ULTRAFINE PARTICLES ALTER ENDOTHELIAL PHENOTYPE THROUGH
OXIDANT SIGNALING

Samantha Jean Snow

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Approved by:

Martha Sue Carraway, PhD

David Diaz-Sanchez, PhD

Alisa Wolberg, PhD

Ilona Jaspers, PhD

Frank Church, PhD

ABSTRACT

SAMANTHA JEAN SNOW: Ultrafine particles alter endothelial phenotype through oxidant
signaling
(Under the direction of Dr. Martha Sue Carraway and Dr. David Diaz-Sanchez)

Mechanisms that underlie the strong association between air pollution exposure and
cardiovascular (CV) morbidity and mortality remain unknown. Particulate matter (PM) is a
major component of air pollution and ultrafine (UF) particles, which are the smallest
diameter particle, are of particular importance in CV dysfunction caused by exposure to air
pollution. UF particles can deposit in the heavily vascularized region of the lung and the
soluble components of UF particles (soluble UF) are able to cross from the lung into the
circulation and adversely affect cells of the vasculature such as endothelial cells. Endothelial
cell activation, as characterized by an increase in reactive oxygen species (ROS) production,
initiation of coagulation, and induction in inflammation, is a pathophysiological mechanism
that could link inhaled air pollutants to vascular dysfunction. This project will test the
hypothesis that soluble UF cause altered endothelial cell phenotype through activation of
oxidant signaling that mediates procoagulant and proinflammatory responses. EA cells, an
immortalized endothelial cell line, and primary human coronary artery endothelial cells
(HCAEC) were assessed for their production of ROS, procoagulant activity, and
proinflammatory responses following exposure to non-cytotoxic doses of soluble UF. We
show that exposure to soluble UF results in immediate increases in extra- and intracellular
H_2O_2 production from NADPH oxidase (NOX) enzymes in endothelial cells. Furthermore,
soluble UF PM increased the expression of proinflammatory mediators and induced

endothelial procoagulant activity via a tissue factor (TF)-dependent mechanism. Pretreatment with antioxidants and NOX inhibitors attenuated the soluble UF-induced upregulation of the procoagulant protein TF, the proinflammatory cytokine IL-1β, and the oxidant stress-inducible protein HO-1, linking the procoagulant and proinflammatory responses to ROS formation from NOX enzymes. These novel findings provide mechanistic insight into the endothelial dysfunction and enhanced thrombosis that underly increased risk for CV morbidity and mortality associated with air pollution exposure.

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LIST OF ABBREVIATIONS

^{99m}Tc	^{99m}Technetium
A549	Human Lung Adenocarcinoma Epithelial Cell Line
ANOVA	Analysis of Variance
AP-1	Activating Protein-1
APC	Activated Protein C
β-actin	Beta-Actin
BSA	Bovine Serum Albumin
CAT	Calibrated Automated Thrombography
cDNA	Complementary Deoxyribonucleic Acid
COPD	Chronic Obstructive Pulmonary Disease
CV	Cardiovascular
DAPI	4', 6-diamidino-2-phenylindole
DEP	Diesel Exhaust Particles
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
DPI	Diphenyleneiodonium
DUOX	Dual Oxidase
E-selectin	Endothelial Adhesion Molecule 1
EA	EA.hy926 Endothelial Cell Line
ECL	Enhanced Chemiluminescence
EDTA	Ethylene Diamine Tetraacetic Acid
EGM-2	Endothelial Growth Medium
EGTA	Ethylene Glycol Tetraacetic Acid
EPA	Environmental Protection Agency

EPCR	Endothelial Protein C Receptor
ETP	Endogenous Thrombin Potential
FBS	Fetal Bovine Serum
FV	Factor V
FVa	Activated Factor V
FVIIa	Activated Factor VII
FVIII	Factor VIII
FVIIIa	Activated Factor VIII
FX	Factor X
FXa	Activated Factor X
FXI	Factor XI
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HCAEC	Human Coronary Artery Endothelial Cells
HCL	Hydrochloric Acid
HO	Heme Oxygenase
HO-1	Heme Oxygenase-1
H_2O_2	Hydrogen Peroxide
HRP	Horseradish Peroxidase
HUVECs	Human Umbilical Vein Endothelial Cells
ICAM-1	Intercellular Adhesion Molecule-1
IgG	Immunoglobulin G
IL-1β	Interleukin-1 Beta
IL-6	Interleukin-6
IL-8	Interleukin-8

KCN	Potassium Cyanide
LDH	Lactate Dehydrogenase
MCP-1	Monocyte Chemoattractant Protein-1
mRNA	Messenger Ribonucleic Acid
NaCl	Sodium Cloride
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
Nrf2	Nuclear Factor (Erythroid-Derived 2)-Like 2
NF-κB	Nuclear Factor-Kappa B
NO	Nitric Oxide
NOX	Nicotinamide Adenine Dinucleotide Phosphate Oxidase
NOX-1	Nicotinamide Adenine Dinucleotide Phosphate Oxidase-1
NOX-2	Nicotinamide Adenine Dinucleotide Phosphate Oxidase-2
NOX-4	Nicotinamide Adenine Dinucleotide Phosphate Oxidase-4
O_2	Oxygen
$\cdot O_2$	Superoxide Anion
$\cdot OH$	Hydroxyl Radical
p53	Protein 53
PAI-1	Plasminogen Activator Inhibitor-1
PARs	Protein Activated Receptors
PBS	Phosphate Buffer Saline
PC	Protein C
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PG1	Peroxy Green 1
PFP	Platelet-Free Plasma

PM	Particulate Matter
PM_{10}	Coarse Particulate Matter
$PM_{2.5}$	Fine Particulate Matter
$PM_{0.1}$	Ultrafine Particulate Matter
R^3-IGF-1	R^3-Insulin-like Growth Factor-1
RFU	Relative Fluorescence Units
rhEGF	Recombinant Human Epidermal Growth Factor
rhFGF-B	Recombinant Human Fibroblast Growth Factor-Basic
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
siRNA	Small Interfering Ribonucleic Acid
SOD	Superoxide Dismutase
Soluble UF	Soluble Components of Ultrafine Particles
SP1	Specificity Protein 1
TF	Tissue Factor
TFPI	Tissue Factor Pathway Inhibitor
TiO_2	Titanium Dioxide
TNF-α	Tumor Necrosis Factor-Alpha
TM	Thrombomodulin
UF	Ultrafine
VEGF	Vascular Endothelial Growth Factor

CHAPTER 1

INTRODUCTION

In the early 20th century, a series of air pollution incidents in Meuse Valley, Belgium (1930), Donora, Pennsylvania (1948), and London, England (1952) illustrated the adverse effects individuals experience during severe air pollution episodes. These events occurred in heavily industrialized cities during a meteorological inversion that concentrated the already severe air pollution and resulted in increased hospitalizations and elevated rates of mortality (Stanek et al., 2011). These incidents raised public awareness about the severity of air pollution, led to new regulations across the developed world, and spurred investigators to conduct epidemiological and toxicological studies to establish the relationship and mechanisms behind air pollution exposure and adverse health effects.

For decades, research has illustrated the association between air pollution exposure and adverse pulmonary effects, but it wasn't until the 1990s when epidemiological (Dockery et al., 1993) and toxicological studies (Costa and Dreher, 1997) began linking air pollution exposure with adverse cardiovascular (CV) health effects. Subsequent epidemiological studies have demonstrated that exposure to air pollution results in increased hospitalizations for circulatory diseases (Poloniecki et al., 1997), increased risk of myocardial infarctions (Peters et al., 2001), and increased risk of CV disease mortality (Pope et al., 2004). It was also found that these adverse effects were more pronounced in susceptible populations, such as the elderly and individuals with preexisting CV or pulmonary diseases (Brook et al., 2004). Additional toxicological studies have shown that exposure to air pollution induced

systemic oxidative stress in mice (Araujo et al., 2008), accelerated atherosclerotic progression in susceptible mouse (Sun et al., 2005) and rabbit models (Suwa et al., 2002), and enhanced experimental arterial and venous platelet rich-thrombus formation in hamsters (Nemmar et al., 2003).

The enactment of the Clean Air Act in 1970 required the Environmental Protection Agency (EPA) to set National Ambient Air Quality Standards for six criteria air pollutants including particulate matter (PM), ozone, carbon monoxide, sulfur dioxide, nitrogen oxides, and lead. These air pollutants were deemed to be ubiquitous in the United States and were suspected or known to induce adverse health effects in humans or the environment (Suh et al., 2000). Of these six criteria air pollutants, PM and ground level ozone present the most widespread threat to human health and are known to commonly exceed the federal standards set by the EPA (Laumbach, 2010).

PM is categorized based on the diameter of the particle: coarse particles (PM_{10}) are 10 to 2.5 μm, fine particles ($PM_{2.5}$) are 2.5 to 0.1 μm, and ultrafine particles (UF; $PM_{0.1}$) are <0.1 μm. PM is derived from an assortment of natural and manufactured sources including forest fires, soil, dust, volcanic emissions, vehicular exhaust, smokestacks, and other industrial sources (Poschl, 2005). PM is a heterogeneous mixture with composition varying depending on several factors such as geographical location, time of day, season, and local emission sources. Common constituents of PM include transition metals, elements, inorganic ions, volatile organic compounds, and polycyclic aromatic hydrocarbons that are adsorbed on a carbon core (Polichetti et al., 2009). Urban UF particles are primarily associated with motor vehicle exhaust from both diesel and gasoline engines, but can also be formed by chemical reactions in the atmosphere (Sioutas et al., 2005).

The EPA currently monitors and regulates coarse and fine PM according to the Clean Air Act, but increasing evidence suggests that UF particles, which are not currently regulated, are of particular importance in CV effects of air pollution (Nel, 2005). UF particles make up only a small portion of ambient PM by mass concentration; however, they constitute the majority of particle number per unit mass and have a significantly high surface area-to-mass ratio as compared to coarse and fine particles. This property suggests that UF particles are capable of adsorbing large amounts of toxic substances on the surface. In addition, the small diameter of the particle makes it more likely to deposit in the alveolar region of the lung due to Brownian motion and diffusion transportation (Kreyling et al., 2004). UF particles are also able to stay in the lung longer than the larger fine and coarse particles because their small diameter makes them more likely to avoid being recognized or phagocytized by alveolar macrophages, a critical lung defense mechanism (Gonzalez et al., 1996).

The specific mechanisms that explain how inhalation of PM through the lung leads to adverse CV health effects are still being determined. There are three broad hypotheses that are currently being investigated to explain this association. These mechanisms include 1) imbalance of the autonomic nervous system that leads to alterations in heart rate variability and cardiac arrhythmias, 2) induction of pulmonary inflammatory cytokines and reactive oxygen species (ROS) that induces systemic inflammation and oxidative stress, and 3) direct activation of cells of the vasculature from PM agents that have crossed into the circulatory system (Figure 1.1) (Brook, 2008).

Figure 1.1
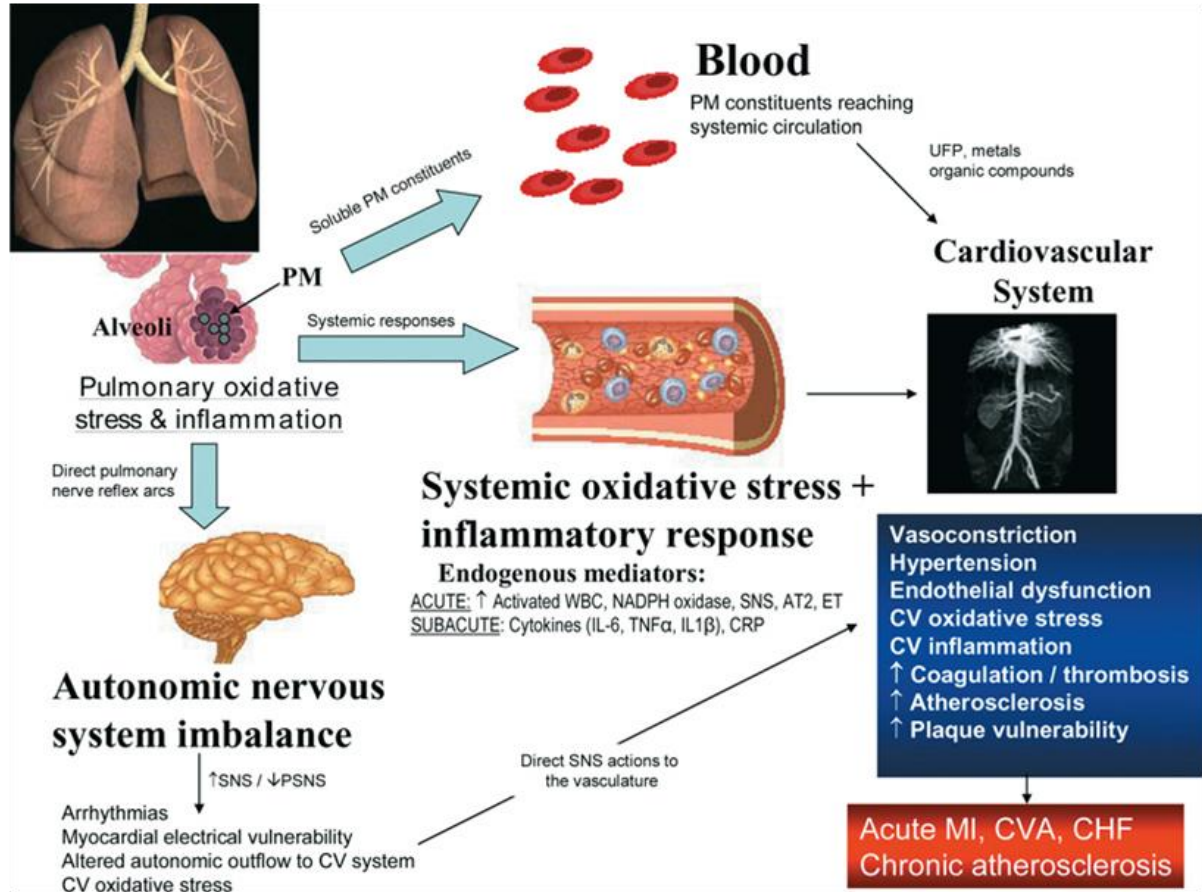
Figure 1.1 Broad biological pathways whereby PM may cause CV events. (Brook, 2008)

These theories are not necessary mutually exclusive and can act in conjunction with one another and multiple studies have been conducted that support each of these hypotheses. The general pathway(s) that an exposure will initiate depends upon several characteristics of the exposure, including whether it is an acute or chronic exposure, whether it is a multipollutant or single exposure, and the size and composition of the particles if the exposure involves PM. For instance, inhaled coarse and fine PM deposit in the larger airways of the lung, whereas UF PM deposits primarily in the heavily vascularized alveoli region of the lung where its components may interact with the circulatory system and thereby follow the third general pathway described above.

It remains unclear as to whether UF sized particles can cross from the lung into the vasculature. A study by Nemmar et al. exposed humans to an aerosol of radioactive ^{99m}technetium (^{99m}Tc) labeled UF carbon particles followed by imaging to determine particle distribution following inhalation (Nemmar et al., 2002). They found a majority of the radioactivity remained in the lungs, but also recorded levels of radioactivity in the blood, urine, bladder, and liver, suggesting extrapulmonary translocation of ^{99m}Tc labeled UF carbon particles. These researchers have reported similar results using animal models following exposure to radioactive labeled UF particles (Nemmar et al., 2001). However, there are several caveats to these studies. For instance, the investigators were unable to detect the radioactive labeled UF particles in the blood using electron microscopy. In addition, a study conducted by Mills et al., which also exposed humans to ^{99m}Tc labeled UF carbon particles, found no evidence of extrapulmonary translocation of the radioactive labeled particles (Mills et al., 2006). They instead explain the findings of Nemmar et al. by suggesting leaching of the radiolabel from the UF particle and/or contamination of free radiolabeled ^{99m}Tc-pertechnetate in their aerosol, which is formed using the same generator but with minimal oxygen contamination. These findings are supported by an additional study in which COPD patients were exposed to ^{99m}Tc labeled UF carbon particles with no evidence of extrapulmonary translocation of the radioactive label particles (Brown et al., 2002).

While it is uncertain whether UF particles can enter into the systemic circulation in humans, it is reasonable to postulate that the soluble components of UF particles can cross the thin alveolar-capillary membrane and cause direct effects on cells of the vasculature. For example, transition metals, major components of the soluble fraction, can translocate into the vasculature and extrapulmonary tissues following intratracheal instillation in rats

(Wallenborn et al., 2009; Wallenborn et al., 2007). Additionally, the ability of these metals to translocate from the lung into the systemic circulation was dependent on their solubility in water (Wallenborn et al., 2007). These water-soluble transition metals are capable of leaching off PM particles in the lung lining fluid and translocating into the circulation, possibly via metal transporters or other mechanisms, before being cleared by the liver.

Transition metals, such as vanadium, nickel, iron, copper, chromium, and zinc, are major constituents of the water-soluble fraction of UF particles and may play an important role in the adverse cardiopulmonary effects associated with air pollution exposure (Costa and Dreher, 1997). Transition metals can increase oxidative DNA damage, induce ROS production, activate inflammatory pathways, and induce procoagulant events in both *in vitro* and *in vivo* models (Prahalad et al., 2001; Sangani et al., 2010; Sorensen et al., 2005; Wu et al., 2002). For example, concentrations of 7-hydro-8-oxo-2'-deoxyguanosine in lymphocyte DNA was shown to be correlated with elevated levels of soluble transition metals associated with 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, indicating that soluble components of PM are capable of inducing oxidative stress following air pollution exposure. Additionally, metal oxide nanoparticles induce inflammatory responses in endothelial cells (Gojova et al., 2007). Furthermore, transition metals located in ambient and emission-source air pollution particles were shown to reduce the clotting time in whole blood, indicating that these soluble components are capable of inducing procoagulant events in humans following exposure (Sangani et al., 2010). The mechanisms behind these adverse effects caused by the water-soluble components of air

pollution particles are not known, but one possibility is that the components have direct effects on cells of the vasculature including inducing endothelial activation.

Endothelial cell activation leads to increased vascular coagulation and inflammation, and it plays an important role in the pathogenesis of certain cardiovascular diseases including atherosclerosis and hypertension (Lwaleed et al., 2007; Sprague and Khalil, 2009). Endothelial activation is typically defined by five principal changes that occur including loss of vascular integrity, upregulation of human leukocyte antigen molecules, induction of inflammatory cytokines, expression of leukocyte adhesion molecules, and a change from an antithrombotic to prothrombotic phenotype (Hunt and Jurd, 1998). Endothelial cells are critical components of the vasculature as they line the blood vessels and are involved in hemostatic balance, vasomotor tone, blood cell trafficking, permeability, and immunity. In addition, endothelial cells have a critical role in the coagulation system and are able to express procoagulant factors, anti-coagulant factors, and fibrinolytic proteins (Aird, 2007).

Endothelial cells are key participants in venous and arterial thrombosis formation, which upon rupture may lead to strokes or myocardial infarction. Air particle pollution is associated with CV and cerebrovascular events that are triggered by thrombus formation. Recent studies have illustrated that PM exposure can increase the risk of deep vein thrombosis (Baccarelli et al., 2008), myocardial infarctions (Murakami and Ono, 2006), and fatal strokes (Kettunen et al., 2007). Endothelial activation could link inhaled PM to vascular dysfunction, which make endothelial cells an excellent cellular model to use for studying the mechanisms leading to CV adverse effects due to air particle pollution exposure (Figure 1.2).

Figure 1.2

| Soluble UF Exposure | → | **Endothelial Activation**
↑ ROS Production
Inflammatory Cytokines
Cell Adhesion Molecules
Prothrombotic Phenotype | → | **Vascular Dysfunction**
Thrombus Formation
Increased Permeability
Leukocyte Adhesion | → | **Cardiovascular Events**
Acute Coronary Syndrome
Deep Venous Thrombosis
Thromboembolism
Stroke |
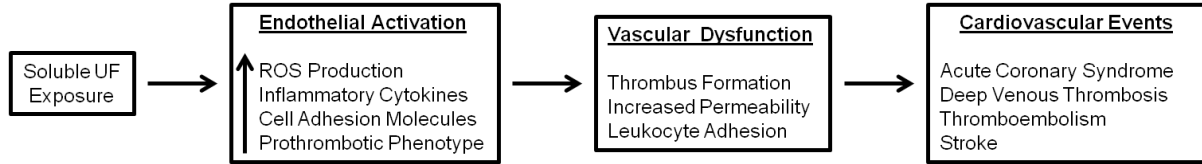
Figure 1.2   Schematic diagram illustrating how exposure to soluble UF can lead to adverse CV events.

Toxicological exposure, vessel wall damage, or pathological activation of cells of the vasculature, including endothelial cells, can initiate the coagulation cascade. The coagulation cascade is traditionally divided into the extrinsic and intrinsic pathways that converge into a common pathway. Altered expression and activity of tissue factor (TF) initiates the extrinsic pathway of the coagulation cascade, which is proposed to be the primary initiator of the coagulation cascade *in vivo*, whereas the intrinsic pathway is thought to be involved in the amplification of the coagulation cascade and recruitment of platelets to the rapidly forming thrombus (Mackman et al., 2007). TF forms a complex with FVIIa to activate FX into FXa. FXa forms the prothrombinase complex with FVa, which activates prothrombin to thrombin. Thrombin is involved in several aspects of the coagulation cascade including activation of cofactors FV, FXI, and FVIII of the intrinsic pathway that leads to amplification of coagulation, activation of platelets that are necessary components of haemostatic plug, and activation of fibrinogen into fibrin that is the basis of the fibrin thrombus (Figure 1.3) (Ott, 2011).

Figure 1.3
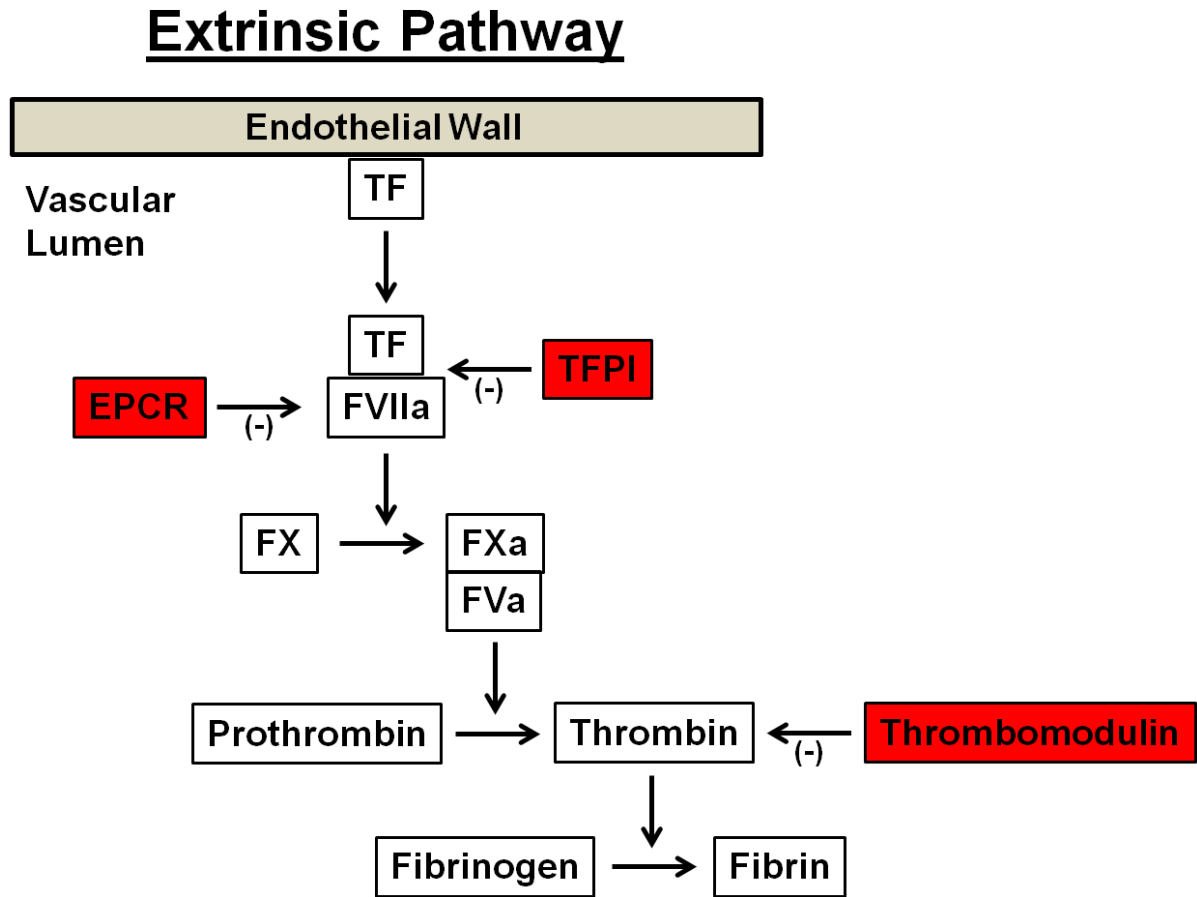
Extrinsic Pathway

Figure 1.3   Schematic diagram illustrating the extrinsic pathway of the coagulation cascade.

