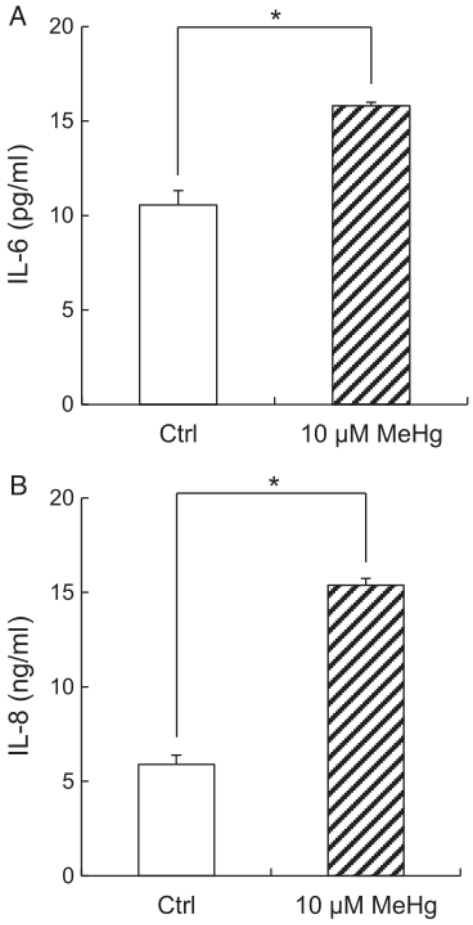

Figure 3. Effects of MeHg on IL-6 and IL-8 protein secretion in U937 macrophages. Cells were treated with 10 μM MeHg for 12 h and protein levels of IL-6 (A) and IL-8 (B) of U937 macrophages were determined by enzyme-linked immunosorbent assay of the culture supernatants. Values represent the mean ± SEM of three experiments. Level of significance is indicated by $*P < 0.05$. Ctrl, control; IL, interleukin; MeHg, methylmercury.

Nuclear factor-κB luciferase reporter activity in methylmercury-treated U937 macrophages

To clarify the molecular mechanism involved in the MeHg-induced IL-6 and IL-8 expressions, we performed luciferase reporter assays. To confirm the functional activation of NF-κB, a luciferase reporter assay containing the NF-κB consensus element was performed (Fig. 5). Significant activation of NF-κB (4.8-fold) was observed in U937 macrophages after 6 h of exposure to 10 μM MeHg. This result indicates there is possible involvement of NF-κB activation in the induction of IL-6 and IL-8 gene expression in the presence of MeHg.

Effect of RelA, RelB and p50 knockdown with siRNA on methylmercury-induced interleukin-6 and -8 expressions in U937 macrophages

To investigate the involvement of NF-κB subunits in MeHg-induced IL-6 and IL-8 expressions, we knocked down the RelA, RelB and p50 by siRNA in U937 macrophages. The requirement of RelB for the regulation and expression of IL-8 induced by dioxin for instance has been shown previously (Vogel *et al.*, 2007). Compared to control siRNA treated cells, the expressions of RelA, RelB and p50 mRNA were downregulated as 35.3, 36.7 and 33.3%, respectively (Fig. 6A). In the RelA and p50 knockdown cells, activation of 10 μM MeHg-induced IL-6 and IL-8 expression were significantly suppressed, whereas this inhibition was not observed in RelB knockdown cells (Fig. 6B). This result indicates that the activation of IL-6 and IL-8 expression by MeHg is at least in part through RelA and p50.

Suppression of interleukin-6 and -8 mRNA expressions by *N*-acetyl-L-cysteine in methylmercury-treated U937 macrophages

We have observed the addition of NAC suppressed the MeHg-induced activation of MCP-1 and IL-6 expressions in U-87 MG cells

