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

Xi Sun^a, Jingbo Pi^b, Wenlan Liu^a, Laurie G. Hudson^a, Ke Jian Liu^a, and Changjian Feng^{a,*}

^aCollege of Pharmacy, University of New Mexico, Albuquerque, NM 87131, USA

^bDivision of Translational Biology, Hamner Institutes for Health Sciences, Research Triangle Park, NC 27709, USA

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 through 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.

Keywords

Arsenic; endothelial cells; heme oxygenase 1; cytokine; monocyte adhesion

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 potentially 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

*Address correspondence to: Changjian Feng, College of Pharmacy, MSC09 5360, 1 University of New Mexico, Albuquerque, NM 87131-0001; Tel: 505-925-4326, Fax: 505-272-6749, cfeng@salud.unm.edu.

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 Southwest, Midwest, and New England in the U.S. (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 high dose of arsenite ($> 10 \mu\text{M}$) in the *in vitro* studies (Bunderson *et al.*, 2002; Tsai *et al.*, 2002; Simeonova *et al.*, 2003; Bunderson *et al.*, 2004). The underlying mechanistic roles of arsenic in cardiovascular diseases and endothelial inflammation are complex and under current investigations (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 through 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% CO₂. 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 6h, the cells were treated with 1ng/ml TNF- α (Invitrogen Inc.) for additional 4h before RNA extraction, or for 6h before monocyte adhesion analyses. For measuring protein expression, the duration of the treatments was increased by another 14h (Fig. S1).

Note that the arsenite dosages (1–5 μ M) 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 24h 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 μ l culture medium, and allowed to grow to 70–80% confluence. The cells were then treated with different concentrations of arsenite (0–5 μ M) for 24h. 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 490nm using the LDH Cytotoxicity Detection Kit (Roche Applied Science, Indianapolis, IN). The cytotoxicity (%) was determined by the equation: $[(OD_{490} \text{ of the treated group} - OD_{490} \text{ of the background control}) / (OD_{490} \text{ of the Triton X-100-treated group} - OD_{490} \text{ of the background control})] \times 100\%$. 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 μ M, 2h). Thirty minutes prior to cell fixation, 5 μ M 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 (1xPBS, 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 (1:1,000 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' and (antisense) 5' CTGTGTTGGCGTACAGGTCTT 3';

HO-1: (sense) 5' CTGAGTTCATGAGGAACCTTCAGAAG3' and (antisense) 5' TGGTACAGGGAGGCCATCAC 3';

ICAM-1: (sense) 5' CAGAGGTTGAACCCACAGT 3' and (antisense) 5' CCTCTGGCTTCGTCAGAATC 3'.

mRNA was reverse transcribed, amplified and quantified by real-time PCR analysis using the SYBR Green PCR Master Kit and RT-PCR reagents (Applied Biosystems, Foster City, CA). Briefly, total RNA were extracted from HUVEC using the TRIzol Reagents (Invitrogen Inc.) according to manufacturer's protocol. 1.0 µg RNA was reverse-transcribed with random primers in a 20 µl final reaction volume using RETROscript® Kit (Ambion Inc., Austin, TX). 0.5 µl RT products were then amplified with 7900HT Fast Real-time PCR system (Applied Biosystems) in a 10 µl final reaction volume using SYBR® Green PCR Master Mix (Applied Biosystems) under the following conditions: 2min at 50°C and 10min at 95°C, followed by a total of 40 cycles of two temperature cycles (15s at 95°C and 1min at 60°C). After amplification, a melting curve was generated and data analysis was performed by using Dissociation Curves 1.0 software (Applied Biosystems). The relative value of mRNA expression was calculated by the comparative $\Delta\Delta C_t$ method described in the 7900HT manual. Briefly, mean C_t values were normalized to the internal control β -actin, and the difference was defined as ΔC_t . The difference between the mean ΔC_t values of treated and untreated cells was calculated and defined as $\Delta\Delta C_t$.