One important area for research is understanding how air pollution affects specific components of the coagulation cascade such as thrombin generation and fibrin thrombus formation. There are several tools available to assess the functional coagulation balance of endothelial cells following pathological injury or toxicological exposures. The Calibrated Automated Thrombography (CAT) assay was developed to measure thrombin generation for potential use in clinical settings as a global test to assess hyper- and hypocoagulability in blood (Campo et al., 2012; Hemker et al., 2002; Luddington and Baglin, 2004). In recent

studies, this assay has been utilized to determine the effects of air pollution on thrombin generation in animal and human models (Emmerechts et al., 2012; Kilinc et al., 2011; Rudez et al., 2009). For example, Emmerechts et al. demonstrated that elevated levels of coarse PM shortened the lag time of thrombin generation in microparticle-rich plasma obtained from diabetic individuals exposed to ambient air pollution (Emmerechts et al., 2012). In addition, fibrin thrombus formation can be measured as a way to assess the functional coagulation balance following exposure to air pollution. For instance, Metassan et al. have shown increased polymerization of purified human fibrinogen in the presence of UF PM (Metassan et al., 2010).

Activation and upregulation of TF is an important initiating step in thrombin and fibrin formation, and TF is implicated in the effects of air pollution on the coagulation system. Under normal physiological conditions, vascular cells that are in contact with blood typically do not express TF, so the extrinsic coagulation pathway is quiescent. However, under pathological conditions that cause endothelial activation, TF can be presented to the circulation by a variety of vascular cells where it can initiate blood coagulation. TF expression is generally suppressed in endothelial cells but can be induced by inflammation (Levi and van der Poll, 2005), endotoxin (Colucci et al., 1983), and exposure to air pollution (Karoly et al., 2007; Sun et al., 2008) *in vitro*. In addition, TF can be introduced to the circulation via vascular cell-derived microparticles, which are vesicles that are shed from the plasma membranes of cells in response to injury, apoptosis, or activation (Morel et al., 2006). TF-bearing microparticles have been shown to be released from several types of vascular cells including vascular smooth muscle cells, leukocytes, platelets and endothelial cells (VanWijk et al., 2003). Microparticles released from endothelial cells are an additional

indication of endothelial cell activation and are known to play a role in thrombosis and atherosclerosis (Chironi et al., 2009).

There are several checks and balances throughout the coagulation cascade to prevent pathological thrombus formation from occurring. For instance, several anticoagulant proteins such as tissue factor pathway inhibitor (TFPI), thrombomodulin, and endothelial protein C receptor (EPCR) play pivotal roles in counteracting the increased expression or activation of procoagulant proteins that arise from cellular injury or pathological conditions. TFPI is the primary inhibitor of TF-initiated coagulation by binding and inhibiting the TF/FVIIa complex (Figure 1.3) (Bajaj et al., 2001). This inhibition prevents initiation of the extrinsic coagulation cascade until enough TF/FVIIa complex is formed to pass a threshold that would exceed the inhibitory potential of TFPI (Crawley and Lane, 2008). Thrombomodulin is a transmembrane protein present on endothelial cells that binds to thrombin with high affinity. This results in activation of protein C (PC) (Anastasiou et al., 2012) and loss of thrombin's procoagulant ability by reducing its ability to cleave fibrinogen, activate FV, and trigger platelet activation (Figure 1.3) (Van de Wouwer et al., 2004). EPCR is a transmembrane protein constitutively expressed on the surface of endothelial cells that binds FVIIa, which prevents the formation of the TF/FVIIa complex (Figure 1.3), and binds PC (Navarro et al., 2011). The binding of PC to EPCR enhances the rate of PC activation by the thrombomodulin-thrombin complex by roughly 20-fold (Stearns-Kurosawa et al., 1996). Upon activation, activated PC (APC) inactivates the procoagulant proteins FVIIIa and FVa by cleaving peptide bonds, which results in the downregulation of thrombin generation (Van de Wouwer et al., 2004). APC also inhibits PAI-1 which promotes fibrinolysis, reduces inflammation by attenuating activation of the transcription factor NF-κB, and blocks p53-

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mediated apoptosis (Anastasiou et al., 2012; Cheng et al., 2003). The balance of these factors is critical, and enhanced procoagulant activity or inhibition of anticoagulant activity could indicate an activated endothelial cell phenotype, resulting in a prothrombotic state (Gilmour et al., 2005).

There is strong cross-talk between the coagulation and inflammatory pathways that involves inflammatory mediators activating the coagulation cascade or suppressing anticoagulant proteins, and coagulation proteins inducing inflammation (Levi et al., 2004a). Inflammation predominately induces coagulation by activating TF and the extrinsic coagulation pathway leading to thrombin generation (van der Poll et al., 2011). For example, proinflammatory cytokines, namely TNF-α and IL-1β, have been shown to induce TF expression and increase procoagulant activity in endothelial cells (Dinarello, 1991). Inflammation is also capable of impairing several anticoagulant pathways (Levi and van der Poll, 2005). Proinflammatory cytokines have been shown to down-regulate the anticoagulant protein TM, which in turn prevents the formation of the thrombomodulin-thrombin complex that is necessary for the activation of PC (Nawroth and Stern, 1986). Inflammation also reduces levels of the anticoagulant protein antithrombin, the main inhibitor of thrombin and FXa (Levi et al., 2004a), and down-regulates TFPI, the main inhibitor of TF (Delvaeye and Conway, 2009).

Similarly, procoagulant mediators are well known to induce inflammatory responses. For instance, thrombin activated platelets have been shown to release the CD40 ligand, which leads to upregulation of inflammatory cytokines and increased expression of cell adhesion molecules in endothelial cells (Henn et al., 1998). Furthermore, TF has been thoroughly documented as having a critical role in linking these two pathways (Levi and van

der Poll, 2005). The TF/FVIIa complex can bind protease activated receptors (PARs), leading to the induction of cell signaling pathways that result in increased expression of proinflammatory cytokines (Cunningham et al., 1999). Thrombin and FXa are also capable of binding to PARs and activating these signaling pathways (Levi et al., 2004b). In addition, it was shown in baboons that inhibition of TF activity prevents inflammation-induced thrombin generation (Taylor et al., 1991) and genetically altered mice with low levels of TF had attenuated levels of proinflammatory cytokines following exposure to endotoxin (Pawlinski et al., 2004).

Aberrant induction of TF has been associated with multiple CV disease pathologies including venous thrombosis, atherosclerosis, and diabetes (Lwaleed et al., 2007; Manly et al., 2010). TF can be induced by a variety of stimuli that primarily activate phospholipase C, resulting in a cascade of signaling events that eventually cumulate in the upregulation of TF expression (Herkert and Gorlach, 2002). TF is also a redox-regulated protein, and TF gene expression and protein levels have been shown to be modulated by ROS in vascular cells (Herkert et al., 2004). Additionally, TF mRNA expression is attenuated in the presence of antioxidants following exposure to ionizing radiation and inflammatory cytokines, further illustrating the critical role ROS plays in TF regulation (Szotowski et al., 2007). ROS is thought to induce TF through activation of transcription factors resulting in induction of TF mRNA, and the *TF* gene in primary endothelial cells has been shown to contain binding sites for the redox-sensitive transcription factors NF-κB, AP-1, and SP1 (Herkert and Gorlach, 2002; Moll et al., 1995).

ROS are produced in response to a variety of both physiological and pathological stimuli (Frey et al., 2008; Sprague and Khalil, 2009). The initial step in ROS generation is

production of the superoxide anion ($\cdot$O$_2$) from molecular oxygen (O$_2$). Superoxide is transformed into a subsequent ROS, hydrogen peroxide (H$_2$O$_2$), a reaction that is facilitated by the enzyme superoxide dismutase (SOD) (Mills et al., 2007). H$_2$O$_2$ can then be cleaved by catalase into water and oxygen. In the presence of transition metals, H$_2$O$_2$ can also be converted into the very reactive hydroxyl radical ($\cdot$OH) (Droge, 2002) (Figure 1.4).

Figure 1.4

$$O_2 \longrightarrow O_2^{\cdot-} \xrightarrow{\text{SOD}} H_2O_2 \xrightarrow{\text{Catalase}} H_2O + O_2$$

$$H_2O_2 \xrightarrow{\substack{\text{Transition} \\ \text{Metals}}} {}^{\cdot}OH$$
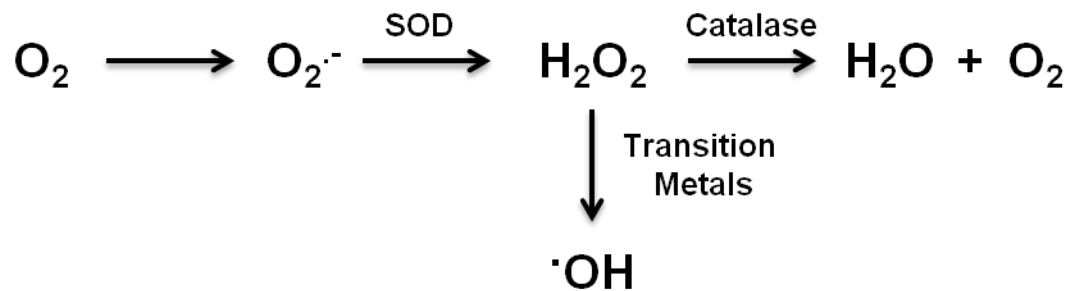
Figure 1.4 Pathways of ROS production.

Cellular generation of ROS is important physiologically because these molecules can be used as both intra- and intercellular signaling molecules (D'Autreaux and Toledano, 2007). Superoxide is relatively unstable in aqueous conditions and is rapidly transformed into H$_2$O$_2$ either by SOD or spontaneously, so it is generally thought that signaling events occur through the H$_2$O$_2$ molecule (Li and Shah, 2004). H$_2$O$_2$ is capable of oxidizing cysteine residues in proteins to cysteine sulfenic acid or disulfide, which can lead to protein phosphorylation and initiation of signaling cascades (Rhee et al., 2000). Additionally, H$_2$O$_2$ has been shown to activate transcription factors, such as NF-κB (Schmidt et al., 1995), AP-1 (Karin and Shaulian, 2001) and p53 (Thomas et al., 2006) , leading to induction of redox-sensitive genes.

H$_2$O$_2$ is a membrane soluble molecule is detectable both extra- and intracellularly (Rhee et al., 2010). Extracellular H$_2$O$_2$ release can be measured using the impermeant

Amplex Red reagent (*N*-acetyl-3,7-dihydroxyphenoxazine) (Li et al., 2006). H_2O_2 reacts with Amplex Red in the presence of horseradish peroxidase (HRP) to produce resorufin, a highly fluorescent molecule that can be measured using a fluorescent plate reader (Figure 1.5) (Rhee et al., 2010). The Amplex Red reagent is specific for H_2O_2 and is highly sensitive with the ability to detect 5pmol of H_2O_2 per 100 μL sample (Bartosz, 2006). The Amplex Red reagent is not membrane permeable, so it is used to detect both extracellular H_2O_2 that was made on the plasma membrane of the cell and intracellular H_2O_2 that diffused through the cellular membrane into the extracellular space. Intracellular H_2O_2 can be measured by Peroxy Green 1 (PG1), a small-molecule fluorophore that is membrane soluble (Miller et al., 2007). PG1 is a boronate-based probe that fluoresces upon removal of the boronate group by H_2O_2 (Figure 1.5) (Miller et al., 2007). PG1 is highly specific for intracellular H_2O_2 and is able to detect physiologically relevant levels of H_2O_2 in live, individual cells during real time using confocal microscopy (Cheng et al., 2010).

Figure 1.5

Amplex Red

Amplex Red reagent   Resorufin
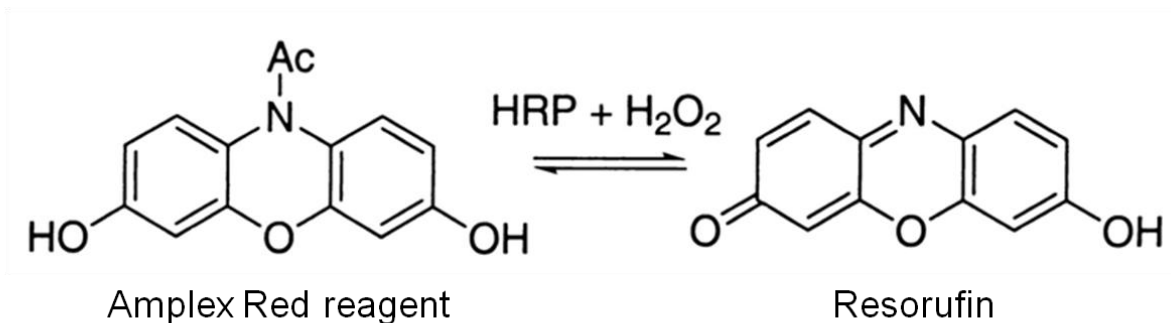
PG1
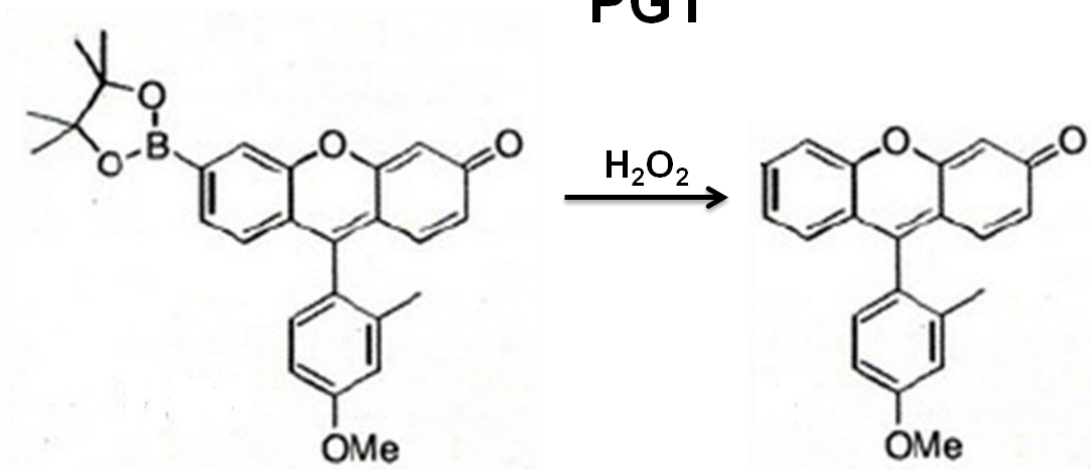
Figure 1.5 Schematic diagram illustrating activation of the Amplex Red reagent and PG1 by H_2O_2. Images were modified from Wentworth et al. and Miller et al. (Miller et al., 2007; Wentworth et al., 2000).

ROS concentrations are tightly controlled by enzymatic (i.e. catalase, SODs, heme oxygenase (HO), thioredoxin) and non-enzymatic antioxidants (i.e. uric acid, glutathione, vitamins A, C, and E) (Li and Shah, 2004). However, under pathophysiological conditions, ROS can be produced at an elevated rate, overwhelming these antioxidant systems and resulting in oxidative stress that leads to cellular damage. For instance, elevated levels of

H_2O_2 have been shown to induce cellular injury by causing damage to key cellular molecules such as DNA (Imlay et al., 1988), proteins (Knock and Ward, 2011), and lipids (Kellogg and Fridovich, 1975). Furthermore, elevated levels of ROS are implicated in the pathogenesis and progression of several CV diseases including atherosclerosis, hypertension, and diabetes (Madamanchi et al., 2005).

Endothelial cells can generate ROS in a variety of enzymatic and non-enzymatic ways, such as the mitochondrial electron transport chain, xanthine oxidases, and NADPH oxidase (NOX) enzymes (Droge, 2002; Li and Shah, 2004). In addition, transition metals, such as iron, copper, and chromium are capable of undergoing redox cycling and producing intracellular ROS through Fenton reactions (Jomova and Valko, 2011). Of these potential sources, NOX enzymes are proposed as the key generator of ROS production in the vasculature (Babior, 2000; Mohazzab et al., 1994).

NADPH oxidases are a family of enzymes comprised of 7 major members, NOX1-5 and Duox1-2 (Frey et al., 2008). NOX-2 was the first to be characterized, where it was discovered on the plasma membrane of phagocytic cells and shown to produce a respiratory burst of oxidants for microbial defense (Babior, 1984). Since that discovery, the NOX family of enzymes have been reported in an assortment of non-phagocytic cells including fibroblasts, vascular smooth muscle cells, and endothelial cells among others (Brown and Griendling, 2009). Endothelial cells express mainly the NOX-2 and NOX-4 isoforms (Li and Shah, 2004), but expression levels of the NOX isoforms varies among different endothelial cell types (Guzik et al., 2004).

NOX isoforms are differentially expressed and regulated depending on the type of tissue in which they are located (Altenhofer et al., 2012). Furthermore, NOX enzymes can

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be found in various subcellular locations and expression of these enzymes can dictate participation in distinct signaling pathways (Gough and Cotter, 2011). For example, the intracellular distribution of NOX-4 is broad and has been shown to be variably located in the perinuclear space, endoplasmic reticulum, mitochondria, and nucleus of endothelial cells (Lassegue and Griendling, 2010; Lassegue et al., 2012) and has been reported to be involved in several cellular processes including vasodilation, cell migration, and proliferation (Pendyala et al., 2009; Petry et al., 2006; Ray et al., 2011).

Most NOX isoforms produce a basal level of ROS that can be further induced by an array of stimulants including cytokines, shear stress, G-protein coupled agonists, growth factors, and hypoxia (Griendling et al., 2000). NOX-derived ROS function in basic physiological processes such as oxygen sensing, endothelial permeability, differentiation, and tissue development; however, pathological activation of NOX enzymes can lead to elevated levels of ROS, resulting in oxidative stress and cellular injury (Brown and Griendling, 2009; Frey et al., 2009). For instance, ROS from NOX enzymes induced TF mRNA expression in vascular smooth muscle cells following agonist stimulation by thrombin or platelet-derived products from activated human platelets (Gorlach et al., 2000; Herkert et al., 2002). Furthermore, recent studies indicated that NOX-induced oxidative stress in endothelial cells plays an important role in the pathogenesis of CV disorders including hypertension, atherosclerosis, and diabetes (Madamanchi et al., 2005). NOX enzymes are considered important therapeutic targets when trying to control overproduction of ROS (Schramm et al., 2012) and inhibition of these enzymes is a potential approach to combat adverse CV health effects caused by air pollution exposure.

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Exposure to air pollution is strongly associated with CV morbidity and mortality but the mechanisms behind these associations are currently not known. Endothelial cell activation, increased ROS production, and initiation of coagulation and inflammation are pathophysiological mechanisms that could link inhaled air pollutants to vascular events. I hypothesize that exposure to soluble components of UF particles causes altered endothelial cell phenotype through activation of oxidant signaling that mediates procoagulant and proinflammatory responses resulting in a prothrombotic phenotype (Figure 1.6).

Figure 1.6
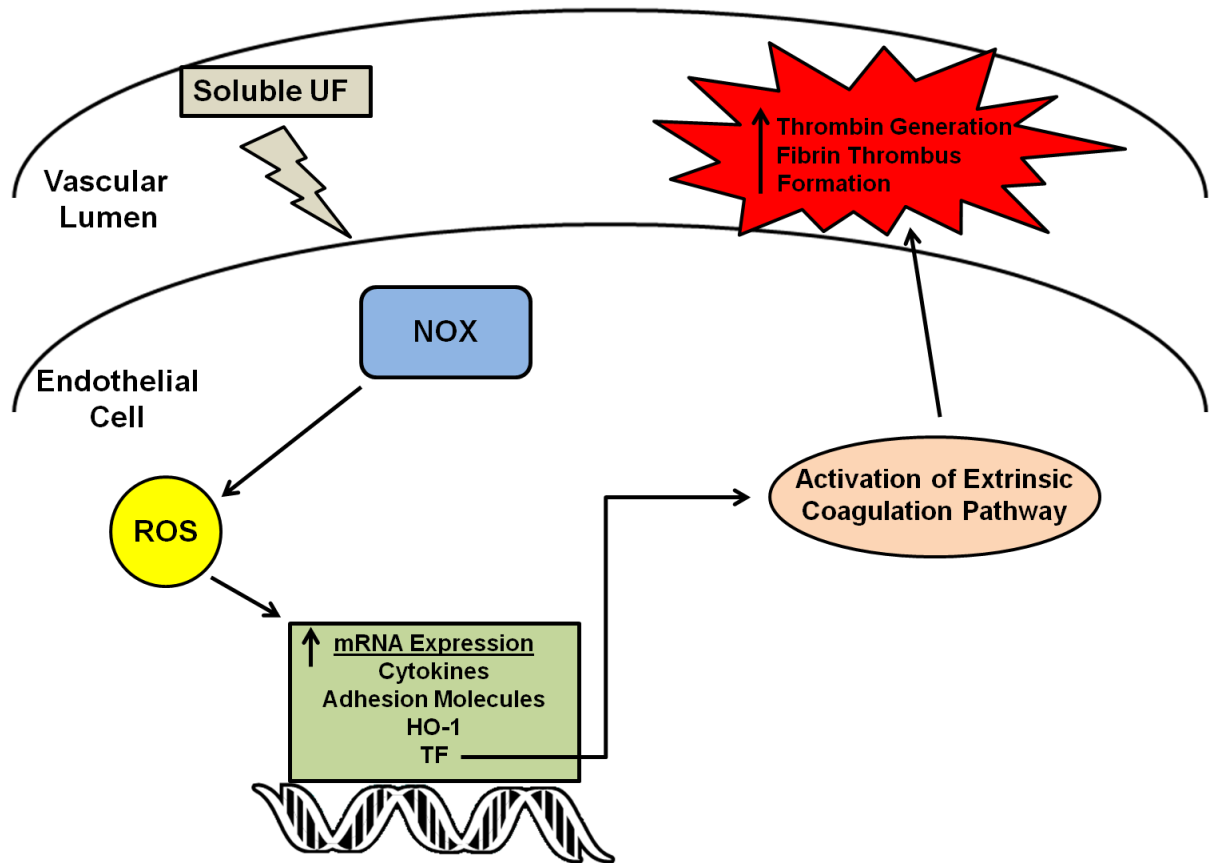
Figure 1.6 Schematic diagram illustrating endothelial activation following exposure to soluble UF.

CHAPTER 2

SOLUBLE COMPONENTS OF ULTRAFINE PARTICLES STIMULATE H$_2$O$_2$ PRODUCTION IN ENDOTHELIAL CELLS

2.1 Introduction

A growing body of evidence shows a strong association between exposure to air pollution and cardiovascular (CV) morbidity and mortality. Exposure to air pollution leads to increased hospitalizations for circulatory disorders (Poloniecki et al., 1997), accelerates the progression of atherosclerosis (Suwa et al., 2002), and increases the risk of myocardial infarctions (Peters et al., 2001). In addition, elevated levels of air pollution are associated with increased risk of CV-related mortality (Pope et al., 2004). The World Health Organization estimates that exposure to ambient air pollution causes several million premature deaths worldwide each year, with particulate matter (PM) exposure accounting for approximately 800,000 of those deaths (Anderson et al., 2012).

PM is a major component of ambient air pollution and is derived from a variety of sources that are both natural and manufactured. Classification of PM is determined by diameter of the particle: coarse particles (PM$_{10}$) are 10 to 2.5 μm, fine particles (PM$_{2.5}$) are 2.5 to 0.1 μm, and ultrafine particles (UF; PM$_{0.1}$) are <0.1 μm (Poschl, 2005). PM is formed out of extremely small particles and liquid droplets with composition varying depending on geographical location, local pollution sources, time of day, season, and size of particle (Polichetti et al., 2009). Coarse particles are typically derived from soil and sea salts and have been shown to contain endotoxin that induces proinflammatory responses (Brooks et al., 2002), whereas fine and UF particles are predominantly derived from combustion sources

and are comprised of volatile organic compounds, polycyclic aromatic hydrocarbons, transition metals, and other constituents adsorbed on a carbonaceous core (Polichetti et al., 2009).

