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Air Pollution Upregulates Endothelial Cell Procoagulant Activity via Ultrafine Particle-Induced Oxidant Signaling and Tissue Factor Expression

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Air pollution exposure is associated with cardiovascular events triggered by clot formation. Endothelial activation and initiation of coagulation are pathophysiological mechanisms that could link inhaled air pollutants to vascular events. Here we investigated the underlying mechanisms of increased endothelial cell procoagulant activity following exposure to soluble components of ultrafine particles (soluble UF). Human coronary artery endothelial cells (HCAEC) were exposed to soluble UF and assessed for their ability to trigger procoagulant activity in platelet-free plasma. Exposed HCAEC triggered earlier thrombin generation and faster fibrin clot formation, which was abolished by an anti-tissue factor (TF) antibody, indicating TF-dependent effects. Soluble UF exposure increased TF mRNA expression without compensatory increases in key anticoagulant proteins. To identify early events that regulate TF expression, we measured endothelial H_2O_2 production following soluble UF exposure and identified the enzymatic source. Soluble UF exposure increased endothelial H_2O_2 production, and antioxidants attenuated UF-induced upregulation of TF, linking the procoagulant responses to reactive oxygen species (ROS) formation. Chemical inhibitors and RNA silencing showed that NOX-4, an important endothelial source of H_2O_2, was involved in UF-induced upregulation of TF mRNA. These data indicate that soluble UF exposure induces endothelial cell procoagulant activity, which involves *de novo* TF synthesis, ROS production, and the NOX-4 enzyme. These findings provide mechanistic insight into the adverse cardiovascular effects associated with air pollution exposure.

Key words: **air pollution; NADPH oxidases; reactive oxygen species; tissue factor; thrombin generation.**

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Epidemiological studies reveal a strong association between air pollution exposure and increased cardiovascular (CV) morbidity and mortality (Pope *et al.*, 2004, 2006). Exposure to air pollution can increase thrombus formation *in vivo* in humans (Lucking *et al.*, 2008) and animal models (Nemmar *et al.*, 2003), and may lead to thrombotic events such as acute myocardial infarctions (Nawrot *et al.*, 2011; Peters *et al.*, 2004) and venous thromboembolism (Baccarelli *et al.*, 2008). One of the major components of air pollution is particulate matter (PM), which is categorized based on the diameter of the particles: coarse (10–2.5 μm), fine (2.5–0.1 μm), and ultrafine (UF; <0.1 μm). The United States Environmental Protection Agency currently monitors and regulates coarse and fine PM, but increasing evidence suggests that UF particles, which are not currently regulated, are particularly important mediators of CV effects of air pollution (Nel, 2005). Although UF particles comprise only a small portion of ambient PM by mass concentration, they constitute the majority of particle number and have a large surface area-to-mass ratio. UF particles can deposit in the alveolar region of the lung due to their small diameter (Kreyling *et al.*, 2004). These extremely small particles are not efficiently cleared by alveolar macrophages (Gonzalez *et al.*, 1996), and they may persist in the lung, increasing the potential to cause adverse effects. Furthermore, soluble components of UF particles (soluble UF) could enter the vasculature by traversing the alveolar-capillary barrier (Wallenborn *et al.*, 2007). These soluble components, which include transition metals, induce oxidative stress and procoagulant activity in animal and human models (Araujo *et al.*, 2008; Sorensen *et al.*, 2005); however, the mechanisms behind these responses have not been delineated.

A causal link between vascular effects of PM exposure and procoagulant responses is suggested by the finding that soluble UF exposure increases tissue factor (TF) mRNA expression in human pulmonary artery endothelial cells (Karoly *et al.*, 2007). TF is not highly expressed on endothelial cells under normal

physiological conditions, but surface expression is induced by various stimuli, including inflammatory cytokines, endotoxin, and hypoxia (Chu, 2005). Altered expression and activity of TF is of particular interest because, once activated, this membrane-bound protein is the primary initiator of the extrinsic coagulation pathway, resulting in thrombin generation and thrombus formation. Elevated TF levels are associated with adverse CV effects including thrombosis and atherosclerosis (Mackman *et al.*, 2007).

TF expression is redox-regulated, and TF mRNA and protein levels are modulated by reactive oxygen species (ROS) in vascular cells (Herkert and Gorlach, 2002). Endothelial cells produce ROS in response to several physiological and pathological stimuli, including exposure to UF particles (Mo *et al.*, 2009). One major cellular source of ROS production is the NADPH oxidase (NOX) family of enzymes (Lassegue *et al.*, 2012). Endothelial cells express mainly NOX-2 and NOX-4 isoforms with variable expression levels between endothelial cell types (Guzik *et al.*, 2004). Nox enzymes produce basal physiological levels of ROS, and NOX activity is augmented upon agonist stimulation producing increased ROS levels that play a role in pathophysiological responses (Lassegue *et al.*, 2012). For example, thrombin enhances TF expression and procoagulant activity in vascular smooth muscle cells (VSMC) by stimulating ROS production by NOX (Herkert *et al.*, 2002). Furthermore, NOX-induced oxidative stress in vascular cells is involved in the pathogenesis of CV disorders (Madamanchi *et al.*, 2005), potentially through these procoagulant mechanisms.

The objective of our study was to determine if soluble UF induces procoagulant responses in human endothelial cells and to define the mechanisms behind these effects. We show here that endothelial cells exposed to soluble UF induce faster onset of thrombin generation and fibrin clot formation via TF upregulation, which involves ROS production and the NOX-4 enzyme.

MATERIALS AND METHODS

Reagents and chemicals. Human coronary artery endothelial cells (HCAEC), endothelial growth medium (EGM-2), and EGM-2 Bullet Kit were obtained from Lonza (Walkersville, MD). All other chemicals and reagents were from Sigma Chemical Company (St. Louis, MO) unless otherwise stated.

