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Pharmacological inhibition of NF-kB activity prevents methylmercury induced glial IL-6 release

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ABSTRACT

Methylmercury (MeHg) is an environmental toxin that can alter a number of cellular events in cells derived from the nervous system. We previously demonstrated that MeHg could cause interleukin-6 (IL-6) release in various glial cell types. Activation of phospholipase C and phospholipase A_2 were required for this event. Using primary mouse glial cultures as an experimental model, this study was designed to further determine intracellular signaling pathways involved in this IL-6 induction. Specifically, we tested the hypothesis that NF-kB activation was critically important for MeHg induced IL-6 production. Results using various NF-kB inhibitors (BAY-117082, TPCA-1, 15d-PGJ_2 and curcumin) indicated that NF-kB activation was required for MeHg induced IL-6 production. Furthermore, BAY-117082, 15d-PGJ_2 and curcumin showed various degrees of protective effects against a cytotoxic concentration of MeHg.

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INTRODUCTION

We reported earlier that methylmercury (MeHg) could induce interleukin-6 (IL-6) release in various primary or transformed glial cells[1,2]. Our previous studies further indicated that activation of the phospholipase C (PLC)[3], release of calcium[3] and activation of the cytoplasmic phospholipase A_2 (PLA_2)[4] by MeHg were required for IL-6 induction. On the other hand, activities of protein kinase C and mitogen-activated protein kinases were not necessary for this event[3]. The current study was designed to further determine the intracellular pathways that led to MeHg induced IL-6 induction.

IL-6 promoter is under the control of several transcription factors[5]. Among them, NF-kB is best characterized in astrocytes[6]. Activation of NF-kB is controlled by IkB (Inhibitor of NF-kB). Phosphorylation of IkB by IkB kinase (IKK, especially IKK-2) can lead to ubiquitination and subsequent degradation of IkB. As a result, the activated NF-kB complex is translocated into nucleus and activates responsive promoters including the IL-6 promoter[5]. See Figure 4 (boxed area) for an abbreviated illustration of the relationship among these molecules.

Activation of the NF-kB pathway can be inhibited by several distinct pharmacological agents through various mechanisms. For example, cytokine induced NF-kB activation is inhibited by BAY 11-7082. This agent can inhibit cytokine induced IkB phosphorylation and the degradation of IkB[7]. BAY 11-7982 is subsequently reported to inhibit IkB phosphorylation by inhibiting IKK[8]. TPCA-1, a structurally different compound from BAY 11-7082, is also a potent inhibitor of IKK, and can prevent LPS induced NF-kB activation and IL-6 production[9].

The prostaglandin derivative, 15-deoxy-delta 12,14-prostaglandin J_2 (15d-PGJ$_2$), can inhibit NF-kB activation by various mechanisms, including a direct inhibition of IKK and covalent binding of a subunit of the NF-kB molecule[10-14]. It was reported that 15d-PGJ$_2$ could prevent LPS/IFNγ induced IL-6 release in primary rat astrocytes by blocking IKK activity and the subsequent NF-kB activation[15]. LPS induced[16] and IL-1β induced[17] IL-6 release from epithelial cells were also inhibited by 15d-PGJ$_2$ through its inhibition on the NF-kB activation.

Curcumin, a broad spectrum pharmacological agent derived from the rhizomes of *Curcuma longa*[18], can also inhibit NF-kB activation through its ability to inhibit IKK[19]. The ability of curcumin to inhibit NF-kB activation is responsible for its inhibition of IL-6 release in several experimental systems[19,20].

If NF-kB activation is required for MeHg induced IL-6 release in astrocytes, all of the IKK inhibitors discussed above should block IL-6 release. Thus, the primary purpose of this study was designed to test the hypothesis that activation of the NF-kB pathway was responsible for MeHg induced IL-6 release. There are reports indicating that PI-3 kinase activity is involved IL-6 generation in some experimental systems. PI-3 kinase inhibitors are reported to decrease[21] or increase[22] IL-6 generation. Based on these reports, the secondary aim of this study was to explore whether inhibitors of PI-3 kinase could affect MeHg induced IL-6 release.

