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Crucial role of Toll-like receptors in the zinc/nickel-induced inflammatory response in vascular endothelial cells

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ABSTRACT

Our previous studies indicated that zinc induced inflammatory response in both vascular endothelial cells and promonocytes. Here, we asked if other metals could cause the similar effect on vascular endothelial cells and tried to determine its underlying mechanism. Following screening of fifteen metals, zinc and nickel were identified with a marked proinflammatory effect, as determined by ICAM-1 and IL-8 induction, on human umbilical vein endothelial cells (HUVECs). Inhibiting protein expression of myeloid differentiation primary response protein-88 (MyD88), a Toll-like receptor (TLR) adaptor acting as a TLR-signaling transducer, significantly attenuated the zinc/nickel-induced inflammatory response, suggesting the critical roles of TLRs in the inflammatory response. Blockage of TLR-4 signaling by CLI-095, a TLR-4 inhibitor, completely inhibited the nickel-induced ICAM-1 and IL-8 expression and NFκB activation. The same CLI-095 treatment significantly blocked the zinc-induced IL-8 expression, however with no significant effect on the ICAM-1 expression and a minor inhibitory effect on the NFκB activation. The finding demonstrated the differential role of TLR-4 in regulation of the zinc/nickel-induced inflammatory response, where TLR-4 played a dominant role in NFκB activation by nickel, but not by zinc. Moreover, inhibition of NFκB by adenovirus-mediated IκBα expression and Bay 11-7025, an inhibitor of cytokine-induced IκB-α phosphorylation, significantly attenuated the zinc/nickel-induced inflammatory responses, indicating the critical of NFκB in the process. The study demonstrates the crucial role of TLRs in the zinc/nickel-induced inflammatory response in vascular endothelial cells and herein deciphers a potential important difference in NFκB activation via TLRs. The study provides a molecular basis for linkage between zinc/nickel exposure and pathogenesis of the metal-related inflammatory vascular disease.

Introduction

Since the middle of the 19th century, production of heavy metals increased markedly for more than 100 years, with concomitant emissions to the environment (Jarup, 2003). Heavy metals are ubiquitous environmental pollutants and contamination of heavy metals in air, food, water and soil is a global threat to the environment. Association of metal contamination and its adverse impact on human health, e.g., atherogenesis, has been well documented. Heavy metals, unlike organic pollutants, do not decay and therefore have the potential to be accumulated in the environment.

Cytokines influence the development of atherosclerosis with the formation of complex atherosclerotic plaques, which may in turn lead to acute thromboembolic complications such as myocardial infarction or stroke (Young et al., 2002). Recent studies have highlighted the central role of vascular endothelium in modulating inflammatory response (Lusis, 2000) and atherosclerosis is thought to be a chronic inflammatory disease of the vessel wall (Ross, 1999). Endothelial dysfunction is a critical early step in atherogenesis (Ross, 1999; Sattar, 2004). Moreover, increasing evidence indicates that chronic obstructive pulmonary disease, asthma, and atherosclerosis are associated with systemic inflammatory cytokine changes. Various pathophysiological stimulators induce cytokine release, including modified low-density lipoprotein (LDL) (Berliner et al., 1993; Hulthe and Fagerberg, 2002), free radicals (Xu et al., 1996), hemodynamic stress (Sakai et al., 1999; Sterpetti et al., 1993), and hypertension (Humbert et al., 1995). Thus, inflammation and endothelial dysfunction have been shown to intimately link to the pathogenesis of cardiovascular disease.

Determination of environmental risk factors, such as heavy metals, for vascular dysfunction is one of our major research focuses. Our previous studies showed that arsenic enhanced TNF-α-induced adhesion molecule expression in vascular endothelial cells through the regulation of redox-sensitive transcription factors, including NF-κB (Tsou et al., 2005), ZnO particles induced ICAM-1 expression in vascular endothelial cells via an NFκB dependent pathway (Tsou et al., 2010), and zinc ions alone were sufficient to induce similar levels of ICAM-1 expression as ZnO particles (Yeh et al., 2011). We also found that zinc induced chemokine and

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inflammatory cytokine release from human promonocytes possibly via activation of multiple immune response-related transcription factors (Tsou et al., 2011). These studies suggest that the links between metal exposure, inflammation, endothelial dysfunction, and cardiovascular disease might be of particular importance; the metal-induced vascular inflammation may play key roles in the development of cardiovascular diseases such as atherosclerosis.

