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Effect of Superoxide Dismutase Mimetic, *AEOL 10150* on the Regulation of the Endothelinergic System In Lungs & Heart of Rats exposed to Air Pollutants

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Effect of Superoxide Dismutase Mimetic, *AEOL 10150* on the Regulation of the Endothelinergic System In Lungs & Heart of Rats exposed to Air Pollutants

Devi Ganesh

Thesis submitted to the

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Abstract

Epidemiological studies have associated cardiopulmonary morbidity and mortality with air pollution. Inhalation of pollutants increases plasma levels of the vasoconstrictor peptide endothelin (ET)-1 and its precursor bigET-1 in experimental animals and human subjects, and induces an oxidative stress in the lungs. Changes of circulating ET-1 is attributed to increased de novo synthesis in lung endothelial cells and spillover in the systemic circulation. Clinical studies indicate that excess ET-1 can be detrimental to individuals with cardiovascular and pulmonary diseases. My hypothesis is that oxidative stress pathways in the alveoli mediate the regulation of the endothelinergic system in response to inhalation of air pollutants. I have tested this hypothesis in male Fischer-344 rats by blocking a potential superoxide surge during or after inhalation of pollutants with a superoxide dismutase (SOD) mimetic drug, *AEOL 10150.* Rats were injected with 2mg/kg of *AEOL 10150* two hours prior to inhalation exposure for four hours to pollutants, and sacrificed immediately or 24 hours post exposure. Treatment with the SOD mimetic abrogated the increase in expression of preproET-1 mRNA and ECE-1 mRNA and plasma ET-1 levels caused by the air pollutants. This suggests that oxidative stress pathways contribute to the regulation of endothelinergic system.

Dedication

"Do your duty to the best of your abilities for Me, without any selfish motive, and remember Me at all times --- before starting a work, at the completion of a task, and while inactive --- the power of God is within you at all times and is constantly doing all the work using you as a mere instrument"..... Srimad Bhagavad Geeta

To my family

Acknowledgements

I owe my heartfelt thanks to my husband for supporting me throughout my academic journey and my little kids who add meaning to my accomplishment. My gratitude is always there for my parents and brother who are always proud of me and to my in-laws who were always looking forward to my graduation. Thanks to my brother's and bro-in-law's families encouragement throughout my academic pursuit. My family's prayers, love and support means a great deal to me.

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Abbreviations

aa	Aminoacid
BAL	Bronchoalveolar lavage
BP	Blood pressure
ECE	Endothelin converting enzyme
eNOS	Endothelial nitric oxide synthase
EHC	Environmental health centre
ET	Endothelin
HPLC	High performance liquid chromatography
H_2O_2	Hydrogen peroxide
iNOS	Inducible nitric oxide synthase
MMP	Matrix metalloprotease
NADPH	Nicotinamide adenine dinucleotide phosphate
NS	Not significant
NO	Nitric oxide
nNOS	Neuronal nitric oxide synthase
O_3	Ozone
$ONOO^-$	Peroxynitrite
PCR	Polymerase chain reaction
PM	Particulate matter
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SODm	Superoxide dismutase mimetic

1 INTRODUCTION

Worldwide epidemiological studies show a consistent increase in cardiac and respiratory morbidity and mortality from exposure to particulate matter (PM). Particulate matter is a key ingredient of polluted air and is estimated to kill more than 500,000 people each year (Nel, 2005). Ambient air PM is divided into three major classes based on their size known as aerodynamic diameter: coarse (PM_{10}, 2.5-10μm), fine ($PM_{2.5}$, <2.5μm), and ultrafine ($PM_{0.1}$, >0.1μm) (Kang, 2002). All PM contain biological material, organic compounds, hydrocarbons, ions, acid aerosols, reactive gases, and metals adsorbed or attached to a typically carbonaceous core (Shore et al, 1995). Extensive epidemiological studies suggest a link between air pollution and daily mortality rates as well as between overall mortality and long-term exposure. Consistent associations have been demonstrated with both respirable particles (<10μm in diameter; PM_{10}) and fine particles that reach the deep lung (<2.5μm; $PM_{2.5}$) (Kunzli et al, 2000; Pope et al, 2002; Samet et al, 2000). A potential link between air particulates and cardiovascular disease in particular is suggested by several time series studies showing that elevated PM_{10} and $PM_{2.5}$ levels are associated with an increase in cardiovascular hospital admissions (Dockery et al, 2001; Schwartz, 1999). Ozone (O_3) is one of the most toxic components of the photochemical air pollution mixture (Gryparis et al, 2004). Short-term respiratory irritant symptoms, lung dysfunction, and bronchoalveolar inflammation have been well documented in controlled O_3 exposures (Lippman, 1993) and in natural outdoor exposures (Kinney et al, 1996).

Seaton et al (1995) proposed a general hypothesis that exposure to inhaled particles induces alveolar inflammation, leading to exacerbation of preexisting lung disease, increased blood coagulability, and an associated increased risk of cardiovascular events. Urban particles have been shown to increase immunoreactive endothelins in plasma of healthy F344 rats (Bouthillier et al, 1998). Vincent et al (2001a) have proposed that the mechanisms by which ambient particles affect cardiovascular function involve changes in vasoregulation and have shown that exposure to urban particulate matter caused an increase in ET-3, ET-1 and blood pressure in rats. PM is also known for inducing vasoconstriction in pulmonary arteries of rats (Batalha et al, 2002). Calderon-Garciduenas et al (2003) have shown about 20% increase in serum endothelins in Southwest Metropolitan Mexico City children exposed to complex mixture of air pollutants. Thomson et al (2004) have demonstrated that air pollutants increase gene expression of ET-1 in lungs of rats. Vincent et al (2001b, unpublished data) have demonstrated increases in the most potent vasoconstrictor, plasma ET-1 and 3 after short-term exposure to $PM_{2.5}$and O_3 in humans. Short-term exposure to a mix of fine particulate air pollution ($PM_{2.5}$) and at levels observed in urban environment induces artery vasoconstriction in healthy adults (Brook et al, 2002).

Endothelin has multiple cardiovascular actions, including vasoconstriction, leading to maintenance of basal vascular tone and BP (Haynes and Webb, 1998) and accentuating BP elevation in more severe, sodium-sensitive hypertension (Schiffrin, 2001). Short-term O_3 exposure within a period of 1 to 2 days is related to acute coronary events in middle-aged adults without heart disease (Ruidavets, 2004). Elevated $PM_{2.5}$ concentrations have also been associated with a transient risk of acute myocardial infarction within a few hours and 1-day

exposure (Bhatnagar et al, 2004). There is accumulating evidence that PM_{10} and $PM_{2.5}$ also have intrinsic ability to cause oxidative stress in cell-free systems (Gilmour et al, 1996) in cells exposed in vitro (Jimenez, 2000) and in exposed animals (Costa et al, 1997). The following paragraphs outline a brief background about endothelins and oxidative stress and its relevance to air pollution and cardiovascular diseases.