MATERIAL AND METHODS

Chemicals

InSolution™ Bay 11-7082, InSolution™ IKK-2 inhibitor IV (2-[(aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide) (also known as TPCA-1) and wortmannin were purchased from Calbiochem (Gibbstown, NJ, USA). 15d-PGJ$_2$ was from Caymon Chemical (Ann Arbor, MI, USA). LY 294002 was from Alexis Biochemicals (Farmingdale, NY, USA). Methylmercury chloride, curcumin and other general chemicals were from Sigma (St. Louis, MO, USA) unless otherwise stated.

Cell cultures

Mixed mouse cerebral glia derived from 1-2 days old C57BL/6 mice were prepared as described previously[4,23]. Cells were plated in 75 cm² flasks, expanded once into 150 cm² flasks before plating into multi-well culture plates for experiments. Astrocytes constituted the majority of cells in these cultures because more than 90% of culture surface was covered by cells positive for glial fibrillary acidic protein (GFAP) staining[23]. A small number of microglia were present in these cultures. However, they had only a very minor contribution to IL-6 release detected in this mixed glia culture system, as reported earlier[4]. 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 24-well culture plates in this growth medium at 140,000 cells/well (700 µl medium per well) for IL-6 measurement and concurrent viability assay (Figures 1, 3) or into 96-well plates at 20,000 cells/well (100 µl medium per well) for viability assay (Figure 2). Two hours after plating, each testing agent (at 10x final concentration) was added to wells to reach final concentration indicated. Following overnight (≥ 18 hrs) incubation, the medium was switched to DMEM/F12 (without testing agent) supplemented with 1% newborn calf serum and 2.5 mM glutamine for overnight MeHg treatment.

Assays

For IL-6 measurement, culture medium from each well was collected after overnight MeHg treatment, stored at -70°C for later IL-6 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 by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay[1,24]. 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[1].

As described previously[3,4], IL-6 release caused by 5 µM MeHg was defined as 100% (designated at "MeHg control"), and was used to determine the effect of each testing agent. Because there was always a possibility that pretreatment of cells with a pharmacological agent could have a positive or negative effect on MeHg cytotoxicity, 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.

Statistical analysis

Unless otherwise stated in the figure legend, results were pooled from 12 replicate samples derived from 4 independent experiments (Figures 1, 3) or from 3 independent experiments (Figure 2) and expressed as mean ± SEM. Statistical analyses were performed by one-way ANOVA. The Bonferroni test was used for post-hoc analysis, and $p < 0.05$ was considered as significant difference.

RESULTS

Inhibitors of NF-kB activation prevented MeHg induced IL-6 release

Four structurally different inhibitors of NF-kB activation, namely, BAY 11-7082, TPCA-1, 15d-PGJ$_2$, and curcumin, were used in this set of experiments. Pretreatment of cells with BAY 11-7082 led to a concentration-dependent decrease of IL-6 release (Figure 1A, solid circles, right axis). This agent at 0.125 µM inhibited IL-6 release to ~74% of that induced by 5 µM MeHg. The viability assay indicated that 5 µM MeHg did not affect cellular viability. Pretreatment of cells with BAY 11-7082 at 0.125, 0.5 or 1 µM followed by 5 µM MeHg increased cellular viability slightly (Figure 1A, open circles, left axis). However, the viability of each treatment was close to 100% of control.

TPCA-1 also led to a significant reduction of MeHg induced IL-6 release (Figure 1B, solid circles, right axis). A significant decrease could be observed at 0.625 µM TPCA-1. At 10 µM, this agent reduced the IL-6 re-

lease to ~19% of MeHg control. TPCA-1 at this concentration range had little effect on the cytotoxicity induced by MeHg (Figure 1B, open circles, left axis).

The next set of experiments indicated that 15d-PGJ$_2$ was a very potent inhibitor of MeHg induced IL-6 release (Figure 1C, solid circles, right axis). At 0.5 µM, this agent reduced IL-6 release to ~24% of MeHg control. Viability assay indicated that 2 µM 15d-PGJ$_2$ prevented cytotoxicity caused by MeHg (Figure 1C, open circles, left axis).