On the basis of these results, it was of importance to ask if any other metal could also cause the similar inflammatory effects on vascular endothelial cells. Moreover, the present study aimed to determine the potential membrane receptor targets of these proinflammatory metals. Here we demonstrated the crucial role of TLRs in the zinc/nickel-induced inflammatory responses in vascular endothelial cells, providing a molecular basis for the pathogenesis of inflammatory vascular diseases induced by environmental metal exposure.

Materials and methods

Materials Metal compounds used in this study included sodium arsenite ($NaAsO_2$, Sigma-Aldrich S7400), cesium chloride (CsCl, Merck 102041), iron sulfate ($FeSO_4$, Merck 103965), potassium hexacyanoferrate ($K_4Fe(CN)_6$, Sigma-Aldrich P3289), sodium selenite (Na_2SeO_3, Sigma-Aldrich S5261), chromium oxide (CrO_3, Sigma-Aldrich 27081), potassium dichromate ($K_2Cr_2O_7$, Sigma-Aldrich P5271), cadmium chloride ($CdCl_2$, Aldrich 202908), zinc acetate ($Zn(CH_3COO)_2$, J.T. Baker 4296), copper nitrate ($Cu(NO_3)_2$, Sigma-Aldrich 31288), lead chloride ($PbCl_2$, Aldrich 203572), mercury chloride ($HgCl_2$, Sigma-Aldrich M1136), manganese chloride ($MnCl_2$, Merck 105934), magnesium chloride ($MgCl_2$, Merck 105833), and nickel chloride ($NiCl_2$, Sigma N6136). Polymyxin B (PxB) (P4932), lipopolysaccharides (LPS) (L6529), and Bay 11-7082 (B5556) were obtained from Sigma-Aldrich. Antibodies against ICAM-1 (sc-7891), IκBα (sc-847), and phospho-IκBα (p-IκBα) (sc-7977-R) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies against IL-8 (GTX27747), myeloid differentiation primary response protein-88 (MyD88) (GTX112987), and green fluorescent protein (GFP) (GTX113617) were obtained from GeneTex, Inc (Irvine, CA). Mouse monoclonal antibody against α-actin (MAB1501) was purchased from Chemicon Int. Inc. (Temecula, CA) and CLI095 (tlrl-cli95), a TLR-4 signaling inhibitor, was from InvivoGen (San Diego, CA). Lipofectamine RNAiMAX (Invitrogen 13778-150) was obtained from Life Technologies. The siRNA sequence for MyD88, MyD88 siRNA614, was designed and selected using Invitrogen BLOCK-iT RNAi Designer and synthesized into double-strand oligonucleotides of 25 nucleotides started from the 614 position. A negative siRNA control, Stealth RNAi™ siRNA Negative Control, Med GC Duplex #2 (Invitrogen 12935112), was obtained from Life Technologies. Human umbilical vein endothelial cells (HUVECs) were prepared and maintained as previously described (Tsou et al., 2010).

Construction, amplification, and infection of recombinant adenoviruses Construction and amplification of recombinant adenoviruses, AdV-NFκB-Luc, AdV-GFP, and AdV-IκBα, were performed as previously described in detail (Tsou et al., 2010). Briefly, AdV-NFκB-Luc construct carries a firefly luciferase gene driven by a promoter containing five copies of the NFκB response element and a TATA box. AdV-GFP carries a built-in CMV-driven green fluorescent protein (GFP) tracer. In addition to a built-in GFP tracer, AdV-IκBα carries a wild-type IκBα under the CMV promoter.