Cell culture. HCAEC were cultured in EGM-2 media supplemented with the EGM-2 Bullet Kit. Cells were grown to confluence and used between passages 5–8.

UF particles. UF particles collected from February to May 2007 in Chapel Hill, NC were analyzed for chemical components (Table 1) by the Research Triangle Institute (Research Triangle Park, NC) as previously described (Becker *et al.*, 2005). The water-soluble fraction of UF particles was obtained as previously described (Karoly *et al.*, 2007).

Calibrated automated thrombography assay. Platelet-free plasma (PFP) from healthy donors was prepared as previously described (Machlus *et al.*, 2009). Cellular procoagulant activity was measured as previously described with alterations (Campbell *et al.*, 2009). Briefly, HCAEC were cultured on 96-well plates and exposed to soluble UF for 6 h. Following exposure, cells and PFP were incubated with an inhibitory anti-human TF monoclonal inhibitory antibody or control mouse IgG for 15 min at 37°C. PFP with antibodies, MP reagent containing phospholipids (4μM; 60% phosphatidylcholine/20% phosphatidylethanolamine/20% phosphatidylserine; Diagnostica Stago, Parsippany, NJ), and a fluorogenic thrombin substrate (Z-Gly-Gly-Arg-AMC; Diagnostica Stago) were then added to the cells (Machlus *et al.*, 2009). Reactions were calibrated against wells containing a thrombin calibrator (α2-macroglobulin/thrombin complex; Diagnostica Stago). Thrombin generation was measured using a Fluoroskan Ascent fluorometer (ThermoLabsystem, Helsinki, Finland). Thrombinoscope software version 3.0.0.29 (Thrombinoscope BV, Maastricht, Netherlands) was used to calculate lag time (first time point after the thrombin concentration exceeds one-sixth peak height), peak (greatest amount of thrombin generation at a single point in time), time to peak (time to reach the maximum peak height), and endogenous thrombin potential (ETP; total amount of thrombin generated during the test).

Turbidity assay. HCAEC were cultured and exposed to soluble UF as described above and fibrin clot formation measured as previously described (Gray *et al.*, 2011). Clot formation onset (time to reach inflection point before turbidity increase) and V_{max} (slope of the line fitted to maximum rate of turbidity increase) were calculated using Softmax Pro Software version 1.21 (Molecular Devices, Sunnyvale, CA).

Real-time quantitative PCR. Relative gene expression in HCAEC was obtained using quantitative RT-PCR as previously described (Karoly *et al.*, 2007). Total RNA was isolated from HCAEC using an RNeasy kit (Qiagen, Valencia, CA) as per manufacturer's protocol. RNA was quantified using a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). Oligonucleotide primer pairs and fluorescent probes for β-actin (forward, 5′-CCTGGCACCCAGCACAAT-3′; reverse, 5′-GCCGATCCACACGGAGTACT-3′; probe, 5′-ATCAAGATCATTGCTCCTCCTGAGCGC-3′) and TF (forward, 5′-TTGGCACGGGTCTTCTCCTA-3′; reverse, 5′-CGAGGTTTGTCTCCAGGTAAGG-3′; and probe, 5′-AGAACCGGTGCTCTCCACATTCCCTG-3′) were designed using Primer Express (Applied Biosystems, Foster City, CA) and obtained from Integrated DNA Technologies (Coralville, IA). Thrombomodulin, endothelial protein C receptor (EPCR), tissue factor pathway inhibitor (TFPI), GAPDH, and NOX-4 primer pairs and fluorescent probe sets were obtained as Taqman pre-developed assay reagents from Applied Biosystems. Standard curves generated from a serially diluted standard

TABLE 1
Components of UF PM (ng/mg) Collected in Chapel Hill, NC from February to May 2007

Component	ng/mg	Component	ng/mg
Aluminum	855.0	Nitrate	44100.0
Arsenic	55.8	Nitrite	579.0
Bromide	1824.0	Selenium	76.5
Chromium	12.3	Silicon	3480.0
Copper	108.0	Sulfur	99300.0
Iron	624.0	Titanium	21.5
Lead	79.5	Vanadium	57.0
Nickel	34.2	Zinc	735.0

pool of cDNA prepared from cultured human endothelial cells exposed to 100 ng/ml TNF-α for 6 h were used to determine the relative abundance of mRNA levels, which were normalized to relative abundance of β-actin mRNA.

H_2O_2 measurement. Extracellular H_2O_2 release was measured using the Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine; Invitrogen, Carlsbad, CA) as previously described (Li *et al.*, 2006). Following analysis, cellular protein was collected and measured using the Bio-Rad protein reagent (Bio-Rad, Richmond, CA) as per manufacturer's protocol.

Peroxy Green 1 (PG1; kindly provided by Dr Christopher Chang, University of California-Berkeley), a small-molecule fluorophore that is more responsive to H_2O_2 than other ROS (Miller *et al.*, 2007), was used to measure intracellular H_2O_2 as previously described (Cheng *et al.*, 2010).

siRNA transfection. HCAEC were transfected with 90nM concentration of NOX-4 or scrambled no. 5 siRNA using siPORT Amine transfection agent (Ambion, Austin, TX) as per manufacturer's protocol. Cells were used for experiments 48 h post-transfection.

Statistical analysis. Statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA). A t-test or one-way ANOVA analysis followed by Bonferroni's post-hoc test was used to compare exposed cells with control cells. A p-value < 0.05 was considered statistically significant.