Experiments using curcumin indicated that pretreatment of cells with this agent caused concentration-dependent decrease of IL-6 release. The IL-6 release decreased to ~81%, ~67%, ~36% or ~5% of MeHg control when cells were pretreated with 1.25, 2.5, 5 or 10 µM curcumin (Figure 1D, solid circles, right axis). MeHg caused a significant decrease of cell viability to ~62% of control in this set of experiments. Curcumin pretreatment prevented MeHg cytotoxicity significantly in a concentration-dependent manner (Figure 1D, open circles, left axis).

Effect of NF-kB inhibitors on MeHg induced cytotoxicity

While results from the previous set of experiments indicated that NF-kB inhibition prevented MeHg induced IL-6 release (Figures 1A-1D, solid circles), they also suggested that NF-kB inhibition might prevent MeHg induced cytotoxicity (Figures 1A-1D, open circles). However, since 5 µM MeHg itself caused little cytotoxicity in most cases (Figure 1A-1C), it was difficult to conclude whether NF-kB inhibitors could indeed prevent MeHg induced cytotoxicity. To get a more definite answer, cells were pretreated with each NF-kB inhibitor overnight, followed by 10 µM MeHg, a concentration of MeHg that could cause significant cytotoxicity, and then the viability of each treatment was assessed.

Results from the first set experiments using BAY 11-7082 indicated that 10 µM MeHg alone reduced cell viability to ~41% of control. Pretreatment of cells with 0.5 µM to 2 µM BAY 117082 significantly increased cell viability (Figure 2A). This protection, however, was not exhibited by TPCA-1 (Figure 2B). 15d-PGJ$_2$ could lead to a concentration-dependent increase of cell viability in cells treated with MeHg. While MeHg itself reduced cell viability to ~36% of control, 15d-PGJ$_2$ pretreatment at 0.125, 0.25, 0.5, 1 or 2 µM raised the viability to ~44%, ~52%, ~65%, ~80% or ~90%,

respectively (Figure 2C). Similar protection could also be observed in cells pretreated with curcumin (Figure 2D). Based on these results, it was concluded that while some NF-kB inhibitors tested were protective against MeHg cytotoxicity; however, this was not a reproducible property of NF-kB inhibitors.
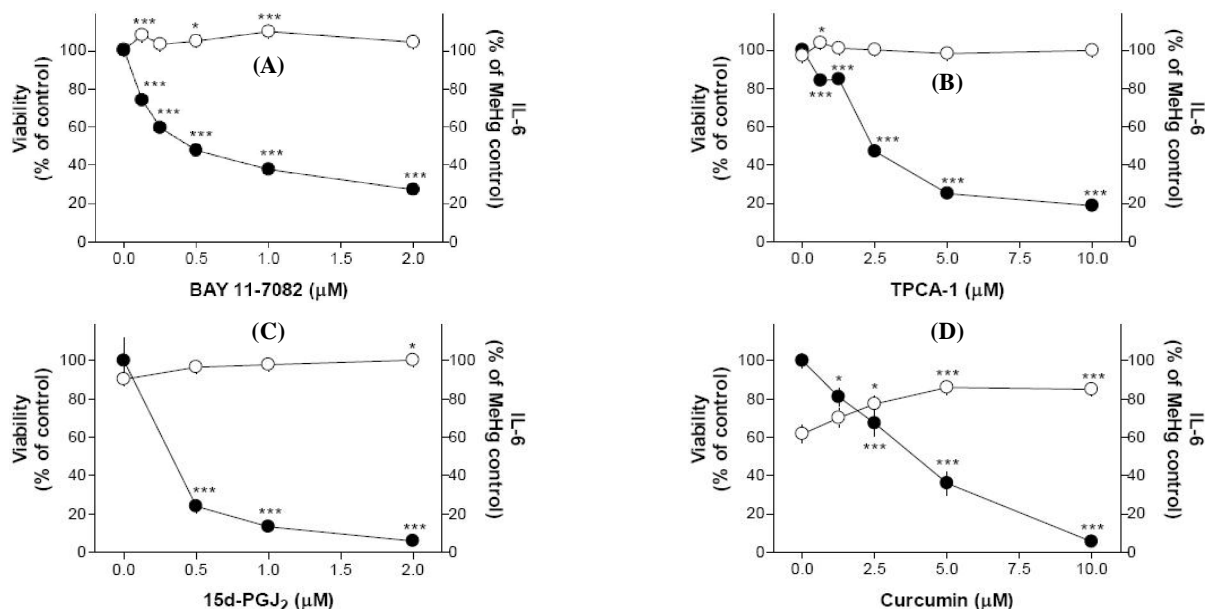

