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Methylmercury-induced IL-6 release requires phospholipase C activities

Jason Y. Chang [a,b,*]

[a] Department of Neurobiology and Developmental Sciences, College of Medicine, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA
[b] Department of Ophthalmology, College of Medicine, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA

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ABSTRACT

Methylmercury (MeHg) is a neurotoxin capable of causing severe damage to the CNS, especially in the developing fetus. Glia in the CNS release a number of cytokines that are important for proper CNS development and function. We reported earlier that MeHg could induce interleukin-6 (IL-6) release in primary mouse glia. This finding is significant considering previous reports indicating that sustained IL-6 exposure could be detrimental to cerebellar granule neurons, one of the major cellular targets of MeHg cytotoxicity. By using pharmacological antagonists against phophatidycholine- and phosphoinositol-specific phospholipase C, the current study indicated that phospholipase C activity was necessary for MeHg-induced IL-6 release. Results from pharmacological antagonists further suggested that the calcium signaling initiated by phospholipase C appeared essential for this event. In contrast, protein kinase C activity did not appear to be important. Even though mitogen-activated protein kinases were important for IL-6 release in some experimental systems, these enzymes did not appear to be required for MeHg-induced IL-6 release in glia. Based on these data and those reported by us and others, there is a possibility that MeHg-induced phospholipase C activation initiates a calcium signaling that causes phospholipase A_2 activation. This, in turn, leads to arachidonic acid and lysophosphatidyl choline generation, both of which are potent inducers for IL-6 release.

Glia in the central nervous system (CNS) release a number of cytokines, which are important for proper CNS development and function [8]. Insults to the CNS can alter the pattern of cytokine release. For example, methylmercury (MeHg) can increase interleukin (IL)-6 release from the glial cells of the CNS. This is observed in several glial cell lines derived from various species including those from human [5], rat [5] and mouse [11] as well as primary cultures of mouse glia [6]. Results from human glioma cells indicate that MeHg specifically induces IL-6 release without causing the release of either tumor necrosis factor (TNF)-α or IL-1β in the same cultures [5]. Interestingly, even though some cytotoxicity is associated with MeHg induced IL-6 release, cytotoxicity itself caused by a heavy metal is not a sufficient cause for IL-6 release. For example, even though $CdCl_2$ (another toxic metal) and $HgCl_2$ (another toxic mercury compound) cause cytotoxicity, they do not cause IL-6 release [6]. Sustained glial IL-6 release can be detrimental to cerebellar granule neurons [14,22], one of the major cellular targets of MeHg cytotoxicity [4,9]. This is because pretreatment of cerebellar granule neurons with IL-6 can increase glutamate-induced death in this neuronal population. Thus, IL-6 release caused by MeHg has significant pathological importance.

MeHg is known to activate cytosolic phospholipase A_2 (PLA_2) activity in a variety of cell types [20,31] including CNS glial cells [25]. Results from our previous study indicate that PLA_2 activation is required for MeHg to induce IL-6 release [6]. This is because inhibition of PLA_2 activity by various pharmacological inhibitors prevents MeHg-induced IL-6 release from primary mouse glia. The current study was designed to explore the events upstream of PLA_2 activation that lead to MeHg-induced IL-6 release.

There is evidence that phospholipase C (PLC) activation is an upstream event that leads to PLA_2 activation [16]. Specifically, MeHg can induce phosphatidylcholine-specific phospholipase C (PC-PLC) activation. This is followed by several events including generation of diacylglycerol (an activator of protein kinase C, PKC), an increase in intracellular calcium levels and PLA_2 activation. The pharmacological inhibitor of PC-PLC, D609, cab block all these events subsequent to PC-PLC activation in cells treated with MeHg [16]. In addition to PC-PLC, MeHg can also activate the phosphoinositol-specific PLC (PI-PLC). This is because MeHg can increase intracellular phosphoinositol levels [24], a product of PI-PLC activation.

