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Cadmium but not Mercury Suppresses NF-κB Activation and COX-2 Expression Induced by Toll-like Receptor 2 and 4 Agonists

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Abstract

Toll-like receptors (TLRs) induce innate immune responses by recognizing conserved microbial structural molecules. All TLR signaling pathways culminate in the activation of nuclear factor kappa-B (NF-κB) leading to the induction of inflammatory gene products such as cyclooxygenase-2 (COX-2). Deregulated activation of TLRs can lead to the development of severe systemic inflammation. Divalent heavy metals, cadmium and mercury, have been used for thousands of years. While cadmium and mercury are clearly toxic to most mammalian organ systems, especially the immune system, their underlying toxic mechanism(s) remain unclear. Here, we report biochemical evidence that cadmium, but not mercury, inhibits NF-κB activation and COX-2 expression induced by TLR2 or TLR4 agonists, while cadmium does not inhibit NF-κB activation induced by the downstream signaling component of TLRs, MyD88. Thus, the target of cadmium to inhibit NF-κB activation may be upstream of MyD88 including TLRs themselves, or events leading to TLR activation by agonists.

Keywords: Cadmium, Mercury, Toll-like receptor, Cyclooxygenase-2, Nuclear factor-κB

Divalent heavy metals such as cadmium [Cd (II)] and mercury [Hg (II)] have been used for thousands of years. They are known occupational hazards and air pollutants[1]. Although the toxicity of these metals has been known for a long time, their exposure has still increased in some areas of the world. Cadmium compounds are currently used in polyvinyl chloride products, color pigments, alloys, and, increasingly, in rechargeable nickel-cadmium batteries[1]. Cigarette smoking is also a major source of cadmium exposure; in non-smokers, food is the most important source of cadmium exposure[2,3]. Cadmium is present in most foods, but the concentration of cadmium in foods varies greatly. Cadmium exposure may cause kidney damage and bone fracture[4,5], and the metal has been linked to kidney cancer[6,7].

Mercury compounds are used in dental amalgams, thermometers, and instruments for measuring blood pressure. Food and dental amalgams are the most important source of mercury exposure in the general population[8,9]. Claims linking amalgam-associated mercury with a variety of illnesses remain contentious, and are not yet based on reputable scientific evidence[10,11]. Nevertheless, mercury compounds can cause nervous system damage[12] and coronary heart disease[13].

The innate immune system is the first line of defense in host protection against invading microbial pathogens[14]. Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns and elicit innate immune responses such as the induction of inflammatory cytokines[14-16]. Currently, at least 13 TLRs have been identified in mammalian cells. These detect pathogen-associated molecular patterns (PAMPs) derived from various microbial pathogens such as viruses, bacteria, protozoa, and fungi[14]. The binding of agonists to TLRs activates intracellular signaling cascades that involve the recruitment of myeloid differential factor 88 (MyD88) or toll-interleukine-1 receptor domain-containing adapter inducing interferone-β (TRIF) leading to the activation of transcription factor nuclear factor-κB (NF-κB) or interferon (IFN)-regulatory factor (IRF3)[14]. MyD88 is the immediate downstream adaptor molecule recruited by activated TLRs through their TIR domain. The interaction of MyD88 with the

TIR domain of TLRs leads to the activation of IκB kinase (IKK) complex resulting in the activation of the NF-κB transcription factor. Activation of this transcription factor up-regulates the expression of numerous pro-inflammatory gene products including cytokines and cyclooxygenase-2 (COX-2)[17].

TRIF is another immediate downstream adaptor molecule recruited by TLR3 or TLR4[14]. TRIF activates downstream kinases, TNF receptor-associated factor (TRAF) family member-associated NF-κB activator (TANK)-binding kinase 1 (TBK1) and inhibitor-κB kinase-ε (IKKε), leading to activation of IRF3. IRF3 activation induces interferon-β (IFNβ) and IFN-inducible genes such as inducible nitric oxide synthase (iNOS)[18]. Activation of TRIF pathway also leads to delayed NF-κB activation[14].