Figure 1 : Prevention of NF-kB activation blocked MeHg induced IL-6 release. Cells were pre-treated with various concentrations of testing agents overnight (≥ 18 hrs) then treated with 5 μM MeHg (without testing agent). The IL-6 level (solid circles, right axis) and viability (open circles, left axis) of each culture were then determined as described in the Materials and Methods. Results indicated that all NF-kB inhibitors tested (BAY 11-7082, Figure 1A; TPCA-1, Figure 1B; 15d-PGJ$_2$, Figure 1C; and curcumin, Figure 1D) could prevent IL-6 induction caused by MeHg. Furthermore, there were indications that some of these agents could increase cellular viability in cultures treated with 5 μM MeHg. *p< 0.05, **p< 0.01, ***p< 0.001.
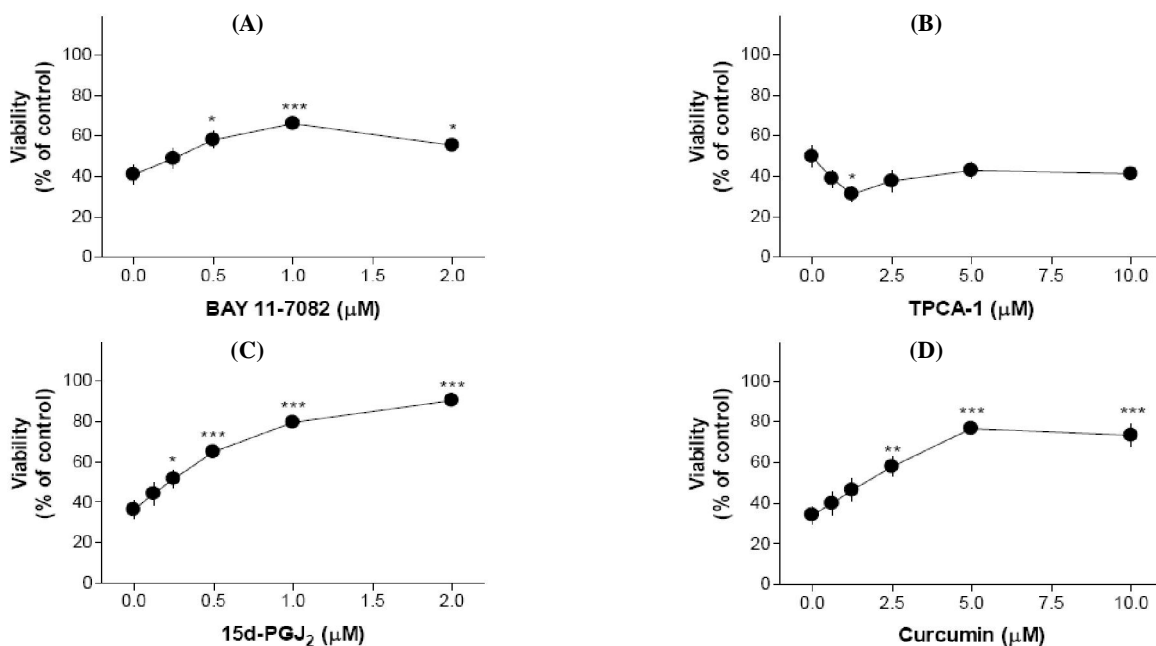

Figure 2 : Effect of NF-kB inhibitors on MeHg induced cytotoxicity. Cells were pretreated overnight with various concentrations of BAY 11-7082, TPCA-1, 15d-PGJ$_2$ or curcumin, then with 10 μM MeHg (without testing agents) overnight. The viability of each well was then determined by the MTT assay. Results indicated that BAY 11-7082 (Figure 2A), 15d-PGJ$_2$ (Figure 2C) and curcumin (Figure 2D) were protective against a cytotoxic concentration of MeHg. TPCA-1 (Figure 2B), however, did not have this protective effect. *p< 0.05, **p< 0.01, ***p< 0.001.