RESULTS

Soluble UF Cause HCAEC to Promote Earlier Onset of TF-Dependent Thrombin Generation and Fibrin Clot Formation

HCAEC were exposed to 10, 50, and 100 μg/ml soluble UF. We found that these doses are non-cytotoxic to the cells (Supplementary fig. 1). Six hours following exposure, cells were assessed for functional coagulation balance using two methods. We used the calibrated automated thrombography (CAT) assay to quantify thrombin generation (Figs. 1A–C) and the turbidity assay to measure the rate of fibrin clot formation (Figs. 1D–F) in PFP. The CAT assay showed that lag time (Fig. 1B) and time to peak (Fig. 1C) significantly shortened with increasing doses of soluble UF, indicating earlier onset of thrombin generation. Soluble UF exposure did not significantly affect thrombin peak or ETP (data not shown). In the turbidity assay, HCAEC exposure to soluble UF led to a significant shortening in the onset time to clot formation (Fig. 1E) and an increase in V_{max} (Fig. 1F), indicating earlier onset and faster rate of fibrin clot formation. These effects on thrombin and fibrin were abolished by addition of an inhibitory anti-human TF antibody. These data suggest soluble UF increases endothelial procoagulant activity via a TF-dependent mechanism.

Soluble UF Increases TF mRNA Expression in HCAEC

To characterize the cause of soluble UF-induced changes in HCAEC cellular procoagulant activity, we quantified mRNA expression of key procoagulant and anticoagulant proteins by RT-PCR following exposure for 6 and 24 h. Exposure to 50 and 100 μg/ml of soluble UF led to a significant ($p < 0.001$) 3.8-fold and 5.1-fold, respectively, increase of TF mRNA expression in HCAEC at 6 h, and increased expression was sustained at 24 h (Fig. 2A). Conversely, we found no significant changes in thrombomodulin, EPCR, or TFPI following soluble UF exposure (Figs. 2B–D). The upregulation of TF and lack of compensatory changes in the anticoagulant proteins provide a mechanistic rationale for the increased cellular procoagulant activity seen in Figure 1.

Soluble UF-Induced TF Upregulation Involves ROS

To characterize the events initiating increased TF production, we treated HCAEC with PEG-SOD and PEG-catalase prior to 6 h soluble UF exposure (50 μg/ml) and measured TF mRNA expression. These cell-permeable antioxidants significantly suppressed TF mRNA upregulation by soluble UF by 55% and 36%, respectively ($p < 0.001$, Fig. 3A). In addition, pretreatment with L-NAME, a nitric oxide synthase inhibitor, did not attenuate TF mRNA levels following exposure (Fig. 3B). These data indicate that ROS production is involved in increasing TF mRNA expression in soluble UF-treated HCAEC.

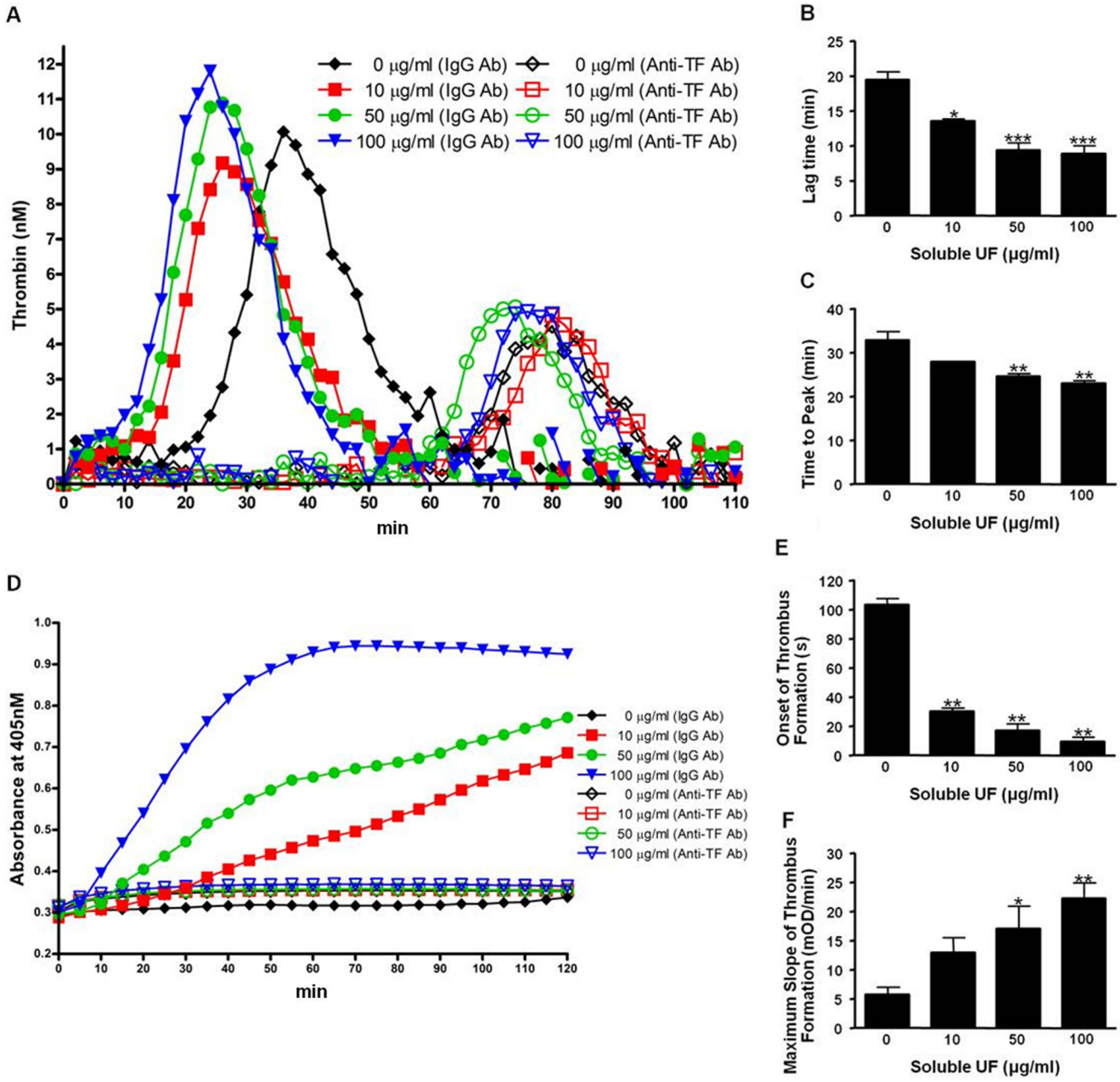

FIG. 1. Soluble UF exposure leads to faster onset of TF-dependent thrombin generation and fibrin clot formation. Endothelial cell coagulation balance was assessed by measuring capacity of HCAEC to induce thrombin generation (A-C) and fibrin clot formation (D-F) in PFP following soluble UF exposure (0, 10, 50, and 100 μg/ml). Thrombin parameters lag time (B) and time to peak (C) and the fibrin clot parameters clot formation onset (E) and V_{max} (F) were measured. Graphs are representative of three separate experiments with samples in triplicate. Error bars indicate ± SE. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with control.