1.1 Endothelin

1.1.1 Biosynthesis of ET

The ET system includes ET-1, ET-2 and ET-3 (Berger and Pacher, 2003). Endothelin-1, a 21 aminoacid peptide was initially isolated from porcine aortic endothelial cells (Yanagisawa et al, 1988) and secreted mostly by vascular endothelial cells and is the predominant isoform expressed in vasculature and the most potent vasoconstrictor known (Agapitov and Haynes, 2002). The aminoacid sequence of ETs is given in Figure 1. The three ETs are expressed in a variety of tissues and other kinds of cells including smooth muscle, neuron, messangium, melanocyte, parathyroid, and amnion cells (Tomoh Masaki, 2005). PreproET-1, a 212 amino-acid peptide is the first product of the ET-1 gene. This precursor is transformed to pro-ET-1 by removal of a short sequence by a signal peptidase, then to big-ET-1 via the activity of a maturing enzyme of subtilisin family (calcium-dependent serine proteases) namely furin (Blais et al., 2002). Human big-ET-1, a 38-aminoacid peptide, is found at detectable levels in the peripheral circulation. The mature $ET\text{-}1_{1\text{-}21}$ is obtained by proteolytic cleavage of big ET-1 by endothelin converting enzymes (ECE). The physiologic importance of the conversion of big ET-1 to ET-1 is emphasized by the higher vasoconstrictor potency of the mature peptide (about 140-fold), while the larger proET-1 does not show any

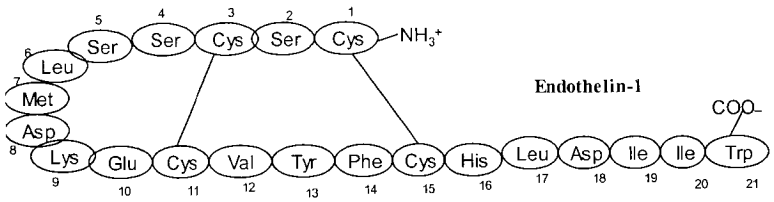
vasomotor action (Rubanyi and Polokoff, 1994). The ECE is a zinc-dependent metalloendopeptidase and the three isoforms identified to date are ECE-1, ECE-2 and ECE-3 of which ECE-1 is the most physiologically implicated, because of its wide action and expression. Indeed, other moieties, such as mast cell chymase and matrix metalloproteinase II, have been suggested to be involved in the production of ET intermediates, such as ET-1 (1-31) and ET-1 (1-32), respectively. Other enzymes, such as the neural endopeptidase (NEP) is not only involved in the degradation and inactivation of ET-1, but is also responsible for the final production of the peptide via the hydrolysis of ET-1 (1-31) (D'Orleans-Juste et al, 2003). Russel and Davenport (1998) have proposed that two distinct exocytic pathways are involved in the transport of ET-1 to the cell surface. ET-1 is stored in Weibel-Palade bodies with other vasoactive compounds and is released at the cell surface following an appropriate stimulus. ET-1 is also sorted into secretory vesicles and continuously released by a cyclic AMP independent constitutive pathway which might be contributing to the normal physiological tone. ET-1 biosynthesis is stimulated by elevated levels of LDL cholesterol and glucose, vasoconstrictors, cytokines like interleukin-1 beta, tumor necrosis factor-alpha (Corder et al, 1995), growth factors like vascular endothelial growth factor, basic fibroblast growth factor, platelet derived growth factor (Matsuura et al, 1998; Boulanger et al, 1991; Hahn et al, 1990), thrombin and adhesion molecules like intracellular adhesion molecule, vascular cell adhesion molecule (Schwarting et al, 1996). Inhibitors of ET-1 synthesis include NO, prostacyclin, artrial natriuretic peptides and estrogens (Levin, 1995).

1.1.2 Mechanism of action of ET

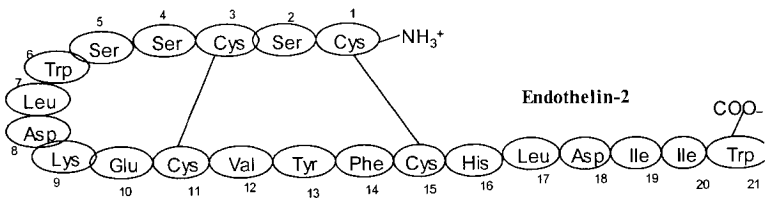
Endothelin-1 acts through the activation of G_i –protein coupled receptors. The binding of ET-1 to ET_A receptors activates phospholipase C, which leads to an accumulation of inositol triphosphate and intracellular calcium and in turn, to long lasting vasoconstriction. The activation of ET_A receptors also induces cell proliferation in different tissues. ET_A receptors are found in the medial smooth muscle layers of the blood vessels, and atrial and ventricular myocardium (Hosoda et al, 1991). In contrast, the activation of endothelial ET_B receptors stimulates the release of NO and prostacyclin, prevent apoptosis and inhibits ECE-1 expression in endothelial cells. ET_B receptors are localized on endothelial cells and to some extent, smooth muscle cells and macrophages (Ogawa et al, 1991). ET_B receptors also mediate the pulmonary clearance of circulating ET-1 and the reuptake of ET-1 by endothelial cells (Luscher and Barton, 2000). The regulation and processing of ET-1 is represented in Figure 2.

Figure 1. Amino acid sequence of human endothelins and sarafotoxin c.

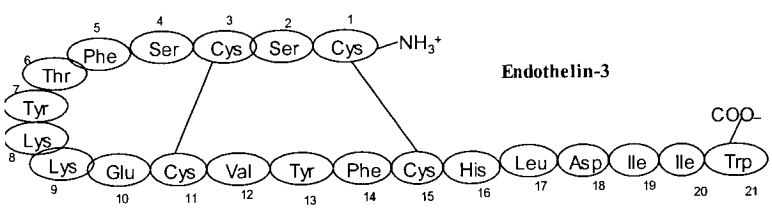
The structure of peptides isolated from the venom of the Israeli burrowing snake, *Actrapsis engaddensis*, termed sarafotoxins (STXs), was shown to have remarkable similarities to ETs. Amino acid substitutions with respect to ET-1 are indicated (Nussdorfer, 1999).



