Inorganic mercury-induced MIP-2 expression is suppressed by N-acetyl-L-cysteine in RAW264.7 macrophages

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Abstract. Macrophages play an important role in neurotoxicity caused by methylmercury exposure through inflammatory responses. Methylmercury is known to demethylate to inorganic mercury in the brain, and macrophages are likely to be involved in this process. However, the inflammatory responses of macrophages against exposure to inorganic mercury are unclear. In the present study, inflammatory cytokine expression profiles were examined in the presence of non-toxic doses of inorganic mercury (Hg^{2+}) using RAW264.7 macrophages, focusing on the expression of C-X-C motif chemokine 2 (MIP-2)/platelet-derived growth factor-inducible protein KC (KC) and C-C motif chemokine 12 (MCP-5). Furthermore, the suppressive effect of N-acetyl-L-cysteine (NAC) on inorganic mercury-induced MIP-2 expression was also examined. Inorganic mercury-induced mRNA expression was measured using reverse transcription-quantitative PCR. The mRNA expression of MIP-2 and MCP-5 was significantly upregulated by exposure to 20 μM Hg^{2+} (non-toxic levels), but not that of KC. The suppressive effect of NAC on these cytokine expression levels was examined by its addition to the culture medium together with Hg^{2+} (co-treatment), and pre- and post-treatments in which the cells were treated with NAC before and after Hg^{2+} exposure, respectively. Hg^{2+}-upregulated MIP-2 expression was suppressed by NAC regardless of the time sequence of the treatment, suggesting that the suppressive role of NAC in Hg^{2+}-induced inflammation manifests as a possible chelator and antioxidant/reactive oxygen species scavenger.

Introduction

Minamata disease is a toxic nervous disease resulting from the consumption of seafood contaminated with methylmercury (MeHg) compounds (1). In the brain of patients with Minamata disease, the accumulation of macrophages was observed around brain lesions, and Hg was detected in neurons, neuroglial cells and macrophages, indicating the potential involvement of macrophages in MeHg-induced neurotoxicity (2). Furthermore, the infiltration of CD204-positive macrophages in the brains of MeHg-exposed KK-Ay mice has been reported (3,4). Acute and chronic brain inflammation induces brain injury and neurodegenerative disorders (5), and macrophages and microglia are potent modulators of repair and regeneration in the central nervous system (6). Microglia, in addition to peripherally derived macrophages and perivascular macrophages, participate in inflammatory responses. Therefore, clarification of the role of inflammation due to Hg compounds in macrophages is important for understanding the pathophysiology of acute and chronic exposure to MeHg.

Exposure to a non-cytotoxic dose of MeHg increases the expression of various cytokines, such as interleukin (IL)-6, IL-8 and monocyte chemoattractant protein (MCP-1), which have been reported in human U-87MG astrocytoma/glioblastoma and U937 macrophage cell lines (7,8). *In vivo* studies using mice also reported the activation of MCP-1 expression through MeHg exposure in the brain (9,10). A recent study reported that a non-toxic dose of MeHg induced the expression of macrophage inflammatory proteins C-X-C motif chemokine 2 (MIP-2) and C-C motif chemokine 12 (MCP-5), which

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are murine functional homologs of human IL-8 and MCP-1, respectively, in RAW264.7 macrophages (11).