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Effect of PI-3 kinase inhibitors on MeHg induced IL-6 release

Given the reports that PI-3 kinase may be involved in IL-6 generation in some experimental systems[21,22], we explored whether PI-3 kinase was involved in MeHg induced IL-6 release. The PI-3 inhibitors Wortmannin and LY294002 were used in this series of experiments. Conflicting results were obtained such that wortmannin appeared to decrease (Figure 3A, solid circles) while LY294002 appeared to increase (Figure 3B, solid circles) IL-6 generation under identical experimental conditions. We thus could not conclude whether PI-3 kinase was involved in MeHg induced IL-6 release. Neither agent appeared to affect MeHg cytotoxicity (Figures 3A, 3B, open circles)
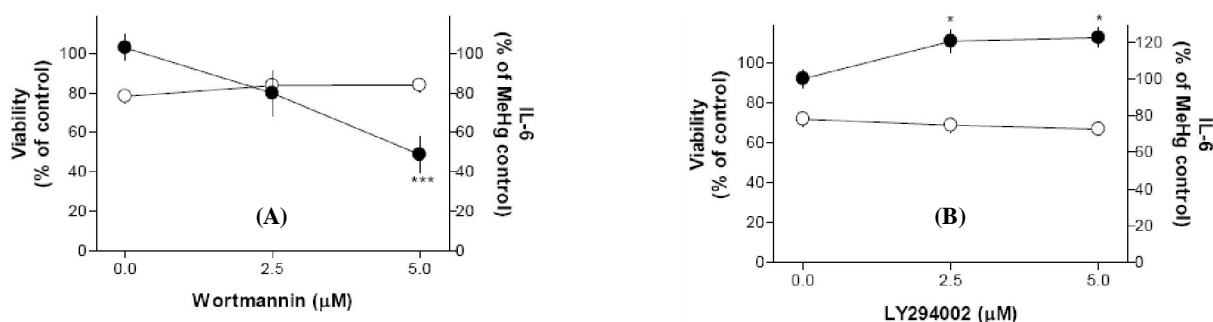

Figure 3 : Effect of PI-3 kinase inhibitors on MeHg induced IL-6 release. Cells were treated with wortmannin or LY294002 and then with MeHg under the experimental conditions described in Figure 1. Results indicated that while wortmannin could prevent MeHg induced IL-6 release (Figure 3A, solid circles), LY294002 (Figure 3B, solid circles) could potentiate the IL-6 generation. Neither agent affected MeHg cytotoxicity (open circles). *p< 0.05, ***p< 0.001.

DISCUSSION

MeHg is an environmental toxin that can severely damage various human organ systems[25], including the nervous system[26-28]. In addition to direct toxicity on neurons, there is evidence that damages to glia caused by MeHg also contribute the overall toxicity toward the nervous system[29,30]. MeHg could induce IL-6 release from glia[1,2,31]. Eskes et al. suggested that this IL-6 release was neuroprotective[31]. On the other hand, sustained glial IL-6 release can be detrimental to cerebellar granule neurons[32,33], one of the major cellular targets of MeHg cytotoxicity[34,35]. The patho/physiological role of IL-6 release caused by MeHg in humans or in animals remains to be determined.

Based on previous studies concerning the role of NF-kB in IL-6 induction[5,6] (see Introduction), this study was designed to test the hypothesis that NF-kB activation was required for MeHg induced IL-6 release. Results from Figure 1 with four structurally different agents clearly showed that NF-kB activation was required for IL-6 induction caused by MeHg. While MeHg can modulate a variety of cellular events, none was shown to depend on NF-kB activation. The finding from this study showing NF-kB was important for a MeHg induced cellular event was novel.

Results from Figure 2 indicated that some of the NF-kB inhibitors could prevent MeHg induced cytotoxicity. Specifically, BAY 117082 (Figure 2A), 15d-PGJ$_2$ (Figure 2C) and curcumin (Figure 2D) were protective against MeHg induced cytotoxicity. TPCA-1, however, was not protective (Figure 2C). We thus could not conclude that NF-kB inhibitors, as a group, could prevent MeHg cytotoxicity. The protective effect of 15d-PGJ$_2$ observed in this study was consistent with our previous reports[1,23]. Specifically, we showed that this agent could inhibit MeHg induced mitochondrial depolarization, glutathione depletion and cell death[23]. The mechanisms by which BAY 117082 and curcumin exhibited protective effect against MeHg cytotoxicity are currently under investigation.