Soluble UF Exposure Causes H_2O_2 Production in HCAEC

To characterize ROS production by endothelial cells, we measured extracellular and intracellular H_2O_2 production in soluble UF-treated HCAEC. Using the Amplex Red assay to quantify extracellular H_2O_2 release, we found an 11.8-fold ($p < 0.05$) and 19.7-fold ($p < 0.001$) increase in extracellular H_2O_2 levels following HCAEC exposure to 50 and 100 μg/ml soluble UF, respectively (Fig. 4A). Using the chemical indicator PG1, we observed a significant and progressive dose-dependent increase in intracellular H_2O_2 in cells exposed to 50 (21%, $p < 0.05$) and 100 μg/ml (32%, $p < 0.001$) (Fig. 4B). We then visualized the distribution of intracellular H_2O_2 production in individual

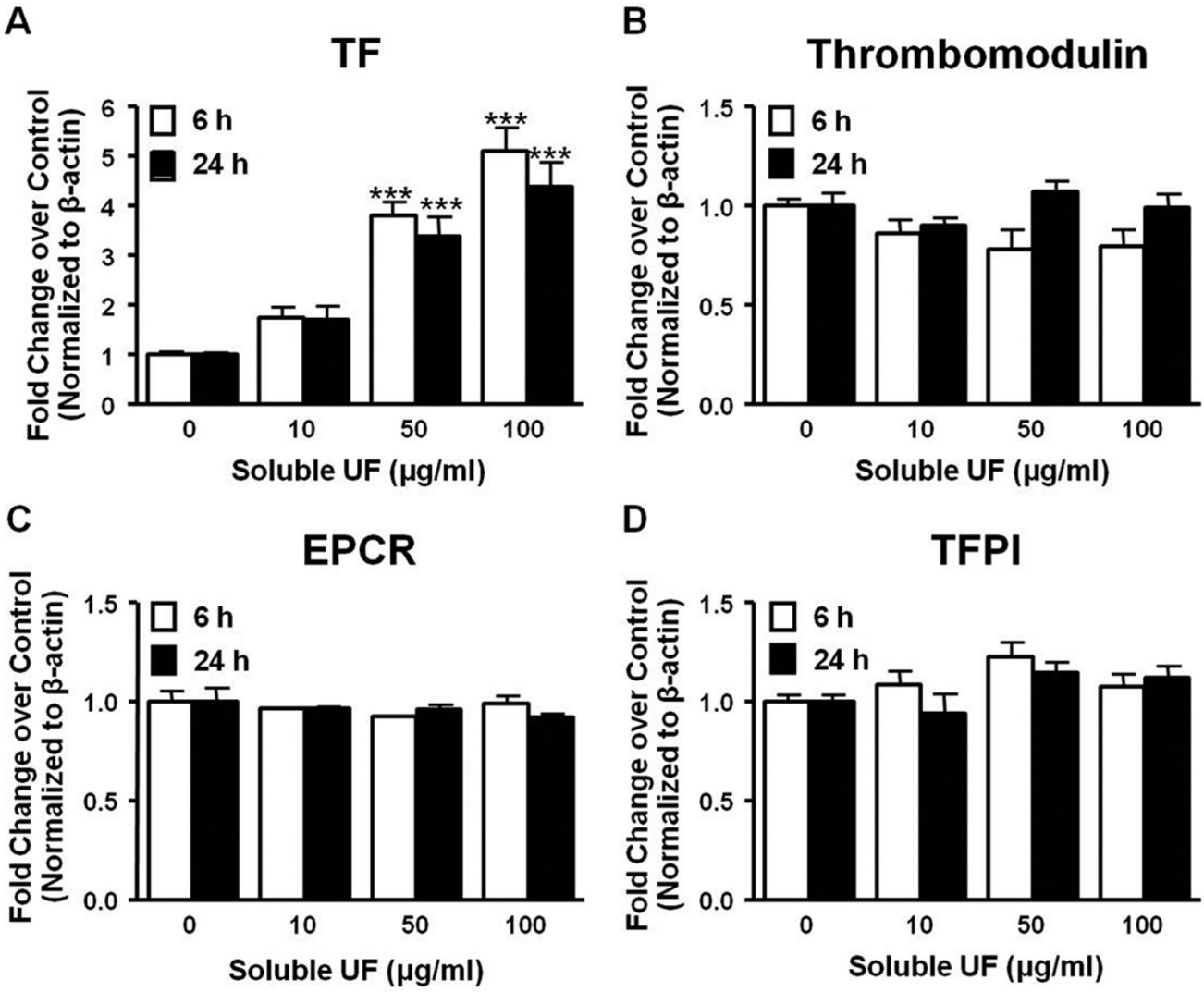

FIG. 2. Soluble UF exposure leads to TF upregulation. (A-D) HCAEC were exposed to soluble UF (0, 10, 50, and 100 μg/ml) for 6 or 24 h. TF (A), thrombomodulin (B), EPCR (C), and TFPI (D) mRNA expression were quantified by RT-PCR. Error bars indicate ± SE of $n = 3$ separate experiments with samples in triplicate. ***$p < 0.001$ compared with control.

living cells in real time using confocal microscopy. Soluble UF (100 μg/ml) induced intracellular H_2O_2 in HCAEC in a diffuse, cytoplasmic distribution as indicated by increased PG1 fluorescence intensity (Fig. 4C). Quantitative analysis of the confocal images indicated a significant increase ($p < 0.001$) in fold change over baseline in soluble UF-exposed HCAEC (Fig. 4D). These data indicate that soluble UF exposure results in immediate increases in extracellular and intracellular H_2O_2 production in HCAEC.