Epidemiological studies illustrated that exposure to elevated levels of coarse and fine PM induced adverse CV effects (Harrison and Yin, 2000; Polichetti et al., 2009). The Environmental Protection Agency currently monitors and regulates PM_{10} and $PM_{2.5}$, but increasing evidence suggests that UF particles are a key factor in the adverse CV effects due to air pollution exposure (Nel, 2005). UF particles make up only a small portion of ambient PM by mass concentration; however, they constitute the majority of particle number per unit mass (Donaldson et al., 2001). For example, at the same mass concentration, UF particles have a 6-fold increase in number concentration as compared to fine particles, resulting in significantly greater collective particle surface area (Stanek et al., 2011). This high surface area-to-mass ratio allows for the adsorption of toxic substances such as organic compounds and transition metals (Sioutas et al., 2005). In addition, the small diameter of UF particles makes them more likely to deposit in the alveolar region of the lung based on Brownian motion and diffusion transportation as opposed to coarse and fine particles that tend to deposit in the upper airways of the lung (Kreyling et al., 2004). Because of their small size, UF particles remain longer in the lung following deposition since they are more likely to avoid being recognized and phagocytized by alveolar macrophages as compared to the larger coarse and fine particles (Gonzalez et al., 1996). Even though UF particles may not leave the lung to directly mediate adverse CV effects (Brown et al., 2002; Mills et al., 2006; Nemmar et al., 2002), soluble components of UF particles (soluble UF) could cross from the lung into

the circulation and directly interact with cells of the vasculature such as endothelial cells (Wallenborn et al., 2007).

Transition metals are a major component of the soluble fraction and may play an important role in the adverse CV effects associated with air pollution exposure (Costa and Dreher, 1997). Transition metals can increase oxidative DNA damage in lymphocyte DNA and induce reactive oxygen species (ROS) production in endothelial cells, indicating that soluble components of PM are capable of inducing oxidative stress following exposure to air pollution (Montiel-Davalos et al., 2012; Sorensen et al., 2005).

Elevated levels of ROS under pathological conditions have been linked to the development of several CV diseases (Madamanchi et al., 2005). Endothelial cells can produce ROS in response to an array of both physiological and pathological stimuli from a variety of both enzymatic and non-enzymatic sources (Droge, 2002). The major sources of ROS production in endothelial cells include the mitochondrial electron transport chain, xanthine oxidases, and NADPH oxidases (NOX), and of these, NOX enzymes are proposed to be the key generator of ROS production following exposure to toxic substances such as UF particles (Li and Shah, 2004; Mo et al., 2009). Furthermore, NOX-induced oxidative stress in endothelial cells is implicated in the progression of various CV disorders including hypertension, atherosclerosis, and diabetes (Bengtsson et al., 2003)

We hypothesize that UF particles cause ROS production in endothelial cells through activation of NOX enzymes by the soluble components. There were several objectives to this study. We set out to 1) determine the relevant size, fraction, and dose of PM to use throughout the project, 2) identify the primary cellular source for PM-induced ROS production, and 3) determine the components responsible for the adverse effects following

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exposure. We show here that exposure to non-cytotoxic doses of soluble UF increase ROS production in endothelial cells that is dependent on activation of NOX enzymes and transition metals.

2.2 Materials and Methods

2.2.1 Reagents and Chemicals

EA.hy926 (EA cells), are an immortalized endothelial cell line derived by fusing human umbilical vein endothelial cells with A549 cells, a human lung adenocarcinoma epithelial cell line (Edgell et al., 1983). EA cells were obtained from University of North Carolina's Tissue Culture Facility (Chapel Hill, NC). Dulbecco's modified Eagle's medium (DMEM)-high glucose, fetal bovine serum (FBS), and antibiotic-antimycotic (100X) were obtained from Gibco (Grand Island, NY). 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.

2.2.2 Cell Culture

EA cells were cultured in DMEM-high glucose medium supplemented with 10% FBS and 1% antibiotic-antimycotic mix. HCAEC were cultured in EGM-2 media supplemented with the EGM-2 Bullet Kit (2% FBS, 0.4% rhFGF-B, 0.1% gentamicin sulfate amphotericin-B, 0.1% rhEGF, 0.1% heparin, 0.1% ascorbic acid, 0.1% R^3-IGF-1, 0.1% VEGF, and 0.04% hydrocortisone). HCAEC were obtained from two adult donors with no known history of CV disease. Cells were grown to confluence and used between passages 5-8.

2.2.3 Ultrafine Particles

Coarse, fine, and UF particles were collected from February to May 2007 in Chapel Hill, NC as previously described (Becker et al., 2005). UF particles were analyzed for chemical components by the Research Triangle Institute (Research Triangle Park, NC) and the composition of these particles is expressed relative to UF particle mass (ng/mg; Table

2.1). The soluble fraction of these particles was acquired by suspending the particles in PBS at the desired concentration and centrifuging the resultant suspension for 30 minutes at 20,000 $x\,g$. The supernatant from the pelleted particles was collected and used as the soluble fraction. The pellet was then re-suspended in the same volume of PBS and used as the insoluble fraction.

2.2.4 Extracellular H_2O_2 Measurement using the Amplex Red Assay

Extracellular H_2O_2 release was measured using the Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine; Invitrogen, Carlsbad, CA), which reacts with H_2O_2 in the presence of horseradish peroxidase to produce resorufin, a highly fluorescent molecule. EA cells and HCAEC were cultured on 12-well plates and exposed to coarse, fine, or UF PM (0, 50, and 100 µg/mL). Immediately following exposure, Amplex Red (150 µM) and HRP (0.8 U/ml) were added, and plates were analyzed at 5 min intervals for 30 min on a Bioassay HTS7000 plate reader (Perkin-Elmer, Wellesley, MA) with HTSoft version 1.0 software (PE Applied Biosystems, Weiterstadt, Germany). Excitation wavelength was 535 nm and emission fluorescence was 590 nm. H_2O_2 was quantified by subtracting the baseline fluorescence from the final fluorescence, and normalizing these relative fluorescence units (RFU) to a standard curve of H_2O_2 (0-1 nm). Following analysis, the endothelial cells were washed with PBS and cellular protein was collected with lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X-100, pH 7.4) containing 1:100 Protease Inhibitor Cocktail Set 1 (Calbiochem, La Jolla, CA). Protein concentrations of cell lysates were measured using the Bio-Rad protein reagent (Bio-Rad, Richmond, CA) according to the manufacturer's protocol.

<u>2.2.5 Measurement of Cytotoxicity</u>

Cytotoxicity was determined by measuring supernatant lactate dehydrogenase (LDH) levels using the colorimetric CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega Corporation, Madison, WI) according to the manufacturer's protocol. EA cells and HCAEC were exposed to soluble UF (0, 10, 50, and 100 μg/mL) for 6 or 24 hours. Supernatant from cells treated with saponin (0.5%) to disrupt the cellular membrane was a positive control for the assay.

<u>2.2.6 Statistical Analysis</u>

Statistical analysis was performed using commercially available software (GraphPad Prism, version 4.03 for Windows; GraphPad Software, San Diego, CA). A one-way ANOVA analysis followed by Bonferroni's post-hoc test was used to compare exposed cells to control cells. A p-value < 0.05 was considered statistically significant.

2.3 Results

2.3.1 Coarse, fine, and UF PM exposure increases extracellular H_2O_2 generation in endothelial cells

To determine the effect particle size has on PM-induced ROS production in endothelial cells, we exposed EA cells, an immortalized endothelial cell line, to coarse, fine, or UF particles (100 μg/mL). Cells were exposed to the whole particle, and the soluble and insoluble fractions. Extracellular H_2O_2 release was quantified over 30 min immediately following exposure using the Amplex Red assay. We found that the whole particle of coarse, fine, and UF PM significantly ($p<0.001$) increased extracellular H_2O_2 production by 2.5-fold, 5.4-fold, and 4-fold, respectively, compared to control cells (Fig. 2.1A-C). The soluble fraction of UF particles induced a 3.7-fold increase ($p<0.001$) in extracellular H_2O_2 production, which is roughly equivalent to that induced by the whole UF particle, whereas the insoluble fraction of UF particles did not induce a significant increase in H_2O_2 production in the EA cells. While the soluble fractions of coarse and fine PM increased H_2O_2 production as well, we demonstrate that the insoluble fraction of these particles also significantly ($p<0.001$ and $p<0.05$, respectively) increased extracellular H_2O_2 production. These data indicate that components of both the soluble and insoluble fractions of coarse and fine PM induce ROS production in EA cells, whereas only components of the soluble fraction of UF particles are responsible for the increased H_2O_2 release. This experiment was repeated in human coronary artery endothelial cells (HCAEC) in order to assess the response in a primary cell model. The studies illustrated that soluble UF induced a 15.8-fold increase ($p<0.05$) in extracellular H_2O_2 production in HCAEC, whereas the insoluble fraction did not cause a significant change (Fig. 2.1D). This response in primary cells was substantially

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augmented as compared to that in the EA cell line. Given the responses, we selected the soluble fraction of UF particles for my project to evaluate endothelial cell responses to PM exposure.

Figure 2.1
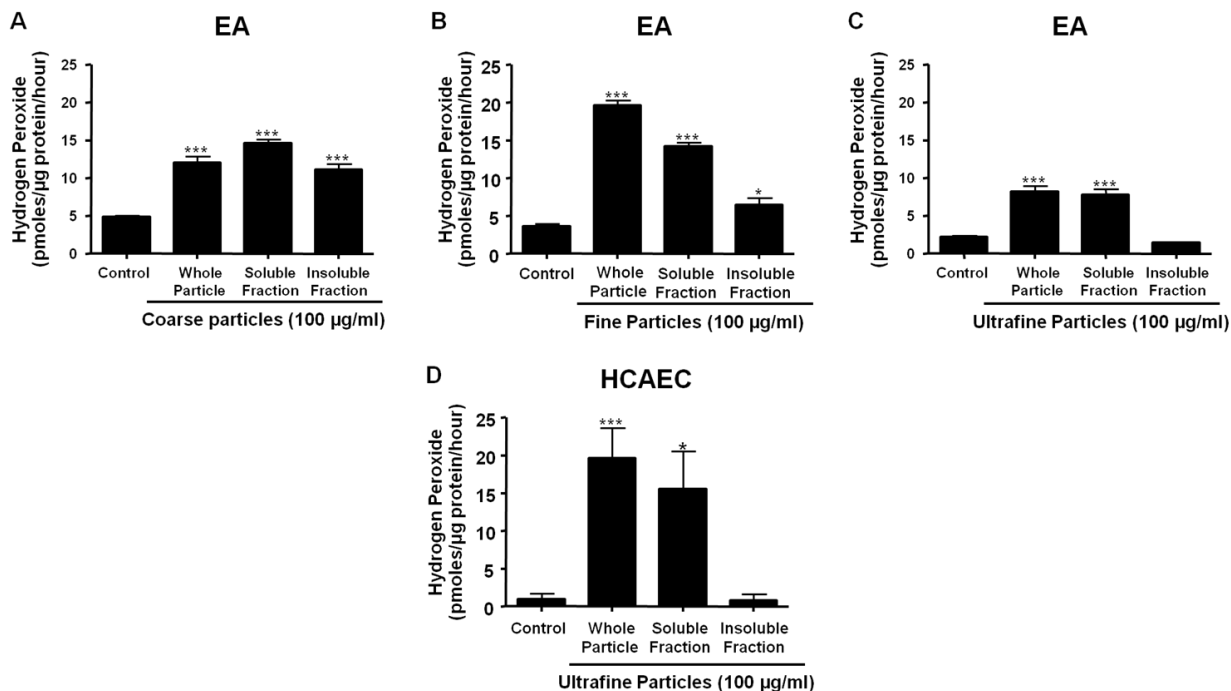
Figure 2.1 Exposure to soluble UF induces rapid H_2O_2 generation by EA and HCAEC.

EA (A-C) and HCAEC (D) were exposed to the whole particle, soluble fraction, and insoluble fraction of coarse (A), fine (B), and UF (C-D) PM (100 µg/mL) and extracellular H_2O_2 release was measured using the Amplex Red reagent. n = 3 independent experiments with samples in triplicate. $*$ = p<0.05, $***$ = p<0.001 compared to control.

2.3.2 Soluble UF exposure causes a dose- and time-dependent increase in extracellular H_2O_2

generation in endothelial cells

To ascertain the potential cytotoxicity over a range of soluble UF concentrations, LDH release was quantified following exposure to 10, 50, and 100 μg/mL soluble UF for 6 and 24 hrs. Soluble UF did not induce cytotoxicity in the EA (Fig. 2.2A) or HCAEC (Fig. 2.2B). In addition, extracellular H_2O_2 production by both the EA and HCAEC increased in a dose- and time-dependent manner following exposure to soluble UF (50 and 100 μg/mL) (Fig. 2.2C-F). These data indicate that exposure to soluble UF increases extracellular H_2O_2 release in a concentration- and time-dependent manner at doses that are non-cytotoxic to endothelial cells. For a majority of the remaining experiments in this project, we will expose the endothelial cells to the intermediate (50 μg/mL) or high (100 μg/mL) dose of soluble UF.

Figure 2.2
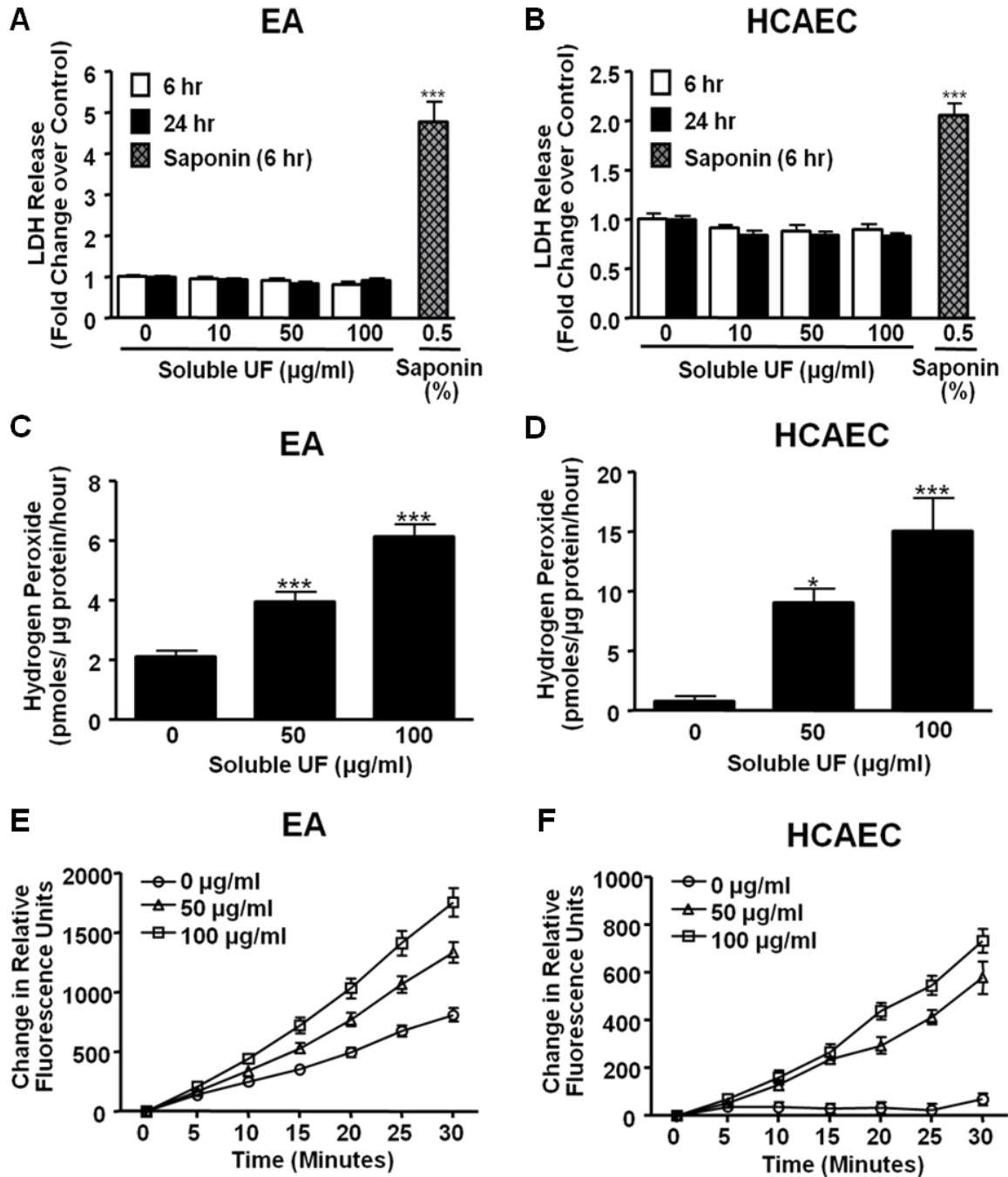
Figure 2.2 Non-cytotoxic doses of soluble UF increase H_2O_2 production in a dose- and time-dependent manner in EA and HCAEC. EA (A) and HCAEC (B) were exposed to soluble UF (0, 10, 50, and 100 µg/mL) or the detergent saponin (0.5%) as the positive control

for 6 or 24 hrs. LDH release was measured as a marker of cytotoxicity using the CytoTox 96 Non-Radioactive Cytotoxicity Assay. n = 3 independent experiments with samples in triplicate. EA (C, E) and HCAEC (D, F) were exposed to soluble UF (0, 50, and 100 µg/mL) and the Amplex Red reagent was used to measure the dose response (C-D) and time response (E-F) of extracellular H_2O_2 release. n = 3 independent experiments with samples in duplicate. $* = p<0.05$, $*** = p<0.001$ compared to control.

2.3.3 Soluble UF-induced ROS production is from NOX enzymes

To determine the cellular source of soluble-UF induced H_2O_2 production, we measured extracellular H_2O_2 production by EA cells in the presence of chemical inhibitors of several key sources of ROS production in endothelial cells. Soluble UF-induced extracellular H_2O_2 release was not inhibited by allopurinol, a xanthine oxidase inhibitor (Fig. 2.3A) or inhibitors targeting the mitochondrial respiratory chain complex I (Fig. 2.3B) and IV (Fig. 2.3C). However, DPI, a NOX inhibitor, reduced extracellular H_2O_2 production in EA cells exposed to 50 and 100 μg/mL soluble UF by 57% and 59% respectively (Fig. 2.3D). These experiments were repeated in primary endothelial cells. Pretreatment with DPI significantly ($p<0.05$) attenuated extracellular H_2O_2 production in HCAEC exposed to 50 and 100 μg/mL soluble UF by 54% and 38% respectively (Fig. 2.3E). These data implicate NOX enzymes as a major source of H_2O_2 production in endothelial cells following exposure to soluble UF.

Figure 2.3
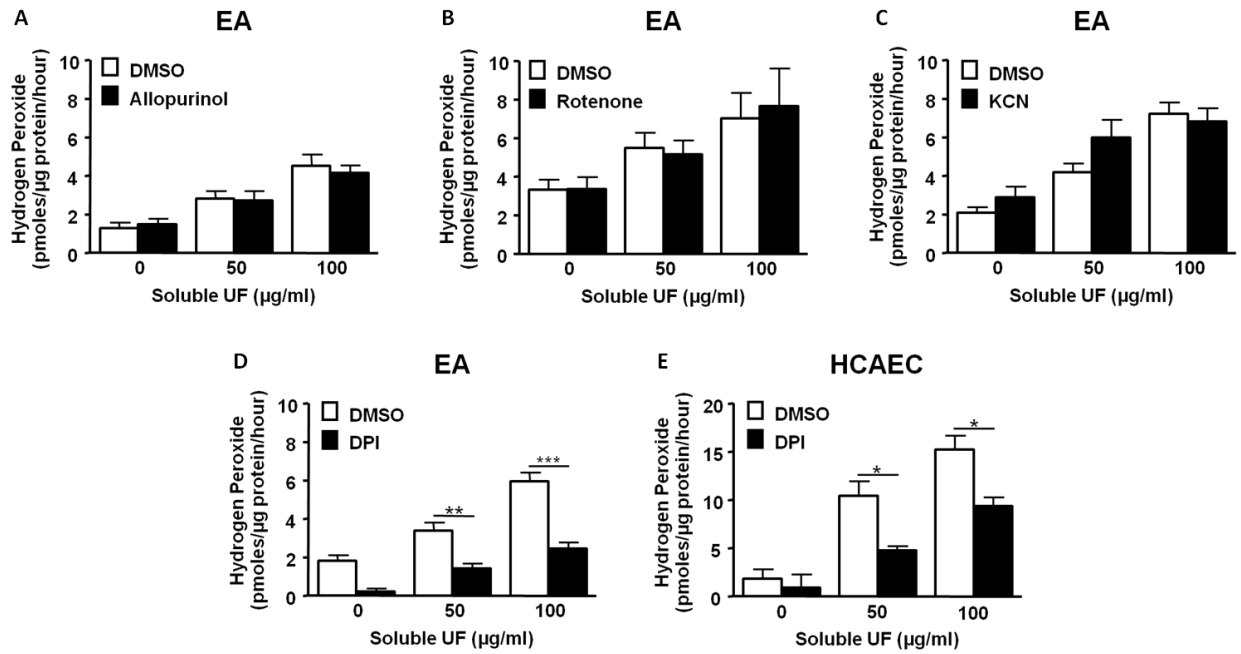
Figure 2.3 Soluble UF-induced ROS production is dependent on NOX enzymes.

Extracellular H_2O_2 was measured using the Amplex Red reagent in EA (A-D) and HCAEC (E) pretreated with the xanthine oxidase inhibitor allopurinol (A, 20 µM), the mitochondrial respiratory chain complex I inhibitor rotenone (B, 10 µM), the mitochondrial respiratory chain complex IV inhibitor KCN (C, 10 µM), or the NOX inhibitor DPI (D-E, 50 µM) for 30 minutes followed by exposure to 50 or 100 µg/mL soluble UF. n = 3 separate experiments with samples in duplicate. * = p<0.05, ** = p<0.01 *** = p<0.001

2.3.4 Soluble UF-induced extracellular H_2O_2 generation in HCAEC is dependent on

transition metals

We next wanted to measure the UF particle-associated components to provide information on compounds present in the soluble fraction that could lead to increased ROS production. The UF particles were analyzed at the Research Triangle Institute and components are expressed relative to UF particle mass (ng/mg; Table 2.1). The measured levels of these elements and ions are comparable to those found in Chapel Hill UF particles previously collected and analyzed in 2001 and 2002 (Becker et al., 2005). Transition metals, such as chromium, copper, iron, nickel, vanadium, and zinc, were present in the UF particles. To determine if these transition metals have a role inducing extracellular H_2O_2 production, we measured the extracellular H_2O_2 release from HCAEC following exposure to 50 μg/mL soluble UF that had been pretreated with the metal chelator deferoxamine for 15 min. Pretreatment with deferoxamine (100 μM) reduced extracellular H_2O_2 production in soluble UF-exposed HCAEC by 45% (p<0.001; Fig. 2.4). These data indicate that transition metals play a partial role in the adverse effects induced in endothelial cells following exposure to soluble UF and suggest that other soluble components are also involved.

Table 2.1

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

Table 2.1 Components of UF PM (ng/mg) collected in Chapel Hill, NC from February to May 2007.