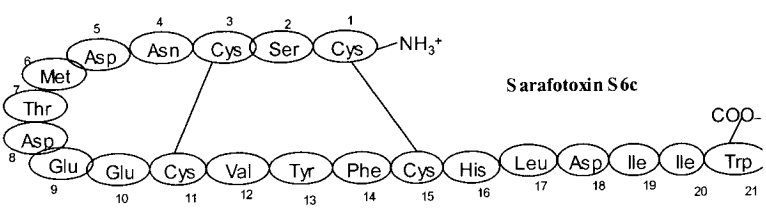
Endothelin-1



Endothelin-2



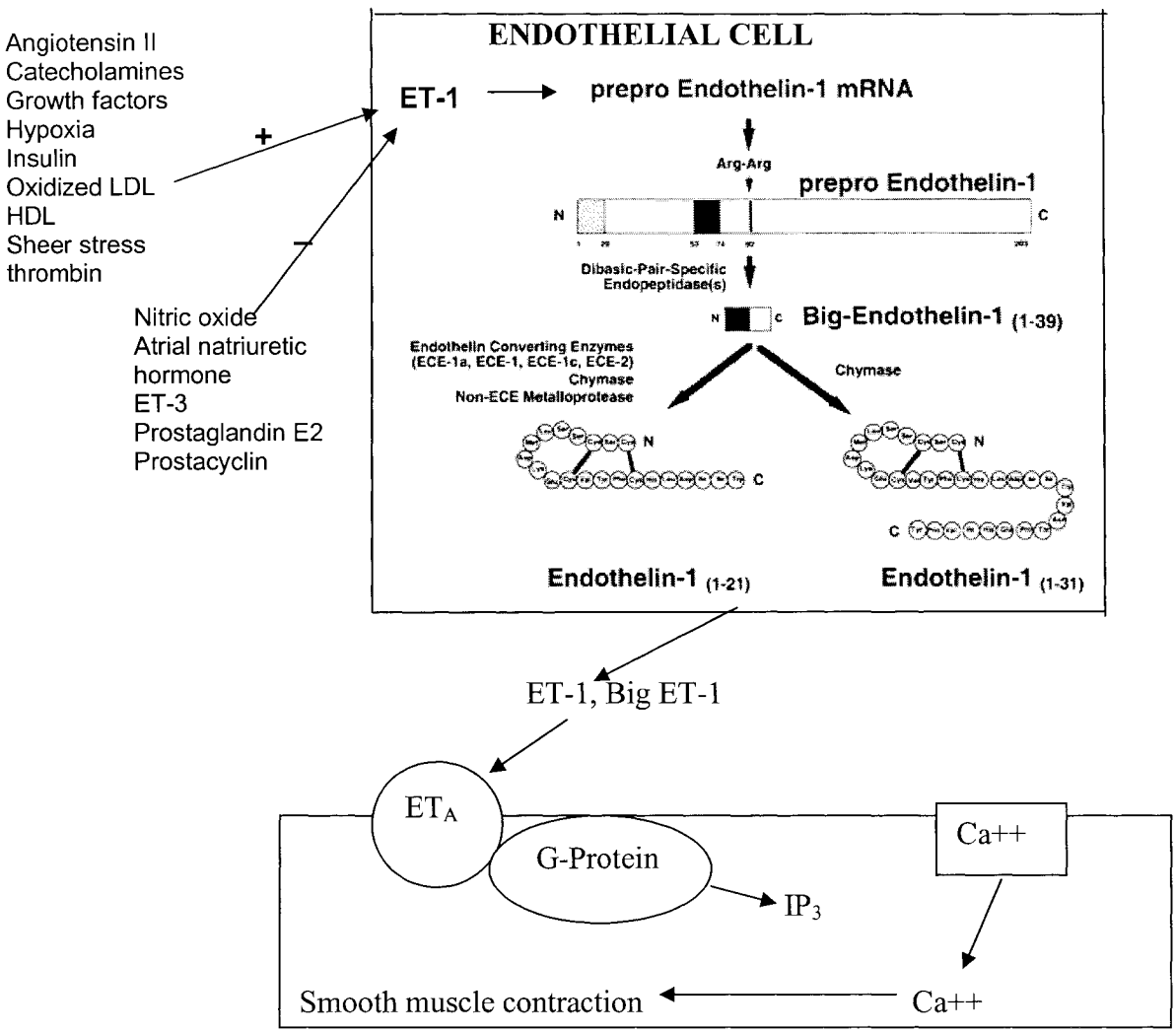
Endothelin-3



Sarafotoxin S6c

Figure 2. Regulation, processing, and secretion of proteins related to ET-1 in endothelial cells

Hormones and vascular factors modulate the synthesis of the preproendothelin-1 by the ET-1 gene by regulating the binding of transcription factors such as GATA-2 and AP-1 to specific elements on the ET-1 gene promoter. The mRNA is translated to a 203-amino-acid preproendothelin-1 protein, which is then converted to a 38-amino-acid prohormone, big ET-1. The ECE converts big ET-1 to the 21aa ET-1 peptide. ET-1 and some big ET-1 are secreted mainly toward the adjacent smooth-muscle layer of the wall of the blood vessel. Smaller amounts of the peptides are secreted into the lumen of the vessel. The list at the top left of the figure contains items that stimulate transcription, and the list at the top right contains items that inhibit transcription (Levin, 1995).


Angiotensin II
Catecholamines
Growth factors
Hypoxia
Insulin
Oxidized LDL
HDL
Sheer stress
thrombin
+
−
Nitric oxide
Atrial natriuretic hormone
ET-3
Prostaglandin E2
Prostacyclin
ENDOTHELIAL CELL
ET-1
prepro Endothelin-1 mRNA
Arg-Arg
prepro Endothelin-1
N
C
Dibasic-Pair-Specific Endopeptidase(s)
Big-Endothelin-1 (1-39)
Endothelin Converting Enzymes (ECE-1a, ECE-1, ECE-1c, ECE-2)
Chymase
Non-ECE Metalloprotease
Chymase
Endothelin-1 (1-21)
Endothelin-1 (1-31)
ET-1, Big ET-1
ETA
G-Protein
IP3
Ca++
Smooth muscle contraction
Ca++

1.1.3 Endothelin and cardiovascular pathophysiology

Endothelin is a potent vasoconstrictor associated with vascular endothelial dysfunction and adverse cardiovascular prognosis at increased systemic level (Levin, 1995). Plasma concentration of ET is increased in humans with congestive heart failure and the level correlates with the severity of disease (Rodeheffer et al, 1992) and risk of subsequent cardiac death in CHF patients (Galatius-Jensen et al, 1996; Tsutamoto et al, 1995). In a human study, plasma levels of ET-1 measured after myocardial infarction were found to be a strong predictor of 1 year survival independent of clinical and biochemical variables previously associated with a poor prognosis (Omland et al, 1994). In rats with experimentally induced myocardial ischemia, the release of ET-1 is greatly augmented during reperfusion, suggesting a role for ET-1 in reperfusion injury (Brunner et al, 1992). High serum levels of ET-1 have been used as an important marker of heart failure (Monge, 1998). Endothelial dysfunction characterized by loss of nitric oxide dependent vasodilation may simultaneously involve augmented ET activity in disease states including atherosclerosis (Galley and Webster, 2004). The earliest stages of atherosclerotic vascular disease are characterized by endothelial dysfunction, in which arterioles paradoxically constrict or fail to dilate in response to a physiologic stimulus such as exercise (Quyumi, 1998). This has been attributed to a relative deficiency in NO- mediated smooth muscle relaxation and vasodilation. There may also be an excess of vasoconstrictive factors, such as ETs and angiotensin II (Warner, 1999). Pulmonary release of ET-1 contributes to an elevation of plasma ET-1 and to vasoconstriction. Endothelins appear to be involved directly in the pathogenesis of cardiovascular diseases through their vasopressor and mitogenic mechanisms, and elevated

circulating ETs may also affect cardiac arrhythmia and dysrhythmia, enhance myocardial ischemia and promote infarct extension, and contribute to increased systemic and pulmonary vascular resistence, vascular dysfunction, renal impairment in diabetes and chronic heart failure patients (Best and Lerman, 2000; Spieker et al, 2001; Zouridakis et al, 2001). ET-1 also causes bronchoconstriction and has been implicated in the development of acute asthma, primary pulmonary hypertension and pulmonary fibrosis (Levin, 1996). Anti-endothelin strategy has been developed in the cardiovascular medicine, particularly in the treatment of heart failure (Spieker et al, 2001). It has been demonstrated that plasma ET-1 levels are higher in these pathologies and that treatments with antagonists of ET receptors are beneficial (D'Orleans-Juste et al, 2003). In two recent clinical trials, the dual endothelin receptor antagonist bosentan has shown beneficial effects in treating pulmonary hypertension. (Kuntzen et al, 2005). Finally, long-term ET_A blockade reduces the extent of atherosclerosis without affecting blood pressure or plasma cholesterol; it also restores NO-mediated endothelium dependent relaxation and prevents increased vascular ET-1 (Barton et al, 1998).

1.2 Oxidative stress

The lung is particularly exposed to various inhaled toxic products whose toxicity can at least be partly mediated by the generation of free radicals (Housset, 1994). Free radicals are molecules capable of independent existence, which contain one or more single electrons in an orbital. Since electrons are usually more stable when paired, radicals are generally more reactive than non-radicals. Reactive oxygen species (ROS) refers to a range of toxic species such as superoxide radical ($O_2^{\cdot -}$) hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl),

singlet oxygen($^1O_2$), hydroxyl radical (HO ·). Nitric oxide (NO·) is a well known example of a nitrogen centred free radical (Heunks, 2000). The NO radical (NO·) is produced in higher organisms by the oxidation of one of the terminal guanido-nitrogen atoms of L-arginine. This process is catalyzed by one of the three isoforms of the enzyme nitric oxide synthase (NOS): neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) (Palmer, 1988). Simultaneous generation of NO· and $O_2^{\cdot-}$ favors the production of reactive nitrogen species (RNS) peroxynitrite anion ($ONOO^-$) (Tarpey and Fridovich, 2001) and this product may account for some of the deleterious effects associated with NO· production (Beckman et al, 1990). Figure 3 represents the derivation of ROS.