Soluble UF-Induced TF mRNA Expression Involves the NOX-4 Enzyme

To determine the cellular source of soluble UF-induced H_2O_2 production, we measured extracellular H_2O_2 production by HCAEC in the presence of chemical inhibitors targeted towards the major sources of endothelial ROS production. DPI, a NOX inhibitor, reduced extracellular H_2O_2 production in soluble UF-exposed (50 μg/ml) HCAEC by 54% ($p < 0.05$) (Fig. 5A). Soluble UF-induced extracellular H_2O_2 levels were not affected by inhibitors of xanthine oxidases (allopurinol) or mitochondrial sources (KCN, rotenone), which are other important sources of endothelial ROS production (data not shown). These data indicate that NOX enzymes are likely the major source of soluble UF-induced H_2O_2 production. To determine if NOX enzymes mediate the increase in TF mRNA, we pretreated HCAEC with DPI followed by a 6 h soluble UF exposure (50 μg/ml). Pretreatment with the NOX inhibitor led to a 50% attenuation ($p <$

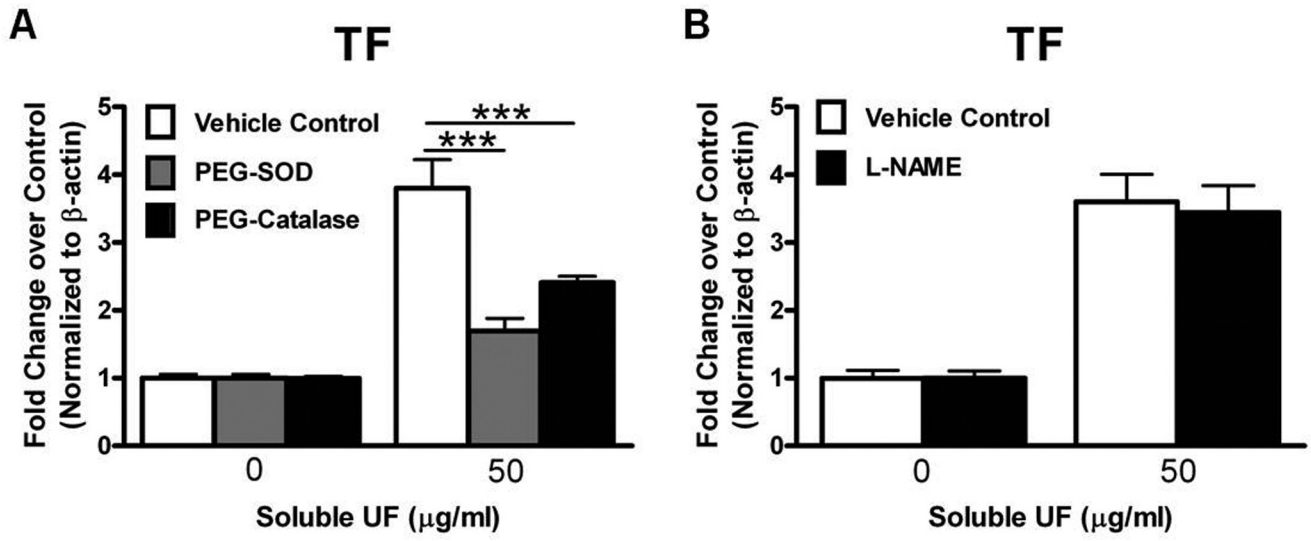

FIG. 3. TF upregulation by soluble UF is ROS dependent. HCAEC were pretreated with 100 U/ml PEG-SOD or PEG-catalase for 1 h (A) or 1mM L-NAME for 30 min (B) prior to a 6 h exposure to 0 or 50 μg/ml soluble UF. TF mRNA expression was quantified by RT-PCR. Error bars indicate ± SE of $n = 3$ separate experiments with samples in triplicate. ***$p < 0.001$ compared with control.

0.01) of the increased TF mRNA levels in exposed cells (Fig. 5B).

Because HCAEC predominately express the NOX-4 isoform (Yoshida and Tsunawaki, 2008), we more specifically probed the role of NOX-4 in soluble UF-induced TF mRNA upregulation by transfecting HCAEC with NOX-4 siRNA. Transfection resulted in 84% knockdown of the target gene mRNA levels as compared with the scrambled siRNA control after 48 h (Fig. 5C). HCAEC were then transfected with NOX-4 or scrambled siRNA for 48 h prior to soluble UF exposure for 6 h. Compared with cells transfected with scrambled siRNA, NOX-4 silencing significantly ($p < 0.001$) attenuated soluble UF-induced TF mRNA upregulation by 56% (Fig. 5D). Together, these data indicate that following soluble UF exposure, ROS and the NOX-4 enzyme are involved in upregulating TF mRNA expression and endothelial cell-dependent procoagulant activity.

DISCUSSION

There is strong epidemiological evidence supporting a causal relationship between air pollution exposure and adverse CV health effects in humans that include clinical events mediated by thrombosis (Pope *et al.*, 2004, 2006). An important but understudied area in this field is the effect of inhaled air pollutants on vascular cells via soluble components that can translocate into the vasculature. Here we report that the water-soluble components of UF PM induce a procoagulant phenotype in endothelial cells, supporting earlier onset of thrombin generation and faster fibrin clot formation, and that these activities are driven by TF upregulation. We further show that this increase in TF mRNA is regulated by increased ROS production and the NOX-4 enzyme, and can be attenuated by treatment of cells with antioxidants or by inhibiting NOX-4. These novel findings provide mechanistic insight into the enhanced thrombosis and endothelial dysfunction that might underlie increased risk for CV morbidity and mortality associated with air pollution exposure.