Monocyte adhesion assay

Cytokine-induced monocyte adhesion to arsenite-treated endothelial cells was measured using fluorescently labeled monocytic U937 cells as described in the literatures (Ishikawa *et al.*, 1997; Soares *et al.*, 2004; Chen *et al.*, 2006; Pae *et al.*, 2006). The human leukemic monoblast cells U-937 (ATCC, Manassas, VA) were cultured in the RPMI-1640 medium (ATCC) supplemented with 0.2% (wt/vol) NaHCO₃ and 0.03% (wt/vol) L-glutamine containing 100U/ml penicillin, 100 mg/ml streptomycin, and 10% (vol/vol) FBS; the cells were collected and suspended in PBS at a concentration of 5×10^6 cells/ml. To fluorescently label the U937 cells, BCECF/AM solution (Molecular Probes) was then added to a final concentration of 2 μ M, and the mixture was incubated at 37°C for 30 min; the cells were washed three times with PBS and re-suspended in RPMI media at a concentration of 2×10^6 cells/ml. HUVEC were grown in 24-well plates to 70% confluence, and pretreated with arsenite (5 μ M, 6h) prior to exposure to TNF- α (1ng/ml, 6h). HUVEC were washed with RPMI three times to remove arsenite and TNF- α before the labeled U937 was added to the HUVEC monolayer at a concentration of 5×10^5 cells/microplate well. The mixture was incubated at 37°C for 30min followed by four rinses with RPMI media to remove arsenite and TNF- α before adding U937 cells, to avoid potential toxicities of arsenite and TNF- α against U937 cells. Adherent U937 cells were visualized via fluorescence microscopy. The adherent U937 cells were counted in the high-power field (40 $\times$ phase-contrast objective) per well, and the data were represented by the relative adherent ratio as compared to TNF- α treatment alone. Experiments at each sample conditions were performed in triplicate.

HO-1 small interfering RNA (siRNA) transfection

HUVEC were transfected with HO-1 siRNA Mix (Santa Cruz Biotechnology Inc.) using DharmaFECT transfection reagent (Dharmacon Inc., Lafayette, CO) according to the manufacturer's instructions. Briefly, HO-1 siRNA (25nM) was added to serum-free medium containing transfection reagents and incubated for 20min. HUVEC cultured in 6-well plates (50–60% confluence) were incubated with the siRNA mixture for 7h. Media were changed, and the cells were incubated for another 24h for maximum inhibition of the HO-1 gene. Un-transfected cells and cells transfected with transfection reagents alone were used as controls. Cells were then treated with arsenite, and analyses of the U937 adhesion, HO-1 and ICAM-1 expressions were conducted as described above.

Statistics

All statistical analyses were performed using Sigma Stat 3.0 (SPSS Inc., Chicago, IL), with $p < 0.05$ taken as significant. One-way analysis of variance (ANOVA) followed by an appropriate post hoc comparison was used to evaluate the effect of exposure time and concentration of arsenite on HO-1 mRNA level (Fig. 1), and the effect of arsenite on TNF- α -induced monocyte adhesion and ICAM-1 expression in non-transfected (Fig. 2) and HO-1 siRNA transfected cells (Figs. 3C and 3D). Two-way ANOVA followed by an appropriate post hoc comparison was used to evaluate the effect of HO-1 siRNA on HO-1 expression under arsenite exposure (Fig. 3A). Each experiment was performed independently for at least three times. All results were expressed as means $\pm$ SE.

Results

Arsenite induces HO-1 expression in HUVEC

It has been shown that arsenite upregulates expression of HO-1 and other stress responsive genes in human and rodent cells (Liu *et al.*, 2001; Andrew *et al.*, 2003; Rea *et al.*, 2003; Zheng *et al.*, 2003; Xie *et al.*, 2004; Benbrahim-Tallaa *et al.*, 2005; Lee *et al.*, 2005a; Prawan *et al.*, 2005; Wei *et al.*, 2005; Cooper *et al.*, 2007). In the current study, when HUVEC were