Radical formation in the body occurs by several mechanisms, involving both endogenous and environmental factors. Environmental sources include O_3 and H_2O_2 from air pollution and cigarette smoke (Bowler and Crapo, 2002a). The superoxide anion is formed by the univalent reduction of triplet-state molecular oxygen ($^3O_2$). This process is mediated by enzymes such as NAD(P)H oxidases and xanthine oxidase or nonenzymatically by redox-reactive compounds such as the semi-ubiquinone compound of the mitochondrial electron transport chain (Deby and Goutier, 1990). Although $O_2^{\cdot-}$ and H_2O_2 themselves are moderate oxidants, both species are critical for the formation of potent cytotoxic radicals in biological systems through their interaction with other molecules. For instance, the lysosomal enzymes myeloperoxidase (MPO) from neutrophils and monocytes/macrophages and the eosinophil peroxidase (EPO) catalyse the oxidation of halides (Cl^-, Br^-, and I^-) by H_2O_2 to form hypohalous acids (HOCl or HOBr). MPO produces predominantly hypochlorous acid (HOCl) whereas EPO produces more hypobromous acid (HOBr). Hypohalous acid

production is important in the host defense against infectious agents, but during this reaction the hydroxyl radical ($OH^{\cdot}$) is also produced, which is a powerful and indiscriminate oxidant (Caramori and Papi, 2004).

Because radicals have the capacity to react in an indiscriminate manner leading to damage to almost any cellular component, an extensive range of antioxidant defences, both endogenous and exogenous, are present to protect cellular components from free radical induced damage (Young and Woodside, 2001). Superoxide dismutases (SODs) convert superoxide enzymatically into hydrogen peroxide (Fridovich, 1978). In biological tissues superoxide can also be converted nonenzymatically into the nonradical species hydrogen peroxide and singlet oxygen ($^1O_2$) (Steinbeck et al, 1993). Alternatively, hydrogen peroxide may be converted into water by the enzymes catalase or glutathione peroxidase. In the glutathione peroxidase reaction glutathione is oxidized to glutathione disulfide, which can be converted back to glutathione by glutathione reductase in an NADPH-consuming process (Droge, 2002).

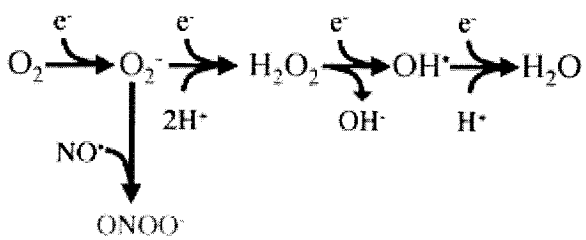
Under normal circumstances, formation of $O_2^{\cdot -}$ is kept under tight control by SOD enzymes (Figure 4). These include the Mn enzyme in mitochondria (MnSOD, SOD2) and Cu/Zn enzyme present in the cytosol (CuZnSOD, SOD1) or extracellular surfaces (EC-SOD, SOD3). In acute and chronic inflammation, the production of $O_2^{\cdot -}$ is increased at a rate that overwhelms the capacity of the endogenous SOD enzyme defense system to remove them. The result of such imbalance results in $O_2^{\cdot -}$ mediated damage. Oxidative stress has been defined as an imbalance in favor of pro-oxidants and disfavor of antioxidants, potentially leading to damage of biomolecules (Risom et al, 2005). ROS can damage cellular proteins,

lipids, membranes, and DNA (Li et al, 2004). In the presence of reduced transition metals (e.g., ferrous or cuprous ions), hydrogen peroxide is reduced by Fenton reaction and cleaves to generate OH^- and highly reactive hydroxyl radical (·OH) (Chance, 1978). Hydroxyl radical initiates lipid peroxidation (Kumarathasan et al, 2003a) and isoprostanes are prostaglandin-like substances that are produced in vivo by free-radical-induced peroxidation of arachidonic acid (Montuschi et al, 2004). Depending on the microenvironment, NO can be converted to various other reactive nitrogen species (RNS) such as nitrosonium cation (NO^+), nitroxyl anion (NO^-) or peroxynitrite ($ONOO^-$) (Stamler, 1992). Peroxynitrite is a strong oxidant capable of modifying most biological molecules and compounds, including aminoacids as tyrosine, tryptophan, cysteine, and methionine. Direct reactions of ONOO with CO2, transition metals, and SOD have been found to catalyze the nitration of tyrosine residues (Turko and Murad, 2002). 3-Nitrotyrosine has been revealed as a relevant biomarker of NO-dependent oxidative stress (Radi, 2004). The lipid peroxidation and protein nitration induced by ONOO- are some of the earliest atherogenic events (Griendling et al, 2003).

Figure 3. . Derivation of reactive oxygen species.

Sequential electron *(e–)* addition to oxygen results in the formation of ROS: superoxide*(O2–)*, hydrogen peroxide *(H2O2)*, and hydroxy radical *(OH)*. Superoxide combines with nitric oxide *(NO) to* form peroxynitrite *(ONOO–)*. (Bowler and Crapo, 2002b).

Reactive Oxygen Species



1.2.1 PM and Oxidative Stress

The mechanism of PM induced health effects are believed to involve inflammation through the generation of ROS (Xiao et al, 2003) and oxidative stress (Schins et al, 2004). Through its metal, semi-quinone, lipopolysaccharide, hydrocarbon, and ultrafine constituents, PM may exert oxidative stress on cells in the lung by presenting or by stimulating the cells to produce ROS (Tao et al, 2003). The reaction of ozone with polyunsaturated fatty acids results in the generation of carbon-centered radicals as well as aldehydes and H_2O_2. Hydrogen peroxide generates hydroxyl radical, the final product of ozone-induced cascade reactions (Kumarathasan et al, 2002). Failure to overcome oxidative stress leads to the activation of additional intracellular signaling cascades that regulate the expression of cytokine and chemokine genes (Silbajoris et al, 2000). Growing evidence indicates that chronic and acute overproduction of ROS under pathophysiologic conditions is integral in the development of cardiovascular diseases (Madamanchi, 2005). Increases in the levels of ROS have been associated with several cardiac pathologies, including reperfusion injury (Guarnieri et al, 1980). Experimental perfusion of isolated systems demonstrated that oxidative stress significantly increases the incidence of arrhythmias (Hearse and Tosaki, 1987), an effect that is prevented by perfusion with antioxidants (Llesuy et al, 1995).

1.3 PM, ET and Oxidative stress

Increased oxidative stress has been implicated in the pathogenesis of a variety of cardiovascular and pulmonary diseases in humans and animal models, including those with systemic hypertension and underlying cardiovascular complications (Alexander, 1998). High levels of ROS can be cytotoxic. For example, vessel injury can lead to the adherence of

neutrophils to the endothelium, the activation of NADPH oxidase, and the generation of extracellular superoxide, which can damage endothelial cells (Harrison, 1999). Griendling et al (1994) have shown that ROS play a role in angiotensin signaling and this leads to intracellular production of superoxide; superoxide can combine with NO to form ONOO- decreasing NO levels, and in theory leads to vasospasm. Endothelin-1 is produced and cleared in the lung and is generated in response to the presence of ROS (free radicals) and their metabolites (Haynes and Webb, 1998). This leaves open the possibility that pollutants could induce an excess production of ET-1. Tzeng et al (2003) have shown that motorcycle exhaust particle extracts can enhance vasoconstriction in organ culture of rat aortas via ROS and Ma et al (2001) have shown that oxidative stress can impair endothelium function.