After a long period (>20 years) of MeHg exposure, the proportion of MeHg in total mercury (T-Hg) was reported to be in the range of 0.48-2.67% in the occipital pole, calcarine region, posterior central gyrus, anterior central gyrus, white matter of the frontal lobe, pallidum and cerebellum of patients with Minamata disease, indicating that MeHg had been demethylated to inorganic mercury (Hg^{2+}) in the brain (12). MeHg demethylation in macrophages with the involvement of reactive oxygen species (ROS) signaling pathways was reported in both *in vivo* and *in vitro* studies (13,14), suggesting that the accumulation of Hg^{2+}might cause inflammation in the chronic stages of MeHg exposure. Therefore, clarification of the inflammatory response in macrophages against Hg^{2+} exposure is important to understand the pathophysiology of the late stages of MeHg exposure. Previous studies reported that exposure to Hg^{2+} activated the expression of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α (15,16) and IL-1 (16), and decreased IL-1-receptor antagonist and IL-10 expression in human peripheral blood mononuclear cells (16). Recently, Wu *et al* (17) reported that the administration of Hg^{2+} (HgCl$_2$, 33.6 mg/kg for 7 days) caused a significant delay in body weight gain and induced the expression of MIP-2 and platelet-derived growth factor-inducible protein KC (KC), a functional IL-8 homolog of C-X-C motif chemokine ligand (CXCL)1, in the livers of Kunming mice. However, the effect of a non-toxic dose of Hg^{2+} on MIP-2, KC and MCP-5 expression in macrophages remains to be elucidated.

As Hg compounds express their toxicity through the inflammatory pathway, a promising strategy to protect the body from the harmful effects of Hg is treatment with an anti-inflammatory agent. The potential applications of N-acetyl-L-cysteine (NAC) to facilitate recovery after various neurological disorders, such as traumatic brain injury, cerebral ischemia, and in the treatment of cerebrovascular vasospasm after subarachnoid hemorrhage, have been examined (18). NAC is a well-known antioxidant, as well as an anti-inflammatory agent, that can work to reduce the toxic effects of heavy metals such as arsenic (19). By inhibiting the upstream signaling of transcription factor production, NAC increases glutathione levels intracellularly and/or acts as a free radical scavenger, which results in the decrease in ROS (19-21). NAC has also been reported to work as a chelating agent to accelerate the urine-based excretion of MeHg in mice (22). Studies have shown that NAC suppresses MeHg-induced IL-6 and MCP-1 expression in U-87MG cells (7), IL-6 and IL-8 expression in U937 macrophages and U-87MG cells (8), and MIP-2 expression in RAW264.7 macrophages (11).

Based on this background information, the activation of MIP-2, KC and MCP-5 expression in macrophages was examined in the presence of Hg^{2+} to clarify the involvement of inflammatory responses upon MeHg exposure. Furthermore, the effects of NAC on Hg^{2+}-induced cytokine expression levels were also examined.

Materials and methods

Cell culture. An initial concentration of 2x10^4 RAW264.7 cells (Sumitomo Dainippon Pharma Co., Ltd.) were cultured in DMEM (Sigma-Aldrich; Merck KGaA) containing penicillin (100 U/ml), streptomycin (171.90 μmol/l), 1% L-glutamine (Sigma-Aldrich; Merck KGaA) and 10% heat-inactivated FBS (Nichirei Biosciences) at 37˚C in a 5% CO$_2$ humidified incubator.

Cytotoxicity assay. Hg^{2+} (HgCl$_2$) stock solution (10 mM) (Merck KGaA) was dissolved in Dulbecco's PBS (Sigma-Aldrich; Merck KGaA) and kept at 4˚C. It was diluted with cell culture medium immediately prior to being added to the cells. NAC (Wako Pure Chemical Industries, Ltd.) was dissolved in FBS-free DMEM, and the pH was adjusted to 7.4 by adding NaOH.

The cells were cultured (2x10^4 cells/well) for 24 h in 96-well plates and then incubated with medium containing Hg^{2+} (0.1-100 μM) or NAC (0.1-100 mM) for 24 h to check the cytotoxicity level using a WST-Cell Counting Kit-8, according to the manufacturer's protocol (Wako Pure Chemical Industries, Ltd.). The WST-8, highly water-soluble tetrazolium salt, is reduced by dehydrogenase activity in the cells to give a yellow-colored formazan dye, which is soluble in the tissue culture media. The amount of the formazan dye, generated by the activity of dehydrogenases in the cells, is directly proportional to the number of living cells. The yellow color was quantified within 1-2 h at 450 nm absorbance using a microplate spectrophotometer (TriStar LB941; Berthold Technologies GmbH and Co. KG) and culture medium was used as a standard to adjust the absorbance values of the samples. The mean values and standard errors (SEs) were based on four independent experiments.