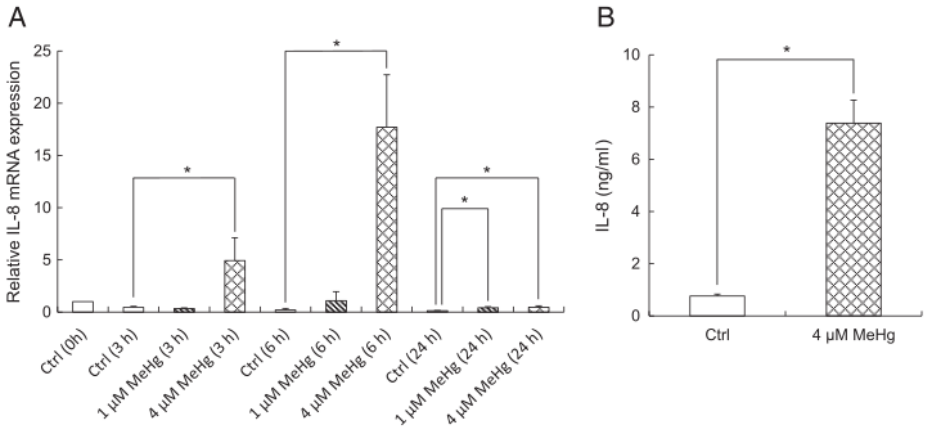

Figure 4. Effects of MeHg on IL-8 mRNA expression and protein secretion in U-87 MG cells. (A) Cells were treated for 3, 6 and 24 h with 1 or 4 μM methylmercury. IL-8 mRNA expression of U-87 MG cells were analyzed by real-time polymerase chain reaction. (B) Cells were treated with 4 μM MeHg for 12 h and protein secretion of IL-8 in U-87 MG cells were determined by enzyme-linked immunosorbent assay of the culture supernatants. Values represent the mean ± SEM of three experiments. Level of significance is indicated by $*P < 0.05$. Ctrl, control; IL, interleukin; MeHg, methylmercury.

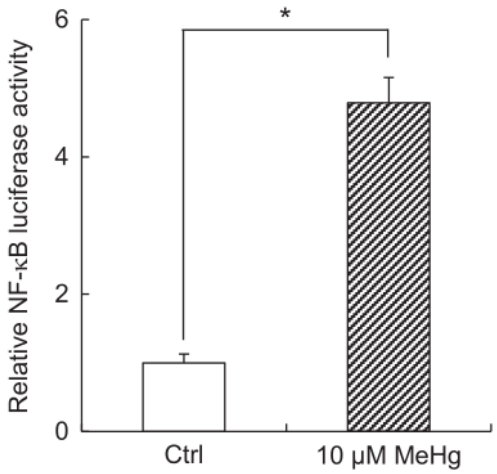

Figure 5. NF-κB luciferase reporter activity in U937 macrophages. Cells were treated with 10 μM MeHg for 6 h. Values represent the mean ± SEM of three experiments. Level of significance is indicated by $*P < 0.05$. Ctrl, control; MeHg, methylmercury; NF-κB, nuclear factor kappaB.

(Muniroh *et al.*, 2015). Therefore, we examined whether activation of MeHg-induced IL-6 and IL-8 mRNA expression in U937 macrophages at 6 h was suppressed in the presence of NAC. As a result, activation of IL-6 and IL-8 mRNA expression in the presence of 10 μM MeHg was completely blocked by 5 mM NAC (Fig. 7A,B).

Suppression of Nrf2 protein expression by *N*-acetyl-L-cysteine in methylmercury-treated U937 macrophages

Nrf2 expression was reported to be activated by MeHg and had a protective role against MeHg toxicity (Ni *et al.*, 2010; Toyama *et al.*, 2007, 2011). Western blot analysis indicated that Nrf2 expression in the nucleus was activated in the presence of 10 μM MeHg at 6 h, and its activation was completely suppressed by 5 mM NAC (Fig. 8).

Nrf2 luciferase reporter activity in methylmercury-treated U937 macrophages

To investigate the functional activation of Nrf2 in the presence of MeHg, a luciferase reporter assay containing the Nrf2 consensus element was performed (Fig. 9). Significant activation of Nrf2 was observed after 6 h of exposure to 10 μM MeHg (1.7-fold) and 10 μM *tert*-Butylhydroquinone (3.5-fold; Fig. 9; Li *et al.*, 2005) in U937 macrophages, respectively. This result indicates that the possible involvement of Nrf2 expression in induction of IL-6 and IL-8 gene expression by MeHg.