Our results add to a growing body of evidence linking air pollution exposure to thrombosis. Recent studies demonstrated hypercoagulable effects of air pollution on plasma thrombin generation in human and animal models (Emmerechts *et al.*, 2012; Kilinc *et al.*, 2011). Emmerechts *et al.* demonstrated that elevated levels of coarse PM shortened the lag time of thrombin generation in microvesicle-rich plasma obtained from diabetic individuals exposed to ambient air pollution (Emmerechts *et al.*, 2012). Our results extend these observations to potentially pathologic effects of PM on *cellular* procoagulant activity. Specifically, using the CAT assay, we show that soluble UF exposure decreased lag time in a dose-dependent manner, which has been previously shown to be a sensitive reflection of TF activity (Ollivier *et al.*, 2010). We did not find significant differences in ETP or peak thrombin; however, this is not surprising because these parameters are typically more associated with alterations in levels of soluble clotting factors (Machlus *et al.*, 2009). *In vivo*, we anticipate that these observed changes in plasma (Emmerechts *et al.*, 2012; Kilinc *et al.*, 2011) and cellular procoagulant activity (endothelial cells and possibly platelets) may be additive or even synergistic, ultimately dysregulating multiple thrombin generation parameters simultaneously. Moreover, by disrupting endothelial cell function, soluble UF may also alter expression of endothelial-derived fibrinolytic

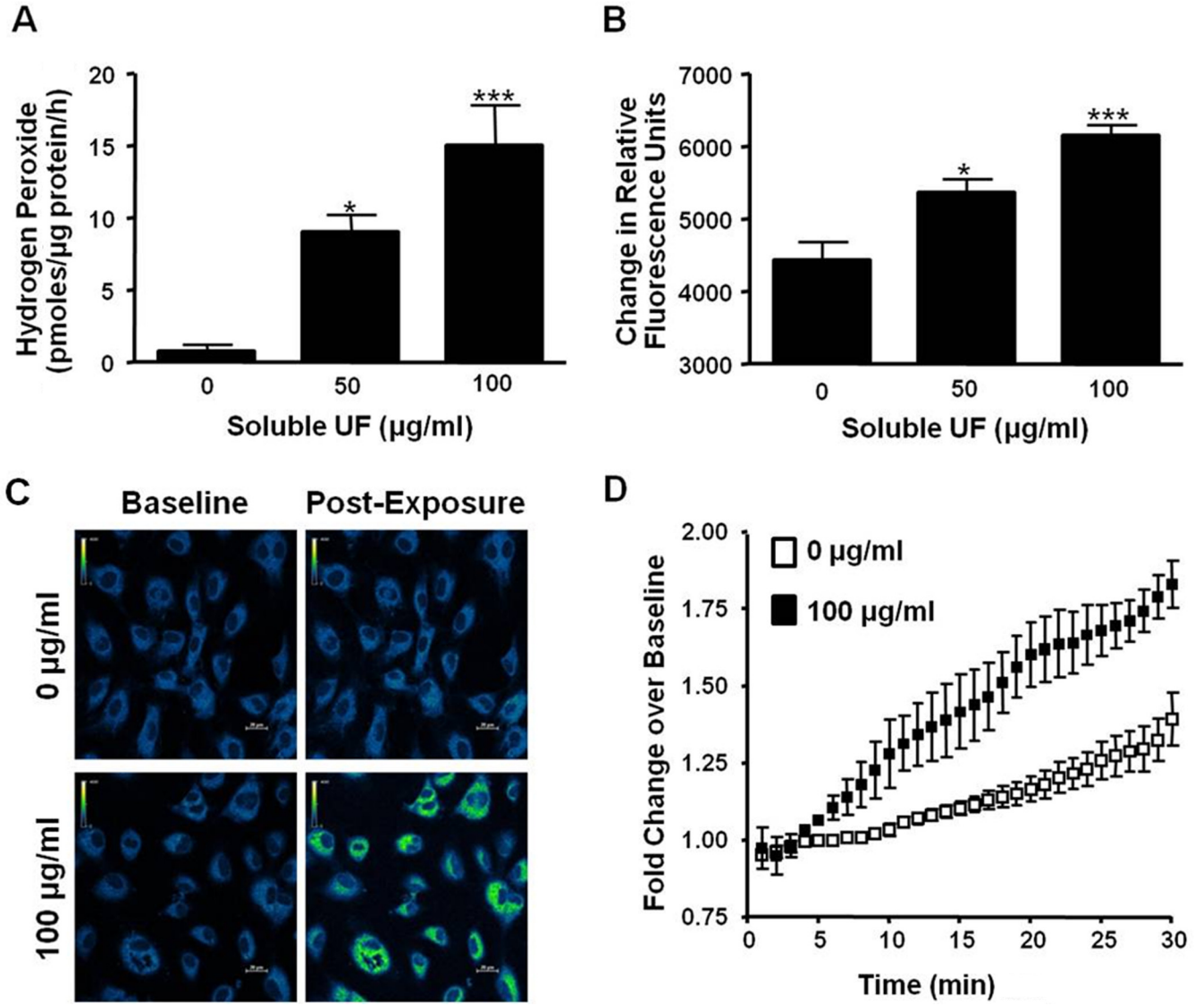

FIG. 4. Soluble UF exposure induces rapid H_2O_2 generation by HCAEC. (A) HCAEC were exposed to soluble UF (0, 50, and 100 µg/ml) and extracellular H_2O_2 release was measured using the Amplex Red reagent. (B) HCAEC were exposed to soluble UF (0, 10, 50, and 100 µg/ml) for 60 min in the presence of the H_2O_2 indicator PG1. Fluorescence was measured with a fluorescent plate reader. Error bars indicate ± SE of $n = 3$ independent experiments with samples in triplicate. (C) PG1 fluorescence was detected by confocal microscopy. HCAEC were pre-incubated with PG1 for a baseline measurement, then exposed to 0 or 100 µg/ml soluble UF for 30 min. Representative images shown in a pseudo-colored scheme are from baseline and 30 min post-exposure. (D) Quantitative data from the confocal images were gathered by outlining individual cells and measuring the Relative Fluorrescense Units (RFU) using Nikon NIS-Elements software. Error bars indicate ± SE of $n = 4$ independent experiments. *$p < 0.05$, ***$p < 0.001$ compared with control.