Treatments with Hg^{2+} and NAC. Based on the results of the cytotoxicity experiments, 10 or 20 μM Hg^{2+} and 1 or 20 mM NAC were used as non-cytotoxic doses. A non-cytotoxic dose was considered to be aconcentration of Hg^{2+}or NAC at which cell viability could be maintained near 100%. Cells were incubated with Hg^{2+} for 3, 6, 12 and 24 h to deduce the optimal time for Hg^{2+}-induced MIP-2, KC and MCP-5 expression. The suppressive effect of NAC was investigated in three different experiments using the following protocols.

Pre-treatment. NAC was added to cells at 23 h into the cell culture period and maintained for 1 h. Cells were then washed with double the volume of culture medium to eliminate the remaining NAC, and then incubated with medium containing Hg^{2+} for 3 h. The incubation time of NAC was determined based on the results of previously published study (11).

Co-treatment. Medium containing both Hg^{2+} and NAC, that was prepared and kept at room temperature for 30 min in advance, was added to cells at 24 h into the cell culture time for 3 h.

Post-treatment. Hg^{2+} was added to the culture medium after 24 h of cell culture, and then washed out after 3-h incubation. Next, the cells were incubated with medium containing NAC for 3 h. The cells were harvested at 30 h into the cell culture time (for all experiments).

Determination of mRNA expression. To harvest the cells, β-mercaptoethanol and RLT buffer from the RNeasy Plus Mini kit (Qiagen, Inc.) was used. The total level of mRNA was analyzed as previously described (7,8,11,23,24). Total RNA

from the cells was extracted using an RNeasy Plus Mini kit (Qiagen, Inc.). Then, total RNA (600 ng) was used to synthesize cDNA using the QuantiTect Reverse Transcription kit (Qiagen, Inc.) following the kit instructions using the ASTEC Program Temp Control system (ASTEC Co., Ltd.) and kept at -80˚C until use. The entire reverse transcription reaction was performed at 42˚C and inactivated at 95˚C. Spectrophotometry was used to determine the cDNA concentration (absorbance at 260 nm, 50 μg/ml; Eppendorf BioPhotometer; Eppendorf). The reverse-transcribed samples were then used for reverse transcription-quantitative(RT-q) PCR.The expression levels of mRNA were quantified with Light Cycler Fast Start DNA MasterPLUS SYBR-Green I (Roche Diagnostics), following the manufacturer's instructions. Primers for specific cytokine genes were obtained from the respective information base in NCBI, using Primer 3 software (http://frodo.wi.mit.edu/primer3/), and the lengths of target sites were 80-300 bp (Sigma-Aldrich; Merck KGaA).

The target genes were amplified using the PCR method, as reported in previous studies (8,23,24). The total volume for one reaction was 20 μl, which consisted of cDNA, primers (0.5 μM) and master mix solution (prepared using the aforementioned kit). The sequences of each gene primer were as follows: β-actin, 5'-CGTGCGTGACATCAAAGAGAAG-3' forward and 5'-ATGCCACAGGATTCCATACCC-3' reverse; MIP-2, 5'-AAGTTTGCCTTGACCCTGAA-3' forward and 5'-AGGCACATCAGGTACGATCC-3' reverse; KC, 5'-AGAACATCCAGAGCTTGAAGGTGTT-3' forward and 5'-GGACACCTTTTAGCATCTTTTGGACA-3' reverse; and MCP-5, 5'-TGGACCAGATGCGGTGAGC-3' forward, and 5'-GGCTGCTTGTGATTCTCCTGTAG-3' reverse. The initial denaturation step was at 95˚C for 10 min, followed by 45 amplification cycles (denaturation at 95˚C for 10 sec, annealing at 60˚C for 10 sec, and elongation at 72˚C for 15 sec).