exposed to arsenite (0–5 μ M, 0–24h), the mRNA levels of HO-1 were significantly increased in a time- and concentration-dependent manner (Fig. 1). A notable increase of HO-1 mRNA was detectable after 3h, with peaked induction after 6h, whereas HO-1 was barely detectable under basal conditions (Fig. 1A); this is consistent with the time course of HO-1 induction by dopamine through an oxidative mechanism in HUVEC (Berger *et al.*, 2000). Western blot analysis confirmed that arsenite exposure significantly increased protein level of HO-1 (see below). Note that cell viability assay showed that the arsenite treatment was nontoxic to HUVEC under such conditions (Fig. S2 in Supplementary Material), indicating that the observed upregulation of HO-1 does not involve nonspecific cytotoxic mechanisms. Based upon these results, we chose to treat HUVEC with arsenite (5 μ M, 6h) for inducing optimal HO-1 expression (see protocols in Fig. S1), in order to study the role of arsenite-induced HO-1 in modulating adhesion molecule expression (see below).

It has been well established that one of the major upstream mediators of HO-1 expression is ROS formation (Cooper *et al.*, 2007). Indeed, arsenite treatment of HUVEC cells induced generation of ROS in a dose-dependent manner, as indicated by increase of the red fluorescence signal resulting from the fluorescent probe oxidation by ROS (Fig. S3). Therefore it is likely that arsenite induces ROS, which act as signaling molecules to induce HO-1 expression in HUVEC.

Arsenite exposure attenuates TNF- α -induced monocyte adhesion

The adhesion of monocyte to the arterial wall is a crucial step in the development of atherosclerosis (Glass and Witztum, 2001). Cytokines facilitate the attachment of monocyte to vascular endothelial surfaces via induction of adhesion molecule expression, such as ICAM-1 (Cybulsky and Gimbrone, 1991). In particular, TNF- α is a dominant pro-inflammatory cytokine that induces adhesion molecule, and this process characterizes early atherogenesis (Cybulsky and Gimbrone, 1991; Takahashi *et al.*, 1996; Libby, 2002). We thus designed the experimental protocols (Fig. S1) for analyzing the effect of arsenite pre-treatment on the TNF- α -induced monocyte adhesion and cell adhesion molecule expression. Such an approach using TNF- α as an inflammatory stimulus is well-accepted in studying mechanism of monocyte adhesion *in vitro* (Soares *et al.*, 2004; Kwon *et al.*, 2007; Mukherjee *et al.*, 2007). We chose to use 1 ng/ml TNF- α to avoid complications due to high dose, and this dose has been shown to effectively induce adhesion molecule expression in HUVEC within 4h (Tsou *et al.*, 2007).

We measured TNF- α -induced monocyte adhesion to arsenite pre-treated endothelial cells using fluorescently labeled monocytic U937 cells (Fig. 2A); quantifications of the images show that arsenite inhibited TNF- α -induced monocyte adhesion to HUVEC by 50% (Fig. 2A). Moreover, arsenite treatment decreased the TNF- α -induced ICAM-1 mRNA (Fig. 2B) and protein (Fig. 2C) expressions by 80% and 40%, respectively. Note that arsenite and TNF- α were removed prior to addition of the U937 cells to avoid their potential toxicity against the U937 cells. Because TNF- α -induced expression of the adhesion molecules such as ICAM-1 is important in mediating monocyte adhesion to endothelial cells (Libby, 2002), our results indicate that arsenite treatment may inhibit TNF- α -induced monocyte adhesion to HUVEC through suppressing ICAM-1 expression.

Arsenite-induced HO-1 is important in suppressing monocyte adhesion

As shown above, arsenite is a potent inducer of HO-1 in HUVEC (Fig. 1). Increasing evidence indicate that HO-1 has important antioxidant and anti-inflammatory properties that result in a vascular anti-atherogenic effect (Morse and Choi, 2002). To determine whether it is arsenite-induced HO-1 that inhibits monocyte adhesion to HUVEC, we applied siRNA strategy to effectively knockdown the arsenite-induced HO-1 expression in HUVEC.

