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Heme Oxygenase-1 Protects Endothelial Cells from the Toxicity of Air Pollutant Chemicals

Akeem Lawal [1,2] , Min Zhang [1, 2], Michael Dittmar [1],
Aaron Lulla [1], Jesus A. Araujo [1, 3]

Short Title: DEP and HO-1

[1] Division of Cardiology, David Geffen School of Medicine, University of California-Los Angeles, 10833 Le Conte Avenue, CHS 43-264, Los Angeles, CA 90095

[2] AL and MZ contributed equally to this work

[3] **Corresponding author**: Jesus A. Araujo, MD, PhD. UCLA Division of Cardiology, 10833 Le Conte Avenue, CHS 43-264, Los Angeles, CA 90095. P.O. Box 951679. Phone number (310) 825-3222, Fax number (310) 206-9133. E-mail address: JAraujo@mednet.ucla.edu

Abbreviations: PM (particulate matter), DEP (diesel exhaust particles), ROS (reactive oxygen species), CVD (cardiovascular diseases), HO-1 (Heme oxygenase-1), DCFDA (2',7'-Dichlorofluorescein diacetate), DHE (Dihydroethidum), CoPPIX (Protoporphrin IX cobalt chloride), SnPPIX (Tin Protoporphrin IX dichloride), A-DEP (Automobile-DEP), F-DEP (Forklift-DEP), HMEC (human microvascular endothelial cells), BAEC (bovine aortic endothelial cells), DCF (dichlorofluorescein), qPCR (quantitative polymerase chain reaction), RFU (relative fluorescence units), LDH (lactate dehydrogenase), IL-8 (interleukin 8), MCP-1 (monocyte chemotactic protein-1), ICAM-1 (intercellular adhesion molecule 1), VCAM-1 (vascular adhesion molecule 1), ATF-4 (activating transcription factor 4), EOM (extractable organic materials).

Abstract

Diesel exhaust particles (DEP) are a major component of diesel emissions, responsible for a large portion of their toxicity. In this study, we examined the toxic effects of DEP on endothelial cells and the role of DEP-induced heme oxygenase-1 (HO-1) expression. Human microvascular endothelial cells (HMEC) were treated with an organic extract of DEP from an automobile engine (A-DEP) or a forklift engine (F-DEP) for 1 and 4 hours. ROS generation, cell viability, lactate dehydrogenase leakage, expression of HO-1, inflammatory genes, cell adhesion molecules and UPR gene were assessed. HO-1 expression and/or activity were inhibited by siRNA or Tin protoporphyrin (Sn PPIX) and enhanced by an expression plasmid or Cobalt protoporphyrin (CoPPIX). Exposure to 25 μg/ml of A-DEP and F-DEP significantly induced ROS production, cellular toxicity and greater levels of inflammatory and cellular adhesion molecules but in a different degree. Inhibition of HO-1 enzymatic activity with SnPPIX and silencing of the HO-1 gene by siRNA enhanced DEP-induced ROS production, further decreased cell viability and increased expression of inflammatory and cell adhesion molecules. On the other hand, overexpression of the HO-1 gene by a pcDNA 3.1D/V5-HO-1 plasmid significantly mitigated ROS production, increased cell survival and decreased the expression of inflammatory genes. HO-1 expression protected HMEC from DEP-induced prooxidative and proinflammatory effects. Modulation of HO-1 expression could potentially serve as a therapeutic target in an attempt to inhibit the cardiovascular effects of ambient PM.

Keywords: Diesel exhaust particles, air pollution, endothelial cells, Heme oxygenase-1, reactive oxygen species, inflammation.

Introduction

Several epidemiological studies have shown that exposure to ambient particulate matter (PM) is associated with adverse health effects resulting in increased mortality, mostly via the exacerbation of cardiovascular ischemic events (Peters et al, 2004; Pope et al, 2004; Miller et al, 2007). Motor vehicle emissions are a major contributor to ambient PM in major cities (USEPA, 1999). For instance, Diesel exhaust particles (DEP), generated via the combustion of diesel fuels by motor vehicle engines and various industries are widely present in urban ambient air and substantially contribute to the fine and ultrafine PM size fractions in urban dwellings (Lloyd and Cackette, 2001). Importantly, we and others have shown that exposure to DEP or DEP organic chemicals can lead to significant cytotoxic and proinflammatory effects in vascular cells such as endothelial cells and macrophages (Hiura et al, 1999; Mundandhara et al, 2006; Gong et al, 2007; Yin et al, 2013a). The generation of reactive oxygen species (ROS) and development of oxidative stress in the artery wall are important in the pathogenesis of atherosclerosis and cardiovascular diseases (CVD) (Araujo, 2011). Thus, vascular oxidative injury can lead to endothelial dysfunction (Haruna et al, 2007), atherosclerotic plaque initiation and progression. Vascular ROS could be triggered and/or enhanced by prooxidant factors such as ambient PM (Araujo, 2011). Indeed, DEP prooxidant actions may be key in the ability to induce harmful cellular effects (Li et al, 2009; Montiel-Davalos et al, 2010; Brook et al, 2004; Kumagai et al, 1997; Lund et al, 2007). DEP have a high content of organic compounds, which can lead to highly electrophilic metabolites and the formation of ROS through redox cascade reactions (Iwamoto et al, 2007). We have shown that ApoE null mice exposed to diesel exhaust for two weeks exhibited increased lipid peroxidation in the bronchoalveolar compartment, blood and liver, together with the development of dysfunctional pro-oxidant and pro-inflammatory HDL (Yin et al, 2013b). It is possible that DEP-induced ROS in vascular cells could be responsible for a significant portion of the vascular effects (Bai et al, 2011; Gong et al, 2007; Hirano et al, 2003; Araujo, 2011).

We and others have shown that DEP can induce the expression of antioxidant genes such as Heme oxygenase (HO-1) in endothelial cells (Gong et al, 2007) and macrophages (Li et al, 2004), at the same time that concentrated ultrafine particles led to the upregulation of HO-1 in the livers of ApoE null mice (Gong et al, 2007), likely in response to the oxidative stress generated. HO-1 expression has been reported to exert cytoprotective, antioxidant, antiapoptotic, anti-inflammatory and possibly immunomodulatory effects in vascular cells, most of which play a major role in the protection against atherogenesis (reviewed by Araujo et al, 2012). HO-1 vascular protective effects have been mostly attributed to its enzymatic activity which comprises the oxidative cleavage of heme groups to generate equimolar amounts of carbon monoxide, biliverdin and ferrous iron (Fe^{2+}) (Abraham and Kappas, 2005). Under certain circumstances, however, HO-1 overexpression could lead to harmful effects (Tronel et al, 2013). Furthermore, deletion of HO-1 in macrophages appear to protect against diet-induced inflammation and insulin resistance in mice (Jais et al 2014). Therefore, it is not known whether HO-1 expression induced by pollutant chemicals in endothelial cells could protect or exacerbate their toxicity. In this study, we evaluated the prooxidative and proinflammatory effects induced by DEP obtained from two different sources on human microvascular endothelial cells. We also examined the role of HO-1 expression in the protection or exacerbation of DEP toxicity.