The data for inter-assay variance (three independent experiments) were analyzed using Light Cycler analysis software version 4.1 (Roche Diagnostics Japan). β-actin gene was used as the reference gene. This experiment used untreated cells harvested after 24 h as a standard, and untreated cells harvested after 30 h as a negative control. Relative gene expression was calculated according to the $2^{-\Delta\Delta Cq}$ method (25).

As a positive control for the relative gene expression experiments, cells were incubated with LPS (3 mM) for 3-6 h and the LPS-induced MIP-2, KC and MCP-5 expression were measured by reverse transcription-quantitative PCR.

Statistical analysis. Mean values and corresponding SEs were calculated using STATA 14.0 software (StataCorp LP). The P-values for the differences between two groups were calculated using the Kruskal-Wallis test and adjusted with Holms method, while the P-values for trends were calculated using the non-parametric trend test. The significance level was indicated by P<0.05.

Results

Cytotoxicity of Hg^{2+} and NAC. After RAW264.7 macrophages were incubated with Hg^{2+} or NAC at different doses for 24 h, cytotoxicity was determined. In our previous studies (7,8), the expression of inflammatory cytokines was activated at
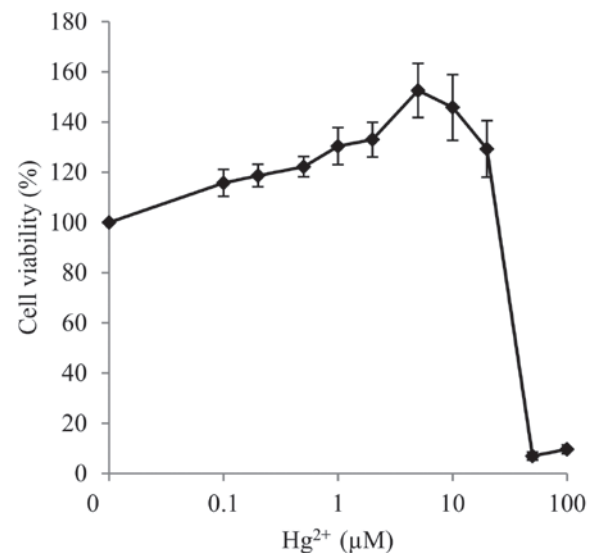

Figure 1. Viability of RAW264.7 macrophages treated with Hg^{2+} (0-100 μM) for 24 h. Values represent the mean ± SEM of four experiments.
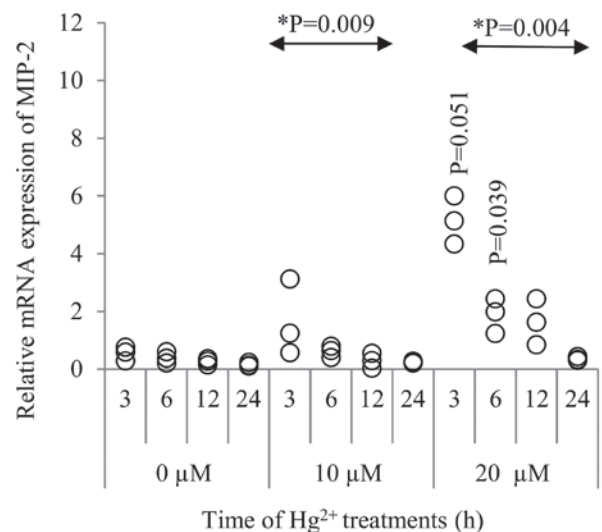