Indeed, the HO-1 siRNA effectively suppressed arsenite-induced HO-1 mRNA (Fig. 3A) and protein (Fig. 3B) expressions in HUVEC. Importantly, knockdown of HO-1 with siRNA abolished the inhibitory effects of arsenite treatment on TNF- α -induced monocyte adhesion (Fig. 3C) and ICAM-1 expression (Fig. 3D). The arsenite-induced differences in monocyte adhesion were not statistically significant after knocking out HO-1 with siRNA (Fig. 3C), in contrast with the notable inhibition of monocyte adhesion to the non-transfected cells by arsenite pre-treatment (Fig. 2A). Note that the siRNA reagent treatment alone did not cause notable changes in the TNF- α -induced cell adhesion and ICAM-1 expression ($p>0.05$) (Figs. 3C and 3D). These results clearly indicate that arsenite-induced HO-1 is important in suppressing TNF- α -induced ICAM-1 expression and monocyte adhesion to HUVEC.

Discussion

Recent research reveals the emerging significance of HO-1 in cardiovascular diseases including atherosclerosis (Immenschuh and Schroder, 2006). HO-1 is the inducible isoform of the first and rate-controlling enzyme of heme degradation. HO-1 is up-regulated by a host of oxidative stress stimuli, and has potent cytoprotective and anti-inflammatory functions via decreasing tissue levels of the prooxidant heme along with production of bilirubin and the signaling gas carbon monoxide (Ryter *et al.*, 2006). Increasing evidence show that HO-1 may counteract the detrimental effects of oxidative stress in the endothelium (Dulak *et al.*, 2008). Recent advances have been made in understanding the role of endothelial HO-1 in the regulation of the inflammatory response, including the modulation of leukocyte recruitment and transmigration through the endothelial barrier (Stocker and Perrella, 2006). Furthermore, experimental evidence from various cell culture and animal models suggests an association of HO-1 with the complex sequence of events in atherosclerosis (Chung *et al.*, 2008). Therefore it is of current interest to study the role of endothelial HO-1 in ROS-involved development of atherosclerosis.

Because monocyte adhesion to endothelial cells is important in the development of atherosclerosis (Libby, 2002), we focused on the mechanistic role of HO-1 in monocyte adhesion to arsenite-treated endothelial cells. We found that arsenite upregulated HO-1 expression in HUVEC in a dose- and time-dependent manner (Fig. 1). Note that the arsenite treatment was nontoxic to HUVEC under such conditions (Fig. S2), indicating that the observed upregulation of HO-1 does not involve nonspecific cytotoxic mechanisms. To determine the role of arsenite-induced HO-1 in monocyte adhesion to endothelial cells, we measured TNF- α -induced monocyte adhesion and ICAM-1 expression in arsenite-pretreated HUVEC cells. Our results clearly show that arsenite pretreatment significantly suppressed monocyte adhesion to TNF- α -induced endothelial cells (Fig. 2A). Moreover, co-incubation HUVEC with TNF- α and arsenite significantly inhibited the mRNA (Fig. 2B) and protein (Fig. 2C) expressions of ICAM-1, compared to cells treated with TNF- α alone. These results indicate that arsenite inhibits TNF- α -induced monocyte adhesion to HUVEC through suppressing ICAM-1 expression. Importantly, the inhibitions afforded by arsenite were diminished in HUVEC transfected with HO-1 siRNA (Figs. 3C and 3D). Taken together, these results indicate that induction of HO-1 by arsenite inhibits monocyte adhesion through suppressing ICAM-1 expression, as proposed in Fig. S4 in Supplementary Material. Such an inhibition via HO-1 upregulation is consistent with the potent anti-inflammatory and cytoprotective effects of HO-1 in a number of *in vivo* models of inflammation (Soares *et al.*, 2004).

There is lack of consistency in the effects of HO-1 siRNA on TNF- α -induced monocyte adhesion (Fig. 3C) and ICAM-1 expression (Fig. 3D) under the TNF- α +As/siRNA treatments. This suggests possible involvement of other factors in the effects of arsenite on TNF- α -induced ICAM-1 expression. One of the factors could be NF- κ B because its

activation is required for TNF- α -mediated up-regulation of ICAM-1 in endothelial cells (Zhou *et al.*, 2007). Selective intervention of NF- κ B-activating pathway inhibits the TNF- α -induced inflammatory response in HUVEC and monocyte adhesion (Gutiérrez *et al.*, 2007). Future study is needed to investigate the signaling events in monocyte adhesion to HUVEC under arsenite exposure.