Materials and Methods

Chemicals

Gelatin, Heparin, 2',7'-Dichlorofluorescein diacetate (DCF-DA), Dihydroethidum (DHE), Protoporphrin IX cobalt chloride (CoPPIX), RIPA buffer and Thiazolyl blue tetrazolium (MTT) were obtained from Sigma-Aldrich (St. Louis, MO). Endothelial Cell Growth Supplement (ECGS) was purchased from Fisher Scientific (Pittsburgh, PA). MCDB131, RPMI-1640, Lipofectamine™ 2000, L-Glu, trypsin, Dulbecco's phosphate buffered saline 1X and penicillin-streptomycin were purchased from Invitrogen (Carlsbad, CA). Sodium pyruvate was obtained from Irvine Scientific (Santa Ana, CA). Fetal Bovine Serum (FBS) was from Hyclone (Logan, UT). Tin Protoporphrin IX dichloride (SnPPIX) was obtained from Tocris Bioscience (Elisville, MO). Ammonium Persulfate, 2-Mercaptoethanol, N,N,N',N'-tretra-methyl-ethylenediamine (TEMED), Laemmli Sample buffer, 30% Acrylamide/Bis Solution and Protein Dye Reagent Concentrate were purchased from Bio-Rad (Hercules, CA). HO-1 mouse monoclonal and Goat anti-mouse antibody-HRP conjugate antibody were purchased from Assay designs (Ann Arbor, MI). GAPDH rabbit polyclonal antibody and Goat anti-rabbit antibody-HRP conjugate antibody were purchased from Santa Cruz Biotechnology. ECL Plus Western Blotting Detection System was obtained from GE Healthcare (Buckinghamshire, UK).

Preparation of DEP organic extracts

A-DEP was obtained as a generous gift from Dr. Andrew Saxon at UCLA. It was originally generated at the National Institute of Environmental Studies (Tsukuba, Ibaraki, Japan). These particles were collected from four-cylinder Isuzu diesel engine (Isuzu, Hokkaido, Japan) as previously described (Hiura et al, 1999) and the chemical composition of these particles has been previously described (Raveh et al, 2000; Gong et al, 2007). F-DEP (Standard reference material 2975, SRM 2975) was purchased from the National Institute of Standards and Technology (NIST, Gaithersburg, MD). F-DEP has been well characterized and

used for comparisons with other types of DEP. F-DEP was collected from a Industrial forklift engine and has been analysed by NIST to have 50% DEP volume< 19.4 µm. A-DEP and F-DEP methanol extracts were prepared as previously described (Hiura et al, 1999). Briefly, the particles were suspended in methanol (200 mg/ 50 ml methanol) and sonicated on ice for 5 min. The suspension was centrifuged for 10 min at 2,500 xg at 4^{o}C. The supernatant was transferred into a new tube and centrifuged again for 10 min at 2,500 xg at 4^{o}C. The methanol supernatant was collected in a pre-weighed 50 ml polypropylene tube and dried under nitrogen gas. The dried DEP extract was dissolved in DMSO at a final concentration of 100 µg/µl and stored in the dark at -80^{o}C until use.

Cell culture and treatments

Human microvascular endothelial cells (HMEC) were grown in MCDB 131 media supplemented with 15% FBS and 1% glutamine/penicillin-streptomycin, 1.5% sodium pyruvate, ECGS (120 µg/ml) and heparin (50 µg/ml). The cells were cultured in a gelatin pre-coated 10 ml flask and were maintained at 37^{o}C in a humidified atmosphere of 5% CO_2 and 95% air. Prior to treatment, cells were replated in appropriate cell culture plate and allowed attachment for 24 hr. Treatments conducted in 6-well plates (1 well = 9.6 cm^2) containing approximately 1.5 × 10^6 cells/well in a monolayer distribution, within a total volume of 2 ml/well. HMEC were treated with either 25 µg/ml of A-DEP or F-DEP freshly prepared in complete growth media (supplemented with 1% Fetal bovine serum, FBS) just before use. In the inhibitor and inducer studies, HMEC were pre-treated with either 10 µM CoPPIX or 10 µM SnPPIX (prepared in 0.1M NaOH and pH adjusted to 7.4) for 24 hr at 37^{o}C. After the incubation, the cells were washed with PBS and then exposed to 25 µg/ml of A-DEP or F-DEP. The cells were then harvested for various assays. All DEP treatments were done for 4 hr except when assessing ROS generation via DCFH oxidation where the cells were treated for 1 hr. Bovine aortic endothelial cells (BAECs), passages of 10 to 17 were purchased from VEC Technologies and were cultured in MCDB 131

media containing penicillin-streptomycin-glutamine and 10% FBS. BAECs were seeded into 6-well plates the day before treatment and experiments for determination of ROS by DHE fluorescence.

Cell Viability

Cell viability was measured colorimetrically as previously reported (Mosmann, 1983). Briefly, cells were seeded at density of 10^5 cells/ well in a 96 well for 24 hr. The cells were then incubated for 4 hr with either A-DEP or F-DEP at a concentration of 5, 10, 25 and 50 µg/ml. After the 4 hr incubation, 20 µl of MTT (1.2 mg/ml in media) was added into each well and incubated for 2 hr at 37^{o}C. After the incubation period, the media was aspirated and 100 µl of DMSO was added to each well. The cells were further incubated for 10 min at 37^{o}C and the absorbance was measured in triplicates in plate reader at 560 nm.

Cell transfection

HMEC cells were plated in antibiotic free media in a 6-well plate at 1 x 10^5 cells/well a day before transfection. Cells were transfected with Lipofectamine™ 2000 in Opti-MEM I Reduced Serum Medium. Each well was transfected for 5 hr with 4.0 µg of either HO-1 expression plasmid (pcDNA 3.1D/V5-HO-1) or the empty vector (pcDNA 3.1D/V5-His/lacZ) for the targeted over expression of HO-1. The HO-1 expression plasmid was generated by cloning HO-1 cDNA into Hind III and Pme I sites of pcDNA 3.1D/V5-His/lacZ vector (Invitrogen). The HO-1 expressing cassette was released from the vector via restriction digestion and gel-purified. Transfection efficiency was determined using the Genlantis X-Gal Staining™ kit (San Diego, CA) according to the manufacturer's instructions. The percentage of cells with blue stain was examined under the microscope and more than 50% efficiency was obtained (supplementary figure 1). The targeted knockdown of HO-1 was achieved by transfecting cells for 5 hr with HO-1 siRNA (Qiagen, Valencia, CA) at a final concentration of 40 nM. A non-targeting siRNA Scrambled was used as control (Qiagen, Valencia, CA). The over expression and

knockdown of the HO-1 gene was assessed by qPCR and Western blot analysis at 3 days after transfection.

ROS measurement

The level of ROS was determined by DCF and DHE fluorescence. For DCF fluorescence, we used a previously reported method (Negre-Salvayre et al, 2002) with some modifications. This method involves the monitoring of the DCF fluorecence produced by the conversion of the non-fluorescence compound, dichlorodihyrofluorescein (DCFH), into the fluorescent dichlorofluorescein (DCF) at 485 nm excitation and 530 nm emission. Briefly, HMECs were loaded with 100 µg/ml Dichlorodihydrofluorescein diacetate (DCF-DA) working solution prepared in media and left to incubate for 30 min at 37^{o}C. After the incubation, the cells were washed twice with PBS before the addition of A-DEP or F-DEP. After 1 hr incubation, the media was aspirated and replaced with new complete media. Fluorescence intensity was determined with a plate reader (SynergyMx, BioTek, Vermont, USA) at an excitation wavelength of 485 nm, emission wavelength of 530 nm. For DHE fluorescence, we used a previously reported method (Owusu-Ansah et al, 2008) with some modifications. BAECs were cultured in monolayers in 6-well tissue culture plates, treated with A-DEP for 4 hours at 37^{o}C, washed with PBS and then incubated with Dihydroethidium (DHE) at a final concentration of 30 µM for 5 minutes at 37^{o}C. Cells were then washed three times with DMEM media. Cells were fixed slightly for 5 minutes in 7% formaldehyde in 1XPBS and rinsed once in 1X PBS right after fixation. 2 ml of DMEM phenol free media were added into each well. Light and fluorescence imaging were done with a Carl Zeiss Cell Observer Z.1® microscope together with the AxioVision 4.8.2 image acquisition and analysis software package (Carl Zeiss MicroImaging GmbH) data acquisition. The images were taken with a 10X eyepiece plus 20X objectives, total of 200X magnification. Cy3 (red) fluorescent channel with filter sets of 525-50 excitation/605-70 nm emission combination for DHE fluorescence and phase I transmission paired with 10X eyepiece/10X objective was used for light microscopy. The central positions for each well of a 6-well culture plate were

used and mosaic pictures of each sample were taken with 3 rows x 3 columns for a total of 9 frames with 15% overlap between neighbouring frames. Optimal fixed exposure times of 1500 ms for the red fluorescent channel and 1 ms for phase I channel in multidimensional image acquisition for each sample were used for imaging, to suit the purpose of inter-well fluorescence signal intensity comparison. Fluorescence was quantified by ImageJ software (NIH). The amount of fluorescence in each merged mosaic image was expressed as relative fluorescence units (RFU).