The health effects of cadmium and mercury have been extensively studied globally. These metals are toxic to most mammalian organ systems, especially the immune system[19]. The high affinity of both heavy metals for sulfhydryl groups is instrumental in the inhibition of NF-κB binding to DNA *in vitro*[20]. However, little is known about the effect of these heavy metals on key signaling steps prior to NF-κB-DNA binding. The present study was designed to explore the effects of heavy metals on TLR signaling pathways, which play an important role in innate immunity.

Cadmium, but Not Mercury, Inhibits NF-κB Activation Induced by TLR2 or TLR4 Agonists

Agonist-induced activation of TLRs can induce the activation of NF-κB, which subsequently mediates the inducible expression of a variety of genes involved in immune and inflammatory responses including COX-2, iNOS, TNFα, IL-1 and IL-6[21-23]. Divalent metals such as cadmium and mercury that have high affinities for thiol groups inhibit the binding of NF-κB to DNA *in vitro*[20]. However, little is known about the effect of these metals on key signaling steps prior to NF-κB-DNA binding. Therefore, to identify whether cadmium or mercury modulates TLR-mediated signaling pathways, the induction of NF-κB activation by several TLR agonists was used as a readout for the activation of TLRs. Cadmium inhibited the activation of NF-κB induced by macrophage-activating lipopeptide 2-kDa (MALP-2, a TLR2 and TLR6 agonist) or lipopolysaccharide (LPS, a TLR4 agonist) in a dose-dependent manner as determined by the luciferase reporter gene assay, while mercury did not (Figure 1A and 1B).

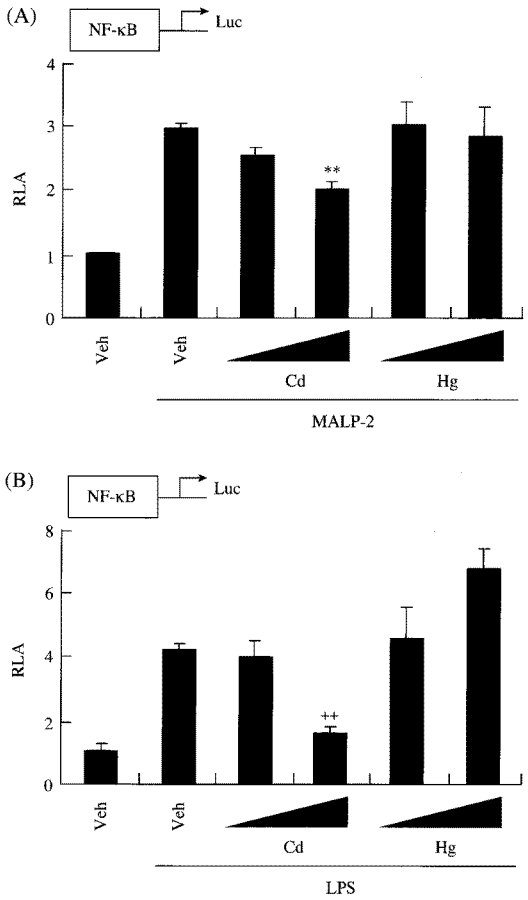

Figure 1. Cadmium but not mercury suppressed the NF-κB activation induced by MALP-2 or LPS (A,B). RAW264.7 cells were transfected with NF-κB luciferase reporter plasmid and pre-treated with cadmium (10, 30 μM) or mercury (10, 30 μM) for 1 h and then treated with MALP-2 (10 ng/mL) (A) or LPS (10 ng/mL) (B) for an additional 8 hrs. Cell lysates were prepared and luciferase enzyme activities were determined using the Luciferase Assay System. Values are mean ± SEM (n=3). **, Significantly different from MALP-2 alone (A), $P<0.01$. ++, Significantly different from LPS alone (B), $P<0.01$. Veh, vehicle; Cd, cadmium; Hg, mercury.