Another study showed that arsenite enhances TNF- α -induced expression of cell adhesion molecule in HUVEC (Tsou *et al.*, 2005). It is important to note that the reported increase of cell adhesion molecule expression was very modest at comparable arsenite concentrations (1.3-fold increase at 5 μ M arsenite), and that the cell adhesion studies were not reported (Tsou *et al.*, 2005). In this study, for the first time, we established an important mechanistic link between the functional monocyte adhesion properties of HUVEC and the induction of HO-1 by arsenite. Our study is thus directly relevant to molecular targets and signaling pathways that account for the biological effects of arsenic on endothelial cells. The difference between that of Tsou and our study may be due to the different experimental conditions. In particular, the percentages of serum in the culture media were different: we used 2% FBS, whereas 20% FBS was used in Tsou's study (Tsou *et al.*, 2005). Arsenic may synergistically interact with other mitogen(s)/growth factors in the serum, as suggested by a recent finding that ERK/MAPK activation is necessary but not sufficient for optimal arsenite-induced HO-1 expression (Cooper *et al.*, 2007). In addition, the origins of the HUVEC cells are different: Tsou *et al.* prepared the cultured HUVEC by collagenase digestion of umbilical veins, whereas we used pooled HUVEC cells in this study. Pooled cells in our study respond to arsenite treatments without specificity difference.

The underlying mechanistic roles of arsenic in cardiovascular diseases are complex and remain primarily unknown. Conflicting results have been reported from *in vitro* studies on endothelial cells under arsenic exposure. For example, high dose of arsenite ($\gg 10\mu$ M) was reported to induce oxidative DNA damage in HUVEC (Bau *et al.*, 2002) and bovine aorta endothelial cells (Liu and Jan, 2000), whereas low dose of arsenite ($< 1\mu$ M) stimulates endothelial cell growth (Kao *et al.*, 2003). These *in vitro* studies suggest a biphasic dose-response relationship induced by arsenic in endothelial cells and other cell lines, in which arsenic at low dose induced adaptive response whereas high amounts of arsenic produced adverse effects, including excess ROS formation and oxidative damage (Snow *et al.*, 2005; He *et al.*, 2007). Our study suggested that low dose short-term arsenite exposure inhibits the cytokine-induced monocyte adhesion to human endothelial cells through HO-1 induction. Epidemiological studies have shown that specific inflammatory mediators are related to arsenic-induced cardiovascular diseases (Simeonova and Luster, 2004; Chen *et al.*, 2007). Note that environmental arsenic exposures are characterized by chronic exposures to low levels of arsenic. Importantly, recent *in vivo* studies also indicated that endothelial inflammation may play a role in arsenic-related vascular effects (Simeonova *et al.*, 2003; Bunderson *et al.*, 2004; Straub *et al.*, 2007); the animals were exposed to low dose arsenic for weeks/months. Further mechanistic studies of human endothelial cells under chronic arsenic exposure will be required to better understand the role of modulated HO-1 induction in the arsenic-induced endothelial inflammation.

In summary, our results indicate that HO-1 induction by arsenite inhibits the cytokine-induced monocyte adhesion to human endothelial cells through suppressing cell adhesion molecule expression. These results need to be extended by future functional and mechanistic studies under chronic arsenic exposure to provide insights into how the modulated HO-1 pathway may be involved in the pathogenesis of atherosclerosis. These experiments need considerable efforts, and are outside of the scope of the present study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Arsenite induces HO-1 expression in HUVEC. The cells were treated with (A) 5μM arsenite for 0–24h, or (B) different arsenite doses for 6h. Total RNA were extracted and reverse transcribed. The expression level of HO-1 was detected by real-time PCR, and normalized as the ratio of density values to the house-keeping β-actin. The mRNA level was presented as relative HO-1 mRNA levels as compared to the control without arsenite treatment. One-way ANOVA with Dunnett's multiple comparison test indicates * $p < 0.05$ vs control. Panels are representative of 3 or more experiments. All results were expressed as means±SE.

Fig. 2.