enzymes (e.g., tissue plasminogen activator), with additive negative consequences for clot stability. Further study of these intriguing possibilities may reveal additional therapeutic targets for reducing deleterious effects of air pollution on clot quality.

TF expression is generally suppressed in endothelial cells under normal physiological conditions, but is induced by a variety of stimuli *in vitro* (Chu, 2005; Karoly *et al.*, 2007) and possibly *in vivo* (Mackman *et al.*, 2007). Activation of TF from injury or pathological conditions is typically balanced by expression of anticoagulant proteins such as TFPI (Crawley and Lane, 2008); however, our findings show lack of parallel upregulation of these anticoagulant proteins, resulting in a procoagulant environment that favors thrombin generation. The *TF* gene in endothelial cells contains binding sites for redox-sensitive transcription factors (Herkert and Gorlach, 2002). Accordingly, we found that in HCAEC, TF mRNA levels following soluble UF exposure involved the ROS molecules H_2O_2 and superoxide rather than NO. This finding supports previous studies showing attenuated TF mRNA expression in the presence of antioxidants

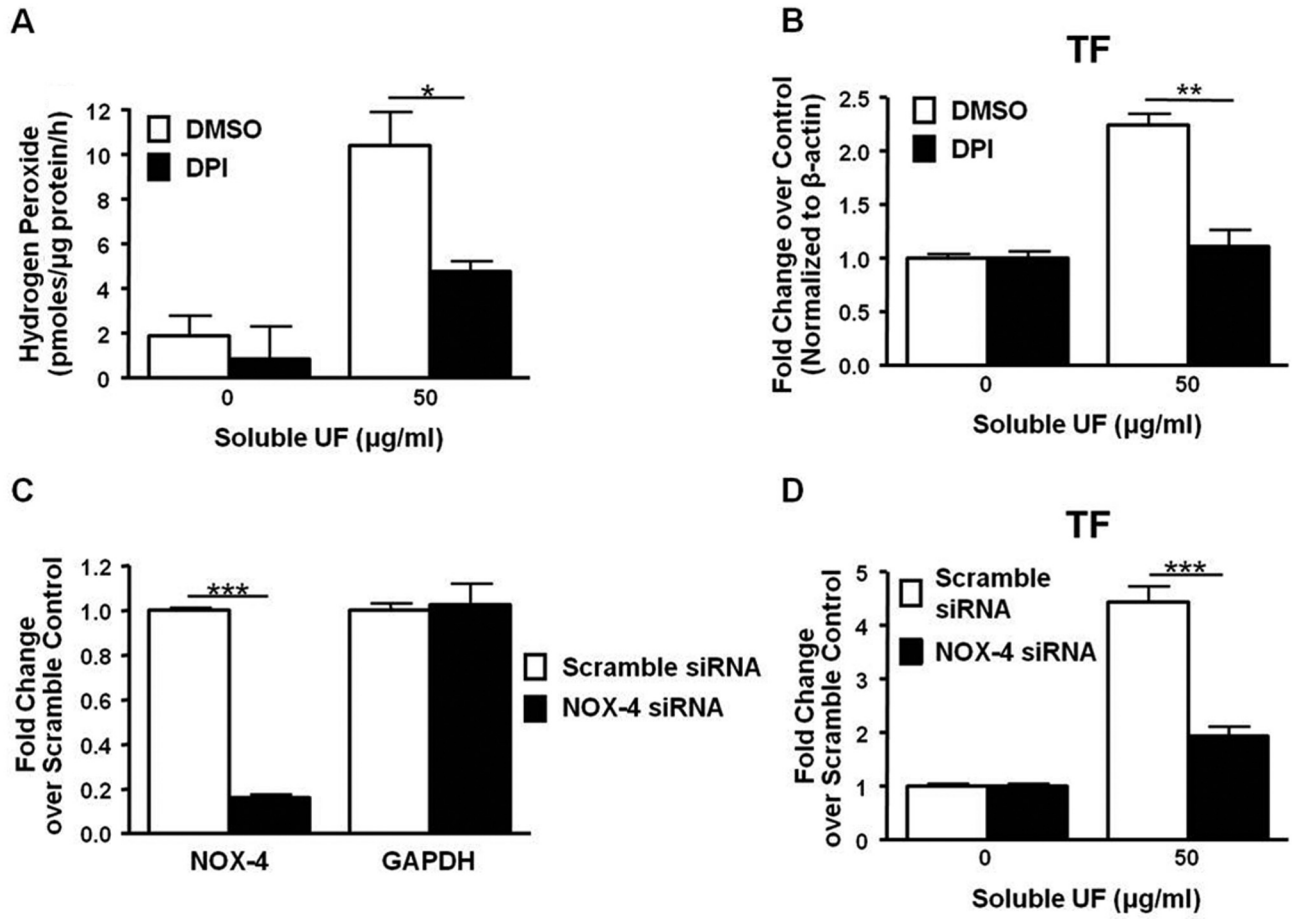

FIG. 5. TF mRNA expression following soluble UF exposure requires ROS production and NOX-4. HCAEC pretreated with the NOX inhibitor DPI (50µM) for 30 min followed by exposure to 0 or 50 µg/ml soluble UF were used to measure (A) extracellular H_2O_2 and (B) TF mRNA expression. (C) HCAEC were transfected with NOX-4 or scramble siRNA for 48 h. mRNA expression of the target gene NOX-4 and non-target gene GAPDH were quantified by RT-PCR. (D) HCAEC were transfected with NOX-4 or scramble siRNA for 48 h and then exposed to 0 or 50 µg/ml soluble UF for 6 h. TF mRNA expression was quantified by RT-PCR. Error bars indicate ± SE of $n = 3$ separate experiments with samples in duplicate. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with control.

following exposure to ionizing radiation and inflammatory cytokines (Szotowski *et al.*, 2007).