Cadmium, but Not Mercury, Suppresses COX-2 Expression Induced by TLR2 or TLR4 Agonists

Next, we determined whether cadmium or mercury inhibits COX-2 expression induced by MALP-2 or LPS. COX-2 is one of the target genes regulated through the activation of NF-κB in macrophages. Cadmium suppressed the expression of COX-2 induced by MALP-2 or LPS in RAW264.7 cells as determined by a COX-2 immunoblotting assay, while mercury did not (Figure 2A and 2B). Our results suggest that cadmium suppresses activation of NF-κB induced by TLR2 or TLR4 agonists resulting in the inhibited ex-

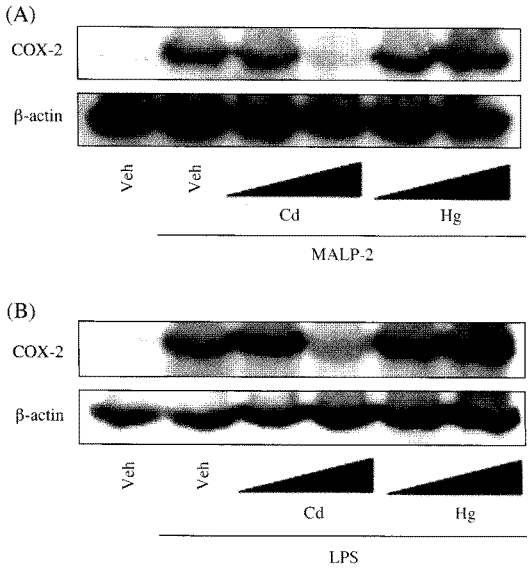

Figure 2. Cadmium but not mercury suppressed the COX-2 expression induced by MALP-2 or LPS (A,B). RAW264.7 cells were pretreated with cadmium (10, 30 μM) or mercury (10, 30 μM) for 1 hr and then further stimulated with MALP-2 (10 ng/mL) (A) or LPS (10 ng/mL) (B) for 8 hrs. Cell lysates were analyzed for COX-2 and β-actin protein by immunoblots. Veh, vehicle; Cd, cadmium; Hg, mercury.

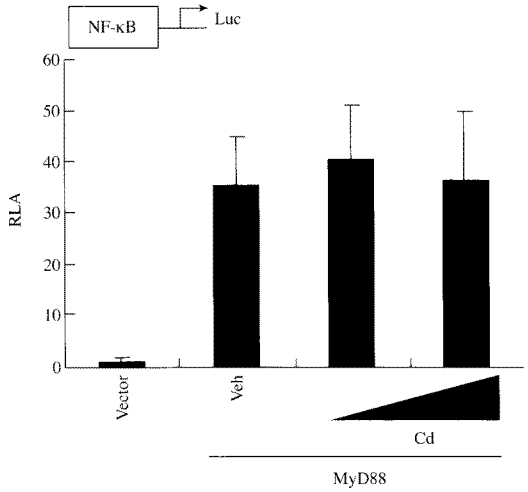

Figure 3. Cadmium did not inhibit the activation of NF-κB induced by the overexpression of MyD88. 293T cells were co-transfected with NF-κB-luciferase reporter plasmid and an expression plasmid for MyD88. pcDNA was used as a vector control for MyD88. After 3 hr, cells were treated with cadmium (10, 30 μM) for 18 hr. Luciferase enzyme activities were determined using the Luciferase Assay System. Values are mean ± SEM (n=3). Veh, vehicle; Cd, cadmium; Hg, mercury.

pression of target genes such as COX-2.

Cadmium Does Not Inhibit NF-κB Activation Induced by MyD88, the Downstream Signaling Component of TLRs

All TLRs except for TLR3 induce the activation of NF-κB through a MyD88-dependent pathway. The latter pathway induces NF-κB activation through the MyD88-IRAKs-TRAF6-TAK1-IKKα/β-IκBα/NF-κB pathway. Therefore, we further investigated whether the inhibitory effects of cadmium on the activation of NF-κB are mediated through the inhibition of the My88 downstream signaling component. Cadmium did not inhibit the activation of NF-κB induced by overexpression of MyD88 in 293T cells (Figure 3), consistent with the suggestion that cadmium inhibits TLR signaling activated by agonists, but not by downstream signaling components of TLRs.

