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Induction of heme oxygenase 1 by arsenite inhibits cytokine-induced monocyte adhesion to human endothelial cells

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ABSTRACT

Heme oxygenase-1 (HO-1) is an oxidative stress responsive gene upregulated by various physiological and exogenous stimuli. Arsenite, as an oxidative stressor, is a potent inducer of HO-1 in human and rodent cells. In this study, we investigated the mechanistic role of arsenite-induced HO-1 in modulating tumor necrosis factor α (TNF-α) induced monocyte adhesion to human umbilical vein endothelial cells (HUVEC). Arsenite pretreatment, which upregulated HO-1 in a time- and concentration-dependent manner, inhibited TNF-α-induced monocyte adhesion to HUVEC and intercellular adhesion molecule 1 protein expression by 50% and 40%, respectively. Importantly, knockdown of HO-1 by small interfering RNA abolished the arsenite-induced inhibitory effects. These results indicate that induction of HO-1 by arsenite inhibits the cytokine-induced monocyte adhesion to HUVEC by suppressing adhesion molecule expression. These findings established an important mechanistic link between the functional monocyte adhesion properties of HUVEC and the induction of HO-1 by arsenite.

Introduction

Ample evidence supports heme oxygenase 1 (HO-1) as a key player in various cardiovascular diseases, especially those in which reactive oxygen species (ROS) have been implicated (Morita, 2005). HO-1 is highly induced by many oxidant producing stimuli, such as heme, heavy metals and UV irradiation, and believed to confer cytoprotective effects (Maines and Gibbs, 2005; Ryter and Choi, 2005). It has been recently proposed that HO-1 and its products function as adaptive molecules in regulating important biological responses, including inflammation, oxidative stress, cell survival, and cell proliferation (Otterbein et al., 2003; Takahashi et al., 2004). With regard to the possible relevance of HO-1 in atherosclerosis, HO-1 and its products potently inhibit adhesion of leukocytes and particularly monocyte to activated vascular endothelial cells (Ishikawa et al., 1997; Chen et al., 2006; Pae et al., 2006). This is an important mechanism for HO-1 protective actions because monocyte adhesion and migration into the vessel wall is an essential event for the development of atherosclerosis (Libby, 2002). Moreover, it has been shown that HO-1 directly modulates the expression of adhesion molecules in endothelial cell, such as intercellular adhesion molecule 1 (ICAM-1) (Soares et al., 2004). ICAM-1 is an immunoglobulin-like protein expressed on the surface of several cell types including endothelial cells and cells involved in the immune response, and plays an important role in the adhesion and migration of leukocytes to the sites of inflammation (Libby, 2002).

Arsenic is a naturally occurring element that is present in food, soil and water (Edelman, 1990). Environmental or occupational exposures to arsenic may result in both acute and chronic toxic effects in humans. In certain geographical areas, including the Southwest, Midwest, and New England in the US (Lewis et al., 1999), inorganic arsenic occurs naturally at toxicologically relevant levels in mineral deposits (McKone and Daniels, 1991; Hewitt et al., 1995). Epidemiological studies around the world have demonstrated a correlation between chronic environmental or occupational arsenic exposure and a risk of cardiovascular diseases including atherosclerosis (Simeonova and Luster, 2004; Navas-Acien et al., 2005; Wang et al., 2007; Tseng, 2008). A recent study indicated an effect of chronic arsenic exposure from drinking water on vascular inflammation that persists over time, and also suggested a potential mechanism underlying the association between arsenic exposure and cardiovascular disease (Chen et al., 2007).