PLC activation leads to two signaling pathways, i.e., PKC and calcium. PI-PLC activation can generate IP_3, which can cause release of intracellular calcium stores. This can subsequently lead to store-

* Correspondence address: Department of Neurobiology and Developmental Sciences, Slot 510, University of Arkansas for Medical Sciences, 4301 W. Markham St., Little Rock, AR 72205, USA. Tel.: +1 501 686 7025; fax: +1 501 686 6382.
E-mail address: changjasony@uams.edu

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activated calcium entry from the extracellular compartment, which serves as a mechanism to replenish the intracellular calcium stores (reviewed in [30]). Even though the precise mechanism is unknown, PC-PLC activation also causes elevation of intracellular calcium levels in several experimental systems [1,26,29] including MeHg treated MDCK cells [16]. This PI-PLC and PC-PLC induced calcium signaling is likely responsible for the observation that MeHg causes elevation of intracellular calcium levels, first by release of intracellular stores, followed by calcium entry from the extracellular compartment [12,13].

Both the PKC pathway and calcium signaling initiated by PLC can activate PLA_2 (reviewed by [28]). Based on this, we hypothesized that MeHg-induced activation of PLC and the subsequent PKC and/or calcium signaling were the signaling pathway that led to PLA_2 activation and the observed glial IL-6 release. Because IL-6 production in other experimental systems can be inhibited by various mitogen-activated protein (MAP) kinase inhibitors [18,23], possible involvement of MAP kinases on MeHg-induced IL-6 was also investigated.

Mixed mouse cerebral glia derived from 1 to 2 day old C57BL/6 mice were prepared as described previously [7]. As reported earlier, astrocytes constituted the majority of cells in these cultures because more than 90% of the culture surface was covered by cells positive for glial fibrillary acidic protein (GFAP) staining [7]. Even though some microglia were present in these cultures, they had only a minor contribution to the IL-6 release detected in this mixed glia culture system [6]. The PLC inhibitors D609, U73122 and ET-18-OCH_3 were purchased from Calbiochem (Gibbstown, NJ, USA). The PKC inhibitors H7 and chelerythine were from Alexis Biochemicals (San Diego, CA, USA). The inhibitor for IP_3-induced calcium release and stored-operated calcium entry, 2-APB, as well as the MAP kinase inhibitors PD 98059 (against ERK), SB 203580 (against p38) and SP 600125 (against JNK) were from Cayman Chemical (Ann Arbor, MI, USA). Methylmercury chloride and other general chemicals were from Sigma (St. Louis, MO, USA) unless otherwise stated. The growth medium was composed of Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12 medium) supplemented with 5% newborn calf serum and 2.5 mM glutamine. To prepare for experiments, cells were plated into culture plates in this growth medium at 140,000 cells/well in 24-well plates with 700 μl medium per well. A 10× concentration of each testing agent was added to each well two hours after plating to reach the final concentration. The only exception to this was SP 600125. Because of its low solubility, it was difficult to make a 10× solution with this agent. Consequently, the final concentration of SP 600125 used was prepared directly in growth medium, and then was used to replace the initial plating medium 2 h after plating. Following overnight incubation, the medium was switched to a medium containing MeHg (but without the testing agent) consisting of DMEM/F12 supplemented with 1% newborn calf serum and 2.5 mM glutamine.

For IL-6 measurement, culture medium from each well was collected after overnight (≥18 h) MeHg treatment, stored at −70 °C for later ELISA analysis. Mouse IL-6 ELISA kits were obtained from eBioscience (San Diego, CA). The assay was set up in duplicates, and a standard curve was run in parallel, per the manufacturer's instructional manual. Cell viability was determined immediately by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [10,11]. The OD of each well was measured by a plate reader (Spectra Max 190, Molecular Devices, Sunnyvale, CA) with a filter setting at 570 nm (reference filter setting was 630 nm). Our previous studies indicated that the MTT viability assay agreed well with results from the trypan blue exclusion viability assay [11].