Figure 2. MIP-2 mRNA expression at 3, 6, 12 and 24 h after Hg^{2+} exposure. Effects of Hg^{2+} on MIP-2 mRNA expression. RAW264.7 macrophages were treated for 3, 6 and 24 h with 10 or 20 μM Hg^{2+}. mRNA expression of MIP-2 was analyzed by reverse transcription-quantitative PCR. Values for three independent experiments and the associated P-values are shown. *P-values for trend. MIP-2, C-X-C motif chemokine 2.

non-cytotoxic, but still relatively close to cytotoxic, doses of MeHg. Since the maximum non-toxic dose of Hg^{2+} is 20 μM (Fig. 1), 10 and 20 μM doses, where cell viability was kept ~100%, were selected for Hg^{2+}-induced cytokine experiments. NAC cytotoxicity experiments showed no change in cell viability up to a dose of 100 mM (results not shown), and doses of 1 and 20 mM were used in following experiments to examine its suppressive effect on Hg^{2+}-induced cytokine expression.

mRNA expression of MIP-2, KC and MCP-5 after Hg^{2+} treatment. As shown in Fig. 2, MIP-2 expression was significantly increased in the presence of 20 μM Hg^{2+} at 3 h (P=0.051) and 6 h (P=0.039) ,when compared to the respective 0 and 10 μM
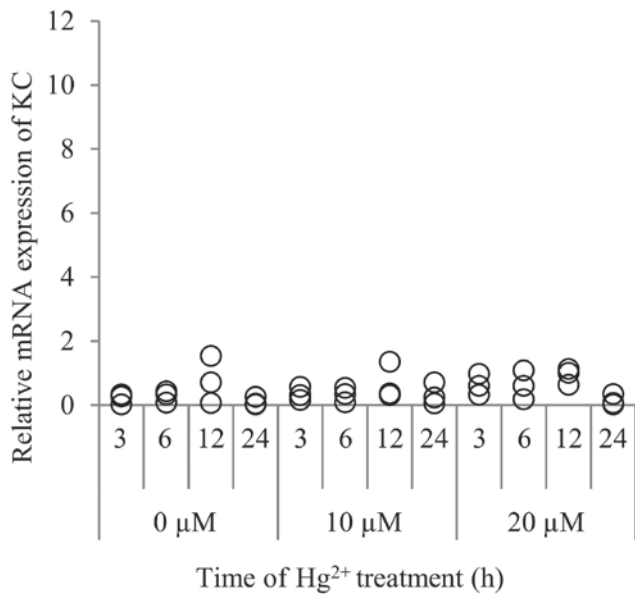
Figure 3. KC mRNA expression at 3, 6, 12 and 24 h after Hg^{2+} exposure. Effects of Hg^{2+} on KC mRNA expression. RAW264.7 macrophages were treated for 3, 6 and 24 h with 10 or 20 μM Hg^{2+}. mRNA expression of KC was analyzed by reverse transcription-quantitative PCR. Values for three independent experiments are shown. KC, platelet-derived growth factor-inducible protein.
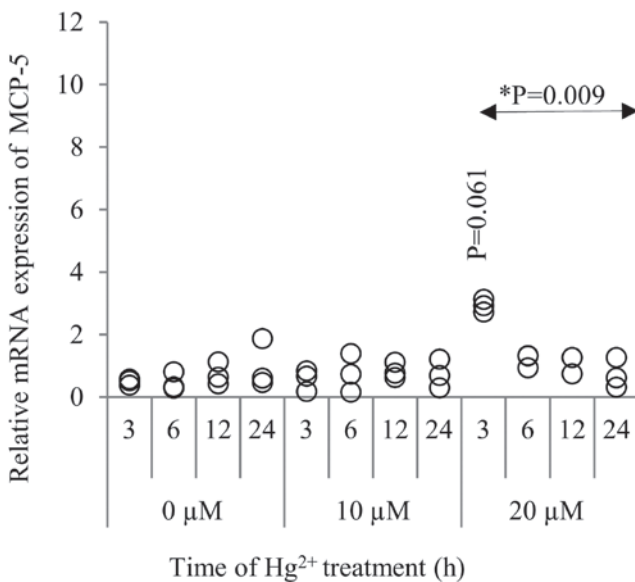