Recent *in vivo* (Simeonova et al., 2003; Bunderson et al., 2004) and *in vitro* (Bunderson et al., 2002; Tsai et al., 2002) studies suggested that arsenic-induced oxidative stress plays an important role in the exacerbation of endothelial inflammation. Because endothelial inflammation is a hallmark of atherosclerosis (Libby, 2002), these studies provided a preliminary pathophysiological basis for atherogenic potential of arsenic. It is important to note that the expression of the inflammatory mediator such as cyclooxygenase-2 was only clearly observed at a high dose of arsenite (>10 μM) in the *in vitro* studies (Bunderson et al., 2002; Tsai et al., 2002; Simeonova et al., 2003;

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Bunderson et al., 2004). The underlying mechanistic roles of arsenic in cardiovascular diseases and endothelial inflammation are complex and under current investigation (Straub et al., 2007; Balakumar et al., 2008; Basu et al., 2008; Chen and Chen, 2008; Klei and Barchowsky, 2008; Prozialeck et al., 2008; Pysher et al., 2008). The exact molecular targets and signaling pathways that account for many of the biological effects of arsenic remain unknown.

The cell has developed several defense strategies, including HO-1, against toxic effects of excess ROS induced by arsenic. For example, arsenite-induced HO-1 has been suggested to be protective against arsenite-induced injury in vascular smooth muscle cells (Lee et al., 2005b). It has been shown that arsenite induces HO-1 in several cell types and tissues; for example, arsenite is a potent inducer of HO-1 in epidermal keratinocytes (Cooper et al., 2007). However, to our best knowledge, no study has been reported on the induction of HO-1 by arsenic in human endothelial cells. This is remarkable because vascular endothelial cells have long been considered as a primary target in the process of vasculopathy induced by arsenic exposure (Engel et al., 1994; Engel and Smith, 1994). Indeed, the endothelial injuries induced by arsenic exposure have been demonstrated as a leading factor causing vascular leakage and dysfunction (Ross, 1999; Chen et al., 2004).

More importantly, cytokine-induced monocyte adhesion is an essential event for the development of atherosclerosis (Libby, 2002), and such important functional studies on endothelial cells under arsenic exposure have not been reported. It is thus important to investigate effect of HO-1 induction by arsenic on cytokine-induced monocyte adhesion to endothelial cells, as reported in this work. The present study indicated that HO-1 induction by arsenite inhibits the cytokine-induced monocyte adhesion to human endothelial cells by suppressing adhesion molecule expression.

Materials and methods

Materials. Fetal bovine serum (FBS), cell culture reagents, dimethylsulfoxide (DMSO), dihydroethidium (DHE), and sodium arsenite were purchased from Sigma-Aldrich (St. Louis, MO). All primary and secondary antibodies of HO-1, ICAM-1 and β-actin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). TNF-α and primers for HO-1 and ICAM-1 were purchased from Invitrogen Inc. (Camarillo, CA).

HUVEC cells culture and arsenite treatment. Pooled human umbilical vein endothelial cells HUVEC (Lonza Bioscience, Walkersville, MD) were cultured in EGM-2 medium (Lonza Bioscience) supplemented with 2% FBS at 37 °C with 5% CO_2. Only 3–6 passage HUVEC were used in this study to avoid age-dependent cellular modifications. HUVEC (2.0×10^5/well) were seeded on 6-well plates and allowed to grow to 70–80% confluence before arsenite/TNF-α treatments.

Experimental protocols (Fig. S1 in Supplementary material) were designed for analyzing the role of arsenite-induced HO-1 in modulating the TNF-α-induced cell adhesion molecule expression and monocyte adhesion. After pre-incubation with arsenite for 6 h, the cells were treated with 1 ng/ml TNF-α (Invitrogen Inc.) for additional 4 h before RNA extraction, or for 6 h before monocyte adhesion analyses. For measuring protein expression, the duration of the treatments was increased by another 14 h (Fig. S1).

Note that the arsenite dosages (1–5 μM) used in this study were close to environmentally relevant concentration under chronic exposures in the US. The non-toxic doses were selected based on cell viability during 24 h arsenite treatment, as determined by lactate dehydrogenase (LDH) release assay (see below).