In this study, ectopical expression of IκBα by adenovirus infection was used to block NFκB activation. Moreover, upon NFκB activation, it was very difficult to detect IκBα phosphorylation due to the rapid polyubiquitination and subsequent degradation of phosphorylated IκBα by the 26S proteasome (Krappmann and Scheidereit, 2005). Because of the abundant IκBα expression by adenovirus system used here, we were able to detect the IκBα phosphorylation by immunoblot analysis. For adenovirus infection experiments, HUVECs were seeded on 6-well dishes at a density of 5×10^5 cells/well one day before infection. Then, the cells were infected with AdV-GFP (as a mock-infected control) or AdV-Iκα at multiplicity of infection (MOI) = 50 or MOI = 5 as indicated one day before metal treatments.

Immunoblot analysis Following treatments, cells were lysed in ice-cold RIPA buffer (50 mM Tris–HCl, pH 7.5, 5 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.25% sodium deoxycholate) containing PMSF, (2 mM), aprotinin (2 μg/ml), leupeptin (2 μg/ml), NaF (2 mM), Na_3VO_4 (2 mM), and β-glycerophosphate (0.2 mM). Cell lysates were subjected to SDS-PAGE and immunoblot analysis, as described previously (Tsou et al., 2007). The blots were probed with a primary antibody (against ICAM-1, IL-8, MyD88, p-IκBα, IκBα, GFP, or actin) and then with a HRP-conjugated secondary antibody. Protein bands in the membrane were visualized in an X-ray film by using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Boston, MA, USA). The protein band intensity was quantified with densitometry scanning of X-ray films using actin as an internal control, unless otherwise indicated.

Cytotoxicity assay HUVECs were seeded into 6-well dishes at a density of 2×10^5 cells/well one day before metal treatments. Following metal treatments, cytotoxicity was determined with MTT assay as previously described in detail (Tsou et al., 2010).

siRNA inhibition of MyD88 protein expression Inhibition of MyD88 protein expression in HUVECs was performed by transfection of cells with MyD88 siRNA614. Briefly, HUVECs were seeded on 6-well dishes at a density of 3×10^5 cells/well for 36 h to 40 h. Then, the cells were transfected with 20 nmol negative siRNA or MyD88 siRNA614 with Lipofectamine RNAiMAX one day before metal treatments.

NFκB luciferase reporter assay To determine the activation of NFκB transcription factor, HUVECs (5×10^5 cells/well in 6-well dishes) were infected with the adenovirus AdV-NFκB-Luc at MOI = 50 for one day. Following adenovirus infection, the infected cells were then subjected to chemical treatments as indicated. After treatments, luciferase activity was determined using the Luciferase Assay System (Promega, Madison, WI), according to the manufacturer's instructions.

Statistical analysis Each experiment was performed independently at least three times. Analysis of variance (ANOVA) with the post-hoc Turkey HSD test was used to examine the difference in protein levels of IL-8 or ICAM-1 in cells treated with different concentration of zinc or nickel (Fig. 2). Paired sample *T* test was used to determine the difference in protein levels (MyD88, IL-8, or ICAM-1) in cells transfected with negative siRNA versus MyD88 siRNA614 (Fig. 3). Paired sample *T* test was used to determine the difference in protein levels (IL-8, ICAM-1, or p-IκBα) and NFκB reporter activity in cells without versus with the CLI-095 treatment (Figs. 4 and 5). Paired sample *T* test was used to determine the difference in protein levels (IL-8 or ICAM-1) in cells infected with AdV-GFP versus AdVIκBα (Fig. 6). Difference was considered statistically significant when $p < 0.05$. Analysis was carried out with the Statistical Package for Social Sciences (SPSS) version 12.0 (SPSS Inc., Chicago, IL, USA).

Results

Screening of the metals with inflammatory effect on vascular endothelial cells

Our previous studies revealed that zinc caused ICAM-1 expression in vascular endothelial cells via an NFκB dependent pathway (Tsou et al., 2010) and induced chemokine and inflammatory cytokine release from promonocytes through activation of multiple immune response-related transcription factors (Tsou et al., 2011). In the present study, we further asked if other metals could also induce the similar