Figure 4. MCP-5 mRNA expression at 3, 6, 12 and 24 h after Hg^{2+} exposure. Effects of Hg^{2+} on MCP-5 mRNA expression. RAW264.7 macrophages were treated for 3, 6 and 24 h with 10 or 20 μM Hg^{2+}. mRNA expression of MCP-5 was analyzed by reverse transcription-quantitative PCR. Values for three independent experiments and the associated P-values are shown. *P-values for trend. MCP-5, C-C motif chemokine 12.

Hg^{2+} groups. Although the MIP-2 mRNA level was gradually decreased after 3 h, this upregulation was observed up to 12 h after Hg^{2+} exposure. When comparing to the control (0 μM Hg^{2+}) groups at different treatment durations, there was a significant time trend in MIP-2 expression when exposed to 10 and 20 μM of Hg^{2+} (P=0.009 and 0.004, respectively).

The expression of KC was not significantly stimulated by Hg^{2+} at either 10 or 20 μM (Fig. 3). There was no significant time trend observed.
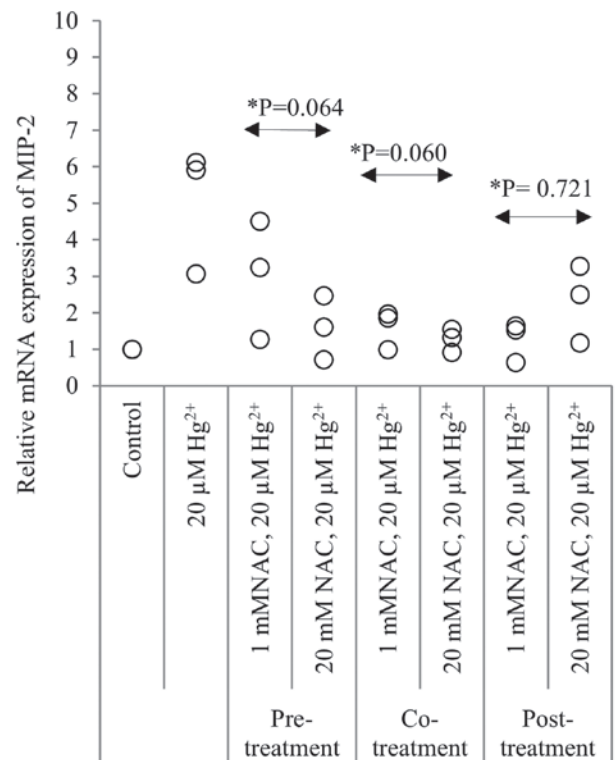

Figure 5. NAC suppresses Hg^{2+}-induced MIP-2 mRNA expression. RAW264.7 cells were treated with 1 or 20 mM NAC at 1 h before (pre-treatment), at the same time as (co-treatment), or 3 h after 20 μM Hg^{2+} (post-treatment) administration, and incubated for 3 h. MIP-2 mRNA expression levels were analyzed by reverse transcription-quantitative PCR. Values were normalized to the expression of β-actin and represent three independent experiments. *P-values for trend. NAC, N-acetyl-L-cysteine; MIP-2, C-X-C motif chemokine 2.

In Fig. 4, MCP-5 was also upregulated significantly by treatment with 20 μM Hg^{2+}, and its peak was noted at 3 h after Hg^{2+} exposure (P=0.061). When comparing to the control (0 μM Hg^{2+}), although the treatment with 10 μM Hg^{2+} did not induce a significant increase in MCP-5 expression, there was a significant time trend in MCP-5 expression at 20 μM Hg^{2+} (P=0.009). To confirm the mRNA expression of these cytokines, RAW264.7 macrophages were treated with 3 mM lipopolysaccharide (LPS) as a positive control, and the upregulation of KC, as well as MIP-2 and MCP-5, was observed after 3 h of incubation with LPS (Fig. S1).