Figure 2.4
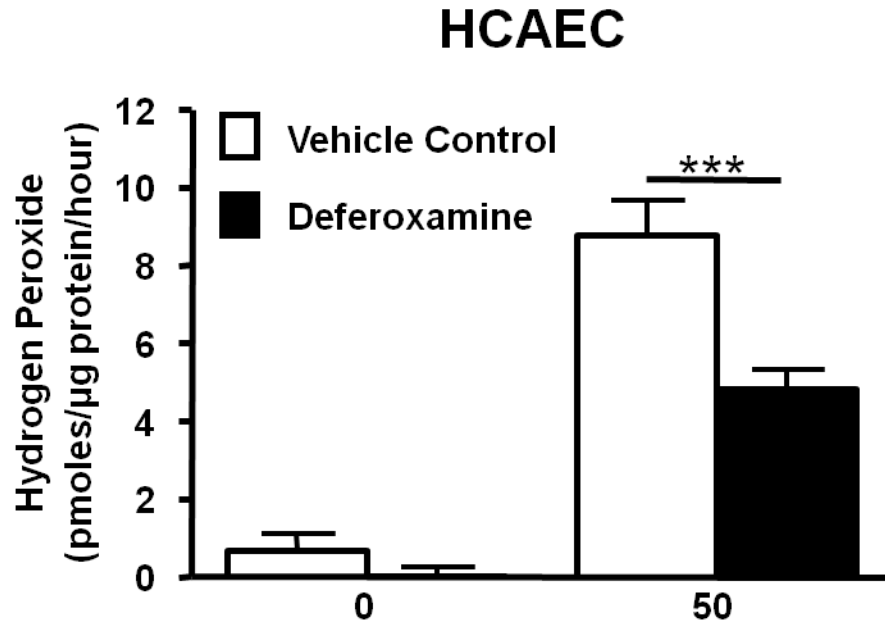
Figure 2.4   Soluble UF-induced H_2O_2 production is dependent on transition metals.
HCAEC were exposed to soluble UF (0 or 50μg/mL) that were pretreated with the metal
chelator deferoxamine (100 μM) for 15 min. Extracellular H_2O_2 release was measured using
the Amplex Red reagent. n = 3 independent experiments with samples in triplicate. *** =
p<0.001 compared to control.

2.4 Discussion

Increasing evidence suggests a strong association between exposure to air pollution and CV morbidity and mortality (Brook, 2008; Polichetti et al., 2009; Pope et al., 2004); however, further investigation needs to be conducted on the adverse vascular effects associated with exposure to components of PM. Here we report that exposure to non-cytotoxic doses of soluble components of UF particles increase extracellular H_2O_2 release in immortalized and primary endothelial cells. We further show that this induced H_2O_2 production is dependent on NOX enzymes and transition metals. These novel findings suggest that particle-induced ROS production in endothelial cells is a plausible mechanism for adverse CV events associated with exposure to air pollution.

Our results add to a growing body of evidence linking PM to the adverse CV effects associated with air pollution exposure. We showed that exposure to coarse, fine, and UF PM increased extracellular H_2O_2 production in endothelial cells. This finding supports previous studies showing that the different sized fractions of PM induce ROS production in bronchial epithelial cells and alveolar macrophages (Becker et al., 2005). We also demonstrated that the soluble and insoluble fraction of coarse and fine PM induced extracellular H_2O_2 production, whereas only the soluble fraction of UF particles increased H_2O_2 release. This finding is important because it further supports the concept that the water soluble components of UF particles, which are capable of translocating to the vasculature, are responsible for the adverse CV effects associated with PM exposure. It is not certain if whole PM particles can translocate from the lung to the vasculature (Mills et al., 2006; Nemmar et al., 2002). Since UF particles are most capable of depositing in the heavily vascularized alveoli (Kreyling et al., 2004) and the soluble fraction of PM is more likely to

traverse the alveolar-capillary barrier to enter the vasculature (Wallenborn et al., 2007), we designed our experiments to evaluate endothelial cell responses to PM exposure using the soluble fraction of UF particles.

Elevated levels of ROS have been shown to induce cellular injury by causing damage to key cellular molecules and have been implicated in the pathogenesis and progression of several CV diseases (Imlay et al., 1988; Kellogg and Fridovich, 1975; Knock and Ward, 2011; Madamanchi et al., 2005). We show here that exposure to soluble UF induces immediate ROS production in endothelial cells; suggesting vascular oxidative stress may occur following air pollution exposure. While there are several major sources of ROS production in endothelial cells, a recent study has shown that mouse pulmonary microvascular endothelial cells exposed to whole UF particles induced ROS production by NOX enzymes (Mo et al., 2009). Our data extend this finding by illustrating that NOX enzymes are the likely source of H_2O_2 production following exposure to water-soluble components of UF particles in human endothelial cells. Inhibition of NOX enzymes is an emerging strategy to combat oxidative stress-mediated diseases (Jaquet et al., 2009) and our data further illustrate the prospect of targeting these enzymes when trying to combat adverse CV effects due to air pollution exposure.

Transition metals are thought to play an important role in the adverse health effects associated with air pollution exposure (Costa and Dreher, 1997). Several studies involving air pollution samples collected when a steel mill in Utah Valley was open (1986), closed (1987), and reopened (1988) demonstrated that metals associated with PM induce adverse pulmonary effects (Stanek et al., 2011). Extracts from 1986 and 1988 contained the highest levels of metals and were shown to induce pulmonary injury and inflammation in rats (Dye et

al., 2001), increase pulmonary inflammation in healthy human volunteers (Ghio and Devlin, 2001), and increase rates of respiratory related hospital admissions (Pope, 1989). In addition, *in vitro* studies have shown that transition metals induce inflammatory responses and increase ROS production in pulmonary and vascular cells (Gojova et al., 2007; Montiel-Davalos et al., 2012; Shafer et al., 2010). For instance, Shafer et al. demonstrated that pretreatment with metal chelators attenuated particle-induced ROS production in rat alveolar macrophages, indicating that transition metals in the water-soluble fraction of coarse and fine PM are responsible for the increased ROS production (Shafer et al., 2010). Our results extend these observations by illustrating that transition metals associated with the soluble fraction of UF particles cause adverse vascular effects by inducing H_2O_2 production in endothelial cells.

There are potential limitations to this study. We are not certain of the PM concentration HCAEC experience following an air pollution exposure. However, individuals living in heavily polluted cities or exposed to natural disasters such as forest fires are capable of experiencing elevated levels of PM similar to the doses used in this study (Becker et al., 2005). In addition, the doses used in this study were not cytotoxic to the cells, indicating that the increased ROS production following exposure to soluble UF is due to endothelial activation rather than cellular death. Endothelial activation could link inhaled air pollutants to vascular events and have been shown to contribute to the pathogenesis of a variety of CV diseases (Szmitko et al., 2003). We used EA cells and HCAEC as our cellular models for studying CV adverse effects due to air pollution exposure. EA cells are an immortalized endothelial cell line derived by fusing human umbilical vein endothelial cells with A549 cells, a human lung adenocarcinoma epithelial cell line (Edgell et al., 1983). These cells

maintain most of the characteristics of endothelial cells, are fast growing, and ideal for establishing protocols, but the effects produced in these cells may not fully represent effects in primary endothelial cells. Therefore, we also used HCAEC as a primary endothelial cellular model. Endothelial cells from the coronary artery are among the first vascular cells to receive fresh blood from the lung and will accordingly be exposed to high concentrations of any soluble components that cross into the circulation from the alveoli.

In summary, we have shown that exposure to soluble UF induces production of extracellular H_2O_2 from NOX enzymes in endothelial cells. This increase in ROS production is dependent upon transition metals located in the soluble fraction. These novel findings support the assertion that particle-induced ROS is a plausible mechanism by which PM exposure induces adverse CV health effects.

CHAPTER 3

SOLUBLE COMPONENTS OF ULTRAFINE PARTICLES INDUCE ENDOTHELIAL PROCOAGULANT ACTIVITY THROUGH OXIDANT SIGNALING

3.1 Introduction

Epidemiological evidence reveals a strong association between exposure to air pollution and increased cardiovascular (CV) morbidity and mortality (Baccarelli et al., 2008; Calderon-Garciduenas et al., 2008; Pope et al., 2004). 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 particles by number and have a significantly large surface area-to-mass ratio (Donaldson et al., 2001). In addition, UF particles can deposit in the alveolar region of the lung because of their small diameter (Kreyling et al., 2004). Because these particles are not as well recognized or phagocytized by alveolar macrophages (Gonzalez et al., 1996), they may remain longer in the lung, which increases the probability of causing adverse effects. Furthermore, soluble components of UF particles (soluble UF) could traverse the alveolar-capillary barrier to enter the vasculature (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 exposure to soluble UF increases tissue factor (TF) mRNA expression in human pulmonary artery endothelial cells (Karoly et al., 2007). Under normal physiological conditions, TF is not highly expressed on endothelial cells, but surface expression of TF can be induced by a variety of stimuli including fibrin, endotoxin, and the proinflammatory cytokines TNF-α and IL-1β (Colucci et al., 1983; Contrino et al., 1997; Dinarello, 1991; Levi and van der Poll, 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 fibrin thrombus formation (Lwaleed et al., 2007). Elevated levels of TF have been associated with adverse CV effects including venous thrombosis and atherosclerosis (Lwaleed et al., 2007; Manly et al., 2010).

TF is a redox-regulated protein, and TF gene expression and protein levels can be modulated by ROS in vascular cells (Herkert and Gorlach, 2002). Endothelial cells produce ROS in response to a variety of 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 (Li and Shah, 2004). Endothelial cells express mainly the NOX-2 and NOX-4 isoforms but expression levels of the NOX family members vary among different endothelial cell types (Guzik et al., 2004). Most NOX homologs produce basal levels of ROS that can be further induced by a variety of agonists (Griendling et al., 2000). ROS can regulate procoagulant activity; for example ROS from NOX enzymes alters TF

expression in platelet-activated vascular smooth muscle cells (Gorlach et al., 2000). In addition, NOX-induced oxidative stress in endothelial cells plays an important role in the pathogenesis of CV disorders (Madamanchi et al., 2005), perhaps through these and other mechanisms.

The objective of our study was to determine if soluble components of UF particles induced 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 thrombus formation via TF upregulation that is dependent upon increased ROS production and the NOX-4 enzyme.

3.2 Materials and Methods

3.2.1 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.

3.2.2 Cell Culture

HCAEC were cultured in EGM-2 media supplemented with the EGM-2 Bullet Kit (2% FBS, 0.4% rhFGF-B, 0.1% gentamicin sulfate amphotericin-B, 0.1% rhEGF, 0.1% heparin, 0.1% ascorbic acid, 0.1% R^3-IGF-1, 0.1% VEGF, and 0.04% hydrocortisone). HCAEC were obtained from two donors with no known history of CV disease. Cells were grown to confluence and used between passages 5-8.

3.2.3 Ultrafine Particles

UF particles collected from February to May 2007 in Chapel Hill, NC were analyzed for chemical components by the Research Triangle Institute (Research Triangle Park, NC) as previously described (Becker et al., 2005). The composition of these particles is expressed relative to UF particle mass (ng/mg) (Table 2.1). The measured levels of these elements and ions are comparable to those found in Chapel Hill UF particles previously collected and analyzed in 2001 and 2002 (Becker et al., 2005). The soluble fraction of these UF particles was acquired by suspending the particles in PBS at the desired concentration and centrifuging the resultant suspension for 30 minutes at 20,000 x g. The supernatant from the pelleted particles was collected and used as the soluble fraction.

3.2.4 Measurement of Cytotoxicity

Cytotoxicity was determined by measuring supernatant LDH levels using the colorimetric CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega Corporation, Madison, WI) according to the manufacturer's protocol. Supernatant from cells treated with saponin (0.5%) to disrupt the cellular membrane was a positive control for the assay.

3.2.5 Calibrated Automated Thrombography (CAT) 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 minor alterations (Campbell et al., 2009). Briefly, HCAEC were cultured on 96-well plates and exposed to soluble UF (0, 10, 50, and 100 µg/mL) for 6 hrs. Following exposure, cells and PFP were incubated with either 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 (4 µM; 60% phosphatidylcholine/20% phosphatidylethanolamine/20% phosphatidylserine; Diagnostica Stago, Parsippany, NJ), and a fluorogenic thrombin substrate (Z-Gly-Gly-Arg-AMC; Diagnostica Stago, Parsippany, NJ) were then added to the cells as previously described (Machlus et al., 2009). Reactions were calibrated against wells containing a thrombin calibrator (α2-macroglobulin/thrombin complex; Diagnostica Stago, Parsippany, NJ). 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).

3.2.6 Turbidity Assay

HCAEC were cultured and exposed to soluble UF as described above. Recalcified (20mM, final) PFP and phospholipids (125 μM, 41% phosphatidylcholine/44% phosphatidylethanolamine/15% phosphatidylserine; Avanti Polar Lipids, Alabaster, AL) were added to the cells at a final volume of 100 μl to initiate thrombus formation (Gray et al., 2011). Fibrin thrombus formation was analyzed by an increase in turbidity at 405 nm with a SpectraMax 340PC plate reader (Molecular Devices, Sunnyvale, CA). Softmax® Pro Software version 1.21 (Molecular Devices, Sunnyvale, CA) was used to calculate thrombus formation onset (time to reach inflection point before turbidity increase) and V_{max} (slope of the line fitted to maximum rate of turbidity increase using 10 points to determine the line).

3.2.7 Real-Time Quantitative PCR

Relative gene expression in HCAEC was obtained using quantitative RT-PCR. Total RNA was isolated from HCAEC using an RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol.   RNA was quantified using a Nanodrop™ 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE).   cDNA was generated as previously described (Karoly et al., 2007). 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) and obtained from Integrated DNA Technologies (Coralville, IA). Thrombomodulin, endothelial protein C receptor (EPCR), tissue factor pathway inhibitor (TFPI), IL-1β, TNF-α, GAPDH, and NOX-4 primer pairs and fluorescent probe sets were obtained as Taqman pre-developed assay reagents from Applied Biosystems (Foster City, CA). Quantitative fluorogenic amplification of cDNA was performed using the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA), primer and probe sets of interest, and Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Standard curves generated from a serially diluted standard pool of cDNA prepared from cultured human endothelial cells exposed to 100 ng/mL TNF-α for 6 hrs were used to determine the relative abundance of mRNA levels. The relative abundance of β-actin mRNA was used to normalize levels in genes of interest.

3.2.8 Immunofluorescence

Confocal microscopy was used for detection of membrane-bound thrombomodulin. HCAEC were exposed to soluble UF (0 or 100 μg/mL) for 6hrs in a double well chamber slide with cover (Nalge Nunc International, Rochester, NY). Following exposure, cells were fixed with methanol:acetone (1:3) for 20 min. HCAEC were incubated overnight with anti-thrombomodulin mouse anti-human antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in 1% BSA. Alexa Fluor 594 goat anti-mouse IgG (Invitrogen, Carlsbad, CA) was used as a secondary antibody. Prolong gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA) was used to mount the slides. Imaging was conducted using a Nikon Eclipse C1Si confocal microscope (Nikon Instruments Inc., Melville, NY, USA).

3.2.9 Extracellular H_2O_2 Measurement using the Amplex Red Assay

Extracellular H_2O_2 release was measured using the Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine; Invitrogen, Carlsbad, CA), which reacts with H_2O_2 in the presence of horseradish peroxidase to produce resorufin, a highly fluorescent molecule. HCAEC were cultured on 12-well plates and exposed to soluble UF (0, 50, and 100 µg/mL). Immediately following exposure, Amplex Red (150 µM) and HRP (0.8 U/ml) were added, and plates were analyzed at 5 min intervals for 30 min on a Bioassay HTS7000 plate reader (Perkin-Elmer, Wellesley, MA) with HTSoft version 1.0 software (PE Applied Biosystems, Weiterstadt, Germany). Excitation wavelength was 535 nm and emission fluorescence was 590 nm. H_2O_2 was quantified by subtracting the baseline fluorescence from the final fluorescence, and normalizing these relative fluorescence units (RFU) to a standard curve of H_2O_2 (0-1 nm). Following analysis, the endothelial cells were washed with PBS and cellular protein was collected with lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X-100, pH 7.4) containing 1:100 Protease Inhibitor Cocktail Set 1 (Calbiochem, La Jolla, CA). Protein concentrations of cell lysates were measured using the Bio-Rad protein reagent (Bio-Rad, Richmond, CA) according to the manufacturer's protocol.

3.2.10 Intracellular H2O2 Detection using PG1

Intracellular H_2O_2 was assessed using a small-molecule fluorophore probe Peroxy Green 1 (PG1; kindly provided by Dr. Christopher J. Chang, University of California-Berkeley), which is highly specific for detection of H_2O_2 (Miller et al., 2007). HCAEC were incubated with 5 µM PG1 for 15 min prior to exposure to soluble UF (0, 50, and 100 µg/mL). Immediately following exposure, PG1 fluorescence was measured in 1 min intervals for a total of 75 min using a PolarStar Optima microplate reader (BMG Labtech,

Durham, NC) with an excitation filter of 485/12 nm and emission fluorescence was read with a 525/30 nm filter. Change in RFU was calculated for each dose by subtracting the baseline fluorescence from the final fluorescence. Live cell imaging was conducted using a Nikon Eclipse C1Si confocal microscope (Nikon Instruments Inc., Melville, NY, USA) and instrument settings as previously described (Cheng et al., 2010). Excitation was provided at 488 nm and emission fluorescence was read with a 525/50 nm filter. Imaging data was collected using Nikon EZ-C1 software and quantified by Nikon Elements (Nikon Instruments Inc., Melville, NY, USA) as previously described (Cheng et al., 2010).

3.2.11 Western Blot Analysis

30 μg of sample protein was mixed with 4X loading buffer (0.5 M Tris-HCL (pH 6.8), 40% glycerol, 8% SDS, 10% β-mercaptoethanol and 0.04% bromophenol blue) and heated for 5 min at 95ºC. Samples were added to the gel in equivalent amounts on adjacent lanes and separated by SDS-PAGE (PAGEr Gold Precast 4-20% Tris-Glycine gels, Lonza, Rockland, ME) with Precision Plus Protein Standards (Bio-Rad, Hercules, CA). Proteins were transferred onto Trans-Blot Transfer Medium Pure Nitrocellulose (Bio-Rad, Hercules, CA) and incubated overnight with anti-NOX-4 primary rabbit antibody (Abcam Inc., Cambridge, MA) in 5% nonfat dry milk. HRP-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a secondary antibody. Protein bands were detected using ECL Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ). Blots were digitized using a Fujifilm LAS-3000 with Multigauge software (Fujifilm U.S.A., Valhall, NY). The blots were stripped using Re-Blot Plus (Chemicon International, Temecula, CA) and re-probed with anti-α-tubulin primary mouse antibody

(Sigma, St. Louis, MO) with HRP-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) used as the secondary antibody.

3.2.12 siRNA Transfection

HCAEC were transfected with 90 nM concentration of NOX-4 or scrambled #5 siRNA (Ambion, Austin, TX) using siPORT Amine transfection agent (Ambion, Austin, TX) according to the manufacturer's protocol. Cells were used for experiments 48 hrs post-transfection.

3.2.13 Statistical Analysis

Statistical analysis was performed using commercially available software (GraphPad Prism, version 4.03 for Windows; 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 to control cells. A p-value < 0.05 was considered statistically significant.

3.3 Results

3.3.1 Soluble UF cause HCAEC to promote faster onset of TF-dependent thrombin generation and fibrin thrombus formation

HCAEC were exposed to 10, 50, and 100 μg/mL soluble UF. We found these doses are non-cytotoxic to the endothelial cells (Fig. 2.2F). 6 hrs following exposure, cells were assessed for functional coagulation balance using two methods. We used the CAT assay to quantify thrombin generation (Fig. 3.1A-E) and the turbidity assay to measure the rate of fibrin thrombus formation (Fig. 3.1F-H) in PFP. The CAT assay showed that lag time (Fig. 3.1B) and time to peak (Fig. 3.1C) were significantly decreased with increasing doses of soluble UF, indicating faster onset of thrombin generation. Exposure to soluble UF did not significantly affect the thrombin parameters ETP (Fig. 3.1D) and peak (Fig. 3.1E). In the turbidity assay, HCAEC exposure to soluble UF led to a significant decrease in the onset time to thrombus formation (Fig. 3.1G) and an increase in V_{max} (Fig. 3.1H), indicating faster onset and rate of fibrin thrombus formation. These effects on thrombin and fibrin were abolished by addition of an inhibitory anti-human TF antibody. These data show that soluble UF PM increases endothelial procoagulant activity and suggests this occurs via a TF-dependent mechanism.

Figure 3.1

A
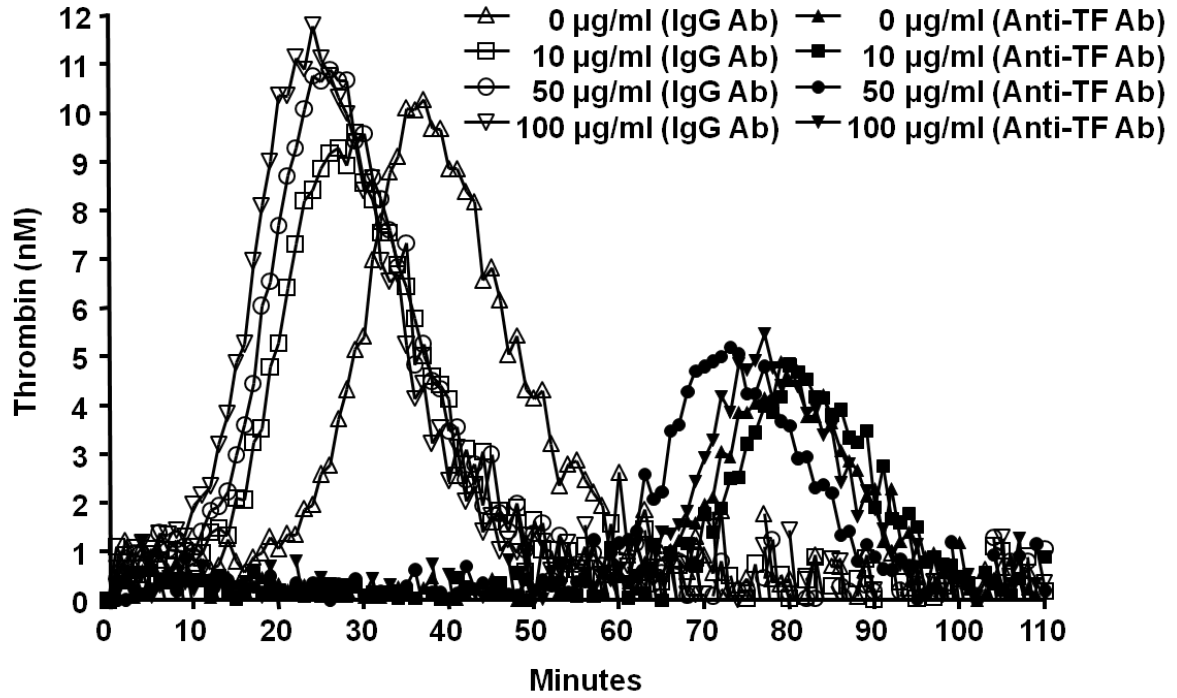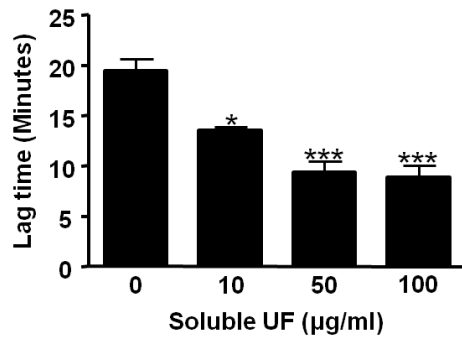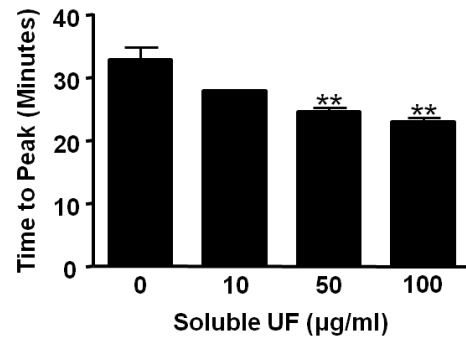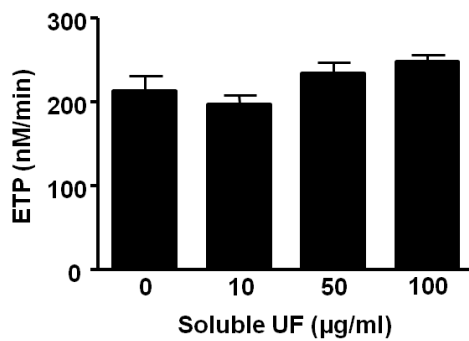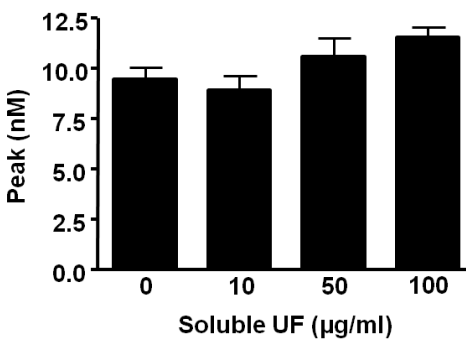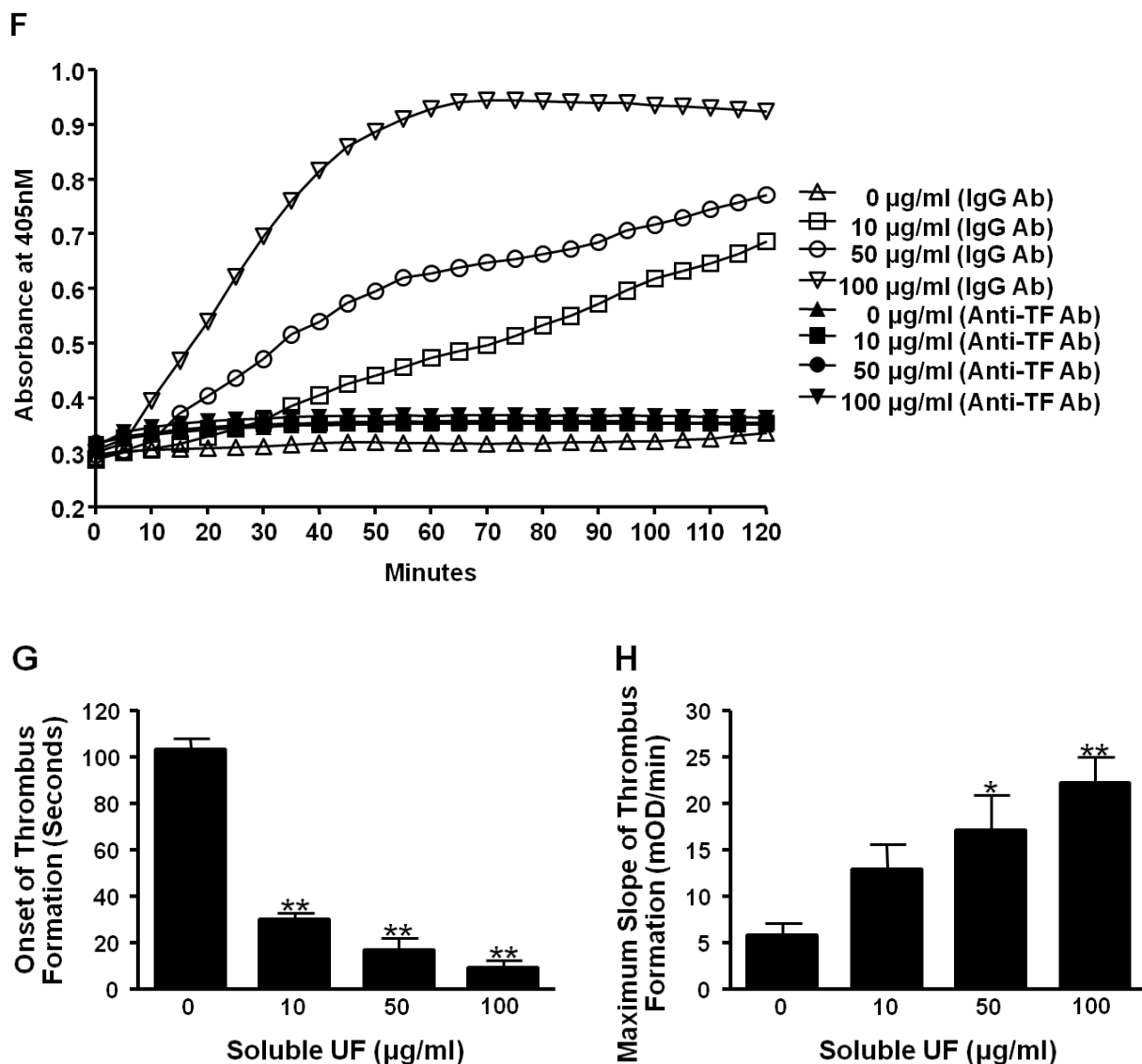
Figure 3.1 Exposure to soluble UF leads to faster onset of TF-dependent thrombin generation and fibrin thrombus formation. The endothelial cell coagulation balance was assessed by measuring the ability of HCAEC to induce thrombin generation (A-E) and fibrin thrombus formation (F-H) in PFP following exposure to soluble UF (0, 10, 50, and 100 μg/mL). Cells and PFP were incubated with either an inhibitory anti-human TF monoclonal antibody or a control mouse IgG. Thrombin parameters (B) lag time, (C) time to peak, (D)