LDH leakage assay

Cell membrane damage was assessed by leakage of lactate dehydrogenase (LDH) from the intracellular space into the cell culture media using the CytoTox-ONE™ Homogeneous Membrane Integrity Assay kit from Promega (Madison, WI). Briefly, Cells were seeded in 96 well plates at a density of 10^5 cells/ well and allowed attachment for 24 hr. The cells were then incubated for 4 hr at 37^{o}C with 25 µg/ml of either A-DEP or F-DEP prepared in serum and pyruvate free media. After the incubation, the cells were further incubated at 22^{o}C for 10 min and equal volume of CytoTox-ONE™ Reagent was added to the cell culture medium. The cells were further incubated for 10 min at 22^{o}C and the reaction was stop by adding 50 µl of Stop Solution (per 100 µl of CytoTOX-ONE™ Reagent added). The plate was properly mixed and fluorescence was taken at excitation wavelength of 560 nm and emission wavelength of 590 nm.

Gene expression

After cells treatment, RNA was harvested using the RNeasy® Mini Kit (Qiagen). cDNA was synthesized by reverse transcriptase method using the High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems) and primers (Roche) to human cDNA are listed in Supplemental Table 1. Real time-Quantitative PCR (qPCR) was carried out using the LightCycler 480 (Roche Molecular Biochemicals) according to manufacturer's protocols. PCR conditions were: 95^{o}C for 3 min, 40 cycles of 95^{o}C for 15 sec, 60^{o}C for 30 sec and 72^{o}C for 30 sec. The

relative amount of cDNA was quantified using a standard curve constructed based on the Crossing Point (Cp) values of each dilution sample. The Cp values were determined using the second derivative analysis (LightCycler Absolute Quantification Software). Samples were normalized to β-actin quantity measured by qPCR for each sample.

Monocyte Adhesion assay

Human acute monocyte leukemia (THP-1) cells were cultured as a suspension at 3.0 x 10^5 cells/ ml of RPMI 640 supplemented with 10% FBS in 75 cm^3 flask. After the cells had reached a density of 10^6 cells/ ml, they were centrifuged at 3,500 xg for 10 min and the pellet was washed with PBS. This process was repeated twice and the cells were resuspended in RPMI (without FBS) at a density of 5 x 10^6 cells/ ml. 5 µl of 1mM calcein AM (Invitrogen, Eugene, Oregon) was added to the cell suspension at a final concentration of 5 µM and mixed thoroughly. The cells were then incubated for 37^{o}C for 30 min in 50 ml polypropylene tubes. After the incubation, the cells were washed twice with pre-warmed (37^{o}C) RPMI and resuspended in RPMI at a density of 5 x 10^6 cells/ml. 300 µl of the calcein-labelled cell suspension was added to the pre-treated (25 µg/ ml A-DEP or F-DEP exposed) HMEC cells in 24 well plate. The cells were further incubated for 10 min at 37^{o}C. The non-adherent cells were removed by washing with prewarmed RPMI four times and gently swirled. Excess liquid were blotted onto filter paper and 300 µl of PBS was added to each well. The fluorescence was measured using a fluorescein filter set at 494 nm excitation and 517 nm emissions. Imaging of the cells was done using the fluorescent microscope. We also studied whether DEP treatment (6.25 to 50 µg/ml) of THP-1 cells for 4 hours, would induce adhesion to untreated HMECs. LPS at 1 µg/ml was used as a positive control. Cells were labeled with Calcein AM as indicated above prior to the treatments. Since both DEP and LPS treatments induced significant adhesion to both treated and untreated polysterene tissue culture (TC) plates, which made difficult to assess the specific adhesion to HMEC monolayers, we tested THP-1 non-specific adhesibility to the TC plates. Plates

were washed with PBS twice and fluorescence was measured as indicated above.

Western blot analysis

Western blot was carried out on the whole cell extracts after treatment. 30 µg of total protein was separated by SDS-PAGE at 25mV and then transferred to Immun-Blot PVDF Membranes (Bio-Rad, Hercules, CA). HO-1 protein was detected using anti-HO-1 antibody (1:2000) and goat anti-mouse antibody (1:5000) conjugated to HRP according to manufacturer's protocol. GAPDH protein was detected by GAPDH antibody (1:2000) and goat anti-rabbit antibody. GAPDH was used for the normalisation of protein loading and blots were developed with the ECL reagent according to manufacture' protocol.

Statistical Analyses.

All data were expressed as mean ± SEM. Statistical analyses was performed using one-way analysis of variant (ANOVA) with Bonferroni post-hoc test for comparisons between groups. Statistical analyses were carried out using Graphpad Prism Software. Differences were considered statistically significant at the p-value of < 0.05.

Results

DEP from different sources induce cytotoxic prooxidative and proinflammatory effects in endothelial cells

Human microvascular endothelial cells (HMEC) were used to evaluate the biological effects induced by DEP generated from two different sources, automobile (A-DEP) and forklift (F-DEP). We tested DEP cytotoxic effects in a 5-50 µg/ml range. While treatment with both A-DEP and F-DEP for 4 hr led to a dose-dependent decrease in HMEC viability, these effects were more pronounced with the A-DEP (Figure 1A). Thus, compared with controls, A-DEP caused a significant decrease ($p<0.0001$) in HMEC viability across the entire range of concentrations tested (5-50 µg/ml) while F-DEP only significantly decreased viability at 25 and 50 µg/ml ($p<0.05$) (Figure 1A). In addition, at 25 µg/ml, F-DEP led to a 20% decrease in cell viability while A-DEP resulted in twice as much (~ 40%, $p<0.0001$) (Figure 1A). We examined additional parameters at a DEP concentration of 25 µg/ml, as this allowed for > 50% cell survival at 4 hours. This concentration is very likely within the range of realistic exposures to air pollutants. While it is not possible to reconcile in vitro with in vivo dosimetry in the case of endothelial cells, it has been reported that in macrophages and bronchial epithelial cells, in vitro DEP extract concentrations in the dose range 1-100 µg/ml correspond to realistic particle concentrations at hotspots of deposition in the respiratory tract (Phalen et al, 2006). It is possible that similar hotspots could exist in the pulmonary interstitium or cardiovascular tree. We noted that both A-DEP and F-DEP led to significant LDH leakage ($p< 0.01$, Figure 1B), significant oxidation of DCFH ($p<0.0001$), suggestive of increased production of reactive oxygen species (ROS) as judged by a DCF-based assay (Figure 1C), upregulation of HO-1 ($p<0.0005$, Figure 1D&E), increased expression of proinflammatory cytokines such as IL-8 and MCP-1, Unfolded Protein Response (UPR) genes such as ATF4, and cell adhesion molecules like ICAM-1 and VCAM-1 (Figure 2). Interestingly, despite triggering a similar degree of DCFH oxidation (Figure 1C), A-DEP led to a greater upregulation of HO-1 (Figure

1D&E), IL-8, ICAM-1 and VCAM-1 as compared with F-DEP ($p < 0.0001$, Figure 2A), which was accompanied by greater monocyte adhesion ($p<0.0001$) (Figure 2B&C). On the contrary F-DEP induced greater levels of MCP-1 and ATF4 ($p<0.0001$, Figure 2A). Interestingly, DEP treatment (25 µg/ml) of THP-1 monocytes led to decreased monocyte adhesion, probably due to increased cytotoxicity. DEP at lower concentrations (6.25 µg/ml) did not induce cytotoxicity and led to small increase in non-specific cell adhesibility that was significant in some but not in all experiments (data not shown). Overall, these data indicate that DEP induce cytotoxic prooxidative and proinflammatory effects in HMEC in a degree that varies depending on the type and source of DEP, which markedly differ in their chemical composition. For instance, we have shown that A-DEP has a large content of organic material (Gong et al, 2007), reported to be larger than in the F-DEP (DeMarini et al, 2004). Indeed, A-DEP has a much larger percentage of extractable organic materials (EOM) as compared with F-DEP (DeMarini et al, 2004).