Effect of Nrf2 knockdown with siRNA on methylmercury-induced interleukin-6 and -8 expressions in U937 macrophages

To confirm the involvement of Nrf2 in MeHg-induced IL-6 and IL-8 expressions, we knocked down the Nrf2 by siRNA in U937 macrophages. Compared to control siRNA-treated cells, the expressions of Nrf2 mRNA were downregulated by 30.3% (Fig. 10A). No significant suppression of MeHg-induced IL-6 and IL-8 mRNA activation was observed in the Nrf2 knockdown cells, indicating that the activation of IL-6 and IL-8 expression by MeHg is not through the Nrf2 pathway (Fig. 10B).

Discussion

In this study, 10 μM MeHg at a non-cytotoxic dose was shown to activate transient expression of IL-6 and IL-8 in U937 macrophages (Figs. 2 and 3). In an acute case of Minamata disease, total mercury concentrations were 4.6–24.8, 22.6–68.2 and 30–38.8 $\mu g\,g^{-1}$ in the brain, kidney and liver, respectively (Okabe & Takeuchi, 1980). In the brains of 12 patients with acute Minamata disease, the average MeHg concentration was 5 $\mu g\,g^{-1}$; in one patient, the MeHg concentration was 1 $\mu g\,g^{-1}$ even after 14 years of exposure. The average MeHg concentration in the brain of patients with severe Minamata disease was 0.7 $\mu g\,g^{-1}$ over periods of 1.3–18 years (Takeuchi *et al.*, 1989). The biological half-life of MeHg in the brain tends to be longer

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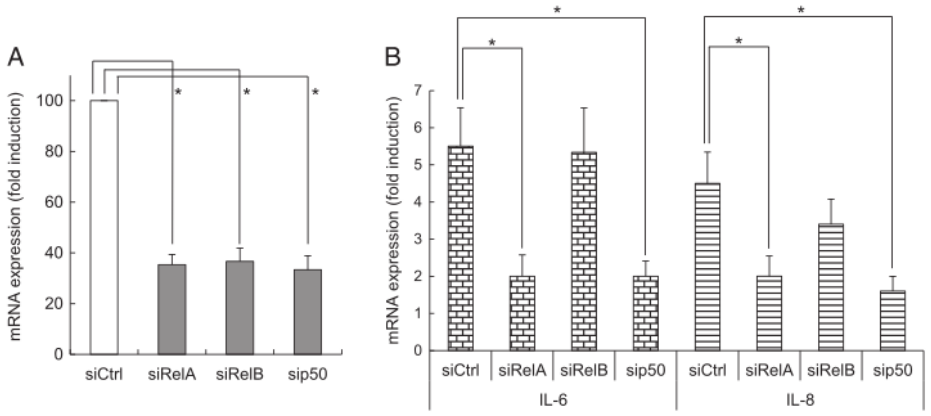

Figure 6. Effect of RelA, RelB and p50 knockdown with siRNA on methylmercury-induced IL-6 and IL-8 expressions in U937 macrophages. (A) RelA, RelB and p50 were knockdown by siRNA in U937 macrophages. mRNA expression of RelA, RelB and p50 in U937 macrophages were analyzed by real-time polymerase chain reaction. Values represent the mean ± SEM of three experiments. Level of significance is indicated by $^*P < 0.05$. (B) Activation of IL-6 and IL-8 expressions by 10 μM methylmercury in RelA, RelB and p50-knockdown U937 macrophages. mRNA expression of IL-6 and IL-8 of U937 macrophages were analyzed by real-time polymerase chain reaction. Values represent the mean ± SEM of three experiments. Level of significance is indicated by $^*P < 0.05$. Ctrl, control; IL, interleukin.