ETP, and (E) peak and the fibrin thrombus parameters (G) thrombus formation onset and (H) V_{max} were measured. Graphs are representative of 3 separate experiments with samples in triplicate. * = p<0.05, ** = p<0.01, *** = p<0.001 compared to control.

3.3.2 Soluble UF increases TF mRNA expression in HCAEC

To characterize the cause of soluble UF-induced changes in HCAEC cellular procoagulant activity, we measured mRNA expression of key cellular pro- and anticoagulant proteins by quantitative RT-PCR following exposure of the cells to soluble UF for 6 and 24 hrs. 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 hrs, and increased expression was sustained at 24 hrs (Fig. 3.2A). Conversely, we found no significant changes in TFPI, EPCR, or thrombomodulin mRNA expression following exposure to soluble UF (Fig. 3.2B-D). Additionally, immunofluorescence staining for thrombomodulin indicated the anticoagulant protein was not shed from the membrane of HCAEC exposed to of soluble UF (100 μg/mL). 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 3.1.

Figure 3.2
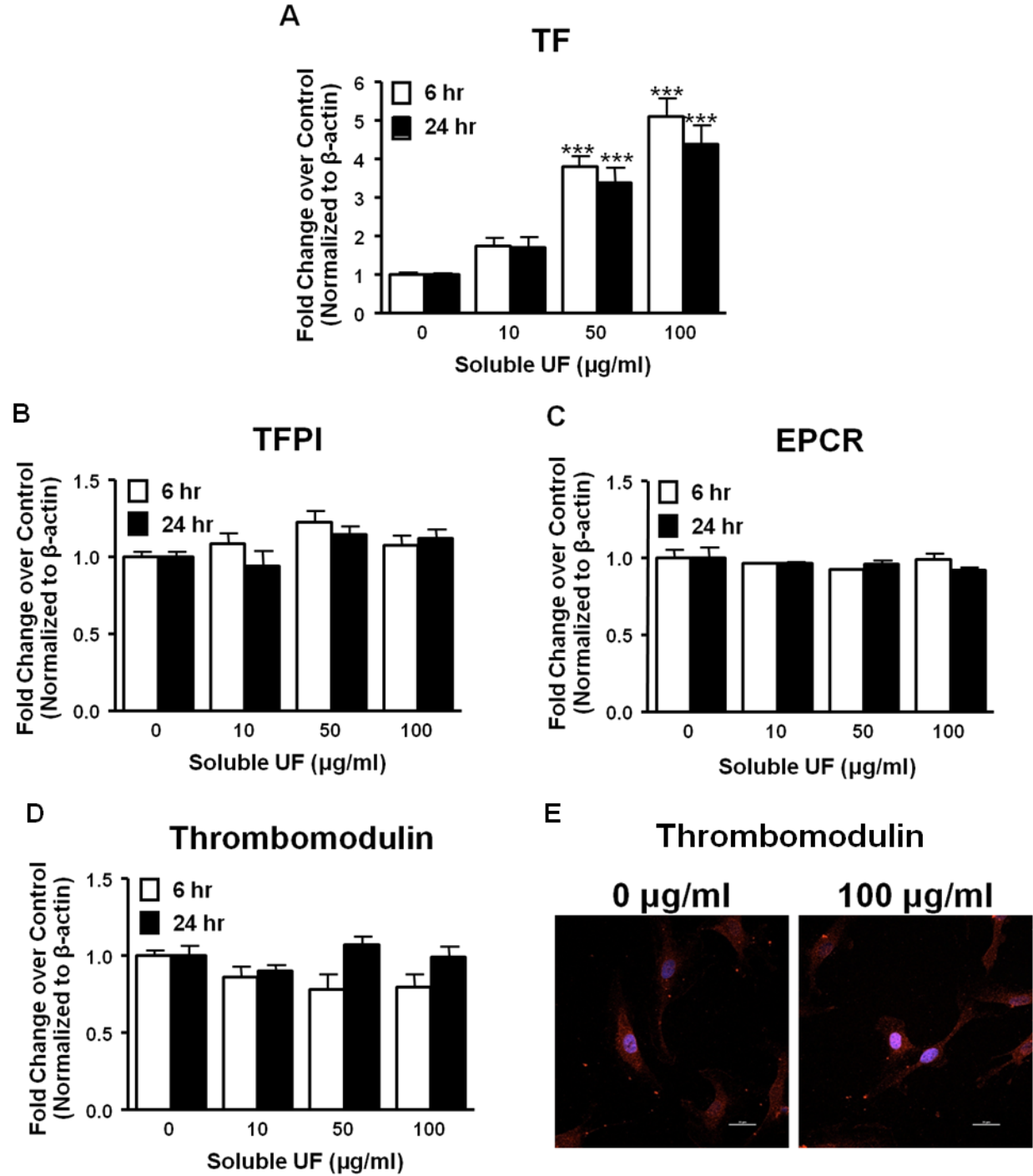
Figure 3.2 Exposure to soluble UF leads to upregulation of TF. (A-D) HCAEC were exposed to soluble UF (0, 10, 50, and 100 µg/mL) for 6 (white bars) or 24 hrs (black bars). Quantitative real time RT-PCR was used to determine mRNA expression of TF (A), TFPI (B), EPCR (C), and thrombomodulin (D). For all mRNA, values were normalized to β-actin expression and are expressed as fold change in exposed cells over control. n = 3 separate experiments with samples in triplicate. (E) Representative images of HCAEC exposed to 0 or 100 µg/mL soluble UF for 6 hrs followed by fixation with a 1:3 methanol:acetone mixture. HCAEC were stained for thrombomodulin (red fluorescence) and nuclei were labeled with DAPI (blue fluorescence). n = 2 separate experiments. ∗∗∗ = p<0.001 compared to control.

3.3.3 Soluble UF-induced TF upregulation is ROS dependent

To characterize the initiating events leading to increased TF production, we first measured the mRNA expression of the proinflammatory cytokines IL-1β and TNF-α which are able to induce TF mRNA expression in endothelial cells (Dinarello, 1991). Fast upregulation of these proinflammatory cytokines preceding an increase in TF would suggest that increases in TF mRNA expression are likely dependent on cytokine regulation following exposure to soluble UF. We exposed HCAEC to 50 µg/mL soluble UF for 2, 4, 6, and 24 hrs and measured the mRNA expression of IL-1β, TNF-α, and TF. There was an early and sustained significant increase in IL-1β (Fig. 3.3A) and TF (Fig. 3.3C) mRNA expression. Conversely, TNF-α mRNA levels (Fig. 3.3B) were not significantly induced following exposure to soluble UF. These data indicate that early induction of proinflammatory cytokines following exposure to soluble UF is not driving TF mRNA expression, suggesting parallel, and possibly independent mechanisms, upregulate proinflammatory and procoagulant proteins in this case.

TF gene expression and protein levels have been shown to be modulated by ROS in vascular cells (Herkert et al., 2004). Since TF is a redox-regulated protein, we designed experiments to determine if ROS regulate TF gene expression changes following exposure to soluble UF. We treated HCAEC with the PEG-SOD and PEG-catalase prior to 6 hr exposure to 50 µg/mL soluble UF and measured TF mRNA expression. These cell-permeable antioxidants significantly (p<0.001) suppressed TF mRNA upregulation by soluble UF by 55% and 36% respectively (Fig. 3.3D). These data indicate that endothelial ROS production are required for increased TF mRNA expression in HCAEC following exposure to soluble UF.

Figure 3.3
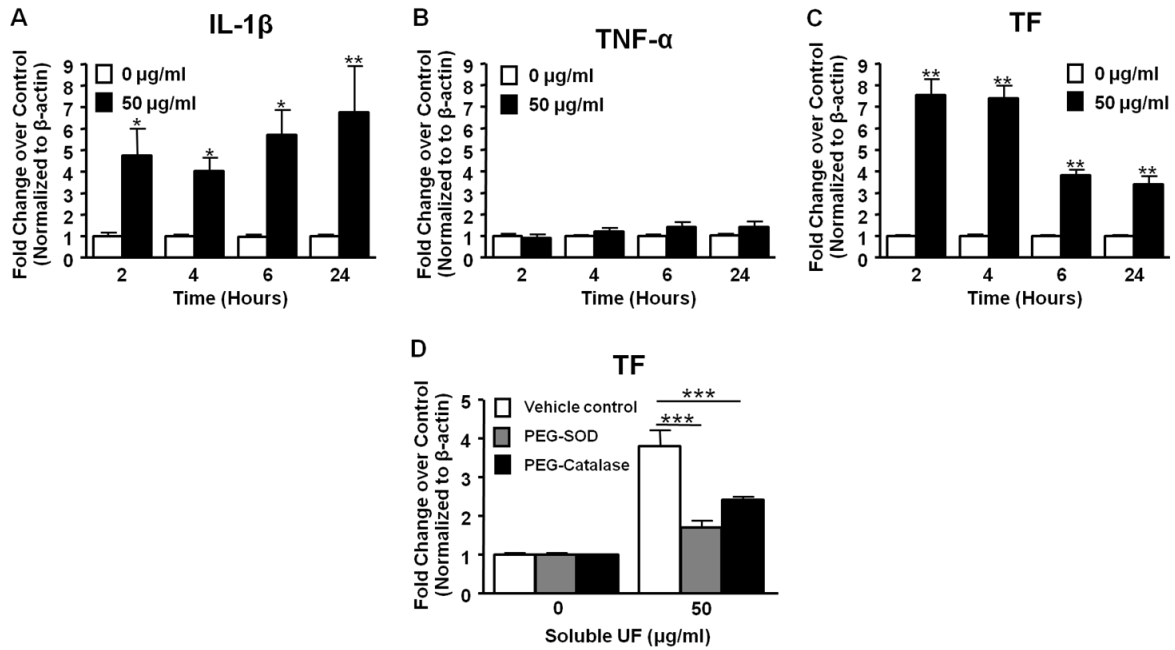
Figure 3.3 Soluble UF-induced upregulation of TF is ROS dependent. (A-C) HCAEC were exposed to 0 or 50 µg/mL soluble UF for 2, 4, 6, or 24 hrs. IL-1β (A), TNF-α (B), and TF (C) mRNA expression was quantified by real time RT-PCR. (D) HCAEC were pretreated with PEG-SOD or PEG-Catalase (100 U/mL) for 1 hr and then exposed to 0 or 50 µg/mL soluble UF for 6 hrs. TF mRNA expression was quantified by real time RT-PCR. For all mRNA, values were normalized to β-actin expression and are expressed as fold change in exposed cells over control. n = 3 separate experiments with samples in triplicate. ∗∗∗ = $p<0.001$ compared to control.

3.3.4 Soluble UF exposure causes H2O2 production in HCAEC

To characterize the cellular events leading to ROS production by endothelial cells, we then measured extracellular and intracellular H_2O_2 production in soluble UF-treated HCAEC. Using the Amplex Red assay to quantitate 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 production following HCAEC exposure to 50 and 100 μg/mL soluble UF, respectively (Fig. 2.2B). Similar results were found for intracellular H_2O_2 production using the chemical indicator PG1. In HCAEC exposed to 50 and 100 μg/mL of soluble UF, we observed a significant and progressive dose-dependent increase in intracellular H_2O_2 in cells at 50 (21%, $p<0.05$) and 100 μg/mL (32%, $p<0.001$) (Fig. 3.4A). We then visualized the distribution of H_2O_2 production in individual 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 increases in PG1 fluorescence intensity (Fig. 3.4B). Quantitative analysis of the confocal images indicate that compared to control cells, soluble UF induced a 2.9-fold increase ($p<0.001$) in RFU (Fig. 3.4C). These data indicate that exposure to soluble UF results in immediate increases in extra- and intracellular H_2O_2 production in HCAEC.

Figure 3.4
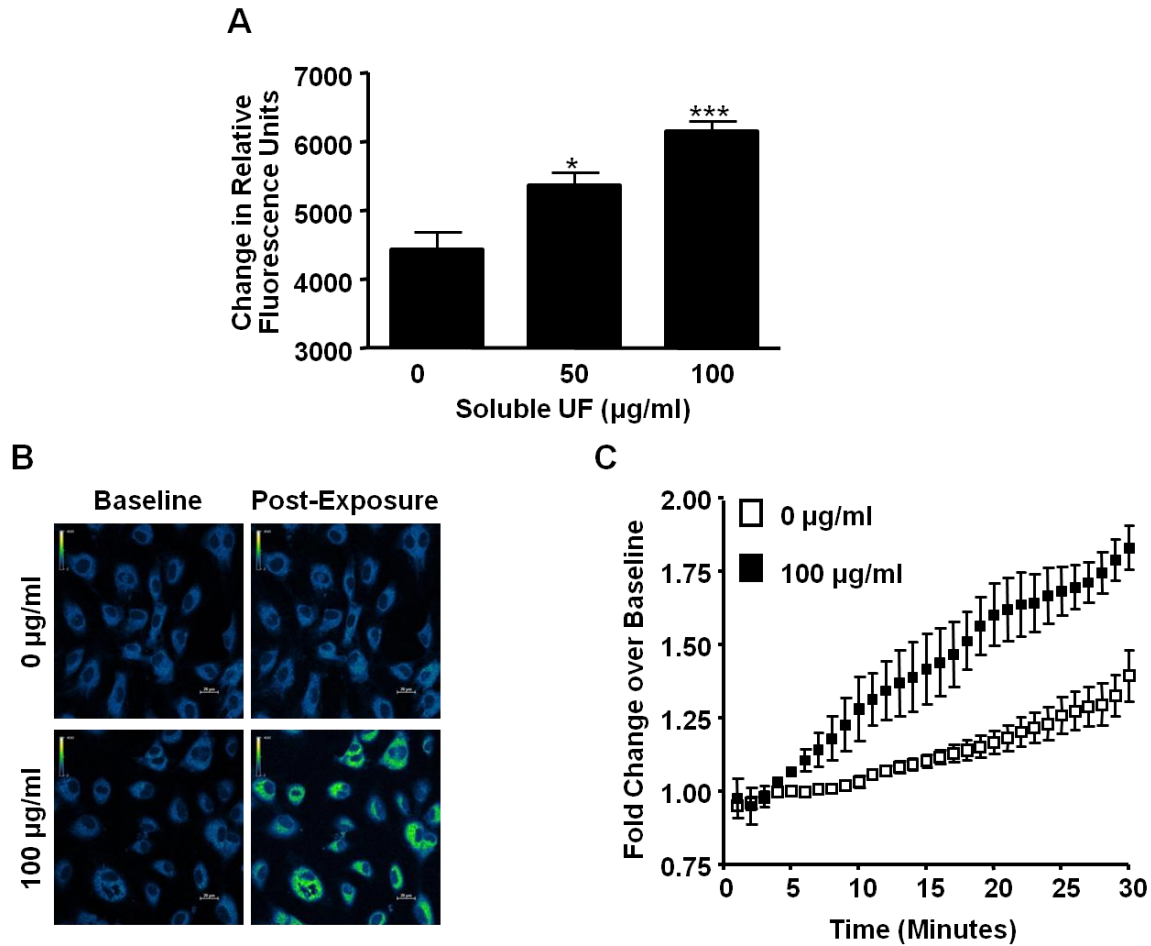
Figure 3.4 Exposure to soluble UF induces rapid H_2O_2 generation by HCAEC. (A) HCAEC were incubated with the H_2O_2 chemical indicator PG1 and then exposed to soluble UF (0, 50, and 100 µg/mL) for 60 minutes. Fluorescence was measured with a fluorescent plate reader. n = 3 independent experiments with samples in triplicate. (B) Confocal microscopy was used to measure PG1 fluorescence in real time. HCAEC were incubated with PG1 followed by a baseline read for 5 minutes. Cells were then exposed to 0 or 100 µg/mL soluble UF for 30 minutes. Representative images in a pseudo-colored scheme are shown from baseline and 30 minutes post-exposure. (C) Quantitative data from the confocal

images were gathered by outlining individual cells and measuring the RFU using Nikon NIS-Elements software. The data are represented as fold change over baseline measurement. n = 4 independent experiments. $*$ = p<0.05, $**$ = p<0.01, $***$ = p<0.001 compared to control.

3.3.5 ROS production from NOX-4 enzyme leads to upregulation of TF mRNA expression

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 of the major sources of ROS production in endothelial cells. We found that DPI, a NOX inhibitor, reduced extracellular H_2O_2 production in both the EA cells and the primary HCAEC exposed to soluble UF (Fig. 2.4D-E). Similar to our findings in the EA cells, soluble UF-induced extracellular H_2O_2 levels were not affected by inhibitors of xanthine oxidases (allopurinol) or mitochondrial sources (KCN, rotenone), which are important additional sources of endothelial ROS production (data not shown), indicating that NOX enzymes are likely a major source of H_2O_2 production following exposure to soluble UF. To determine if NOX enzymes mediate the increase in TF mRNA, we pretreated HCAEC with DPI followed by a 6 hr exposure to 50 μg/mL soluble UF. Pretreatment with the NOX inhibitor led to a 50% attenuation ($p<0.01$) of the increased TF mRNA levels in exposed cells (Fig. 3.5A).

HCAEC predominately express the NOX-4 isoform (Yoshida and Tsunawaki, 2008) and we confirmed that this NOX homolog was present in our endothelial cells (Fig. 3.5B). We more specifically probed the role of NOX-4 in soluble UF-induced TF mRNA production by transfecting HCAEC with NOX-4 siRNA. Transfection resulted in 84% knockdown of the target gene mRNA levels as compared to the scrambled siRNA control after 48 hrs (Fig. 3.5C). HCAEC were then transfected with NOX-4 or scrambled siRNA for 48 hrs prior to exposure to soluble UF for 6 hrs. Compared to cells transfected with scrambled siRNA, NOX-4 knockdown significantly ($p<0.001$) attenuated soluble UF-

induced TF mRNA upregulation by 56% (Fig. 3.5D). Together, these data indicate that following exposure to soluble UF, ROS from the NOX-4 enzyme upregulates TF mRNA expression and endothelial cell-dependent procoagulant activity.