Arsenite attenuates the TNF- α -induced monocyte-endothelial monolayer binding. **(A)** Fluorescent microscopy of endothelial adhesiveness for monocytes (*upper*), and quantification of monocyte adhesion to HUVEC (*lower*); the data were presented by the relative adherent ratio as compared to TNF- α treatment alone. HUVEC were incubated with 5 μ M arsenite for 6h, followed by addition of 1ng/ml TNF- α for another 6h. HUVEC were then washed with RPMI three times to remove arsenite and TNF- α (to avoid their potential toxicity against the U937 cells); fluorescent labeled U937 cells were added, and incubated at 37°C for 30min. **(B)** Arsenite suppresses TNF- α -induced ICAM-1 mRNA expression in HUVEC. The cells were incubated with 5 μ M arsenite for 6h, followed by addition of TNF- α (1ng/ml, 4h). Total RNA were extracted, reverse transcribed, and ICAM-1 mRNA was quantified by real-time PCR; the data were expressed as relative ICAM-1 mRNA levels as compared with untreated control. **(C)** Arsenite suppresses TNF- α -induced ICAM-1 protein expression in HUVEC. The cells were incubated with 5 μ M arsenite for 6h, followed by addition of TNF- α (1ng/ml, 4h). Total proteins were then collected after another 14h incubation, 30 μ g protein was resolved by SDS-PAGE, and ICAM-1 protein level was detected by Western blot (*left*), and quantitized (*right*). One-way ANOVA with Turkey's multiple comparison test indicated significant difference (* p <0.05) between the groups with and without arsenite pre-treatment (panels A–C). Panels are representative of 3 or more experiments. All results were expressed as means $\pm$ SE.

Fig. 3.

HO-1 siRNA abolishes the inhibitory effect of arsenite on TNF- α -induced monocyte adhesion to HUVEC and ICAM-1 expression. **(A)** HO-1 siRNA effectively knocks down arsenite-induced HO-1 mRNA expression in HUVEC. The cells transfected with HO-1 siRNA were treated with 5 μ M arsenite for 6h, and HO-1 mRNA was extracted and quantified. The mRNA data were presented as relative HO-1 mRNA levels compared to the control group of no arsenite/siRNA treatment. Two-way ANOVA with Bonferroni *t*-test indicates significant difference between the arsenite-treated groups with or without HO-1 siRNA transfection (* $p < 0.05$). **(B)** HO-1 siRNA effectively knocks down arsenite-induced HO-1 protein expression in HUVEC. The cells transfected with HO-1 siRNA were treated with 5 μ M arsenite for 20h. HO-1 protein level was analyzed by Western blot. **(C)** HO-1 siRNA abolishes the inhibitory effect of arsenite on TNF- α -induced monocyte-endothelial monolayer binding. Fluorescent microscopy of endothelial adhesiveness for monocytes (*left*), and quantification of the monocyte adhesion to HUVEC (*right*). The data were presented by the relative adherent ratio as compared to the TNF- α /siRNA treated groups. HUVEC cells transfected with HO-1 siRNA were incubated with 5 μ M arsenite for 6h, followed by addition of 1ng/ml TNF- α for another 6h. The HUVEC cells were washed with RPMI three times (to remove arsenite and TNF- α), the U937 cells were then added to the HUVEC, and incubated at 37°C for 30min. The control is HO-1 siRNA transfected HUVEC cells without TNF- α /arsenite treatment. One-way ANOVA with Turkey's test indicates significant difference in the TNF- α -treated groups vs control (* $p < 0.05$), but no significant difference between the TNF- α /siRNA and TNF- α +As/siRNA groups. **(D)** HO-1 siRNA abolishes the suppression of arsenite on TNF- α -induced ICAM-1 protein expression in

HUVEC. The cells transfected with HO-1 siRNA were incubated with 5 μ M arsenite for 6h, followed by addition of 1ng/ml TNF- α . The cells were incubated for another 18h, total proteins were collected, and ICAM-1 was detected by Western blot (*left*), and quantitized (*right*). The control is HO-1 siRNA transfected HUVEC cells without TNF- α /arsenite treatment. One-way ANOVA with Turkey's test indicates * $p < 0.05$ vs control. Panels are representative of 3 or more experiments. All results were expressed as means $\pm$ SE.