CONCLUSION

It was reported that MeHg can induce PLC activation, which led to several events including generation of diacylglycerol (an activator of protein kinase C, PKC), an increase in intracellular calcium levels and PLA$_2$ activation[36]. PLA$_2$ activation can cause generation of arachidonic acid and lysophosphatidyl choline,

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both of which are potent inducers of IL-6 production[37,38]. Our previous studies indicated that inhibitors of PLC, calcium flux and PLA$_2$ could inhibit MeHg induced IL-6 generation. Inhibitors of PKC, however, were not inhibitory in this event[3,4]. The current study further indicated that activation of NF-kB was critical in MeHg induced IL-6 release from glia. Based on these results, we generate a working hypothesis regarding the signaling pathway that is responsible for MeHg induced IL-6 production, as illustrated in Figure 4.
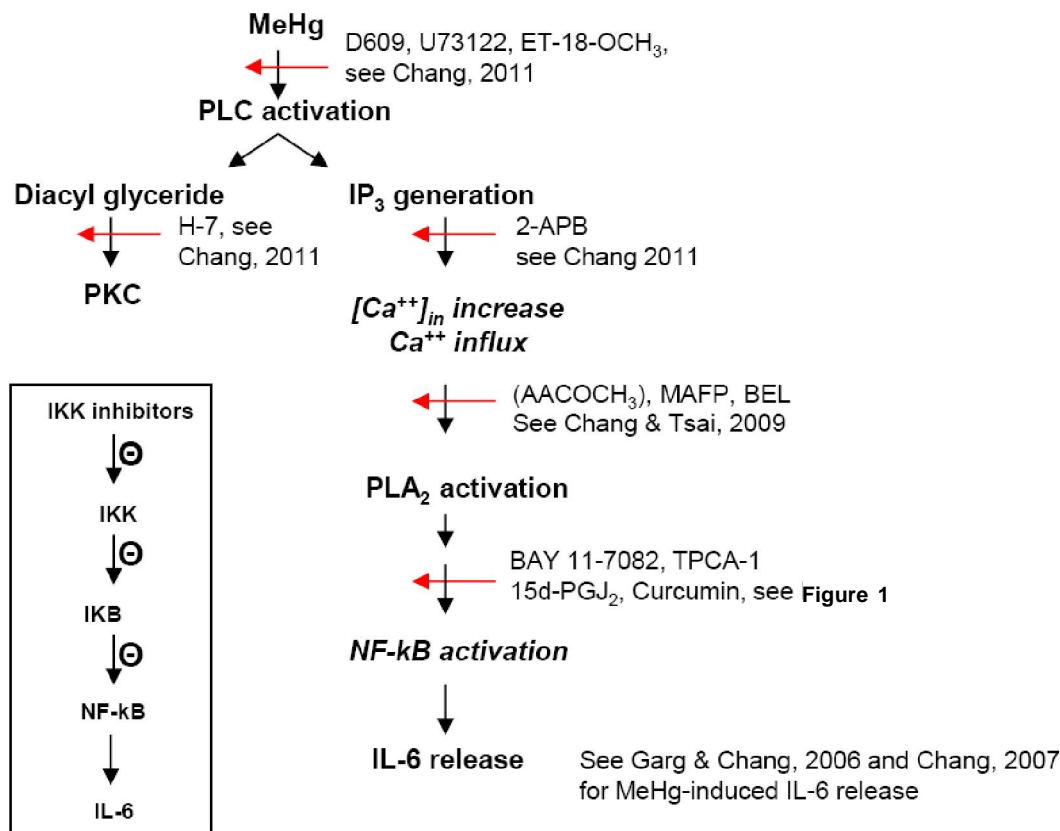

Figure 4 : Working hypothesis of MeHg induced IL-6 release. This figure illustrates the signaling pathways possibly involved in this event. Pharmacological inhibition of PLC, calcium flux, PLA$_2$ and NF-kB prevents MeHg induced IL-6 release. See text for more detailed discussion. Boxed area illustrates the mechanism by which IKK inhibitors prevent NF-kB activation.

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