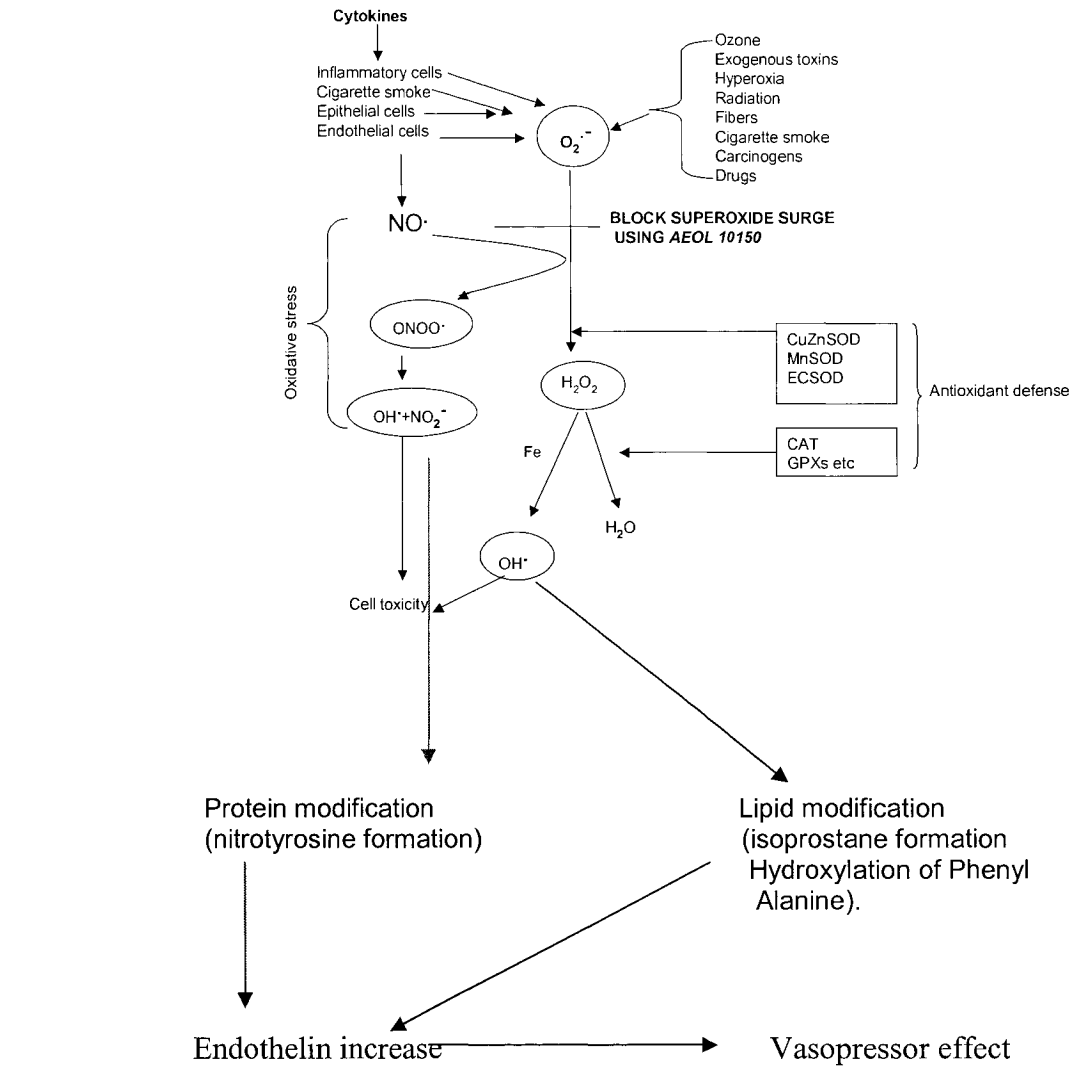
1.4 Rationale and Hypothesis

The hypothesis is that oxidative stress induced by air pollutants is critical in the alteration of the extracellular lining and the initiation of cellular changes in the airways of lungs, leading to activation of the ET system. Blocking the free radical pathway should prevent activation of the ET system by pollutants. Previous studies have shown that pollutants increase ET and causes oxidative stress in lungs. But mechanism of regulation of ET is not known. One critical pathway of endothelial dysfunction could be cell injury by peroxynitrite. Peroxynitrite is formed from nitric oxide and superoxide. In principle, if superoxide bursts are produced by pollutants, then scavenging superoxide flux and preventing peroxynitrite formation should prevent endothelial dysfunction. My plan is to test whether preventing peroxynitrite formation by interruption of superoxide with SODm, *AEOL 10150* can prevent the increase of ET-1 in plasma of rats, as well as prevent upregulation of preproET-1 and

ECE-1 in lungs after exposure to air pollutants. Figure 4 represents the source of ROS and RNS in lung and hypothesis.

Figure 4. Environmental sources of ROS and RNS in the lung and hypothesis

Generation of ROS and RNS by environmental agents and by various cells and the initial reactions of superoxide (O_2^-), hydrogen peroxide (H_2O_2), and nitric oxide (NO·) to generate reactive species that cause cell and tissue injury. Most environmental toxins generate ROS both directly and by activating inflammatory cells. This results in the activation of iNOS with generation of NO· and other RNS. The most important antioxidant mechanisms related to these pathways include superoxide dismutases (SODs), glutathione peroxidases (GPXs), and catalase (CAT). (Kinnula and Crapo, 2003).



(mediated by nitration and hydroxylation pathway?)

1.4.1 Specific Aims

- Evaluate the effect of *AEOL 10150* on circulating ET levels and expression of the ET system genes (ET-1 & ECE-1) in rats.
- Identify a dose of *AEOL 10150* that has a minimal impact on basal levels of plasma ETs and expression of ET system genes.
- Determine the effects of scavenging superoxide with *AEOL 10150* on the regulation of the pulmonary ET system genes and the circulating levels of ET in rats exposed to PM and ozone.

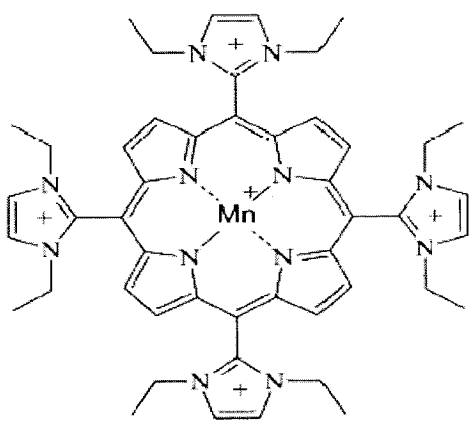
1.5 Superoxide Dismutase Mimetic (SODm) - *AEOL 10150*

The limitations of using natural products like superoxide dismutase and catalase as therapeutic agents to attenuate oxidative stress are their large size, which limits cell permeability, short circulating half-life, antigenicity and expense (Patel and Day, 1999). In certain cases where an enzyme of potential therapeutic benefit does not have the appropriate properties for a drug, a synthetic, small molecule enzyme mimetic can conceivably be designed with chemical and physical characteristics suitable for a therapeutic. The SOD mimetics are *catalytic* drugs and the ability of the SOD mimics to scavenge superoxide in vivo has also been demonstrated by ESR studies. In vitro and in vivo studies have demonstrated that the SOD mimetics have potent anti-inflammatory properties, attenuate myocardial ischemia/reperfusion injury, and prolong the half-life of $NO^{\cdot}$, an antithrombotic

and vascular relaxant. Therefore, the SOD mimetics may find clinical utility in diseases mediated, in part, by superoxide. (Cuzzocrea et al, 2001).