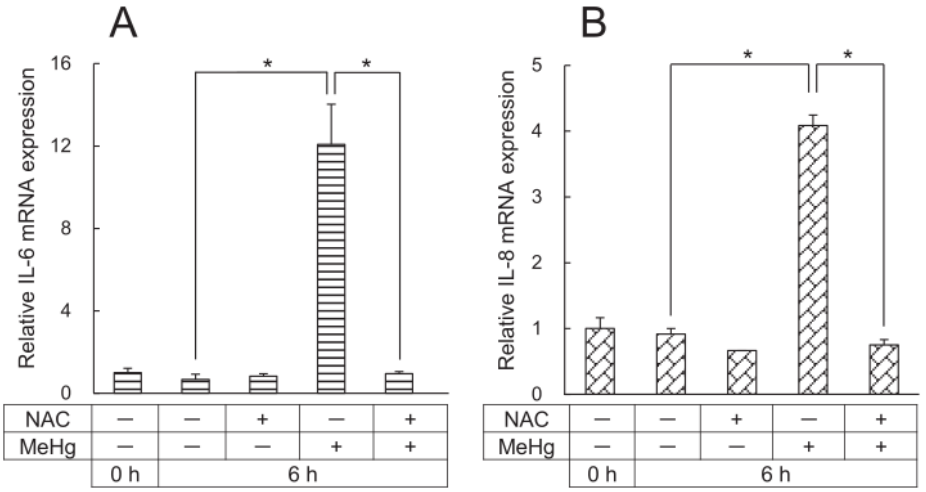

Figure 7. Suppression of IL-6 and IL-8 mRNA expressions by NAC in MeHg-treated U937 macrophages. U937 macrophages were treated with 10 μM MeHg with or without 5 mM NAC for 6 h. mRNA expression of IL-6 (A) and IL-8 (B) of cells were analyzed by real-time polymerase chain reaction. Values represent the mean ± SEM of three experiments. Level of significance is indicated by $^*P < 0.05$. IL, interleukin; MeHg, methylmercury; NAC, *N*-acetyl-L-cysteine.

than those of other tissues such as kidney and liver are. Mercury concentration in hair of patients with Minamata disease was reported to reach 700 μg g^{-1} (Kitamura, 1968), which corresponds to 2.8 μg g^{-1} in blood, assuming a ratio of 250 : 1 between hair and blood (Mahaffey, 2005). In addition, mercury concentration in blood of patients was reported to reach about 4 μg ml^{-1} in MeHg poisoning in Iraq (Bakir *et al*., 1973). Therefore, the concentration of MeHg required to induce the activation of IL-6 and IL-8 cytokines in macrophages in the present study are likely to be relevant *in vivo*. Recently, we observed the infiltration of macrophages with CD204 expression in the brain of MeHg-exposed KK-Ay mice (Yamamoto *et al*., 2014). These observations indicate the importance of understanding the time-dependent responses of macrophages against MeHg exposure to clarify the pathophysiology of MeHg neurotoxicity.

The current study is the first to report the direct exposure of MeHg-induced activation of IL-6 expression in macrophages. Many studies indicated that IL-6 release was activated by MeHg exposure in various cells, including astrocytes. We also observed the activation of IL-6 expression by MeHg in U-87 MG cells (Muniroh *et al*., 2015). Noguchi *et al*. (2013) reported that possible involvement of IL-6 was the protection of neurons against MeHg exposure. IL-6 was reported to influence macrophage recruitment and proliferation in the injured brain (Leskovar *et al*., 2000). Basset and co-workers (2012) reported that MeHg inhibited IL-6 expression treated with PAM(3), a TLR1/2 agonist, in a co-culture of microglia cells and astrocytes. Accordingly, the activation of IL-6 expression by MeHg can have a general effect, and might be involved in many processes of MeHg toxicity, including protection against MeHg exposure.

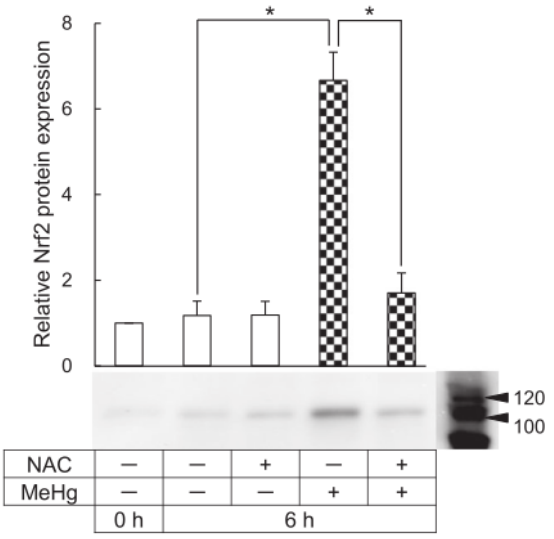

Figure 8. Suppression of Nrf2 protein expression by NAC in MeHg-treated U937 macrophages. U937 macrophages were treated with 10 μM MeHg with or without 5 mM NAC for 6 h. Protein expression of Nrf2 in the nucleus of cells were analyzed by western blotting. Values represent the mean ± SEM of three experiments. Level of significance is indicated by $^*P < 0.05$. MeHg, methylmercury; NAC, *N*-acetyl-L-cysteine; Nrf2, nuclear factor-erythroid 2-related factor 2.