ROS are implicated in the pathogenesis and progression of several CV diseases including atherosclerosis, hypertension, and diabetes (Madamanchi *et al.*, 2005). Additionally, concentrations of 7-hydro-8-oxo-2'-deoxyguanosine in lymphocyte DNA correlated with elevated levels of water-soluble transition metals in fine PM in humans exposed to ambient air pollution (Sorensen *et al.*, 2005). This measurement has been used as a biomarker to assess oxidative damage caused by increased ROS production and suggests air pollution exposure triggers oxidative stress. We showed that soluble UF components lead to significant increases in both extracellular and intracellular endothelial H_2O_2 production immediately following exposure. Our data, using the indicator PG1, indicate that ROS are initially formed intracellularly after soluble UF exposure. This premise is further supported by confocal microscopy, which showed a cytoplasmic distribution of H_2O_2.

NOX is an important source of ROS in vascular cells, and NOX enzymes have been implicated as a source of oxidative stress in numerous CV diseases (Lassegue *et al.*, 2012). Although endothelial cells have several major sources of ROS production, NOX enzymes induced ROS production in mouse pulmonary microvascular endothelial cells exposed to whole UF particles (Mo *et al.*, 2009). Our findings are important because we show that NOX-4 is the likely source of soluble UF-induced H_2O_2 production in human endothelial cells. The intracellular distribution of NOX-4 is broad and has been shown to be variably located in the plasma membrane, endoplasmic reticulum, mitochondria, and nucleus of endothelial cells (Lassegue *et al.*, 2012). Our data further suggest that NOX-4-derived ROS produced following soluble UF exposure is cytoplasmic, but can

escape to the extracellular space. This finding has important implications for paracrine signaling effects by cellular H_2O_2.

NOX-4 is involved in increasing TF mRNA expression in HCAEC by soluble UF. Other researchers have shown that ROS from NOX enzymes induce TF mRNA expression in VSMC after agonist stimulation by thrombin (Herkert *et al.*, 2002). Our data extend this finding to endothelial cells after a more subtle challenge with soluble UF. NOX enzymes are important potential therapeutic targets for strategies to limit overproduction of ROS (Schramm *et al.*, 2012) and our data reinforce the concept that inhibiting these enzymes is a potential approach to combat adverse CV effects from air pollution exposure.

Our study is novel in the use of primary HCAEC to model effects of soluble air pollution components. Because these endothelial cells contact blood immediately from the lungs, they seem likely to receive high levels of any soluble air pollution components that enter the vasculature. Although it is not known whether even the smallest PM can translocate from the lung into the vasculature and cause pathologic effects (Mills *et al.*, 2006; Nemmar *et al.*, 2002), water-soluble components of the particles appear in the vasculature following intratracheal instillation in rats (Wallenborn *et al.*, 2007). Moreover, we previously determined that the insoluble fraction of UF PM does not induce significant adverse responses in HCAEC (Snow and Carraway, 2010). Our study strongly supports the potential for a pathogenic role for the water-soluble components of UF particles and implies that in endothelial cell culture systems, soluble UF exposure is more physiologically relevant than whole UF particle exposures. Furthermore, PM can cause cell death *in vitro* at high concentrations; however, we confirmed that the doses used in this study were non-cytotoxic, indicating that the procoagulant activity following soluble UF exposure was due to endothelial activation rather than cellular death.

Our study has a few limitations that are common to *in vitro* models. First, our study cannot clarify the uncertainty of whether endothelial TF is expressed *in vivo* in humans following PM exposure, even though TF expression does increase *in vitro* and in animals in response to PM (Sun *et al.*, 2008). Furthermore, it has been shown that endothelial TF upregulation occurs in vascular disease in humans (Shet *et al.*, 2003; Thiruvikraman *et al.*, 1996), which is relevant to our postulate that air pollutants induce endothelial activation. Second, our data must be interpreted in light of the lack of current evidence for TF-initiated coagulation as a key early event in adverse CV responses to air pollution. Several negative studies on the role of coagulation considered acute exposures to larger air pollution particles. Yet, a recent study in diabetic individuals did find a trend towards early increased monocyte and platelet TF expression and TF^+ microparticles following smaller UF particle exposure (Stewart *et al.*, 2010), which suggests effects may differ between particles depending on size and associated components. Finally, *in vitro* studies are limited by the uncertainty in predicting PM component levels experienced by vascular cells after inhaling known particle concentrations. However, individuals exposed to indoor smoking, fire smoke, or indoor cook stoves can be transiently exposed to elevated UF particle concentrations on par with those used to generate soluble fractions for this study (Afshari *et al.*, 2005; Baxter *et al.*, 2010; Karoly *et al.*, 2007). Therefore, we estimated levels that would emulate levels of particle components to which vascular cells could be exposed.

In summary, we have shown that exposure of HCAEC to soluble UF leads to increased production of intracellular ROS and activation of the NOX-4 enzyme that regulates TF mRNA. This activation of the extrinsic pathway results in faster onset of thrombin generation and fibrin clot formation. These novel findings support and extend important information regarding mechanism(s) by which PM exposure results in thrombosis and adverse CV health effects.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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