HO-1 expression protects against DEP-induced toxic effects.

We evaluated whether HO-1 expression, highly upregulated by both types of DEP, was beneficial or merely served as a marker of DEP-induced injury. We assessed the effects of HO-1 ablation on A-DEP induced actions by the use of siRNA. About 60% knockdown of HO-1 mRNA expression was achieved using siRNA ($p<0.0001$) as compared with scrambled siRNA by qPCR (Figure 3A), which resulted in a 30-fold decrease in the HO-1 protein level in siHO-1 silenced cells as compared with scrambled controls (Figure 3B). HO-1 knockdown led to a marked enhancement in ROS production induced by A-DEP (Figure 3C). In addition, A-DEP led to upregulation of IL-8 ($p<0.0001$) (Figure 3D) and ICAM-1 ($p<0.001$) (Figure 3E) mRNA expression levels in siHO-1 silenced cells as compared with scrambled controls. These data indicates that HO-1 played an antioxidant, anti-inflammatory and cytoprotective role against DEP-induced effects in HMECs.

HO-1 mediated protection varies with the type of DEP.

Since HO-1 cytoprotective effects have mostly been linked to its enzymatic activity (Araujo et al, 2012), we evaluated whether inhibition of HO enzymatic activity would result in similar effects to those induced by HO-1 siRNA knockdown and if these effects were similar with both types of DEP. HMEC were pre-treated with 10 μM SnPPIX, a known HO inhibitor (Sardana and Kappas, 1987), for 24 hr and then exposed to 25 μg/ml A-DEP or F-DEP for 1 or 4 hours. Pre-treatment with SnPPIX led to enhanced DEP-induced DCFH oxidation after 1 hour exposure (Figure 4A) and increased LDH leakage after 4 hours (Figure 4B) despite not modifying cell survival (Figure 4C). In addition, SnPPIX led to upregulation of IL-8, MCP-1, ICAM-1, and VCAM-1 mRNA expression levels ($p<0.0001$) in the presence of either 25 μg/ml A-DEP or F-DEP (Figure 4D). Interestingly, inhibition of HO-1 by SnPPIX led to a greater enhancement of DEP-induced DCFH oxidation ($p<0.005$, Fig. 4A) and greater upregulation of MCP-1 and ICAM-1 ($p<0.0001$, Figure 4D) in F-DEP as compared with A-DEP. Indeed, Sn pre-treatment resulted in ~ 9.4 and 26.4-fold increase in IL-8, and ICAM-1 mRNA expression after treatment with F-DEP, which was clearly larger than the 1.5 and 4.2-fold increase in mRNA expression of same genes after treatment with A-DEP. This suggests that while HO-1 expression protected against the pro-oxidant and pro-inflammatory effects of both DEPs, the degree of such protection varied with the different type of DEP.

HO-1 overexpression confers protection against DEP-induced cellular effects

In order to evaluate whether HO-1 overexpression would attenuate DEP-induced effects, HMEC were pre-treated with 10 μM CoPPIX, a known pharmacological inducer of HO-1 gene (Araujo et al, 2008; Tsoyi et al, 2009), for 24 hr and then exposed to 25 μg/ml A-DEP or F-DEP for 1 or 4 hrs. Pre-treatment with CoPPIX triggered significant upregulation of HO-1 over the levels of induction exerted by both DEPs (Figure 5A&B), which led to significant improvement in cell viability

(Figure 5C), in spite of not altering DEP-induced DCHF oxidation (Figure 5D). As expected, CoPPIX pre-treatment led to decreased DEP-induced MCP-1 expression (Figure 5E). However, it led to upregulation of IL-8, ICAM-1 and VCAM-1, in the presence of DEP when compared to non pre-treated cells ($p<0.0001$, Figures 5E&F). Thus, while CoPPIX-induced HO-1 expression led to decreased cytotoxicity and lower expression of some inflammatory signals such as MCP-1, it resulted in greater expression of other proinflammatory signals instead.

We hypothesized that some of the CoPPIX-mediated actions could be independent of the induction of HO-1. Therefore, we decided to evaluate the effects of HO-1 overexpression by using a HO-1 expression plasmid, pcDNA 3.1D/V5-HO-1. Transfection of HMECs with pcDNA 3.1D/V5-HO-1 led to significant overexpression of HO-1mRNA ($p<0.0001$) (Figure 6A) and protein (Figure 6B) as compared with cells transfected with the control plasmid, pcDNA 3.1D/V5-His/LacZ. HO-1 overexpression led to significant reduction of DEP-induced ROS generation ($p<0.0001$) (Figure 6C), which was accompanied by improvement of cell viability ($p<0.05$, Figure 6D). Likewise with CoPPIX, HO-1 overexpression led to decreased expression of MCP-1 both at baseline and after treatment with DEP ($p<0.001$, Figure 6E). However, unlike COPPIX, HO-1 overexpression led to decreased levels of IL-8 at baseline (Figure 6E), suggesting that CoPPIX-mediated increased of IL8 was not due to increased expression of HO-1. Importantly, both pharmacological and genetic induction of HO-1 led to increased protection against DEP-induced cytotoxicity.

Discussion

Our data indicate that DEP from two different sources induce significant but different degrees of cytotoxic prooxidative and proinflammatory effects in endothelial cells. Likewise, both DEPs led to significant but different degrees of HO-1 upregulation, which played antioxidant and anti-inflammatory effects that contributed to limit DEP-induced cytotoxicity, especially if HO-1 was overexpressed prior to the treatment with DEP.

DEP toxic effects in the vasculature are important as exposure to air pollution has been associated with endothelial cell dysfunction (Krishnan et al, 2012), myocardial infarction and stroke (Miller et al, 2007), mostly in relation to its particulate matter (PM) components (Brook et al, 2010). Studies with experimental animals are supportive of a causal association between PM exposure and atherosclerosis (Araujo, 2011), reason why it is regarded as a novel cardiovascular risk factor (Brook et al, 2010; Araujo and Brook, 2011). Motor vehicle emissions like Diesel Exhausts (DE) are important contributors to the air pollutants in urban settings such as highly populated cities. Diesel exhaust particles (DEP) represent an important proportion of the fine and ultrafine PM in those settings (Lloyd and Cackette, 2001). Various mechanisms have been proposed to mediate PM-induced cardiovascular effects (Araujo and Nel, 2009). It appears that PM ability to enhance ROS generation is key in the induction of systemic vascular pro-inflammatory effects (Araujo, 2011). Endothelial cells may play an important role in the PM induction of vascular effects as they form a monolayer lining the luminal side of the vasculature and thus, they represent a first point of contact in the vascular wall for toxic chemicals that could access the circulating blood. Inhaled nanoparticles get deposited in the lungs, which could undergo transcytosis across the epithelia of the respiratory tract into the interstitium and access the blood circulation directly or via lymphatics (Oberdorster et al., 2005). However, it has been difficult to document the presence of whole ambient particles encountered in “real life” such as DEP. Alternatively, the particles deposited in the lungs get internalized into the cells by

endocytosis, are processed, and their chemicals could access the pulmonary interstitium, where they could interact with endothelial cells and/or get translocated into the systemic circulation. Indeed, it has been reported that I.T. instillation of ultrafine particles can induce ROS generation in pulmonary endothelial cells using a perfused lung model with Hydroethidine (HE) fluorescence imaging (Mo et al, 2009). Likewise, a DEP organic extract, which concentrate a large portion of the toxicity exerted by whole DEP, is highly potent at inducing prooxidative effects in bronchial epithelial cells and macrophages (Li et al, 2002). We evaluated the differential toxicity of the methanol extract of two different DEPs on HMECs, derived from the combustion of diesel fuels by an automobile vs. forklift engines.