Discussion

Cadmium and mercury have a great predilection to bind to protein sulfhydryl groups[24]. Their primary mechanism of cellular toxicity is through direct binding to thiol groups of proteins. A thiol-based mechanism for the inhibition of protein-DNA interactions by cadmium has been demonstrated in transcription factor IIIA-DNA binding studies, in which the binding site of cadmium was proposed to be the Cys_2Cys_2 zinc finger domain[25]. Other studies demonstrated that cadmium and mercury inhibit the activities of glutathione peroxidase, catalase, and superoxide dismutase through binding of sulfur atoms or the sulfur analog selenium [24,26]. Inhibition of NF-κB-DNA binding by cadmium and mercury appear to be the result of metal-mediated targeting of thiols[20].

Mercury is one of the most potent thiol-binding metals known. Mercury has a great ability to bind to reduced sulfur atoms, especially those on endogenous thiol-containing molecules such as glutathione, cysteine, homocysteine, metallothionein, and albumin[27]. The thiol-binding properties of mercury are responsible for its inhibitory effects on NF-κB activation[28]. Mercury reduces the nuclear translocation of NF-κB and inhibits IκBα phosphorylation and degradation in response to LPS[29]. However, our results showed that mercury did not inhibit MALP-2 or LPS-induced NF-κB activation (Figure 1) and COX-2 expression (Figure 2). A previous study reported that low doses of mercury enhance LPS- and IFNγ-induced iNOS mRNA and iNOS formation in murine macrophage[30]. These

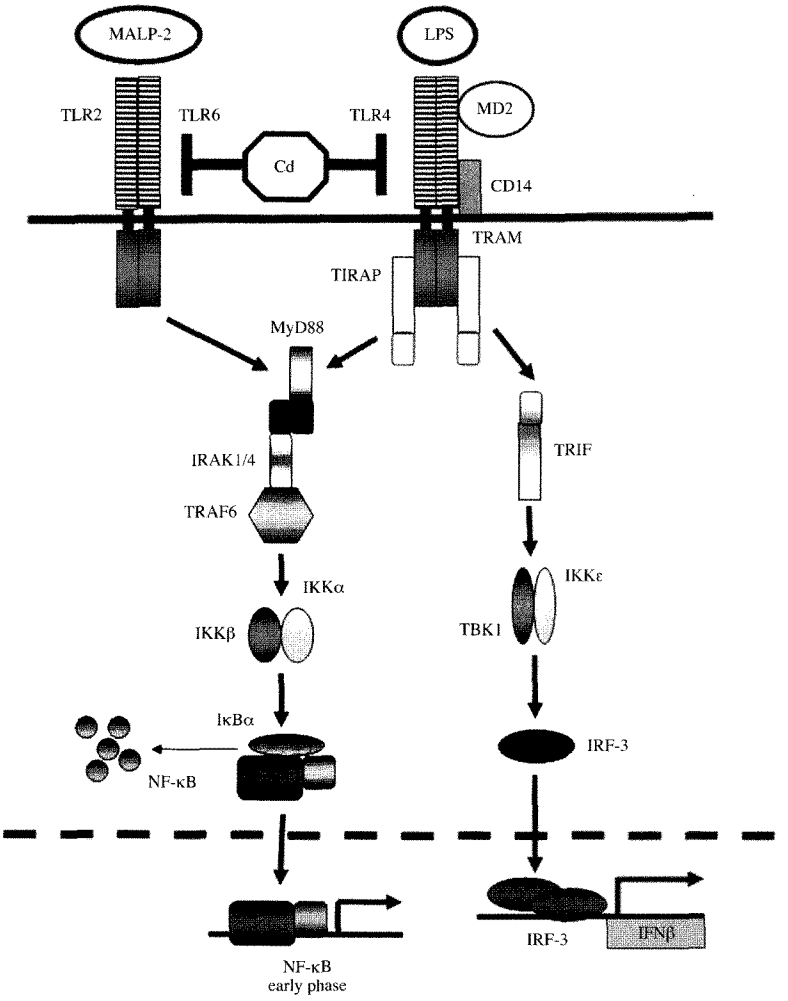

Figure 4. Toll-like receptor (TLR) signaling pathways and possible target of cadmium. TLRs have two major downstream signaling pathways; MyD88- and TRIF-dependent pathways leading to the activation of NF-κB and IRF3.

discrepancies may be due in part to the differences in cell system, dose, and exposure conditions.