Figure 3.5
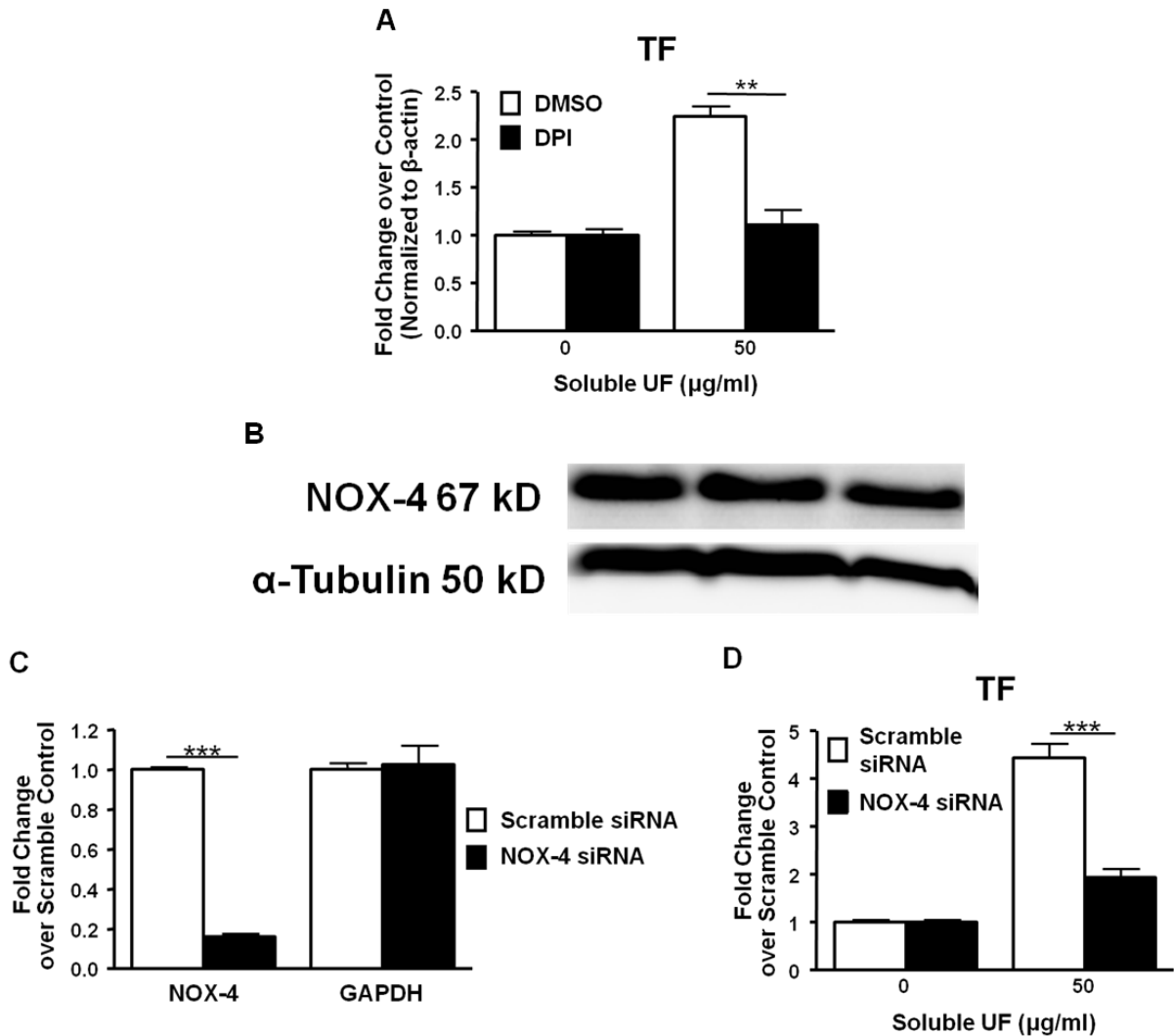
Figure 3.5 TF mRNA expression following exposure to soluble UF requires ROS production and NOX-4. (A) HCAEC were pretreated with DPI (50 µM) for 30 minutes and then exposed to 0 or 50 µg/mL soluble UF for 6 hrs. TF mRNA expression was quantified by real time RT-PCR. (B) HCAEC cellular protein was analyzed for presence of the NOX-4 isoform via Western blotting. Total cellular protein was assessed by probing for α-tubulin. Samples are representative of 3 separate experiments. (C) HCAEC were transfected with NOX-4 or scramble siRNA for 48 hrs. Quantitative RT-PCR was used to determine mRNA

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expression of the target gene NOX-4 and non-target gene GAPDH. (D) HCAEC were transfected with NOX-4 or scramble siRNA for 48 hrs and then exposed to 0 or 50 μg/mL soluble UF for 6 hrs. TF mRNA expression was quantified by real time RT-PCR. For all mRNA, values were normalized to β-actin expression and are expressed as fold change over control. n = 3 separate experiments with samples in duplicate. ∗ = p<0.05, ∗∗ = p<0.01 ∗∗∗ = p<0.001

3.4 Discussion

There is a strong epidemiological evidence supporting a casual relationship between air particle pollution exposure and adverse CV health effects in humans that include clinical events mediated by thrombosis, such as acute coronary syndrome (Peters et al., 2001; Pope et al., 2006), stroke (Kettunen et al., 2007; Wellenius et al., 2012), deep venous thrombosis and thromboembolism (Baccarelli et al., 2008; Martinelli et al., 2012). 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 soluble components of UF PM induce a procoagulant phenotype in endothelial cells, supporting faster onset of thrombin generation and fibrin thrombus formation, and that these activities are driven by upregulation of TF. We further show that this increase in TF mRNA is regulated by increased ROS production from NOX-4, 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 underly increased risk for CV morbidity and mortality associated with air pollution exposure.

Our results add to a growing body of evidence that link air pollution exposure to thrombosis. Recent studies have demonstrated hypercoagulable effects of air pollution on plasma thrombin generation in human and animal models (Emmerechts et al., 2012; Kilinc et al., 2011; Rudez et al., 2009). Emmerechts et al. demonstrated that elevated levels of coarse PM shortened the lag time of thrombin generation in microparticle-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, we showed that exposure to soluble UF resulted in a dose-dependent

decrease in the lag time, which has been previously shown to be a sensitive reflection of TF activity (Ollivier et al., 2010). We did not find a significant difference in ETP or peak thrombin following HCAEC exposure to soluble UF; however, this is not surprising since these parameters are typically more associated with alterations in the 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; Rudez et al., 2009) and cellular procoagulant activity may be additive or even synergistic, ultimately dysregulating multiple thrombin generation parameters simultaneously.

TF expression is generally suppressed in endothelial cells under normal physiological conditions but is induced by inflammation (Levi and van der Poll, 2005), endotoxin (Colucci et al., 1983), and exposure to air pollution (Karoly et al., 2007; Sun et al., 2008) *in vitro* 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 primary endothelial cells contains binding sites for redox-sensitive transcription factors (Herkert and Gorlach, 2002; Moll et al., 1995). Accordingly, we found that in HCAEC, TF mRNA levels following exposure to soluble UF required both H_2O_2 and superoxide. This finding supports previous studies showing that TF mRNA expression is attenuated in the presence of antioxidants following exposure to ionizing radiation and inflammatory cytokines (Szotowski et al., 2007).

ROS are implicated in the pathogenesis and progression of CV diseases including atherosclerosis, hypertension, and diabetes (Madamanchi et al., 2005). Additionally,

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concentrations of 7-hydro-8-oxo-2'-deoxyguanosine in lymphocyte DNA was shown to be 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 oxidative stress following air pollution exposure. We showed that soluble UF components lead to a significant increase in both extra- and intracellular endothelial H_2O_2 production immediately following exposure. Our data strongly suggest that the ROS is made intracellularly as exposure to soluble UF caused an increase in PG1 fluorescence intensity, a chemical indicator specific for H_2O_2 with the ability to cross the cellular membrane (Miller et al., 2007). 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 (Griendling et al., 2000). While there are several major sources of ROS production in endothelial cells, exposure of mouse pulmonary microvascular endothelial cells to whole UF particles induced ROS production by NOX enzymes (Mo et al., 2009). Our findings are important because we show that NOX-4 is a likely source of soluble UF-induced H_2O_2 production in endothelial cells from humans. The intracellular distribution of NOX-4 is broad and has been shown to be variably located in the perinuclear space, endoplasmic reticulum, mitochondria, and nucleus of endothelial cells (Lassegue and Griendling, 2010; Lassegue et al., 2012). Our data further suggest that NOX-4-derived ROS produced following exposure to soluble UF is cytoplasmic, but can escape to the extracellular space. This finding has important implications for paracrine signaling effects by cellular H_2O_2.

Soluble UF-induction of NOX-4 increased TF mRNA expression in HCAEC. Others have shown that after agonist stimulation by thrombin or platelet-derived products from activated human platelets, ROS from NOX enzymes induce TF mRNA expression in vascular smooth muscle cells (Gorlach et al., 2000; Herkert et al., 2002). Our data extend this finding to endothelial cells after a more subtle stimulation with soluble UF. NOX enzymes are important therapeutic targets for strategies trying 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 has several strengths and limitations. First, it is not known whether even the smallest PM can translocate from the lung into the vasculature and cause pathologic effects (Brown et al., 2002; Mills et al., 2006; Nemmar et al., 2002). Indeed, we previously determined that the insoluble fraction of UF PM does not induce significant adverse responses in HCAEC (Figure 2.1D). However, components of the water soluble fraction of particles, which include transition metals, appear in the vasculature following intratracheal instillation in rats (Wallenborn et al., 2009). Our study strongly supports the potential for a pathogenic role for the water-soluble fraction of UF particles and implies that in cell culture systems, exposure to soluble UF is more physiologically relevant than whole UF particle exposures. We used primary human coronary artery endothelial cells as our cellular model because these endothelial cells are among the first vascular cells to receive blood from the lungs and therefore, are likely to receive the highest concentration of any soluble components that transfer from the alveoli into the vasculature. Despite these strengths, this study has potential limitations. PM can cause cell death; however, we confirmed the doses used in this study were not cytotoxic (Figure 2.2B), indicating that the procoagulant activity following

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exposure to soluble UF was due to endothelial activation rather than cellular death. Consequently, our findings suggest the observed effects are likely to be associated with chronic activation of coagulation and inflammation, with substantial potential for long-term pathologic effects. Furthermore, the specific doses of PM experienced by HCAEC are not known. However, individuals exposed to smoke or cooking fumes indoors can be exposed to elevated concentrations of UF particles similar to the doses used in this study(Karoly et al., 2007). These concentrations apply to amounts that would be experienced by alveolar epithelial cells and, by extension, the levels of the water-soluble fraction of UF PM experienced by cells in the vasculature.

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

CHAPTER 4

SOLUBLE COMPONENTS OF ULTRAFINE PARTICLES INDUCE PROINFLAMMATORY RESPONSES IN ENDOTHELIAL CELLS

4.1 Introduction

Cardiovascular (CV) disease is the leading cause of death and disability worldwide (Lauer, 2012), and a growing body of evidence suggests a strong association between exposure to air pollution and CV morbidity and mortality (Brook et al., 2004; Pope, 2009; Simkhovich et al., 2008). Particulate matter (PM) is a major component of air pollution and has been shown to contribute to these adverse CV health effects in both epidemiological and toxicological studies. For instance, individuals living in the greater Boston area had an increased risk of myocardial infarctions when PM concentrations were elevated (Peters et al., 2001). In addition, it was found that exposure to PM accelerated atherosclerotic progression in susceptible mouse (Sun et al., 2005) and rabbit models (Suwa et al., 2002). Although these epidemiological and toxicological studies show a strong relationship between PM exposure and adverse CV health effects, the mechanisms behind these associations are currently not known.

PM is a heterogeneous mixture of extremely small particles and liquid droplets that are derived from a variety of manmade and natural sources (Poschl, 2005). PM is categorized based on the diameter of the particle and increasing evidence suggests that ultrafine (UF) particles, which are the smallest diameter particle, are of particular importance in these adverse CV effects due to air pollution exposure (Donaldson and Stone, 2003). For example, recent studies have shown that exposure to UF particles was associated with

hospitalizations for heart failure in Spain (Dominguez-Rodriguez et al., 2011) and cardiopulmonary and total mortality in Germany (Stolzel et al., 2007). Soluble components of UF particles (soluble UF) can traverse the thin alveolar-capillary membrane to enter the circulation (Wallenborn et al., 2007) and interact with cells of the vasculature including endothelial cells. These soluble components can lead to the formation of reactive oxygen species (ROS; Chapter 2), increase endothelial procoagulant activity (Chapter 3), and potentially cause proinflammatory responses.

Inflammation has been linked to numerous CV disorders including atherosclerosis (Libby, 2002), diabetes (Navarro and Mora, 2006), and chronic heart failure (Yndestad et al., 2006). Several studies have illustrated that exposure to PM leads to upregulation of various mediators of inflammation, such as proinflammatory cytokines and cell adhesion molecules, in lung bronchial epithelial cells (Fujii et al., 2001), alveolar macrophages (Sawyer et al., 2009), and endothelial cells (Aung et al., 2011; Yatera et al., 2008). Many of these inflammatory mediators are regulated by ROS and can be induced following activation of redox-sensitive transcription factors (Lavrovsky et al., 2000). For example, PM exposure induces ROS production from NADPH oxidase (NOX) enzymes, an important source of endothelial ROS production, leading to the upregulation of the proinflammatory cytokines (Mo et al., 2009). Furthermore, there is a strong cross-talk between the coagulation and inflammatory pathways (Levi and van der Poll, 2005) as tissue factor (TF) and thrombin are able to increase expression of proinflammatory cytokines (Cunningham et al., 1999; Levi et al., 2004b).

In addition to upregulating proinflammatory mediators, PM exposure increases the expression of oxidant sensitive molecules such as heme oxygenase 1 (HO-1) in alveolar

macrophages and bronchial epithelial cells (Li et al., 2003). HO-1 catalyzes the degradation of heme to carbon monoxide, biliverdin, and iron. Biliverdin is further reduced to bilirubin, both of which are known to have antioxidant properties (Wu et al., 2011). Furthermore, HO-1 is an inducible protein in response to oxidative stress and has been shown to be regulated by ROS generated from NOX enzymes in murine macrophages exposed to a lipid metabolite derived from prostaglandin D_2 (Hong et al., 2008).

In Chapter 3, we show that endothelial cells exposed to soluble UF induce procoagulant responses that are dependent upon increased ROS production and the NOX-4 enzyme. The objective of this study was to determine if soluble components of UF particles induce proinflammatory responses and HO-1 upregulation in human endothelial cells, and to define the mechanisms behind these effects. We show here that endothelial cells exposed to soluble UF increase mRNA expression of proinflammatory cytokines, cell adhesion molecules, and HO-1 that is dependent upon increased ROS production and NOX enzymes.

4.2 Materials and Methods

4.2.1 Reagents and Chemicals

The immortalized endothelial cell line, EA.hy926 (EA cells), are derived by fusing human umbilical vein endothelial cells with A549 cells, a human lung adenocarcinoma epithelial cell line (Edgell et al., 1983).   EA cells were obtained from University of North Carolina's Tissue Culture Facility (Chapel Hill, NC). Dulbecco's modified Eagle's medium (DMEM)-high glucose, fetal bovine serum (FBS), and antibiotic-antimycotic (100X) were obtained from Gibco (Grand Island, NY). 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.

4.2.2 Cell Culture

EA cells were cultured in DMEM-high glucose medium supplemented with 10% FBS and 1% antibiotic-antimycotic mix. HCAEC were cultured in EGM-2 media supplemented with the EGM-2 Bullet Kit (2% FBS, 0.4% rhFGF-B, 0.1% gentamicin sulfate amphotericin-B, 0.1% rhEGF, 0.1% heparin, 0.1% ascorbic acid, 0.1% R^3-IGF-1, 0.1% VEGF, and 0.04% hydrocortisone). HCAEC were obtained from two donors with no known history of CV disease. Cells were grown to confluence and used between passages 5-8.

4.2.3 Ultrafine Particles

UF particles were collected from February to May 2007 in Chapel Hill, NC as previously described (Becker et al., 2005).   UF particles were analyzed for chemical components by the Research Triangle Institute (Research Triangle Park, NC) and the composition of these particles is expressed relative to UF particle mass (ng/mg; Table 2.1).

The soluble fraction of these particles was acquired by suspending the particles in PBS at the desired concentration and centrifuging the resultant suspension for 30 minutes at 20,000 $x\ g$. The supernatant from the pelleted particles was collected and used as the soluble fraction.

4.2.4 Real-Time Quantitative PCR

Relative gene expression in EA and HCAEC were obtained using quantitative RT-PCR. Total RNA was isolated from the endothelial cells using an RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA was quantified using a Nanodrop™ 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). cDNA was generated as previously described (Karoly et al., 2007). 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) and obtained from Integrated DNA Technologies (Coralville, IA). IL-8, IL-1β, IL-6, TNF-α, MCP-1, GM-CSF, E-selectin, ICAM-1, HO-1, NOX-4, and GAPDH primer pairs and fluorescent probe sets were obtained as Taqman pre-developed assay reagents from Applied Biosystems (Foster City, CA). Quantitative fluorogenic amplification of cDNA was performed using the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA), primer and probe sets of interest, and Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Standard curves generated from a serially diluted standard pool of cDNA prepared from cultured human endothelial cells exposed to 100 ng/mL TNF-α for 6 hrs were used to determine the

relative abundance of mRNA levels. The relative abundance of β-actin mRNA was used to normalize levels in genes of interest.

4.2.5 siRNA Transfection

HCAEC were transfected with 90 nM concentration of NOX-4 or scrambled #5 siRNA (Ambion, Austin, TX) using siPORT Amine transfection agent (Ambion, Austin, TX) according to the manufacturer's protocol. Cells were used for experimentations 48 hrs post-transfection.

4.2.6 Statistical Analysis

Statistical analysis was performed using commercially available software (GraphPad Prism, version 4.03 for Windows; GraphPad Software, San Diego, CA). A one-way ANOVA analysis followed by Bonferroni's post-hoc test was used to compare exposed cells to control cells. A p-value < 0.05 was considered statistically significant.

4.3 Results

<u>4.3.1 Soluble UF induces IL-8 mRNA expression in EA cells</u>

To determine if exposure to soluble UF induces proinflammatory responses in endothelial cells, we first exposed EA cells to soluble UF (10, 50, and 100 µg/mL) for 6 and 24 hrs and measured the mRNA expression of the proinflammatory cytokine IL-8. Exposure to 50 and 100 µg/mL soluble UF led to a significant ($p<0.001$) 2.1-fold and 1.9-fold increase, respectively, of IL-8 mRNA expression in EA cells at 24 hrs post-exposure (Fig. 4.1). These data indicate that exposure to soluble UF leads to proinflammatory responses in EA cells.

Figure 4.1

IL-8
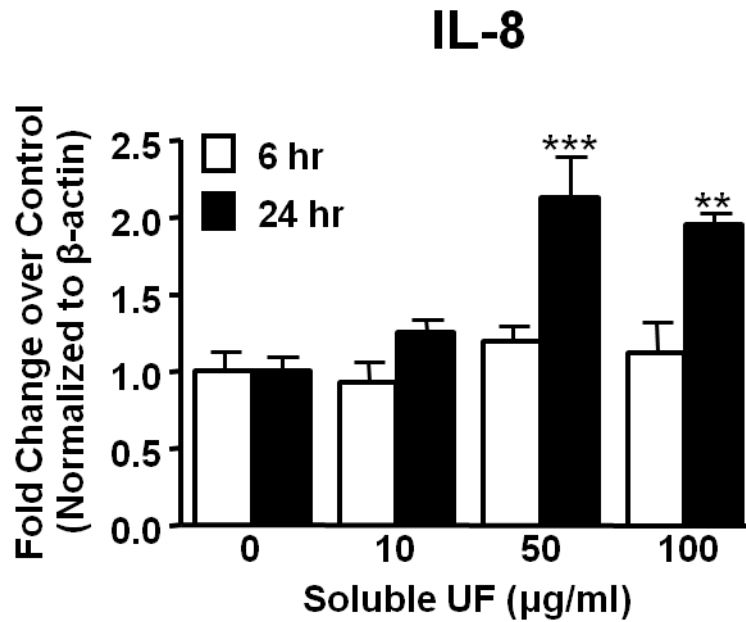
Figure 4.1 Exposure to soluble UF leads to upregulation of IL-8 in EA cells. EA cells were exposed to soluble UF (0, 10, 50, and 100 µg/mL) for 6 (white bars) or 24 hrs (black bars). Quantitative real time RT-PCR was used to determine mRNA expression of IL-8. Values were normalized to β-actin expression and are expressed as fold change in exposed cells over control. n = 3 separate experiments with samples in triplicate. * = p<0.05, ** = p<0.01, *** = p<0.001 compared to control.

4.3.2 Soluble UF induces mRNA expression of proinflammatory cytokines in HCAEC

After illustrating that exposure to soluble UF induces proinflammatory responses in an immortalized endothelial cell line, we exposed HCAEC to soluble UF for 6 and 24 hrs and measured the mRNA expression of several proinflammatory cytokines in order to assess the response in a primary cell model. Exposure to soluble UF (10, 50, and 100 μg/mL) in HCAEC increased the mRNA expression of the proinflammatory cytokines IL-8 (Fig. 4.2A), IL-1β (Fig. 4.2B), MCP-1 (Fig. 4.2C), and GM-CSF (Fig. 4.2D). Although there was not a significant change in IL-6 (Fig. 4.2E) or TNF-α (Fig. 4.2F), there was a trend towards an increase in the mRNA expression of these proinflammatory cytokines following exposure to soluble UF. These data indicate that soluble UF exposure induces proinflammatory responses in primary endothelial cells.

Figure 4.2

Figure 4.2 Exposure to soluble UF leads to upregulation of proinflammatory cytokines in HCAEC. (A-F) HCAEC were exposed to soluble UF (0, 10, 50, and 100 μg/mL) for 6

(white bars) or 24 hrs (black bars). Quantitative real time RT-PCR was used to determine mRNA expression of the proinflammatory cytokines IL-8 (A), IL-1β (B), MCP-1 (C), GM-CSF (D), IL-6 (E), and TNF-α (F).   For all mRNA, values were normalized to β-actin expression and are expressed as fold change in exposed cells over control. n = 3 separate experiments with samples in triplicate. ∗ = p<0.05, ∗∗ = p<0.01, ∗∗∗ = p<0.001 compared to control.

4.3.3 Soluble UF induces mRNA expression of cell adhesion molecules in HCAEC

Altered expression of endothelial cell adhesion molecules have been implicated in the pathogenesis of several CV diseases (Blankenberg et al., 2003). To determine if exposure to soluble UF induces the expression of cell adhesion molecules in endothelial cells, we exposed HCAEC to soluble UF (10, 50, and 100 μg/mL) for 6 and 24 hrs and observed a significant increase in the mRNA expression of the cell adhesion molecules E-selectin (Fig. 4.3A) and ICAM-1 (Fig. 4.3B). Enhanced mRNA expression of these key cell adhesion molecules and proinflammatory cytokines from Figure 4.2 indicate that exposure to soluble UF results in endothelial activation in primary endothelial cells (Figure 1.2).

Figure 4.3
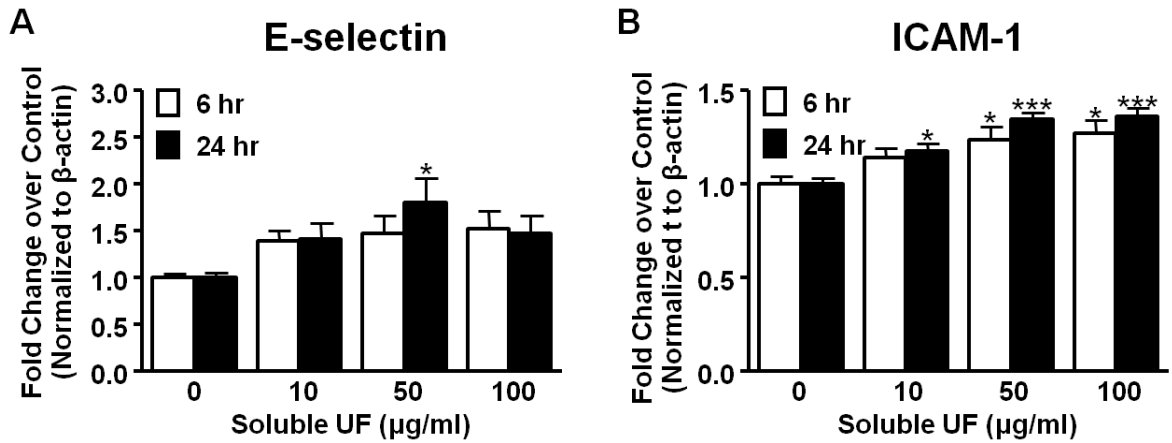
Figure 4.3 Exposure to soluble UF leads to upregulation of cell adhesion molecules in HCAEC. (A-B) HCAEC were exposed to soluble UF (0, 10, 50, and 100 μg/mL) for 6 (white bars) or 24 hrs (black bars). Quantitative real time RT-PCR was used to determine mRNA expression of the cell adhesion molecules E-selectin (A) and ICAM-1 (B). For all mRNA, values were normalized to β-actin expression and are expressed as fold change in exposed cells over control. n = 3 separate experiments with samples in triplicate. ∗ = p<0.05, ∗∗ = p<0.01, ∗∗∗ = p<0.001 compared to control.

4.3.4 Soluble UF increases HO-1 expression in HCAEC

UF exposure has been shown to increase the expression of the oxidant stress-inducible protein HO-1 in macrophages and epithelial cells (Li et al., 2003). To determine if exposure to soluble UF induces HO-1 mRNA levels in primary endothelial cells, we exposed HCAEC to 10, 50, and 100 μg/mL soluble UF for 6 and 24 hrs and measured the mRNA expression. Exposure to 50 and 100 μg/mL soluble UF caused a significant ($p<0.001$) increase in the mRNA expression of HO-1in HCAEC at both time points (Fig. 4.4). These data indicate that exposure to soluble UF increases oxidative stress in endothelial cells resulting in endothelial activation.