Our results indicate that both A- and F-DEP induced different degrees of cytotoxicity and inflammatory responses in HMECs (Figures 1&2), likely in relation to their different chemical composition (DeMarini et al, 2004). This is important since the degree of cardiovascular effects exerted by air pollutants may depend on their chemical composition. We have reported that ambient ultrafine particles exert larger proatherogenic effects than the bigger fine particles likely due, at least in part, to their different chemical composition (Araujo et al, 2008; Araujo and Nel, 2009). Indeed, concentrated ultrafine particles in downtown Los Angeles displayed a greater content of organic carbon and polycyclic aromatic hydrocarbons than fine particulate (Araujo et al, 2008; Araujo and Nel, 2009). Likewise, differences in fuels, engines and combustion processes have been shown to lead to DEPs that are markedly different in their chemical composition, in association with largely different mutagenic potentials in Salmonella (DeMarini et al, 2004). Thus, A-DEP has a content ~ 13 times larger in extractable organic material (26.3%) than F-DEP (2%) (DeMarini et al, 2004). In addition, both whole A-DEP as well as its organic extracts have a markedly higher content of PAHs than F-DEP (DeMarini et al, 2004), which may be a good proxy for the prooxidative potential of DEP in a similar manner how it is for ambient ultrafine particles (Araujo et al, 2008). This is consistent with the work of Marano et al

(Marano et al, 2002) who suggested that DEP-induced toxicity in lung epithelial cells was in relation to their content in organic carbon (OC) as desorption of DEP in the intracellular space resulted in the release of OC and PAHs with the consequent formation of electrophilic intermediates and ROS (He et al, 2008). ROS can lead to Nrf2 activation via the MAPK-NF-kappa B pathway (He et al, 2008; Kim and Vaziri, 2010;) and subsequent upregulation of HO-1 as observed in HMECs in our study, but it can also occur in other cells present in the vasculature such as smooth muscle cells or cells that infiltrate the vascular wall, such as macrophages and neutrophils. Interestingly however, both A- and F-DEP led to a similar increase in DCFH oxidation, suggestive of increased ROS formation, but different degrees of DEP-induced upregulation of HO-1 and cytotoxicity, which suggests that both effects could be due to both ROS-dependent and independent pathways. In addition, while A-DEP induced higher levels of IL-8, consistent with the higher levels of CAMs, cell adhesion and cytotoxicity, F-DEP led to higher levels of ATF4 and MCP-1 instead (Figure 2). This suggests that different components of DEP may activate different proinflammatory pathways.

DEP prooxidative effects in HMECs are consistent with similar effects in macrophages, bronchial epithelial cells, lung microsomes (Hiura et al, 1999; Marano et al, 2002) and human aortic endothelial cells (Li et al, 2010; Montiel-Davalos et al, 2010). DEP-induced upregulation of HO-1 in HMECs is consistent with similar effects in human aortic endothelial cells (Li et al, 2009) and macrophages (Li et al, 2004). We have previously shown that DEP and oxidized phospholipids synergized in the induction of HO-1 as well as other 1,555 genes in HMECs (Gong et al, 2007). Interestingly, concentrated ambient ultrafine but not fine particles led to the upregulation of HO-1 in the liver of ApoE KO mice (Gong et al, 2007), which suggests that the smallest particulate may be more active at inducing HO-1 in-vivo. Our results are also consistent with the observations that DEP lead to enhancement of ROS formation in bovine aortic endothelial cells (Yin et al, 2013a) and Human aortic endothelial cells (Li et al,

2009), via assessment of DCF oxidation or DHE staining (Supplementary Figure 2).

We hypothesized that DEP-induced upregulation of HO-1 could serve as a protective mechanism against DEP harmful effects. Our data indicate that silencing of HO-1 or inhibition of HO enzymatic activity by SnPPIX led to aggravated DEP-induction of ROS production, cytotoxicity and increased expression of proinflammatory genes, in support of our hypothesis. In addition, genetic or pharmacological induction of HO-1 overexpression, further protected against DEP-induced ROS production and cytotoxicity. However, while HO-1 overexpression by the plasmid transfection protected against DEP-induction of proinflammatory genes, CoPPIX treatment did not attenuate it and instead, it aggravated DEP-induction of IL-8 (Figure 5E). These apparently paradoxical results are consistent with a previous study, which showed that 24-hour treatment of HMEC-1 cells with CoPPIX led to enhanced IL-8 mRNA expression despite induction of HO-1 (Loboda et al, 2005). CoPPIX-mediated induction of IL-8 was likely due to other effects collateral to HO-1 induction as plasmid HO-1 overexpression resulted in decreased IL-8 expression instead.

In summary, this study demonstrates that exposure of HMEC to two different DEPs significantly triggered ROS generation, a proinflammatory response and enhanced cytotoxicity, effects that were more pronounced by the DEP with the higher organic content. Exposure to DEP triggered HO-1 induction, which partially protected against DEP harmful effects. Overexpression of HO-1, prior to the treatment with DEP, resulted in additional protection against those effects. Future work is required to determine how HO-1 protects against DEP-induced effects in endothelial cells and whether HO-1 mediated protection occurs in vivo. HO-1 and their byproducts could serve as therapeutic targets in an attempt to ameliorate the cardiovascular effects of air pollution, as it is currently being explored in experimental studies and clinical trials for the treatment of diseases where oxidative stress and/or inflammation play a major role such as coronary

artery disease, pulmonary hypertension, idiopathic pulmonary fibrosis (e.g. Zuckerbraun et al, 2006; Issan et al, 2014), among others.

Conflict of Interest Statement

There are no conflicts of interest associated with this work.

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Figure Legends

Fig.1 DEP is prooxidant and cytotoxic in endothelial cells. (A) HMECs were exposed to 5- 50 µg/ml of either A-DEP or F-DEP for 4 hr and cell viability was determined by MTT. (B) Cells were exposed to 25 µg/ ml of either A-DEP or F-DEP for 4 hr and LDH leakage were determined as described in the Materials and Methods. (C) ROS levels were determined by DCF-DA method after 1 hr exposure to 25 µg/ ml of either A-DEP or F-DEP. (D&E) HO-1 mRNA (D) and HO-1 protein (E) expression levels were determined by RT-qPCR and western blot respectively following 4 hr exposure. Values shown are mean±SEM of three different experiments done in triplicate (n=3). *p<0.05, **p<0.001, ***p<0.0001 as compared with controls. # A-DEP vs F-DEP p<0.0001.