Metalloporphyrin complexes have been shown to mimic the biologic activity of superoxide dismutase (Day and Crapo, 1996) and can pharmacologically augment natural antioxidant defenses (Bowler et al, 2003). The SOD mimetic manganese (III) mesotetrakis (di-N-ethylimidazole) porphyrin (*AEOL 10150*) are low molecular weight, synthetic, redox-active, catalytic antioxidants that act as SOD mimetics and has been shown to attenuate expression of inflammatory genes in stroke (Bowler et al, 2002c). *AEOL 10150* has been shown to be an effective neuroprotective compound. When given i.c.v. to rats, *AEOL 10150* reduced both neurologic deficit and infarct size thereby ameliorating changes caused by temporary focal cerebral ischemia (Sheng et al, 2002). Also, it caused marked reduction in tobacco smoke-induced inflammation and lung injury when given before smoke exposure (Smith et al, 2002). These metalloporphyrin class of antioxidant mimetics exhibit strong antioxidant properties, including scavenging of superoxide, H_2O_2, peroxynitrite, and lipid peroxyl radicals (Kinnula and Crapo, 2003). The manganese moiety of the SOD mimetics functions in the dismutation reaction with superoxide by alternate reduction and oxidation changing in its valence between Mn(III) and Mn(II), much like native SODs, They exhibit definite, but low, catalase-like activity that has been attributed to their extensive conjugated ring system and reversible one-electron oxidations. Their capacity to scavenge peroxynitrite apparently involves the formation of an oxo-Mn (IV) complex that can be reduced to the Mn(III) oxidation state by endogenous antioxidants (Kinnula and Crapo, 2003). Structure of *AEOL 10150* is given in Figure 5.

Figure 5. ***AEOL 10150*** **(Manganese (III) meso-tetrakis (di-N-ethylimidazole) porphyrin)** (Bowler et al, 2002c).



AEOL 10150

2 MATERIALS AND METHODS

2.1 Animals

Pathogen-free Fischer-344 male rats (180-250 g) were obtained from Charles River (St. Constant, Québec, Canada). The animals were housed in individual plexiglass cages on wood-chip bedding under HEPA-filtered air and held to a 12 h dark/light cycle. Food and water were provided ad libitum. All experimental protocols were reviewed and approved by the Animal Care Committee of Health Canada.

2.1.1 Subcutaneous injections

In the dose response study, animals were subcutaneously injected with the 0, 2 and 5mg/kg body weight of the catalytic antioxidant *AEOL 10150* (manganese (III) meso-tetrakis (di-N-ethylimidazole) porphyrin in 1ml/kg body weight of saline. In the particle exposure study, the animals were injected with 2mg/kg of the drug 2 hours before the inhalation exposure to pollutants. The metalloporphyrins are water soluble, manganese meso-porphyrins that are stable and nontoxic. It was provided as a gift from Dr. James Crapo at the National jewish medical center, Denver and from Incara Pharmaceuticals (Research Triangle Park, NC, USA). *AEOL 10150* has a plus-five charge with a SOD activity of ~5,000 units/g and catalase activity of approximately ~1% of purified bovine catalase (weight/weight basis).

2.1.2 Inhalation exposure to air pollutants

The ambient urban particles EHC-93 consist of total suspended particulate matter recovered from filters of the single-pass air-purification system at the Environmental Health Centre

(Tunney's Pasture, Ottawa, Canada) and mechanically sieved using a 36μm mesh filter. The chemical composition, biological reactivity of the particles in cell culture models, and applications in inhalation studies have been described elsewhere (Vincent et al, 1997; Vincent et al, 2001a). Ozone was generated and monitored as previously described (Vincent et al, 1997). Animals were exposed to EHC-93 urban particles, ozone or clean air using a nose-only inhalation system. Rats were trained in nose-only exposure tubes over 5 consecutive days, and then exposed for 4 h to clean air or to the individual pollutants EHC-93 (50 mg/m^3) and ozone (0.8 ppm) essentially as described previously (Vincent et al, 1997; Thomson *et al.*, 2004). The particle size distribution of resuspended EHC-93 in our flow-past nose-only exposure system was multimodal, with two respirable modes at 1.3 μm (aerodynamic diameter, D_{AE}) and 3.6 μm D_{AE} that together comprised 55 % of the mass of the aerosol, and a non-respirable mode at 15 μm D_{AE} that comprised 45 % of the mass (Vincent *et al.*, 2001a). Animals were euthanized immediately after exposure, or following 24 h recovery in filtered air.

2.1.3 Environmental relevance of dose regimen

While the exposure concentrations in this study appear high when compared to ambient conditions, the internal dose of pollutants must be considered in evaluating the data. Knowledge of the quantitative relationship between exposure concentration and delivered dose (the mass of ozone that is absorbed per unit of tissue area at a particular site) is a fundamental starting point in the evaluation of the toxicity of chemicals for intra- and interspecies comparisons (Miller and Graham, 1988). The site in the lung most affected by ozone exposure is the centriacinar region (junction between the alveoli and the conducting

airways). Mathematical models also predict that the centriacinar region receives higher ozone doses than other local regions of the lower respiratory tract (Barry et al, 1985). Rats were exposed at rest to the air pollutants by nose-only route for four hours. It has been estimated that exposure of a human subject to 0.12 ppm of ozone (236 μg O_3/m^3) for 12 hours (85 ng O_3/cm^2), followed by 0.06 ppm ozone for 12 hours (42 ng O_3/ cm^2) would lead to a total daily centriacinar peak dose estimated at 127 ng O_3/ cm^2 (Vincent et al., 2001a). Similarly, assuming a 24 hour exposure to an average PM_{10} concentration of 175$\mu g/m^3$ (Tellex-Rojo et al., 2000), a reference total dose in the pulmonary compartment of humans is estimated as 1.3ng/ cm^2 alveolar surface area (Thomson et al., 2005). The ratio of EHC-93 dose within the respiratory compartment of the rats during the 50mg/m^3 exposure (25 ng/cm2) to the particle dose calculated for a plausible human exposure scenario (1.3 ng/ cm^2) is only 20-fold (Vincent et al., 2001a). Also, the ratio of the centriacinar ozone dose in the animals at 0.8ppm of ozone (427 ng O_3/ cm^2) to the estimated internal dose in a human subject (127ng O_3/ cm^2) under a plausible exposure scenario is only 3.4 fold (Thomson et al., 2005). In general, humans are considered more susceptible to air pollutants than rats. Furthermore, persons with preexisting lung diseases and cardiovascular diseases are more susceptible to air pollution than healthy individuals (Goldberg et al., 2001). Therefore, from the standpoint of evaluation toxicology, the doses of pollutants used in my study are appropriate for the study of mechanisms and are relevant to human health.

2.1.4 Biological samples

Rats were anaesthetized by administration of sodium pentobarbital (60 mg/kg, ip). Blood was collected from the abdominal aorta into vacutainer tubes containing the sodium salt of ethylene diamine tetra acetic acid (EDTA) at 10 mg/ml and phenyl methyl sulfonyl fluoride

(PMSF) at 1.7 mg/ml, mixed gently, and placed on ice (Kumarathasan et al, 2001). Plasma was isolated by centrifugation (2000 rpm for 10 min), aliquoted, and frozen at –80 °C. The animals were then exsanguinated, and the trachea was exposed and cannulated. The diaphragm was punctured to collapse the lungs, and the lungs were filled by intratracheal instillation of warm (37°C) calcium- and magnesium-free Dulbecco's phosphate buffered saline (Sigma Chemical Co., St. Louis, MO) at a ratio of 35 ml/kg body weight. The saline was aspirated and re-injected twice more, and the bronchoalveolar lavage fluid was recovered in cold centrifuge tubes. This first lavage fluid was used for protein assays. The lungs and heart were recovered and flash frozen in liquid nitrogen and stored at–80 °C.