We have previously reported that overnight (≥18 h) treatment of mouse glia with 5 μM MeHg could induce IL-6 release from ∼14 (control) to ∼185 pg/ml [6]. Concurrent analyses indicated that this concentration of MeHg reduced cell viability from 100% (control) to ∼88%. These results indicated that 5 μM MeHg could cause significant (more than 10-fold) IL-6 release with only mild cytotoxicity. We thus used this MeHg concentration for the current study: The level of IL-6 release caused by 5 μM MeHg was defined as 100% (designated as "MeHg control"), and was used for comparison with the level of IL-6 released in the presence of other testing agents. Concurrent assays were performed to determine cell viability under each experimental condition. The viability of untreated cells was defined as 100%, and was used to determine the cytotoxic effect caused by 5 μM MeHg alone as well as a testing agent plus MeHg. For most compounds tested, the results were derived from 4 independent experiments with 3 replicates in each experiment. For chelerythrine and SB 203580, the results were derived from 5 independent experiments with 3 replicates in each experiment. Data were expressed as mean ± SEM. Results of IL-6 measurements were presented as solid circles (use the right *Y* axis); results of viability assay were presented as open circles (use the left *Y* axis). Statistical analyses were performed using one-way ANOVA. The Bonferroni test was used for post hoc analysis; level of significance was expressed as: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

The first set of experiments was performed to test whether D609, a specific PC-PLC inhibitor, could block MeHg-induced IL-6 release from primary mouse glia. Results indicated that D609 at 5, 10 or 20 μM caused concentration-dependent decrease of MeHg-induced IL-6 release such that the IL-6 levels reduced to ∼89%, ∼67% or ∼50% of MeHg control, respectively (Fig. 1A, solid circles, right-side axis). D609 at these concentrations also led to a concentration-dependent increase of cell viability (Fig. 1A, open circles, left-side axis), consistent with the findings of Kang et al. [16].

Subsequent experiments indicated that U73122, a PI-PLC inhibitor, also prevented MeHg induced IL-6 release in a concentration-dependent manner (Fig. 1B). A Significant inhibition of IL-6 release could be observed at 0.25 μM U73122. Another PI-PLC inhibitor, ET-18-OCH_3, also prevented MeHg induced IL-6 release in a concentration-dependent manner (Fig. 1C). This agent at 2 and 4 μM reduced MeHg induced IL-6 release to ∼64% and ∼39% of MeHg control, respectively. These two agents did not affect MeHg cytotoxicity in the concentration range tested. Together with the previous set of experiments, these results using pharmacological agents suggested that PLC activities were important for MeHg to induce IL-6 release from mouse glia. In addition, prevention of MeHg-induced IL-6 release was not necessarily linked to prevention of MeHg-induced cytotoxicity.

We next determined whether the two PLC-activated pathways, i.e., PKC or calcium, were involved in MeHg-induced IL-6 release. The PKC inhibitor chelerythrine at 2 μM had some inhibitory effect (Fig. 2A). It was toxic to cells at higher concentrations. The other PKC inhibitor H7 did not prevent MeHg-induced IL-6 release (Fig. 2B). In contrast, 2-APB (5–40 μM), an inhibitor of IP_3-induced calcium release and store-activated calcium entry [2], prevented MeHg-induced IL-6 release (Fig. 2C). Based on these results, elevation of calcium levels subsequent to PLC activation appeared to be an essential step in MeHg-induced IL-6 release. Even though PLC-dependent IL-6 production in other experimental systems was under the control of MAP kinases [18,23], the inhibition of ERK by PD 98059 (5–20 μM, Fig. 3A), p38 by SB 203580 (520 μM, Fig. 3B) or JNK by SP 600125 (5–20 μM, Fig. 3C) did not prevent MeHg-induced IL-6 release. Thus, these pathways did not appear to be necessary for MeHg-induced IL-6 release.

Results from the current study suggested that MeHg activation of PLC (PC-PLC and PI-PLC) was an important step that led to IL-6 release. The role of PC-PLC in IL-6 release was investigated in other studies. For example, Monick et al. reported that PC-PLC was activated in bacterial lipopolysaccharide (LPS)-treated macrophages [21]. This led to a series of events, such as release of diacylglycerol,