Cadmium is a toxic heavy metal that can damage a variety of organs. Whether cadmium inhibits NF-κB activation is controversial. It was reported that cadmium stimulates the expression of intracellular adhesion molecule-1 (ICAM-1) via NF-κB activation in cerebrovascular endothelial cells[31]. Additionally, cadmium induces COX-2 expression and prostaglandin E2 production via activation of NF-κB, and in turn, ICAM-1 expression[32]. In contrast, cadmium inhibits NF-κB binding to DNA *in vitro*[20]. Cadmium induced apoptosis in rat kidney epithelial cells by suppression of NF-κB activity has been described[33]. Our results also demonstrated that cadmium can suppress ligand-induced NF-κB activation (Figure 1). The discrepancy in the results may be due to the differences in cell types and experimental conditions.

Presently, cadmium suppressed NF-κB activation induced by TLR2 or TLR4 agonists (Figure 1), while not suppressing NF-κB activation induced by the MyD88 downstream signaling component of TLRs (Figure 3). Cadmium has to inhibit NF-κB activation induced by MyD88, because cadmium inhibits NF-κB-DNA binding[20]. However, presently cadmium did not inhibit NF-κB activation induced by MyD88. The

previously reported NF-κB-DNA binding experimental study[20] was performed *in vitro*, but our experiments were done *ex vivo*. It is well-known that a divalent cation such as cadmium cannot pass through a lipid bilayer cell membrane. This lack of membrane permeability may explain the differing results obtained *in vivo* as opposed to the membrane-free *in vitro* system.

Cadmium inhibited the NF-κB activation induced by TLR2 or TLR4 agonists. However, cadmium did not inhibit NF-κB activation induced by the downstream signaling component, MyD88. These results suggest that the target of cadmium is not a downstream signaling component including NF-κB-DNA binding *in vivo*. The target may be components upstream of MyD88 including TLRs themselves or events leading to TLRs activation by agonists (Figure 4). Further studies are needed to elucidate the exact mechanism by which cadmium inhibits NF-κB activation and COX-2 expression induced by TLR agonists.

Materials & Methods

Reagents

Cadmium chloride ($CdCl_2$) and mercury chloride ($HgCl_2$) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were diluted in dimethyl sulfoxide. MALP-2 was purchased from Alexis Biochemical (San Diego, CA, USA). Purified LPS was obtained from List Biological (Campbell, CA, USA). All other reagents were purchased from Sigma-Aldrich unless otherwise indicated.

Cell Culture

RAW264.7 cells (a murine monocytic cell line; ATCC TIB-71) and 293T cells (human embryonic kidney) were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained at 37°C in a 5% CO_2/air environment.

Transfection and Reporter Gene Luciferase Assay

The assays were performed as we have previously described[34,35]. Cells were co-transfected with a luciferase plasmid and a plasmid containing heat shock protein (HSP)70-β-galactosidase as an internal control using SuperFect transfection reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Luciferase enzyme activities were determined using the Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Luciferase activity was normalized by β-galactosidase activity.

Immunoblotting

Immunoblotting was performed as previously described[36,37]. Equal amounts of cell extracts were subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the separated proteins were electrotransferred to a polyvinylidene difluoride membrane. The membrane was blocked to prevent nonspecific binding of antibodies in phosphate-buffered saline containing 0.1% Tween-20 and 3% nonfat dry milk. Immunoblotting was performed with the indicated antibodies and secondary antibodies conjugated to horseradish peroxidase (Amersham Biosciences, Arlington Heights, IL, USA). The reactive bands were visualized with ECL Western blot detection reagents (Amersham Biosciences).

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