Suppressive effect of NAC on Hg^{2+}-induced MIP-2 expression. As MIP-2 showed the highest Hg^{2+}-induced expression among the three cytokines, the effects of NAC treatment on Hg^{2+}-induced MIP-2 mRNA expression were examined (Fig. 5). NAC was added at different timings in pre-, co- and post-treatment experiments, and NAC treatment suppressed Hg^{2+}-induced MIP-2 expression in all protocols. In Fig. 5, a decrease in the trend of MIP-2 expression was seen in the presence of NAC in pre-treatment (P=0.064), co-treatment (P=0.060) and in post-treatment (P=0.721). The concentration of 20 mM NAC did not show a significant suppressive effect on MIP-2 expression in pre-, co- and post-treatment (P=0.281); the comparison was between 1 mM and 20 mM NAC-treated cells in all treatments with 20 μM Hg^{2+}-treated cells.

Discussion

To the best of our knowledge, the current study is the first to report the distinct activation of MIP-2/KC and MCP-5 expression in macrophages following exposure to Hg^{2+}. A non-cytotoxic dose of Hg^{2+}, 20 μM, induced the upregulation of MIP-2 and MCP-5 mRNA expression, which peaked at 3 h after Hg^{2+} treatment. Conversely, this phenomenon was not observed with KC expression, although it is a homolog of IL-8. Similar results were previously found using non-cytotoxic levels of MeHg exposure in RAW264.7 macrophages, showing distinct activation of MIP-2 expression, but not of KC (11).

MIP-2 and KC have been reported to serve complementary roles and functions (26). Tissue-specific and time-dependent expression patterns of MIP-2 and KC also vary, indicating a possible difference in their roles, such as in tissue-specific neutrophil recruitment (27,28). MIP-2 expression was reported to be more active than that of KC in leukocyte recruitment and endothelial cell chemotaxis (29,30), as well as in cyclophilin A-induced neutrophil migration (31). In mice, MIP-2 is 63% identical to mouse KC, and the mouse MIP-2 is 60% identical to human CXCL2 and CXCL1 (32). Based upon the similarities in their protein sequences, it is most likely that mouse KC and MIP-2 are homologs of the human CXCL1 and CXCL2 chemokines, respectively. In mice, a chemokine with protein sequence homology to IL-8 has not yet been identified, to the best of our knowledge, hence it has been suggested that MIP-2 and KC in mice may be functional homologs of human IL-8. Even though they are functional homologs, these chemokines have different functions in response to various stimuli. Therefore, Hg^{2+} may induce MIP-2 and KC expression differently. For example, MIP-2 and KC exhibit differential temporal patterns of expression in the skin of mice following surgical injury (27). These two chemokines are expressed by distinct cell types at different times following injury. At 6 h after skin surgery, KC expression occurs primarily via dermal fibroblasts and endothelial cells, while MIP-2 production occurs later and is restricted to infiltrating inflammatory leukocytes, including neutrophils and monocytes. Similar specific patterns of chemokine expression in different cell types has been shown in *in vitro* experiments using isolated primary- and long-term-cultured cell types. Primary dermal fibroblasts stimulated with IL-1α predominantly express KC and very little MIP-2, and peritoneal exudate neutrophils also produce MIP-2 and KC following stimulation (32). It is clear that various exogenous stimuli can induce KC and MIP-2 expression, and the quantitative ratio of that expression mainly depends on the cell type. This previous study also confirmed that the selective expression of KC over MIP-2 in endothelial cells is due to greater KC gene transcription, and not alterations in the rate of mRNA decay. These results demonstrated that CXC chemokines show different expression patterns in different cell types, and that their expression varies over time.