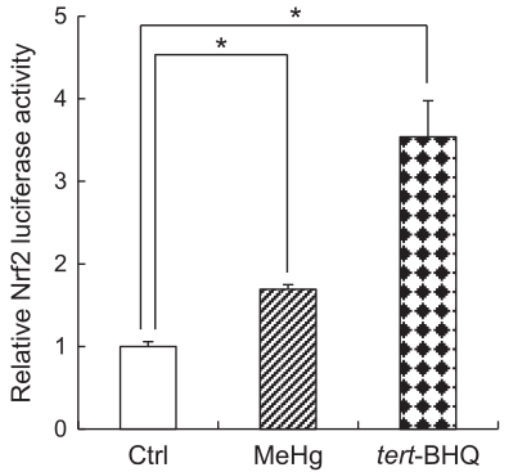

Figure 9. Nrf2 luciferase reporter activity in MeHg-treated U937 macrophages. Cells were treated with 10 μM MeHg or 10 μM *tert*-BHQ for 6 h. Values represent the mean ± SEM of three experiments. Level of significance is indicated by $^*P < 0.05$. Ctrl, control; MeHg, methylmercury; Nrf2, nuclear factor-erythroid 2-related factor 2; *tert*-BHQ, *tert*-Butylhydroquinone.

To our knowledge, the present study is the first report regarding the dose- and time-dependent activation of IL-8 expression by MeHg. Migdal *et al.* reported that the significant IL-8 secretion was not observed in the presence of MeHg or InHg in U937 dendritic model cells (Migdal *et al.*, 2010a,b). The present study showed that the peak of IL-8 mRNA expression occurred at 6 h after MeHg exposure. We measured IL-8 secretion at 12 h after treatment of U937-derived macrophages whereas Migdal *et al.* (2010a,b) measured IL-8 secretion at 24 and 48 h after treatment of U937-derived dendritic cells. The different culture and cell type condition (dendritic cells vs. macrophages) as well as time of treatment may explain the discrepancy between the data by Migdal *et al*. (2010a,b) and the current study. Involvement of IL-8 expression in many neurological disorders has been reported (Hesselgesser & Horuk, 1999). An initial macrophage activation occurs when early warning signals trigger monocyte recruitment (Murray & Wynn, 2011). Beyond the initial activation of macrophages, cooperative actions of multiple cytokine networks increase the output of monocytes driving inflammatory responses. In addition to the contribution in the recruitment of monocytes and macrophages to the injured areas in MeHg-exposed tissues, activation of the IL-8 signaling pathway may be involved in many processes of MeHg toxicity such as peripheral neuropathy.

We addressed the involvement of transcription factors as part of a mechanistic analysis for MeHg-induced IL-6 and IL-8 expressions. The production of IL-8 can be induced by a wide range of stimuli, including arsenite, through an NF-κB-dependent pathway (Yamamoto *et al.*, 2008). In this study, we showed for the first time that the functional expression of p50 is required to medicate the induction of IL-6 and IL-8 expression by MeHg. The RelA and p50 are critical factors to mediate induction of cytokines regulated via the canonical NF-κB signaling pathway (Basak *et al.*, 2008).

The relationship between Nrf2 and NF-κB is not well characterized but the identification of NF-κB-binding sites in the promoter region of the Nrf2 gene suggests a cross-talk between these two regulators of inflammatory and oxidative cellular processes (Sandberg *et al.*, 2014). The results in this study indicated that activation of NF-κB and Nrf2 are the events that occurred at the same time in the presence of MeHg, but the involvement of these pathways in the activation of IL-6 and IL-8 expressions by MeHg were different.