Figure 4.4

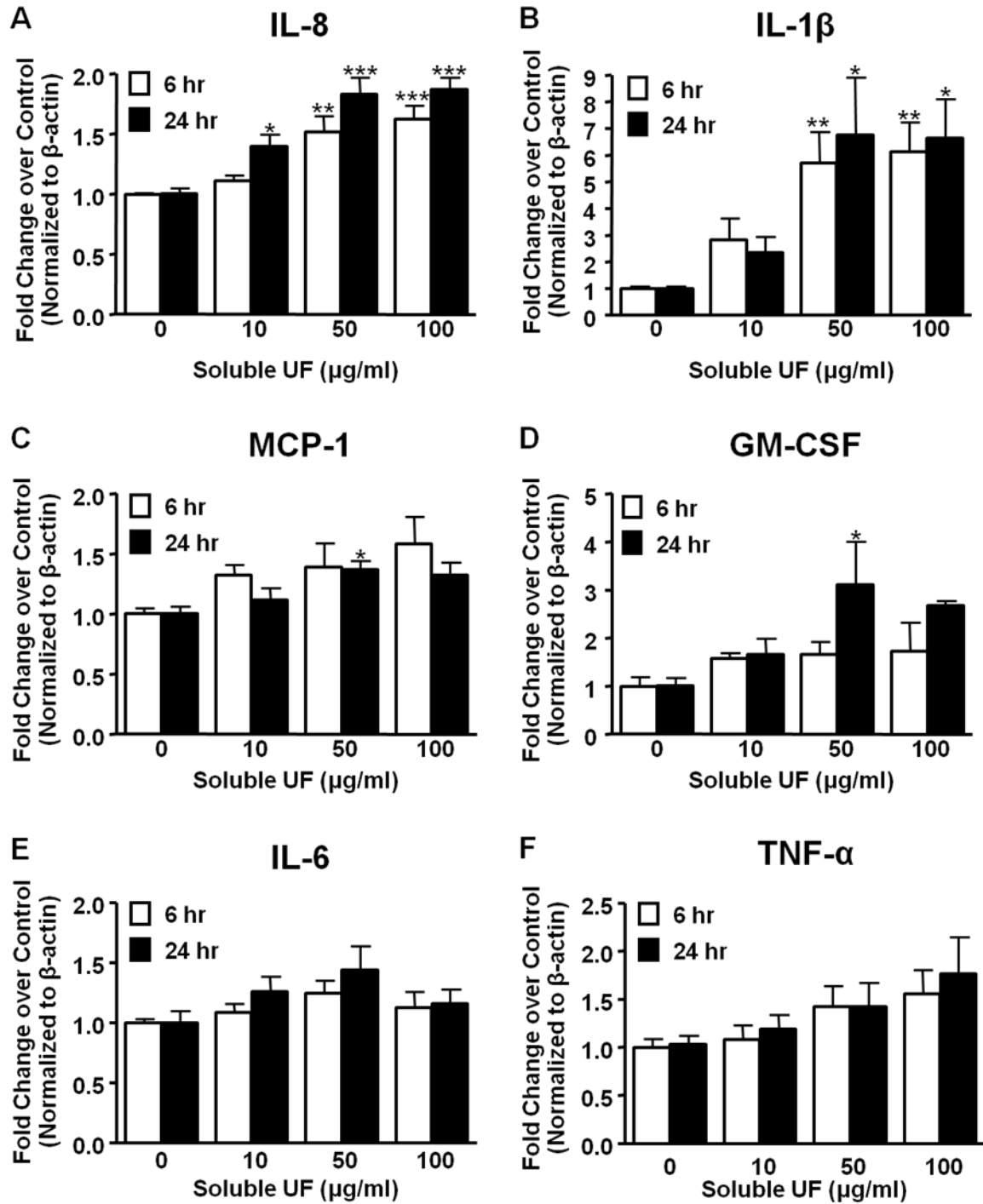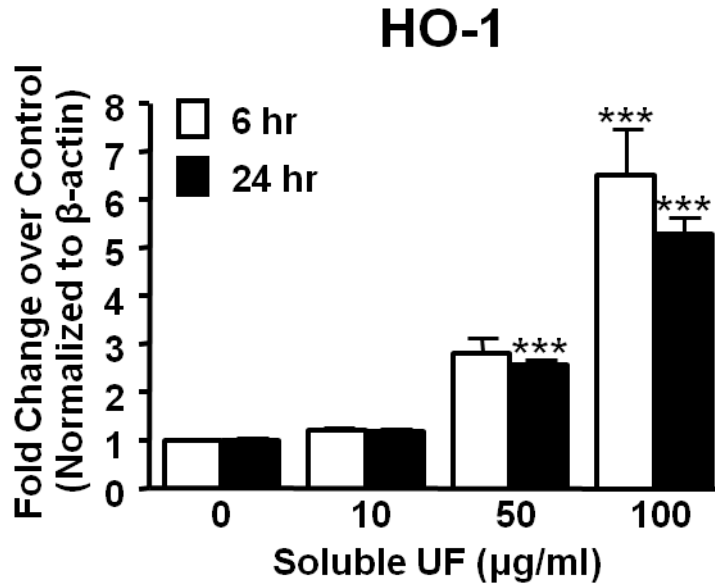**Figure 4.4 Exposure to soluble UF leads to upregulation of HO-1 in HCAEC.** HCAEC were exposed to soluble UF (0, 10, 50, and 100 µg/mL) for 6 (white bars) or 24 hrs (black bars).   Quantitative real time RT-PCR was used to determine mRNA expression of the oxidative stress responsive protein HO-1. Values were normalized to β-actin expression and are expressed as fold change in exposed cells over control. n = 3 separate experiments with samples in triplicate. *** = p<0.001 compared to control.

4.3.5 ROS production from NOX enzymes lead to upregulation of IL-1β and HO-1 mRNA expression

ROS are key intermediates in inducing gene expression of certain proinflammatory cytokines, adhesion molecules, and stress response genes like HO-1 (Lee and Yang, 2012). In Chapter 3, we showed that HCAEC exposed to soluble UF induce procoagulant responses that are dependent upon increased ROS production and NOX-4. To determine if ROS from NOX enzymes mediate the increases in mRNA expression of the key proinflammatory cytokines, cell adhesion molecules, and HO-1 by soluble UF (Fig. 4.2, 4.3, and 4.4), we pretreated HCAEC with DPI followed by a 6 hr exposure to 50 μg/mL soluble UF. Pretreatment with the NOX inhibitor attenuated the increase in IL-1β (Fig. 4.5A) and HO-1 (Fig. 4.5B) mRNA levels by 62% and 57%, respectively, in HCAEC exposed to soluble UF. Conversely, we found no significant changes in the soluble UF-induced mRNA expression of the other proinflammatory markers from Figure 4.2 in HCAEC pretreated with DPI (data not shown).

Since HCAEC predominately express the NOX-4 isoform (Yoshida and Tsunawaki, 2008) and we have confirmed that this NOX homolog is present in our endothelial cells (Fig. 3.5B), we specifically probed the mechanistic role of NOX-4 in soluble UF-induced IL-1β and HO-1 mRNA production by transfecting HCAEC with NOX-4 siRNA. Initial assessment of the NOX-4 siRNA efficiency resulted in an 84% knockdown of the target gene mRNA levels as compared to the scrambled siRNA control 48 hrs after transfection (Fig. 3.5C). Following verification of knockdown, HCAEC were then transfected with NOX-4 or scrambled siRNA for 48 hrs prior to exposure to soluble UF for 6 hrs. Compared to cells transfected with scrambled siRNA, NOX-4 knockdown significantly ($p<0.05$) attenuated

soluble UF-induced HO-1 mRNA upregulation by 30% (Fig. 4.5D); however, we found no significant changes in soluble UF-induced IL-1β mRNA expression in NOX-4 transfected HCAEC (Fig. 4.5C). Together, these data indicate that following exposure to soluble UF, ROS from NOX enzymes upregulate HO-1 and IL-1β mRNA expression, while ROS from NOX-4 specifically contributes to increases in HO-1 mRNA expression.

Figure 4.5
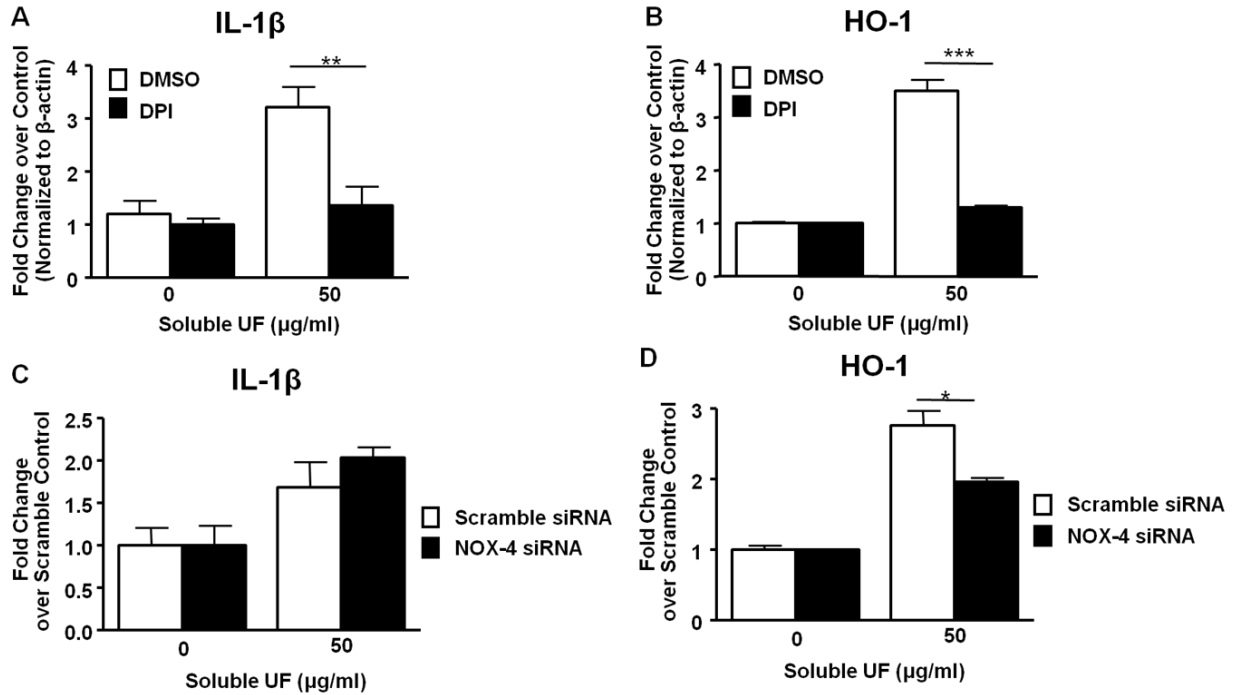
Figure 4.5 IL-1β and HO-1 mRNA expression following exposure to soluble UF requires ROS production from NOX enzymes. (A-B) HCAEC were pretreated with DPI (50 μM) for 30 minutes and then exposed to 0 or 50 μg/mL soluble UF for 6 hrs. IL-1β (A) and HO-1 (B) mRNA expression was quantified by real time RT-PCR. (C-D) HCAEC were transfected with NOX-4 or scramble siRNA for 48 hrs and then exposed to 0 or 50 μg/mL soluble UF for 6 hrs. IL-1β (C) and HO-1 (D) mRNA expression was quantified by real time RT-PCR. For all mRNA, values were normalized to β-actin expression and are expressed as fold change over control. n = 3 separate experiments with samples in duplicate. $*$ = p<0.05, $**$ = p<0.01 $***$ = p<0.001 compared to control.

4.4 Discussion

Increasing evidence shows a strong association between exposure to air pollution and CV morbidity and mortality (Baccarelli et al., 2008; Mann et al., 2002; Pope et al., 2004) but the mechanisms behind this relationship remain unknown. An important area of research that needs to be further investigated is determining if components of PM lead to endothelial activation, which could link inhaled air pollutants to vascular dysfunction. Here we report that exposure to the soluble components of UF particles increase the expression of proinflammatory cytokines and cell adhesion molecules in endothelial cells. We further show that soluble UF exposure induces the expression of the oxidant stress-inducible protein HO-1. These responses, which indicate endothelial activation, were dependent upon increased ROS production and NOX enzymes. These novel findings provide mechanistic insight into the proinflammatory responses and endothelial dysfunction that may contribute to the pathophysiology of CV diseases associated with exposure to air pollution.

Our results add to a growing body of literature indicating that air pollution exposure leads to proinflammatory responses in humans (Ghio and Devlin, 2001), animal models (Ferraro et al., 2011), and *in vitro* models (Aung et al., 2011; Fujii et al., 2001; van Eeden et al., 2001). A majority of these *in vitro* studies examining the adverse cellular responses to PM have generally been investigated in cultured lung epithelial cells and macrophages; however, a recent study by Aung et al. demonstrated that exposure to fine and UF particles induce E-selectin, IL-6, TNF-α, and MCP-1 in human aortic endothelial cells (Aung et al., 2011). Our findings extend this observation by illustrating that exposure to the soluble components of UF particles, which are more biologically relevant as they have a higher

chance of entering the vasculature, increase the gene expression of proinflammatory cytokines and cell adhesion molecules in endothelial cells.

The elevated expression of these proinflammatory mediators and increased endothelial procoagulant activity we demonstrated in Chapter 3 indicate that exposure to soluble UF leads to endothelial activation in cultured primary endothelial cells (Hunt and Jurd, 1998). Endothelial cell activation increases vascular coagulation and inflammation *in vivo*, and is involved in the pathogenesis of several CV diseases including atherosclerosis and hypertension (Lwaleed et al., 2007; Sprague and Khalil, 2009). In addition, endothelial dysfunction contributes to the formation of venous and arterial thrombosis, which upon rupture may lead to myocardial infarctions or strokes (Lowe, 2008). Recent studies have demonstrated that exposure to elevated levels of PM can increase the risk of deep vein thrombosis, myocardial infarctions, and strokes (Baccarelli et al., 2008; Kettunen et al., 2007; Murakami and Ono, 2006), supporting the concept that endothelial activation may contribute to vascular dysfunction following air particle pollution exposure (Figure 1.2).

HO-1 is an oxidant sensitive molecule that is the rate-limiting enzyme in the degradation of heme, resulting in the production of metabolites that have antioxidant properties (Li and Shah, 2004). PM exposure has been shown to increase HO-1 expression in alveolar macrophages and bronchial epithelial cells (Li et al., 2003) and our data extend these findings by illustrating that exposure to soluble UF induces the expression of HO-1 in endothelial cells. Pharmacological induction or overexpression of HO-1 has been shown to exert protective antioxidant, anti-inflammatory, and anticoagulant properties (Fan et al., 2012; Kawamura et al., 2005; Panahian et al., 1999). For example, induction of HO-1 in human aortic endothelial cells attenuated the induced proinflammatory effects and

endothelial dysfunction caused by exposure to oxidized low-density lipoprotein and TNF-α (Kawamura et al., 2005). HO-1 has emerged as therapeutic target for the treatment of CV disorders (Durante, 2010). Our studies demonstrate that exposure to soluble UF induces oxidant production (Chapter 2), increases endothelial procoagulant activity (Chapter 3), and elevates the expression of proinflammatory mediators (Chapter 4) in the presence of increased HO-1 expression; however, targeting this antioxidant protein may still be a viable therapeutic option when trying to combat adverse CV effects associated with air pollution exposure.

Pathological increases in ROS production can lead to oxidative stress and cellular damage resulting in the pathogenesis and progression of a variety of CV diseases (Madamanchi et al., 2005). NOX enzymes are an important source of vascular ROS production and NOX-derived oxidative stress has been implicated in the pathogenesis of several CV disorders including atherosclerosis and diabetes (Griendling et al., 2000). We have previously shown that exposure to soluble UF induces ROS production from NOX enzymes leading to the upregulation of TF and increased endothelial procoagulant activity (Chapter 3). Additional studies have demonstrated that air pollution induces ROS from NOX enzymes that contributes to proinflammatory responses in mouse pulmonary microvascular endothelial cells (Mo et al., 2009) and the upregulation of HO-1 in a human bronchial epithelial cell line (Cheng et al., 2012). Our data further these findings by demonstrating that the soluble components of UF PM can induce proinflammatory responses and HO-1 upregulation via ROS from NOX enzymes in human endothelial cells. It has become apparent that NOX enzymes are critical therapeutic targets when trying to combat oxidative stress-induced vascular diseases (Schramm et al., 2012), and our data further this notion that

targeting NOX enzymes is a viable therapeutic option to address adverse CV effects caused by exposure to air pollution.

In summary, we have shown that exposure to soluble UF increases the expression of proinflammatory mediators and HO-1 in endothelial cells resulting in endothelial activation. These changes were shown to be regulated by ROS from NOX enzymes. These novel findings provide mechanistic insight into the proinflammatory responses and endothelial dysfunction that may contribute to the adverse CV health effects associated with PM exposure.

CHAPTER 5

OVERALL CONCLUSIONS AND SIGNIFICANCE

Increasing evidence suggests a strong association between exposure to air pollution and cardiovascular (CV) morbidity and mortality, but the mechanisms behind this relationship are currently not known. Ultrafine (UF) particles deposit in the heavily vascularized region of the lung, and the soluble components of UF particles (soluble UF) are able to cross from the lung into the circulation and adversely affect cells of the vasculature such as endothelial cells. Endothelial activation and initiation of coagulation are pathophysiological mechanisms that could link inhaled air pollutants to vascular dysfunction. These studies investigated the adverse endothelial responses following exposure to soluble UF. In Chapter 2, we demonstrated that soluble UF induced H_2O_2 release from NADPH oxidase (NOX) enzymes in endothelial cells. This increase in reactive oxygen species (ROS) production was dependent on transition metals present in the soluble fraction. In Chapter 3, we illustrated an increase in intracellular H_2O_2 production from the NOX-4 enzyme that regulated the expression of the procoagulant protein tissue factor (TF) in endothelial cells exposed to soluble UF. These procoagulant responses led to activation of the extrinsic coagulation pathway, which triggered faster onset of thrombin generation and fibrin thrombus formation. In Chapter 4, we showed that exposure to soluble UF increased the expression of proinflammatory cytokines, cell adhesion molecules, and the oxidant stress-inducible protein HO-1 in endothelial cells. These adverse endothelial responses were regulated by ROS from NOX enzymes. Collectively, these studies demonstrate that exposure

to the soluble components of UF particles induced ROS production from NOX enzymes that regulate procoagulant and proinflammatory responses in endothelial cells resulting in endothelial activation and a prothrombotic phenotype (Figure 1.6). These novel findings provide mechanistic insight into the endothelial dysfunction that may contribute to the adverse CV health effects associated with PM exposure.

Endothelial cells are critical components of the vasculature as they are involved in hemostatic balance, blood cell trafficking, and permeability (Aird, 2007). Endothelial activation typically involves increases in cell adhesion molecules, induction and release of proinflammatory mediators, a shift towards a prothrombotic phenotype, and altered permeability (Aird, 2006). Thus, endothelial activation following exposure to air pollution may play a fundamental role in the progression of CV diseases. Over the course of these studies, we have demonstrated that exposure to soluble UF: 1) induces ROS production (Chapter 2), 2) increases endothelial procoagulant activity (Chapter 3), and 3) elevates expression of proinflammatory mediators (Chapter 4); all of which indicate endothelial activation following exposure to particulate air pollution. These pro-oxidant, proinflammatory, and procoagulant responses contribute to a prothrombotic endothelial phenotype and may lead to inappropriate regulation of vascular tone, permeability, coagulation, fibrinolysis, cell adhesion, and proliferation following exposure to air pollution (Calderon-Garciduenas et al., 2008).

To investigate the mechanisms that contribute to soluble UF-induced endothelial activation, we utilized an immortalized endothelial cell line (EA cells) and primary human coronary artery endothelial cells (HCAEC) as *in vitro* models. As an immortalized cell line, EA cells maintained most of the characteristics of endothelial cells, were fast growing, and of

ample supply. Therefore, these cells were ideal for exploring possible adverse endothelial responses following exposure to soluble UF. However, EA cells were derived by fusing human umbilical vein endothelial cells with a human lung adenocarcinoma epithelial cell line, A549 cells (Edgell et al., 1983), and might not fully represent adverse responses induced in primary endothelial cells. In fact, we show that although the trends were analogous, HCAEC had substantially augmented responses in extracellular ROS production (Chapter 2) and TF mRNA expression (data not shown) as compared to that in the EA cell line following exposure to soluble UF.

For our primary endothelial cell model, we used HCAEC obtained from the coronary artery of two male, Caucasian donors with no known history of CV disease. The coronary artery is one of the first blood vessels to receive fresh oxygenated blood from the lung. The endothelial cells that line this blood vessel will accordingly be exposed to relatively high concentrations of any soluble components that traverse from the lung into the vasculature, making it an ideal model to investigate the adverse endothelial responses following exposure to soluble UF. A bulk of the experiments in this project were carried out using primary endothelial cells from only Donor 2 due to lack of commercially available stocks of early passage HCAEC from Donor 1. However, the experiments investigating the changes in mRNA expression of pro- and anticoagulant proteins (Chapter 3) and proinflammatory mediators (Chapter 4) were carried out using endothelial cells from both donors. It is interesting to note that although both donors demonstrated the same trend in expression of these key coagulant and inflammatory proteins, the magnitude of response was different. This can be most clearly identified by examining IL-1β mRNA expression following soluble UF exposure (Fig. 4.2B). The error bars on this graph are considerable due to the differences

in the magnitude of responses between the two donors. For example, Donor 1 had a 12.1-fold increase in IL-1β mRNA expression following exposure to 100 μg/ml soluble UF for 24 hrs, whereas Donor 2 had a 3.8-fold following the same exposure. Although both donors responded to soluble UF exposure by increasing the expression of this proinflammatory cytokine, Donor 1 was more responsive. This divergence in response may be explained by the age difference of the two donors (Donor 1 age – 57; Donor 2 age – 21). This data supports findings by Schneider et al. who observed significant differences in the rate of fibroblast migration, cellular senescence, cell population replication rate, and cell number at confluency from skin fibroblast cultures derived from healthy old and young volunteers (Schneider and Mitsui, 1976). Unknown genetic aspects may also play a role in the variability of adverse responses following an air pollution exposure (Kleeberger, 2003).

This finding brings up an interesting concept to explore in future studies. It is known that individuals will respond differently to the same air pollution exposure. Susceptible populations, such as individuals with preexisting CV or pulmonary disease, the elderly, or small children, are at an increased risk to severe air pollution episodes (Brook et al., 2004). Recent studies have begun comparing the adverse responses of primary airway epithelial cells obtained from asthmatic or non-asthmatic individuals to particulate matter (PM) exposure (Duncan et al., 2012). These researchers have discovered that epithelial cells from the susceptible population exhibit differential responses following exposure to particulate air pollution. Extrapolating further, these data suggest that endothelial cells obtained from individuals with a known history of CV disease, or other susceptible populations, might respond differently to air particle pollution exposure. Comparing the responses of endothelial cells from normal and susceptible populations to a soluble UF exposure would be

an interesting follow up experiment to further elucidate the mechanisms that link air pollution exposure and CV disease.

PM is a major component of air pollution as well as a primary contributor to CV morbidity and mortality associated with ambient air pollution exposure (Anderson et al., 2012). One of the first objectives in this project was to determine the relevant size, fraction, and dose of PM to use throughout the experiments (Chapter 2). Increasing epidemiological evidence suggests that although elevated levels of coarse and fine PM induce adverse CV effects (Harrison and Yin, 2000; Polichetti et al., 2009), UF PM exposure is more significantly correlated with CV and pulmonary diseases (Belleudi et al., 2010; Franck et al., 2011). We demonstrated that the soluble fraction of UF PM induced extracellular ROS production in endothelial cells; whereas, both the soluble and insoluble fraction of coarse and fine PM contributed to increases in ROS production following exposure (Chapter 2). This finding is important because it further supports the concept that the water soluble components of UF particles, which are capable of translocating to the vasculature, are responsible for the adverse CV effects associated with PM exposure.

UF particles are more toxic and inflammogenic compared to larger sized particles of same material when delivered at the same mass dose (Stone et al., 2007). This is most likely due to the significantly greater collective particle surface area that allows for adsorption of toxic substances such as organic compounds and transition metals (Sioutas et al., 2005). In addition, the small diameter of UF particles make them more likely to deposit in the heavily vascularized alveolar region of the lung (Kreyling et al., 2004). UF particles are able to evade critical lung defenses, such as recognition and phagocytosis by alveolar macrophages (Gonzalez et al., 1996) and mucociliary clearance (Kreyling et al., 2006), to remain longer in

the lung and thus increase the possibility of inducing adverse cardiopulmonary effects. For these reasons, we chose to study the water soluble fraction of UF particles for this project.