Cell viability analysis. LDH activity in medium was measured to determine arsenite cytotoxicity, according to the manufacturer's protocol. Briefly, HUVEC were seeded in 96-well plates at a density of 8×10^3 cells/well in 120 μl culture medium, and allowed to grow to 70–80% confluence. The cells were then treated with different concentrations of arsenite (0–5 μM) for 24 h. The medium was harvested from cultures, and centrifuged to remove cell debris. The LDH release was expressed as the percentage of LDH released into the medium compared to the total amount of LDH present in cells lysed with 2% Triton X-100. The activity was monitored through the oxidation of NADH at 490 nm using the LDH Cytotoxicity Detection Kit (Roche Applied Science, Indianapolis, IN). The cytotoxicity (%) was determined by the equation: [(OD_{490} of the treated group − OD_{490} of the background control) / (OD_{490} of the Triton X-100-treated group − OD_{490} of the background control)]×100%. Triplicate wells were used for each sample and the experiments were repeated at least three times to get means and standard deviations.

ROS detection. Intracellular ROS production was monitored by changes in fluorescence intensity resulting from fluorescent probe oxidation (Cooper et al., 2007). Briefly, cells were cultured on glass coverslips in the complete medium till 40% confluent density; the medium was then replaced with serum-free medium, and treated with arsenite (0–5 μM, 2 h). Thirty minutes prior to cell fixation, 5 μM dihydroethidium (DHE, Molecular Probes, Eugene, OR) was added to detect ROS generated upon the arsenite treatments. The coverslips were washed three times with PBS, fixed with paraformaldehyde (3.7%) and mounted on glass slides with Vecta-Shield (Vector Labs Inc., Burlingame, CA). Images were collected with an Olympus IX70 fluorescence microscope. A minimum of three independent samples were analyzed at each conditions.

Protein extraction and Western blotting analysis. Total cell extracts from cultured HUVEC were obtained by lysing the cells in ice-cold RIPA buffer (1×PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide) in the presence of a cocktail of protease inhibitors, PMSF and sodium orthovanadate (Santa Cruz Biotechnology Inc.). After centrifugation, protein contents in the supernatants were quantified using the protein assay kit (Bio-Rad Laboratories, Hercules, CA), and 10–30 μg of total protein for each lane was subjected to SDS-PAGE, using 12% acrylamide gels under reducing conditions. Proteins were subsequently electrotransferred onto a nitrocellulose membrane (Sartorius AG, Gottingen, Germany) following conventional protocols. The nonspecific binding of antibodies was blocked by 5% nonfat dried milk in TBST buffer (Tris-buffered saline and 0.1% Tween 20). Immunodetection of HO-1, ICAM-1, and β-actin proteins was carried out using respective primary antibodies (1:400 in 5% nonfat dried milk in TBST buffer) and horseradish peroxidase (HRP) conjugated secondary antibodies (1:1000 in 5% nonfat dried milk in TBST buffer). Subsequently, the membranes were developed with the SuperSignal West Pico HRP substrate kit (Pierce, Rockford, IL, USA) and photographed on a Kodak 4000 image station (Carestream Molecular Imaging, New Haven, CT, USA). Relative protein levels were expressed as the ratios of densities between proteins and β-actin in the same sample. Equal protein loading was confirmed by re-probing the original membranes with β-actin antibody.

Quantification of transcription levels by real-time polymerase chain reaction (real-time PCR). The primers sequences (see below) were designed upon their gene sequence, and the oligos used for primers were synthesized by Invitrogen.

β-actin: (sense) 5′ TCCTCCCTGGAGAAGAGCTAC 3′
(antisense) 5′ CTGTGTTGGCGTACAGGTCTT 3′;
HO-1: (sense) 5′ CTGAGTTCATGAGGAACTTTCAGAAG 3′
(antisense) 5′ TGGTACAGGGAGGCCATCAC 3′;
ICAM-1: (sense) 5′ CAGAGGTTGAACCCCACAGT 3′
(antisense) 5′ CCTCTGGCTTCGTCAGAATC 3′.