The concentration of Hg^{2+} used in the present study is likely to be able to induce cytokine expression *in vivo* as well, since an acute case of Minamata disease was reported to lead to 4.6-24.8 μg/g (23-124 μM) T-Hg in the brain (2). Non-cytotoxic doses of MeHg have also increased the expression of IL-8 in the human U937 macrophage cell line (8).

A significant trend in the activation of MCP-5 expression by a non-cytotoxic level of Hg^{2+} was also observed in RAW264.7 macrophages. In our previous study, the activation of MCP-5 expression was significantly induced by MeHg exposure at non-cytotoxic doses (11), which indicated that MCP-5 expression maybe an inflammatory marker for exposures to both MeHg and Hg^{2+}. It was observed that the expression of inflammatory cytokines was activated at a non-cytotoxic dose, but that the concentration used was still relatively close to a cytotoxic dose of MeHg (7,8). In the present study, the activation of MIP-2 and MCP-5 expression was observed at 20 μM Hg, indicating that these inflammatory cytokines are expressed in response to a non-cytotoxic dose, and thus may also be at cytotoxic doses of Hg compounds. A limitation of the present study may be that the reproducibility of the results was confirmed only via an inter-assay method, and not by an intra-assay method using the same RNA.

In order to suppress the micro-environmental inflammatory response to Hg^{2+}, further experiments using NAC were conducted to reduce the release of MIP-2. NAC was able to significantly suppress Hg^{2+}-induced MIP-2 expression at all treatment durations. In the pre-treatment protocol, the medium containing NAC was washed out before adding the Hg^{2+}; it was hypothesized that the NAC would primarily function by raising intracellular glutathione (GSH) levels rather than chelating Hg^{2+}. The medium containing Hg^{2+} and NAC for co-treatment was prepared 30 min before the incubation with the cells; therefore, the NAC may have equally acted as an antioxidant, such as by raising intracellular GSH levels, and a chelating agent for Hg^{2+}. In the post-treatment experiment, the cells were incubated with Hg^{2+} for 3 h and the $Hg^{2+}$was washed out before adding the NAC; therefore, the NAC would primarily work by raising intracellular GSH levels rather than chelating Hg^{2+}. The results for the NAC treatments indicated that NAC could suppress Hg^{2+}-induced MIP-2 activation as both an antioxidant and as a chelating agent, depending on the time of addition of NAC.

Activated macrophages promote brain recovery by resolving local inflammation and releasing trophic factors. Conversely, these cells may hinder tissue damage in the brain (33). Infiltration of CD204-positive macrophages was previously observed in the sciatic nerve of MeHg-treated KK-Ay mice, suggesting that macrophages can also serve an important role in the recovery of injured tissues in peripheral nerves, and as a possible target in regenerating peripheral nerves and controlling neuropathies (4). Future studies to identify the specific role of activated MIP-2 and MCP-5 expression after exposure to Hg^{2+}, and the effects of NAC *in vivo*, are warranted.

The present study showed that non-cytotoxic doses of Hg^{2+} induced MIP-2 and MCP-5 expression in RAW264.7 macrophages, indicating the possible involvement of these cytokines in the late stages of MeHg exposure. Among the functional homologs of human IL-8, only MIP-2, but not KC, was demonstrated to be upregulated in response to Hg^{2+} induced in murine macrophages. The suppressive effects of NAC on Hg^{2+}-induced MIP-2 at different treatment times indicated a possible anti-inflammatory effect of NAC, both extracellular as a chelating agent, and intracellular as an antioxidant.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author's contributions

JD, MM, AN and MY conceived the concept, designed the study and were responsible for the interpretation of results, drafting and finalizing the manuscript. AN and CK were involved in data analysis, interpretation of the results and manuscript preparation. MT was involved in the interpretation of the results and preparation of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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