The EPA monitors coarse and fine PM due to the Clear Air Act but do not currently regulate air pollution particles in the UF size range. These particles are extremely small making them difficult to capture on a filter, which contributes to the insufficient amounts of available information pertaining to their concentrations and physical/chemical properties (Sioutas et al., 2005). These data suggest that an emphasis must be placed on developing new technologies to measure ambient levels of UF PM in effort to facilitate a better understanding of their contribution to CV morbidity and mortality.

The emerging nanotechnology industry is incorporating nanoparticles, which are in the same size range as UF PM, into everyday products such as electronics, food, medicines, and cosmetics (Stone et al., 2007). Although applications of engineered nanoparticles have the potential to enhance quality of life by advancing scientific knowledge and improving existing products, they also have the potential to become aerosolized and induce adverse cardiopulmonary effects. Recent studies have demonstrated that an assortment of nanoparticles (i.e. carbon nanotubes, fullerenes, TiO_2) are capable of inducing CV, pulmonary, and systemic adverse responses in *in vivo* models (Shannahan et al., 2012). Nanomaterials, like UF particles, are not currently regulated, and human exposure will only increase as this rapidly growing field expands into new products (Li et al., 2008). Further research needs to be conducted to understand the potential adverse human health effects following exposure to these intentionally engineered UF-sized particles.

Physiological levels of ROS are produced as intercellular signaling molecules to initiate signaling cascades involved in basic cellular functions such as cell growth,

metabolism, and cell death (D'Autreaux and Toledano, 2007). However, under pathophysiological conditions, ROS can be produced at an elevated rate, resulting in oxidative stress and cellular damage that has been implicated in the pathogenesis and progression of several CV diseases (Madamanchi et al., 2005). Moreover, PM exposure increases ROS production and oxidative stress in *in vitro* models (Mo et al., 2009; Montiel-Davalos et al., 2012), animal models (Araujo et al., 2008; Lei et al., 2005), and human volunteers (Sorensen et al., 2005). Our data expands on these studies by demonstrating that exposure to soluble UF induces elevated levels of extracellular (Chapter 2) and intracellular (Chapter 3) endothelial H_2O_2 production immediately following exposure. These findings suggest that PM-induced ROS production is a critical component in the development of CV diseases.

Most signaling events initiated by ROS are thought to occur through the H_2O_2 molecule, which is capable of oxidizing cysteine residues on target proteins to initiate signal transduction cascades (Rhee et al., 2000), and activating transcription factors leading to the induction of redox-sensitive genes (Schmidt et al., 1995). H_2O_2 is a membrane soluble molecule that is detectable both extra- and intracellularly. Our data strongly suggest that soluble UF-induced H_2O_2 production is made intracellularly as endothelial cell exposure to this fraction led to an increase in the fluorescence intensity of PG1, a chemical indicator specific for H_2O_2 with the ability to cross the cellular membrane (Miller et al., 2007). This premise is further supported by confocal microscopy, which showed a cytoplasmic distribution of H_2O_2 (Chapter 3). Our findings indicate soluble UF-induced ROS production derives from the NOX-4 enzyme, which has a broad intracellular distribution and is shown to be variably located in the perinuclear space, endoplasmic reticulum, mitochondria, and

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nucleus of endothelial cells (Lassegue and Griendling, 2010; Lassegue et al., 2012). Determining the subcellular region of NOX-4 is important because location and expression of NOX enzymes can dictate participation in distinct signaling pathways (Gough and Cotter, 2011). However, these data do not provide spatial or temporal information in regards to the specific subcellular location of NOX-4, or the origins of H_2O_2 in HCAEC following exposure to soluble UF. In addition, activation of PG1 with H_2O_2 is irreversible so detection with this probe cannot be used to detect transient changes in H_2O_2 concentration (Rhee et al., 2010). Future studies using organelle-specific fluorescent probes or transduction with genetically-encoded fluorescent indicators targeted towards specific cellular compartments that are reversible and highly specific for H_2O_2, will help elucidate the subcellular location of NOX-4-induced H_2O_2 production (Rhee et al., 2010). These data will further expand our knowledge regarding spatiotemporal and transient production of ROS following exposure to soluble UF in endothelial cells.

Recently, a novel hierarchical cellular response model has been devised to explain the connection between oxidative stress and PM-induced cardiopulmonary adverse responses (Figure 5.1) (Li et al., 2008). This is a 3-tier model that demonstrates increasing cellular damage with elevated levels of oxidative stress. In this model, low levels of oxidative stress induce transcriptional activation of antioxidant and detoxification enzymes such as HO-1, catalase, superoxide, and glutathione peroxidase (Tier 1). Activation of these antioxidants is a protective response, indicating PM-induced ROS production does not automatically cause cellular damage. However, increased levels of oxidative stress can overwhelm these protective enzymes, resulting in inflammatory responses such as activation of proinflammatory cytokines, chemokines, and cell adhesion molecules (Tier 2). Toxic levels

of oxidative stress involve the mitochondria, which can release pro-apoptotic factors that induce apoptosis (Tier 3). Our project supports this cellular response model that was originally developed to estimate the adverse effects of diesel exhaust particle (DEP) exposure and extend it to include endothelial responses following exposure to the soluble components of UF particles. We demonstrate that exposure to soluble UF increased extra- and intracellular ROS production in endothelial cells (Chapter 2 & 3), resulting in the upregulation of antioxidant enzymes (Tier 1; Chapter 4) and proinflammatory mediators (Tier 2; Chapter 4). We do not see an increase in cytotoxicity following exposure, which would be the next step in this model, suggesting the doses used in this project induce endothelial activation rather than cellular death (Tier 3; Chapter 2). This hierarchical cellular oxidative stress model provides a mechanistic pathway that may contribute to adverse cardiopulmonary responses following air particle exposure.

Figure 5.1
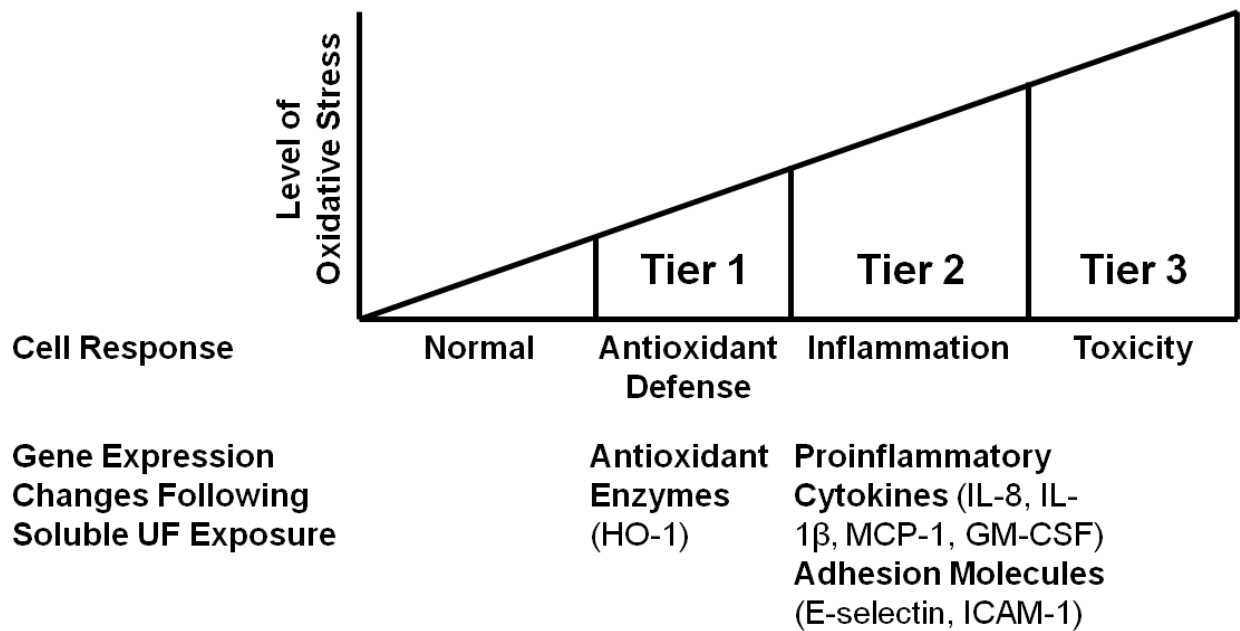
Figure 5.1 Schematic diagram illustrating the hierarchical oxidative stress response. Image modified from Li et al. (Li et al., 2008).

While it is uncertain whether whole UF particles can enter into the systemic circulation in humans, transition metals, which are major constituents of the water-soluble fraction of UF particles, can cross the thin alveolar-capillary membrane and cause direct effects on cells of the vasculature (Wallenborn et al., 2009; Wallenborn et al., 2007). *In vitro* studies have illustrated that transition metals can detach from air pollution particles and subsequently be taken up by human lung epithelial cells to induce proinflammatory responses (Aust et al., 2002). Transition metals are thought to play an important role in the adverse cardiopulmonary effects associated with air pollution exposure by increasing oxidative DNA damage, inducing ROS production, activating inflammatory pathways, and inducing procoagulant events (Costa and Dreher, 1997; Prahalad et al., 2001; Sangani et al., 2010; Sorensen et al., 2005; Wu et al., 2002). We demonstrate in Chapter 2 that transition metals

are involved in the increased ROS production in endothelial cells following exposure to soluble UF. These data support previous studies that determined transition metals increase ROS production leading to induction of inflammatory responses in pulmonary and vascular cells (Gojova et al., 2007; Montiel-Davalos et al., 2012; Shafer et al., 2010). Our results extend these observations by illustrating that transition metals associated with the soluble fraction of UF particles cause adverse vascular effects by inducing H_2O_2 production within endothelial cells. This indicates that soluble components of PM are capable of inducing oxidative stress following exposure to air pollution.

Potential follow up studies to this project would focus on determining the specific transition metal(s) responsible for these adverse effects. Studies have shown that individual metal components like vanadium, copper, cadmium, iron, and zinc, which are present in our UF particles, induce adverse responses in *in vitro* models (Aust et al., 2002; Benton et al., 2011; Cheng et al., 2010; Li et al., 2005). Using inductively coupled plasma atomic emission spectroscopy to detect trace metals, it would be possible to determine the metal uptake in endothelial cells following exposure to the soluble UF (Deng et al., 2009). Subsequently, endothelial cells can then be exposed to environmentally relevant doses of these transition metals to determine the role they play in endothelial activation. These adverse responses are likely not the result of a single soluble component, but rather an unknown combination of transition metals. The resulting data from these experiments would expand the current knowledge of how soluble UF components induce adverse CV health effects.

NOX enzymes are an important source of ROS production in endothelial cells, and pathological activation of these enzymes can lead to oxidative stress and cellular injury (Brown and Griendling, 2009). NOX-induced oxidative stress has been implicated in the

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progression of several CV diseases including hypertension, atherosclerosis, and diabetes (Madamanchi et al., 2005). In our studies, we showed that NOX enzymes are the major source of extracellular H_2O_2 production in endothelial cells following exposure to soluble UF (Chapter 2). In addition, we demonstrated that ROS from NOX enzymes elevated endothelial procoagulant activity (Chapter 3), induced proinflammatory responses (Chapter 4), and increased the expression of the oxidant sensitive molecule HO-1 in endothelial cells (Chapter 4). These data expand upon the findings of previous studies that reported air pollution increases NOX-derived ROS that led to upregulation of TF mRNA expression in vascular smooth muscle cells (Cheng et al., 2010; Gorlach et al., 2000; Herkert and Gorlach, 2002), contributed to proinflammatory responses in mouse pulmonary microvascular endothelial cells (Mo et al., 2009), and increased HO-1 mRNA expression in a human bronchial epithelial cell line (Cheng et al., 2010).

It is interesting to note that neither pretreatment with DPI, a NOX inhibitor, nor deferoxamine, a metal chelator, completely attenuated extracellular H_2O_2 release in HCAEC exposed to soluble UF (Chapter 2). These data imply that there is possibly another cellular source besides NOX enzymes generating ROS following soluble UF exposure in endothelial cells, and that there could be other soluble components besides transition metals inducing ROS production, respectively. Another possibility is that transition metals, which are capable of generating ROS production independently through Fenton reactions (Jomova and Valko, 2011), are inducing ROS through an alternative pathway besides direct activation of NOX enzymes. An ideal follow up experiment would involve exposing endothelial cells with soluble UF pretreated with deferoxamine in the presence of DPI. If pretreatment with both DPI and deferoxamine completely attenuated soluble UF-induced ROS production, it

would indicate that transition metals are inducing ROS separate from the activation of NOX enzymes. These data would further elucidate mechanisms that could link PM exposure to endothelial oxidative stress.

Endothelial cells express mainly the NOX-2 and NOX-4 isoforms but expression levels of the NOX family members vary among the different endothelial cell types (Guzik et al., 2004). HCAEC predominantly express the NOX-4 isoform but also express the NOX-2 and NOX-1 homologs (Yoshida and Tsunawaki, 2008). We demonstrated that transfection with NOX-4 siRNA attenuated soluble UF-induced TF mRNA expression (Chapter 3) and HO-1 mRNA expression (Chapter 4) in HCAEC. However, there was not complete suppression of these soluble UF-induced mRNA increases, suggesting that another source besides NOX-4 is involved in the transcriptional regulation of these genes following exposure. One possibility is that ROS from the NOX-2 enzyme plays a role in the augmented mRNA expression of TF and HO-1 following soluble UF exposure. Similar to the NOX-4 isoform, NOX-2 in endothelial cells can also be involved in agonist-induced vascular oxidative stress (Bendall et al., 2007). In addition, knockdown of either the NOX-4 or NOX-2 enzyme with siRNA increases the mRNA and protein of the other isoform in endothelial cells (Pendyala et al., 2009). This suggests a compensatory mechanism by the cell to ensure that basal levels of ROS can be produced for basic cellular processes. Unlike NOX-4, which is a constitutively active enzyme, NOX-2 is an inducible enzyme that requires the translocation of several cytosolic subunits (Bedard and Krause, 2007). Although these enzymes differ structurally, NOX-2 and NOX-4 both require the small membrane-associated component p22^{phox} for their catalytic activity (von Lohneysen et al., 2008). Knockdown of p22^{phox}attenuated ROS induced TF mRNA expression in vascular smooth muscle cells

activated by platelet-derived products (Gorlach et al., 2000). Future experiments that target this integral subunit in order to knockdown both NOX isoforms would provide additional data as to whether NOX-2 is involved in the adverse responses in HCAEC following soluble UF exposure.

We show that transfection with NOX-4 siRNA attenuated TF mRNA expression following soluble UF exposure, which suggests that NOX-4 is the likely source of soluble UF-induced H_2O_2 production that results in increased endothelial procoagulant activity in HCAEC (Chapter 3). However, we were unable to definitively illustrate that NOX-4 knockdown in HCAEC attenuates the soluble UF-induced ROS production (Chapter 2 & 3) or faster onset of thrombin generation and fibrin thrombus formation (Chapter 3). After several unsuccessful attempts at repeating the experiments with NOX-4 transfected cells, we determined that transfection with siRNA, regardless of whether it was scramble or targeted to NOX-4, slightly activated the endothelial cells to the point where our assays were not sensitive enough to detect the prospective diverse responses between the cells transfected with NOX-4 siRNA or scramble siRNA. Follow up experiments to confirm the role of NOX-4-derived ROS production following soluble UF exposure could involve transducing HCAEC with a lentivirus containing a construct coded for a shRNA designed to knockdown NOX-4 gene expression in order to establish stable suppression of the NOX-4 enzyme. Recent studies have demonstrated that efficient, stable transduction with lentiviral vectors in primary endothelial cells is feasible (Pariente et al., 2008), and suggest that transduction with a NOX-4 lentivirus would help minimize cellular stress and activation prior to soluble UF exposure.

There is strong epidemiological evidence supporting a casual relationship between air particle pollution exposure and cardiovascular and cerebrovascular events triggered by thrombus formation, such as acute coronary syndrome, stroke, deep venous thrombosis, and thromboembolism (Baccarelli et al., 2008; Kettunen et al., 2007; Martinelli et al., 2012; Peters et al., 2001; Pope et al., 2006; Wellenius et al., 2012). Endothelial activation and initiation of coagulation are pathophysiological mechanisms that could link inhaled air particle pollutants to vascular events (Figure 1.2). We show that exposure to soluble UF induced a procoagulant phenotype in endothelial cells, supporting faster onset of thrombin generation and fibrin thrombus formation, and that these activities are driven by upregulation of TF (Chapter 3). Activation of TF from injury or pathological conditions is typically balanced by expression of anticoagulant proteins such as TFPI, EPCR, and thrombomodulin (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 (Chapter 3). Our data add to a growing body of evidence that link air pollution exposure to thrombosis. Recent studies have demonstrated hypercoagulable effects of air pollution on thrombin generation in plasma samples taken from exposed human and animal models (Emmerechts et al., 2012; Kilinc et al., 2011; Rudez et al., 2009). Our results extend these observations to potentially pathologic effects of PM on cellular procoagulant activity. The upregulation of TF and lack of compensatory changes in the anticoagulant proteins, which result in increased endothelial procoagulant activity, provide a mechanistic rationale for the increased cellular procoagulant activity following exposure to soluble UF.

Activation of the extrinsic pathway via TF initiates a cascade of events that cumulates into the activation of fibrinogen into fibrin by thrombin, which is the basis of a fibrin

thrombus (Ott, 2011). Abnormal fibrin structure and stability has been implicated in the progression of several CV diseases associated with thromboembolic events, including deep vein thrombosis, ischemic stroke, and coronary atherothrombosis (Collet et al., 2006; Lisman et al., 2005; Undas and Ariens, 2011). High thrombin concentrations produce dense fibrin networks that are more resistant to fibrinolysis and more prone to thrombosis (Campbell et al., 2009). Recent studies have demonstrated that endothelial cells also play a role in fibrin structure and stability (Campbell et al., 2009). Primary human umbilical vein endothelial cells (HUVECs) exposed to the proinflammatory cytokines TNF-α and IL-1β, increased the rate of thrombin generation and fibrin thrombus formation in platelet-free plasma. In addition, stimulation with the cytokines produced significantly denser fibrin networks than unstimulated HUVECs, indicating a more stable fibrin thrombus that is more resistant to fibrinolysis. Addition of an anti-TF antibody to these cytokine-stimulated HUVECs attenuated the faster onset of thrombin generation, fibrin thrombus formation, and reduced the fibrin network density, indicating these events are TF-dependent. These are similar findings to what we demonstrated in Chapter 3 after a more subtle stimulation with soluble UF. These studies suggest that HCAEC exposed to soluble UF would produce denser fibrin structures that are more prone to thrombosis. This would provide additional evidence that air particle exposure leads to an endothelial prothrombotic phenotype that results in adverse CV events associated with thromboembolisms. Further experiments would need to be conducted in order to confirm this hypothesis.

Increased inflammation plays a critical role in the development and progression of several CV disorders including diabetes (Navarro and Mora, 2006), chronic heart failure (Yndestad et al., 2006), and atherosclerosis (Libby, 2002). Exposure to soluble UF increased

the expression of several proinflammatory mediators in HCAEC, which is a major indicator of endothelial activation (Chapter 4). Our results add to a growing body of literature indicating that air pollution exposure leads to proinflammatory responses in humans (Ghio and Devlin, 2001), animal models (Ferraro et al., 2011), and *in vitro* models (Aung et al., 2011; Fujii et al., 2001; van Eeden et al., 2001). In addition, increasing epidemiological and experimental data have demonstrated that air pollution exposure is a risk factor for atherosclerosis (Araujo and Nel, 2009). For instance, Kunzli et al. determined that for every 10 μg/m^3 rise in fine PM levels, there was a 5.9% increase in carotid intima-medial thickness, a measurement strongly associated with atherosclerosis (Kunzli et al., 2005). Moreover, exposure to coarse PM accelerated progression of atherosclerotic lesions and increased vulnerability to plaque rupture in susceptible rabbit models (Suwa et al., 2002). Endothelial activation is an underlying event in the initiation and progression of atherosclerosis through expression of proinflammatory cytokines and cell adhesion molecules, which attract and provide a pathway for leukocyte trafficking during lesion progression (Libby et al., 2006). Our data demonstrated that soluble UF exposure in endothelial cells induced expression of several key proinflammatory cytokines (MCP-1, IL-8, GM-CSF) and cell adhesion molecules (ICAM-1) involved in the initiation and progression of atherosclerosis (Chapter 4). For example, increased expression of ICAM-1 on the surface of endothelial cells promotes the adherence of monocytes during initiation of atherogenesis (Szmitko et al., 2003). MCP-1 and IL-8 are chemoattractant cytokines that promote migration of inflammatory cells during initiation and progression of atherosclerotic lesions (Apostolopoulos et al., 1996; Harrington, 2000). Furthermore, GM-CSF can enable macrophage maturation and activation during atherogenesis (Smith et al., 1995). Increased expression of these proinflammatory mediators

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in endothelial cells following exposure to soluble UF both support and extend vital information regarding mechanisms by which PM exposure results in the development and progression of atherosclerosis.

The elevated expression of TF, proinflammatory cytokines, adhesion molecules, and HO-1 (Chapter 3 & 4), indicate that exposure to soluble UF leads to endothelial activation in cultured primary endothelial cells (Hunt and Jurd, 1998). Transcriptional regulation of a number of genes studied in this project are known to be redox-sensitive (Haddad, 2002; Herkert et al., 2004; Lavrovsky et al., 2000; Motterlini et al., 2002; Tak and Firestein, 2001). ROS is thought to induce gene expression changes through activation of redox-regulated transcription factors, such as NF-κB, AP-1, SP1, and Nrf2 (Herkert and Gorlach, 2002; Lavrovsky et al., 2000; Li et al., 2011). The *TF* gene in primary endothelial cells has been shown to contain binding sites for the redox-sensitive transcription factors NF-κB, AP-1, and SP1 (Herkert and Gorlach, 2002; Moll et al., 1995). It is interesting to note that although expression of TF can be regulated by the same transcription factors that can control the anticoagulant proteins and proinflammatory mediators examined in this project, our data indicates that the expression of these genes were not regulated by NOX-4-derived ROS following exposure to soluble UF (Chapter 3 & 4). These data suggest activation of an independent mechanistic pathway specific for TF; however, further experiments must be conducted in order to elucidate the transcriptional regulation involved in the augmented expression of TF following exposure to soluble UF.

The data from this project illustrated that soluble UF exposure increased NOX-derived ROS production (Chapter 2) leading to cellular oxidative stress (Figure 5.1) that regulated procoagulant (Chapter 3) and proinflammatory mediators (Chapter 4), resulting in

endothelial activation and a prothrombotic phenotype (Figure 1.6). Combating oxidative stress by targeting ROS or the oxidant source is becoming an apparent approach for treatment of CV disease. Increasing antioxidant capacity through pharmacological induction or overexpression in order to reduce endothelial oxidative stress is a therapeutic strategy used to restore normal endothelial function (Sharma et al., 2012). For example, induction of the antioxidant HO-1 in human aortic endothelial cells attenuated the induced proinflammatory effects and endothelial dysfunction caused by agonist-induced exposure (Kawamura et al., 2005). In addition, there are numerous epidemiological studies demonstrating that dietary and supplemental intake of antioxidant vitamins leads to the reduction of atherogenesis (Diaz et al., 1997). Moreover, NOX enzymes are key sources of vascular oxidative stress, and are emerging as a potential therapeutic target in the limitation of excessive ROS production (Schramm et al., 2012). For instance, newly developed pharmacological inhibitors of NOX-4 (VAS2870 and GKT-136901) have been shown to attenuate agonist-induced oxidative stress in mouse kidney proximal tubule cells (Sedeek et al., 2010) and vascular smooth muscle cells (ten Freyhaus et al., 2006). In general, our findings further support the targeting of ROS, either through increases in antioxidant capacity or direct inhibition of NOX enzymes, which are both viable therapeutic strategies to combat air pollutant-induced adverse CV effects.

It is our belief that this project contributes to the scientific literature addressing the mechanisms behind the association between exposure to air particle pollution and CV disease. We demonstrate that exposure to the soluble components of UF particles induce ROS production in endothelial cells from NOX enzymes that was dependent on transition metals. This PM-induced oxidative stress led to endothelial activation as characterized by elevated expression of proinflammatory mediators and increased endothelial procoagulant

activity.　　These novel findings support and extend important information regarding mechanisms by which PM exposure results in endothelial activation and initiation of coagulation, which may contribute to adverse CV health effects mediated by thrombosis.

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