2.2 Gene expression studies

2.2.1 Reverse transcription of lung and heart RNA samples

Frozen lung and heart samples were homogenized in TRIzol reagent (Invitrogen Canada Inc., Burlington, Ontario, Canada), and total RNA was isolated according to the manufacturer's instructions. RNA was quantified using the RiboGreen RNA Quantitation Reagent and Kit (Molecular Probes, Eugene, Oregon). Total RNA was reverse transcribed using MuLV reverse transcriptase and random hexamers (Applied Biosystems, Mississauga, Ontario, Canada) according to the manufacturer's instructions. Briefly, 1000ng of RNA was added to a reaction mixture of 5 mM $MgCl_2$, 1X PCR Buffer II, 1 mM each dNTP, 1 U/µl RNase Inhibitor, 1 µm random hexamers, and water to produce a final volume of 60μl. The mixture was incubated at 42 °C for 1 h, MuLV reverse transcriptase was inactivated by heating to 99 °C for 5 min, and the reaction was cooled to 5 °C for 5 min followed by storage at –40 °C until used.

2.2.2 Real-time PCR primers

The following primer sequences for β-actin, preproET-1, ECE-1, ET_A receptor, ET_B receptor, eNOS and iNOS are used from Thomson et al, 2005. Double-desalted primers were purchased from Invitrogen, Canada.

β-actin	Sense (2538-): CAC TAT CGG CAA TGA GCG GTT CC; Antisense (2774-): CTG TGT TGG CAT AGA GGT CTT TAC GG
PreproET-1	Sense (362-): GAC AAG GAG TGT GTC TAC TTC TGC; Antisense (446-): GGC TTC CTA GTC CAT ACG GG
ECE-1	Sense (575-): AAA AGG CGC AAG TGT ACT ACC G; Antisense (660-): CTC AAT CAG CTC CAT CAG GG
ETA receptor	Sense (126-): CTA ATC TAA GCA GCC ACG TGG; Antisense (225-): CTA GGC AGG GCC AAA TTA GG
ETB receptor	Sense (948-): GCT GTC CCT GAA GCC ATA GG; Antisense (1022-): AAG CAT GCA GAC CCT TAG GG
eNOS	Sense (547-): CGG TAC TAC TCT GTC AGC TCA GC; Antisense (634-): CAT CCT GGG TTC TGT ATG CC
iNOS	Sense: AAT GGT TTC CCC CAG TTC CTC ACT; Antisense: CTC TCC ATT GCC CCA GTT TTT GA

2.2.3 Real-time PCR analysis of lung and heart gene expression

Master mixes of the reagents were prepared to minimize differences in reagent composition and pipetting errors. cDNA prepared from 1μg of RNA were incubated with 25μl iQ SYBR Green Supermix (Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario, Canada) and 200nM of each primer, and the reagent mixture was brought up to 50μl with DNase/RNase-free water. All reactions were performed in duplicate on 96-well plates in a spectrofluorometric thermal cycler (iCycler iQ, Bio-Rad). PCR runs were initiated by

incubation at 95 °C for 3 min to activate the iTAQ polymerase followed by 40 cycles of 95 °C for 15 s, the appropriate annealing temperature for 15 s (62°C for all the genes and 60°C for ET_B receptor), and 30 s at 72 °C. Fluorescence was monitored at every cycle during the elongation step. A melt curve was conducted following each run to verify product purity. Expression was calculated relative to β-actin using the delta-Ct method (Livak and Schmittgen, 2001), and expressed as fold change relative to air control samples.

2.3 Biochemical analyses

2.3.1 Plasma endothelins

2.3.1.1 Sample preparation

For analyses of endothelins, the samples were processed as follows (Kumarathasan et al, 2001). The conversion of big ET-1 (the inactive precursor that has 38 to 41 amino acids) to ET-1 (the mature peptide that has 21 amino acids) was prevented by adding 3, 4-dichloroisocoumarin (highly reactive serine protease inhibitor) to the plasma samples at a final concentration of 48 mg/mL. Note that ET-2 and ET-3 originate similarly from big ET-2 and big ET-3 precursors, which are cleaved by endothelin-converting enzymes (ECE). Protein was precipitated with 1.5 volume of ice-cold acid acetone (acetone: 1N HCl: water, 40:1:5). Samples were vortexed and clarified by centrifugation at 9800rpm for 10 minutes and the supernatants were concentrated by evaporation under nitrogen flow. The concentrated samples were treated a second time with acid acetone and centrifuged. Molecular weight cutoff filters (30 kDa; Sigma Chemicals, St Louis MO) were washed with 50ml of deionized water by centrifugation at 7500rpm for 10 minutes. The concentrated supernatants were then loaded on the molecular weight cutoff filters and centrifuged at 7500rpm for 30 minutes. The filters were washed with 75 ml of 50% methanol. Filtrates

were dried under a flow of nitrogen. The samples were either stored dry at 40°C or immediately processed for HPLC analysis. The samples were reconstituted with 75 ml phosphate-buffered saline, vortexed, and transferred into 1.5-mL amber glass vials with inserts for HPLC analysis.

2.3.1.2 HPLC analyses

Endothelin-related peptides were measured by HPLC as described by Kumarathasan et al (2001). The HPLC unit consisted of a Gilson solvent delivery system (Mandel Scientific, Guelph Ontario), a Gilson autosampler (model 231 XL; Middleton WI), a Supelcosil LC-318 reverse-phase column (25 cm length, 4.6 mm ID, 5 μm particle size, 300 Å pore dimension; Supelco, Oakville Ontario), and a Shimadzu fluorescence detector (model RF 551; Columbia MD). Injection volume was 20µl. Analytes were resolved using a gradient elution with two solvents: solvent A consisted of 30% acetonitrile and 0.1% trifluoroaceticacid (TFA) in water; solvent B consisted of 90% acetonitrile and 0.1% TFA in water. The mobile phase (flow atl ml/min) was programmed as follows: initial mobile phase 100% solvent A; 3-minute ramp to 92:8, A:B solvent ratio; held for 12 minutes; 10-minute ramp to 100% solvent B; held for 5 minutes; 5-minute ramp to 100% solvent A; held for 3 minutes. Total run time was 38 minutes. The fluorescence detector was optimized for high sensitivity detection with the excitation wavelength set at 280 nm and emission wavelength at 340 nm. A manual integration method was used with a drop-line method of integration applied to shoulders or peak overlaps. The limit of detection of the endothelins was 0.2–0.5 pmol on the column. Linear performance (r2 = 0.99) of the detector established for all endothelins analyzed (big ET-1, ET-1, ET-2, and ET-3) was 1–100 pmol.

2.3.2 Plasma 3-nitrotyrosine

2.3.2.1 Sample preparation

For analyses of plasma 3-nitrotyrosine, the samples were processed as follows (Kumarathasan et al, 2003b). Plasma samples (250μl) in 1.5-mL centrifuge tubes were treated with diethylenetriaminepentaacetic acid (DETPA) (final, 15 mM) and butylated hydroxy toluene (BHT) (final, 43 mM) and then vortexed. Plasma samples (250μl) containing BHT and DETPA were treated with 1.5-fold, by volume, of ice-cold acid–acetone mix (acetone–1 M HCl–water, 40:1:5, v/v) to precipitate proteins. Samples were vortexed and centrifuged at 9800rpm for 10 min to obtain supernatants and were concentrated to 200μl by evaporation under nitrogen flow. The samples were deproteinized and concentrated once again. Molecular weight cut-off filters (30 kDa) were washed with 50μl of deionized water by centrifugation at 7500rpm for 10 min. Concentrated samples were then loaded into the washed molecular mass cut-off filters and centrifuged at 7500rpm for 30 min. After complete drainage of samples, the molecular mass cut-off filters were washed with 100μl aliquots of a methanol–water (50:50, v/v) mix. Filtrates were then dried under a flow of nitrogen. Samples were reconstituted with 200μl of acidified water, vortexed gently and were diluted as required for the analysis by HPLC.