Fig.2 DEP induce proinflammatory genes and monocyte adhesion in HMEC. Cells were exposed to 25 µg/ml of either A-DEP or F-DEP for 4 hr and (A) IL-8 mRNA, MCP-1mRNA, ATF4 mRNA, ICAM-1 mRNA and VCAM-1mRNA levels were quantified by real-time qPCR as described in the Materials and Methods. (B&C) Monocyte adhesion was quantified by flourimeter (B) and (C) immunofluorescence microscope. Values are mean±SEM of three different experiments done in triplicate (n=3). *p<0.05, **p<0.001, ***p<0.0001 as compared with controls. # A-DEP vs F-DEP, p < 0.0001

Fig.3 Silencing of HO-1 exacerbates DEP effects on endothelial cells. HO-1 was silenced in HMEC using HO-1 siRNA (final concentration of 40 nM) and a scrambled control (final concentration of 40 nM) was used to examine the specificity of the silencing as described in Materials and Methods. (A) Real-time qPCR of HO-1 mRNA expression after silencing and (B) Western blot analysis of the HO-1 expression in silenced cells. (C) ROS levels and (D&E) mRNA levels of IL-8 (D), and ICAM-1 (E) were determined in the silenced cells after exposure to 25 µg/ml of A-DEP as described in the Materials and Methods. Values are mean±SEM of three different experiments done in triplicate (n=3). *p<0.05,

**p<0.001, ***p<0.0001, , as compared to scrambled controls. # (p<0.05) significant difference between A-DEP treated and untreated cells.

Fig.4 HO-1 protection from DEP is mediated by its enzymatic activity. HMEC were pre-treated with 10 µM SnPPIX for 24 hr before exposure to 25 µg/ml of either A-DEP or F-DEP (A) ROS levels were determined by the DCF-DA method after 1 hr exposure. (B) LDH leakage were determined after 4 hr exposure as described in the materials and methods. (C) Cell viability were determined by the MTT method after 4 hr exposure, and (D) HO-1, IL-8, MCP-1, ICAM-1 and VCAM-1 gene expressions were determined by RT-qPCR after 4 hr exposure. Values are mean±SEM of three different experiments done in triplicate (n=3). *p<0.05, **p<0.001, ***p<0.0001 for comparisons between SnPPIX pre-treated and non pre-treated cells. # A-DEP vs F-DEP in the presence of SnPPIX (p<0.005).

Fig.5 CoPPIX prevents DEP-induced toxicity. (A&B) HMEC were pre-treated with 10 µM CoPPIX for 24 hr before exposure to 25 µg/ml of either A-DEP or F-DEP for 4 h. HO-1mRNA (A) and HO-1 protein expression (B) were determined by qPCR and western blot respectively. (C) Cell viability was determined after 4 hr exposure to 25 µg/ml of either A-DEP or F-DEP as described in the materials and methods. (D) ROS levels were determined by the DCF-DA method after 1 hr exposure. (E) IL-8mRNA, MCP-1mRNA and (F) ICAM-1mRNA, VCAM-1mRNA were determined by real-time qPCR after 4 hr exposure as described in the Materials and Methods. Values are mean±SEM of three different experiments done in triplicate (n=3). *p<0.05, **p<0.001, ***p<0.0001 for comparisons between CoPPIX pre-treated and non pre-treated cells.

Fig.6 HO-1 over expression inhibits DEP prooxidant effects in vascular effects. HO-1 gene was over expressed in HMEC using HO-1 plasmid (pcDNA 3.1D/V5-HO-1; final concentration of 4 µg) and empty vector as control (pcDNA 3.1D/V5-His/LacZ ; final concentration of 4 µg) as described in Materials and

Methods. (A) Real-time qPCR of HO-1 gene after over expression, (B) Western blot analysis of the over expressed HO-1 in HMEC cells. (C) ROS levels were determined by DCF-DA after 1 hr exposure of the transfected cells to 25 µg/ml of A-DEP (D) Cell viability was determined after 4 hr exposure of the transfected cells to 25 µg/ml of A-DEP (E) mRNA expression of IL-8, and MCP-1 was determined by qPCR. Values are mean±SEM of three different experiments done in triplicate (n=3). *$p<0.05$, **$p<0.001$, ***$p<0.0001$, for comparisons between HO-1 transfected and LacZ controls. # significant difference between A-DEP treated and untreated cells ($p<0.05$).