In this study, the addition of NAC suppressed the MeHg-stimulated cytokine expressions. NAC is known to suppress toxicity of MeHg and other heavy metals such as arsenic (Aremu *et al.*, 2008; Ghani *et al.*, 2014; Samuni *et al.*, 2013). We have reported that NAC suppressed the MeHg-induced cytokine production through both inhibition of reactive oxygen species as well as extracellular chelation of MeHg in U-87 MG cells (Muniroh *et al.*, 2015). Both mechanisms may work independently or in concert to suppress MeHg-induced IL-6 and IL-8 expressions in U937 macrophages. In recent years, clinical trials have employed NAC as an adjunctive treatment of neuropsychiatric disorders, and NAC was proven beneficial as it improved clinical outcome (Samuni *et al.*, 2013). The published reports on the ability of NAC to cross the blood–brain barrier are contradictory (Samuni *et al.*, 2013). Giustarini *et al.* (2012) reported that *N*-acetylcysteine ethyl ester (NACET) increased the lipophilicity of NAC, thus greatly improving its pharmacokinetics. NACET was rapidly absorbed in rats after oral administration but reached low concentration in plasma. After oral administration of NACET in rats, NACET caused an increase in glutathione content of most tissues examined, including the brain. If the MeHg-activated IL-6 or IL-8 expressions in macrophages show harmful effects around the microenvironment in the brain, the NACET may be a useful therapeutic drug to suppress the inappropriate expression of these cytokines in acute cases.

Cell infiltration in the wall of blood vessels were not detected in acute and subacute cases, but a small number of phagocytes containing pigmentary granules was found occasionally in subchronic

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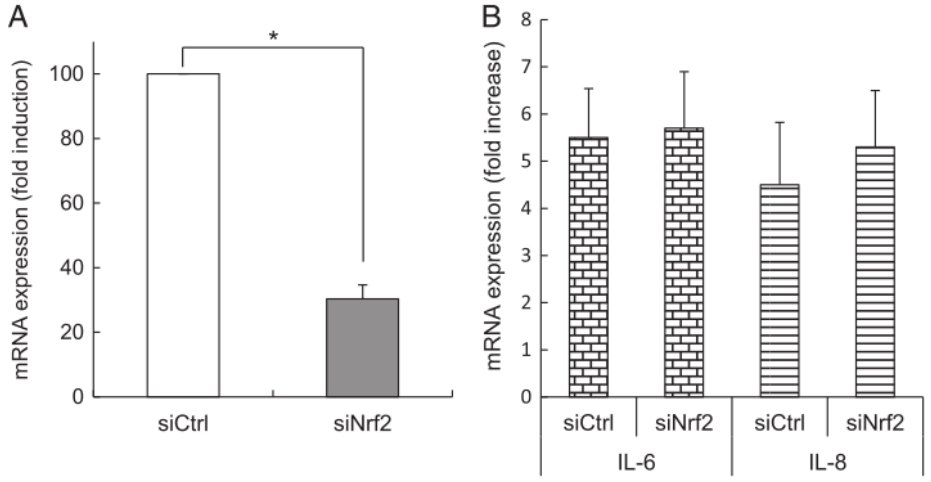

Figure 10. Effect of Nrf2 knockdown with siRNA on methylmercury-induced IL-6 and IL-8 expressions in U937 macrophages. (A) Nrf2 were knockdown by siRNA in U937 macrophages. mRNA expression of Nrf2 in U937 macrophages were analyzed by real-time polymerase chain reaction. Values represent the mean ± SEM of three experiments. Level of significance is indicated by $^*P < 0.05$. (B) Activation of IL-6 and IL-8 expressions by 10 μM methylmercury in Nrf2-knockdown U937 macrophages. mRNA expression of IL-6 and IL-8 of U937 macrophages were analyzed by real-time polymerase chain reaction. Values represent the mean ± SEM of three experiments. Level of significance is indicated by $^*P < 0.05$. Ctrl, control; IL, interleukin; Nrf2, nuclear factor-erythroid 2-related factor 2.

and chronic cases (Takeuchi, 1968). Pathological analyses are very useful to understand the pathophysiology of disease, but in some cases, they may indicate the late stage of the pathogenesis or be a consequence of the pathological condition. The findings of the present study suggest that transiently activated macrophages producing IL-6 and IL-8 may have a critical role in the recruitment of immunocompetent cells such as monocytes and macrophages in time-dependent pathogenesis of MeHg exposure. Moreover, the activation of IL-8 expression in U-87 MG cells suggests that IL-8 activation may be a common alert system after MeHg exposure as described for MCP-1 (Godefroy *et al*., 2012). Future studies to identify the important role of IL-6 and IL-8 activation after exposure to MeHg *in vivo* are warranted.

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Conflict of interest

The authors did not report any conflict of interest.

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