Figure 1

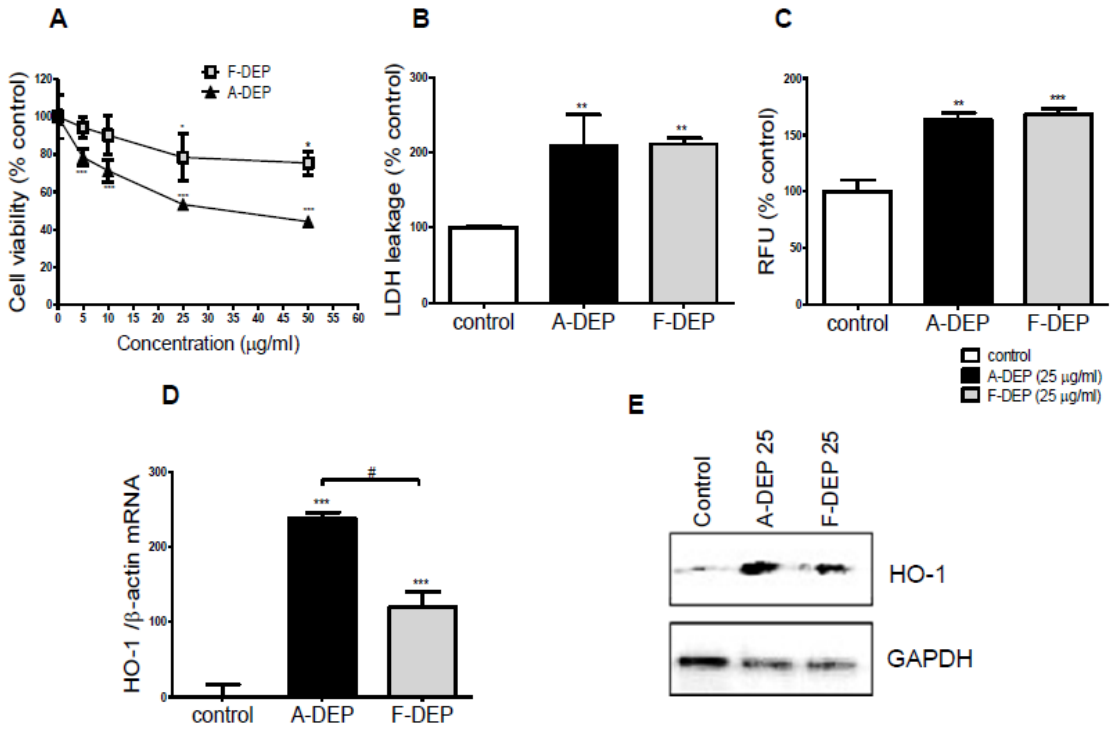

Figure 1

Figure 2

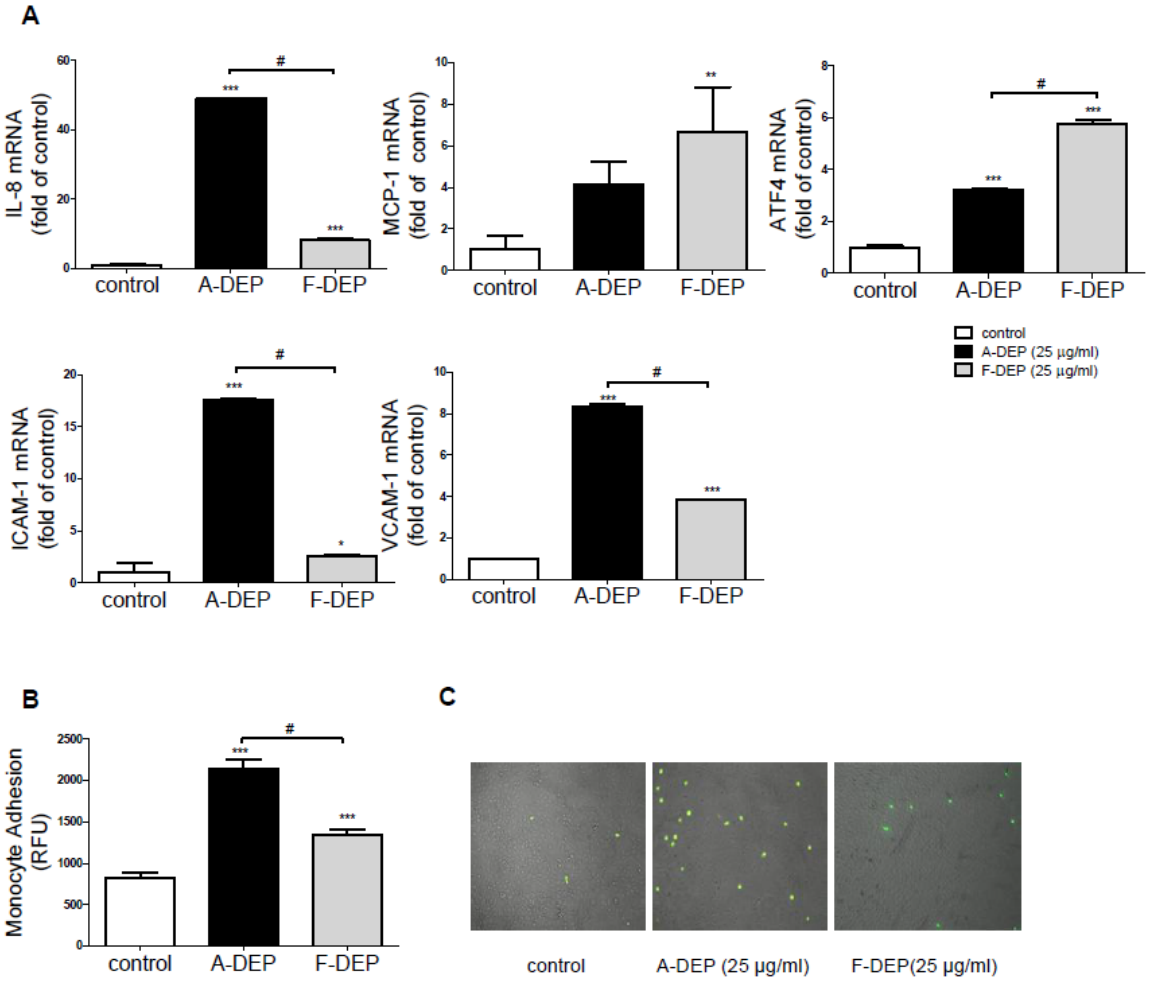

Figure 2

Figure 3

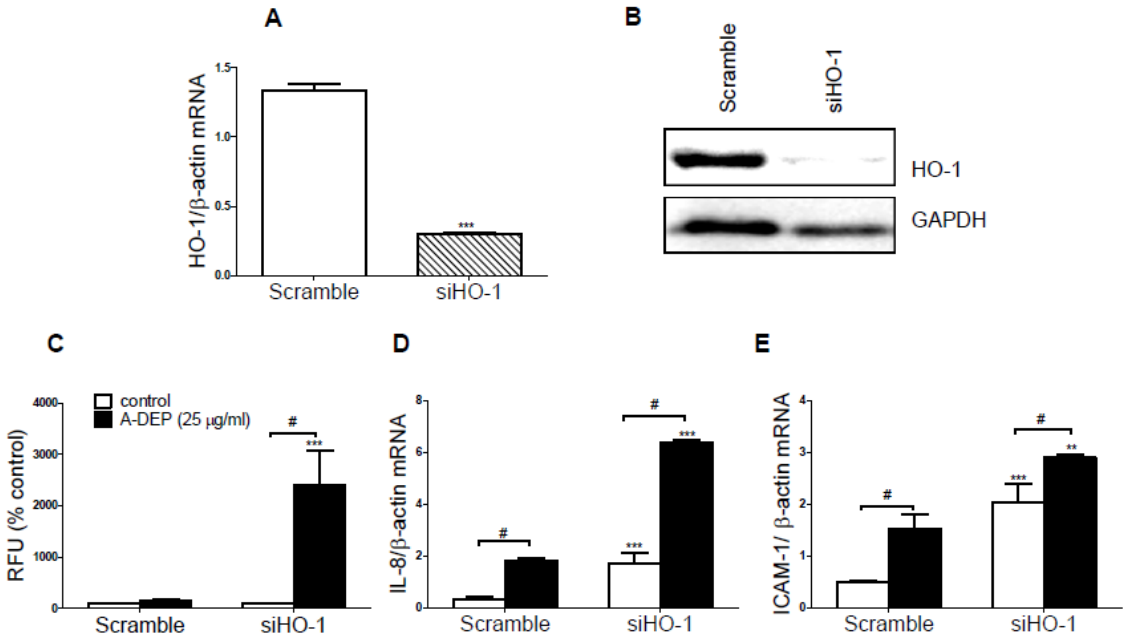

Figure 3

Figure 4

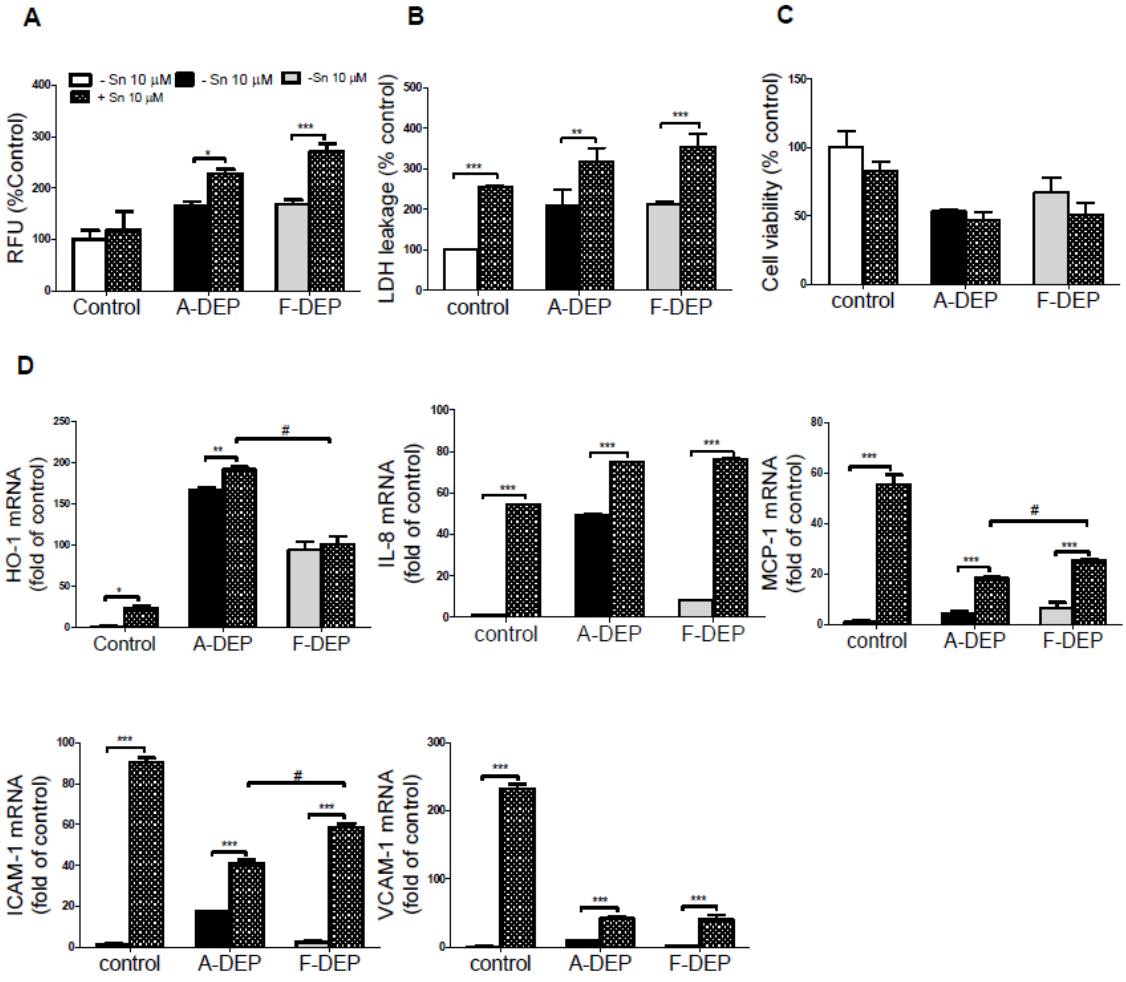

Figure 4

Figure 5

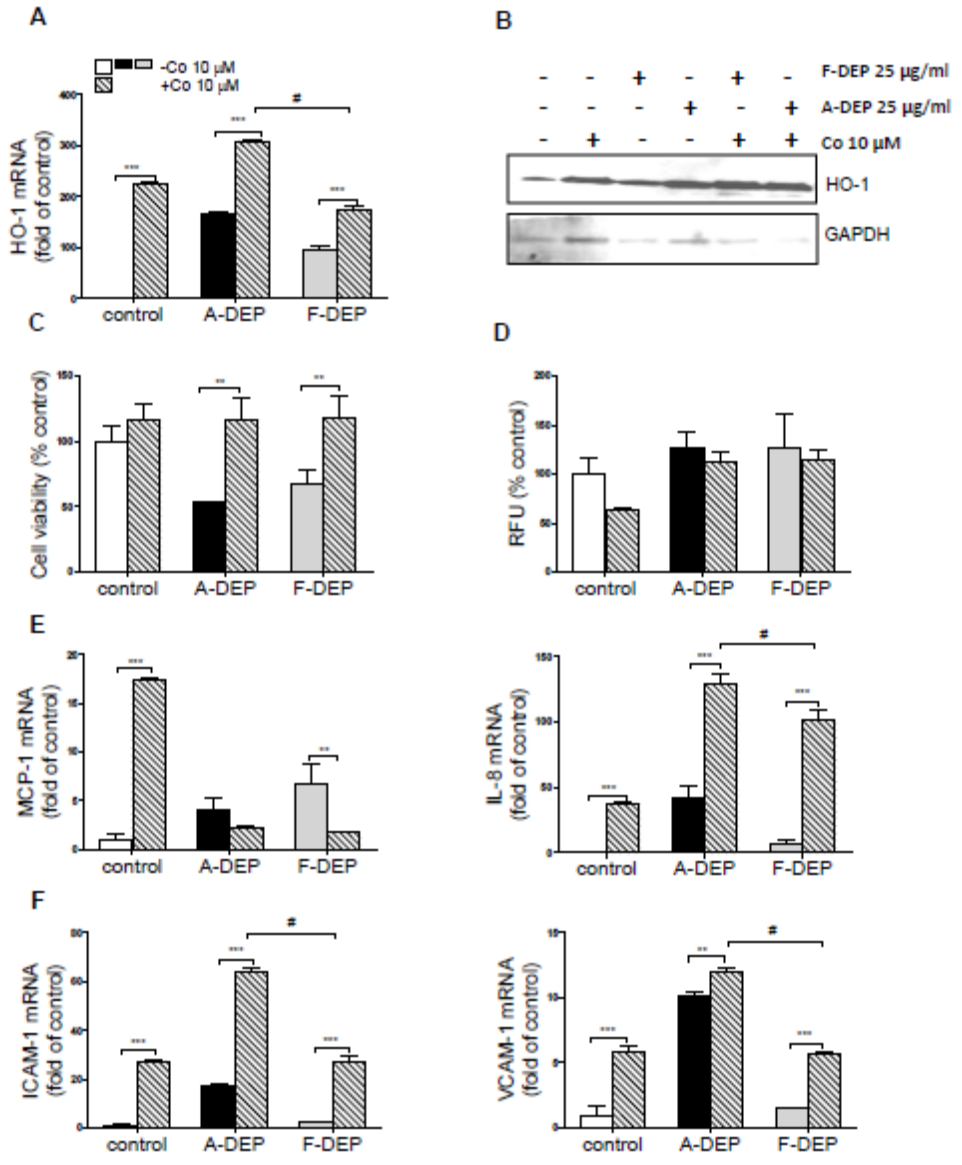

Figure 5

Figure 6

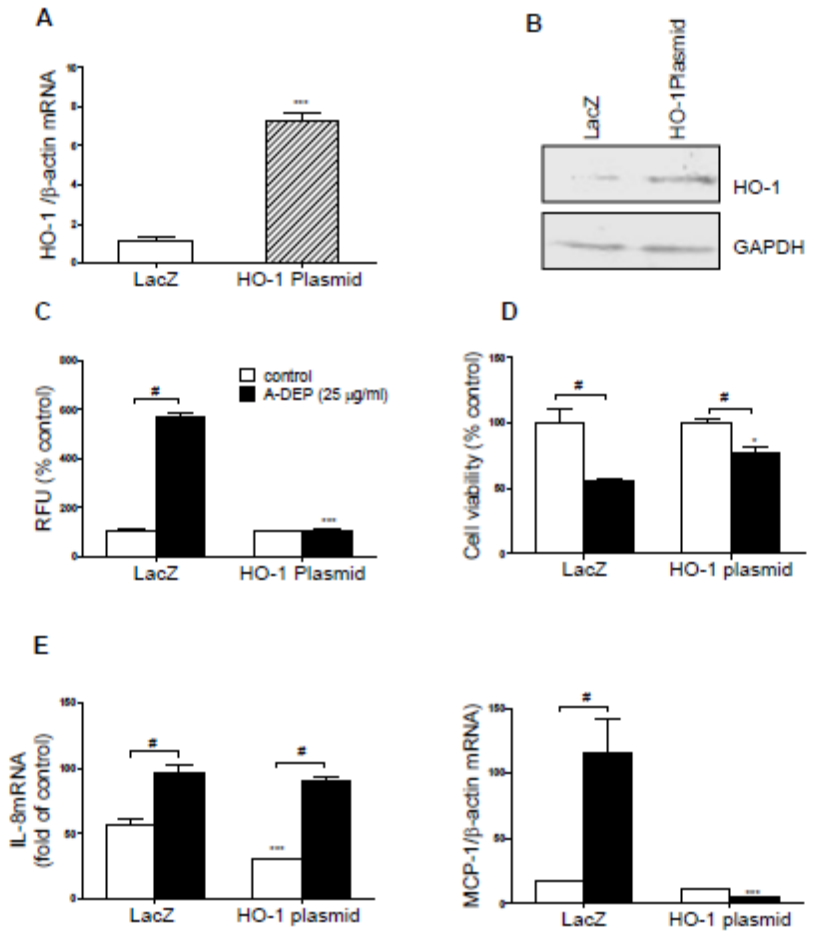

Figure 6

Highlights

- We examined the effects of HO-1 induction on diesel exhaust particles DEP effects in HMEC
- DEP exerts cytotoxic and inflammatory effects on HMEC
- DEP-induced HO-1 protein expression in HMEC
- HO-1 protects against the oxidative stress induced by DEPs
- HO-1 attenuates the proinflammatory effects of DEPs