2.3.2.2 HPLC analyses

3-Nitrotyrosine was analysed by HPLC-Coularray (Kumarathasan et al, 2003b). The HPLC unit consisted of a solvent delivery module (model 582; ESA, Chelmsford, MA, USA), a ESA autosampler (model 542), a ESA model 5600A CoulArray detector and a Supelcosil LC18 reversed-phase column (25 cm×4.6 mm I.D., 5μm particle size; Supelco). The

CoulArray detector unit comprised eight electrodes in series. The mobile phase was prepared as given below. 15.485g of citric acid's trisodium salt, 0.48g of octane sulfonic acid and 272ml of acetate buffer (6.56f of anhydrous sodium acetate and 7.2ml of glacial acetic acid are transferred into a 1l volumetric flask, and then the volume is made up using deionised water. pH of the solution was adjusted to 4.45) are transferred into 2l volumetric flask, and 28ml of methanol and 8.8ml of 1N HCL are added to this solution. The final volume was made using deionised water and then degassed for 20 minutes. Isocratic elution of analytes was carried out at a flow-rate of 1.0 ml/min. The applied voltages on the eight channels were + (0, 150, 300, 450, 600, 650, 700, 800) mV. Injection volume was 25μl. Run time was 38 min for the set of target analytes discussed in this study. Working standard solutions, plasma samples, and spiked plasma were analyzed along with acidified water blanks.

2.3.3 Total Protein

Total Protein in Lavage was determined by the Coomassie blue dye-binding assay using Bovine serum albumin as standard (Vincent et al, 1996). 250 μg/ml working standard solution in PBS was made from a 2 mg/ml BSA stock (1:8 dilution). A standard curve was done with a concentration of 0 to 5ug of BSA/well. 20μl lavage was taken for the analysis. 180μl Coomassie Blue reagent was added to all the wells and agitated 10 minutes on an orbital shaker. Plate was read at 600 nm in a spectrophotometer.

2.3.4 Isoprostanes Assay

Plasma Isoprostanes were determined as per manufacturer's instructions (Cayman's chemical quantification kit, Cayman Chemical Company, MI, USA). Samples were prepared before as below: it was determined from previous assays that a minimum of 12.5µl of

plasma is needed for the results to fall within the linear part of the standard curve of the Cayman immunoassay kit used (i.e. for one reading). Assay was done with 100µl of plasma (100µl) and dilutions are made accordingly to insure precision of results. The samples obtained were purified using C18 *SepPak* cartridges from Waters Inc, USA using the following procedure: For all of the purification steps attach the syringe to the longest end of the SepPak cartridge.

One ml syringe was used to administer 1 ml methanol wash to SepPak cartridge in a drop wise fashion (after this step the cartridge should not go dry until the sample was passed on it). A new syringe was used to wash through 2 x 1 ml deionized water drop wise to displace all of the molecules of MeOH. With a new syringe a sample volume of 100µl was drawn past the needle tip of the syringe and then pushed through the sample. Because sample volume is lower than 250µl, volume was completed to 250µl with dH_2O. 2 x 1ml of air was displaced through the cartridge with the same syringe that was used for the sample.

One ml deionized water wash done and displaced 1 ml of air with the syringe through the cartridge. Methanol syringe was used for 3 x 1 ml methanol elution into 4 ml amber glass vial. This was done slowly and care was taken to make sure the filter cartridge does not sit on the lip of the amber glass vial. Air (2 x 1 ml) was displaced to finish elution in same vial. Same syringe was used that was used for MeOH elution. All samples were dried completely under N_2 flow in the amber glass vials. The drying stage was done the same day as sample processing and stored overnight at -20 ^{o}C. The purified samples can be stored up to two weeks at -20 ^{o}C until further analysis. Since sample volume was 100µl and processed up to

dryness, it was reconstituted in 400µl of 1X enzyme immunoassay buffer (EIA) from kit. From this 4X dilution, 50µl was taken for assay on the plate. Each sample was assayed in duplicate from the reconstituted sample.

2.4 Statistical analyses

Data are expressed as means ± SEM. In the dose response study, the effects of different doses (0, 2, 5mg/kg body weight of *AEOL* of the drug as levels) and time (2hr and 24hr post exposure) as factors were tested for statistical significance by two-way *ANOVA* followed by Holm-Sidak's procedure to elucidate the pattern of significant effects (α=0.05) using Sigma-Stat (Sigma-Stat 3.0, Chicago, Illinois). In the particle exposure study, data were analyzed by 3-way *ANOVA* (α=0.05) with *AEOL* (*-AEOL*, *+AEOL*), exposure (air, ozone, EHC) and time post exposure (immediate, 24 hours) as factors. There was no statistically significant three-way *AEOL* X Exposure X Time factor interaction. Therefore, the data were analyzed by 2-way *ANOVA* using *AEOL* and exposure as factors for each of the recovery time points.

3 RESULTS

3.1 Dose Response Study

3.1.1 Specific aim

The aim of the dose response study is to verify the highest safe dose of *AEOL 10150* that does not alter the basal expression of the endothelin system gene.

3.1.2 Results

3.1.2.1 Total Protein in BAL

The pathophysiological index of a proceeding lung inflammation is increased total protein content obtained in bronchoalveolar lavage (Folkesson, 1991). In this study, no significant changes were noticed in the total protein levels in the BAL at different dose levels of the drug (Figure 6).

3.1.2.2 ET-1 and ECE-1 gene expression in lungs and heart

Pulmonary tissue contains some of the highest concentrations of ET-1 of any organ (Matsumoto et al, 1989). Endothelin can influence cardiac function in a variety of ways including vasoconstriction, mitogenesis, release of atrial natriuretic peptide, and inotropic effects. Vascular endothelium, the conducting system, and cardiomyocites themselves are all potential sources of ET (Highsmith, 1998). The results show a trend in lung and heart ET-1 mRNA expression suggesting an increase in the animals injected with 5mg/kg body weight of the drug after 2 hours post exposure. But the changes were not statistically significant (Figure 7). There were no changes of ECE-1 mRNA expression in the lungs after injection of *AEOL*. However, heart ECE-1 mRNA expression was increased significantly 2 hours and

24 hours after treating with 5mg/kg body weight of *AEOL (Two-way ANOVA, Time X dose, p=0.006; Holm-Sidak: within 2hours: 0mg Vs 5mg, P=0.036, 2 mg Vs 5 mg, P=0.052; within24 hours: 2 mg Vs 5 mg, P=0.029).* Overall, the results indicate that the dose of 2 mg/kg body weight of *AEOL 10150* does not have an impact in the steady state mRNA levels of prepro ET-1 and ECE-1 in the lungs and the heart.

Figure 6. Total protein recovered in bronchoalveolar lavage fluid.
Results are expressed as mean±SE; n=6 animals/group.

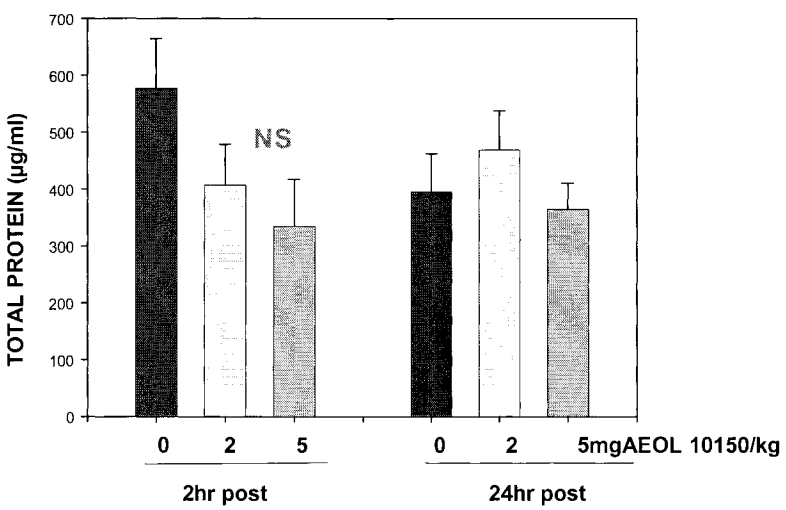
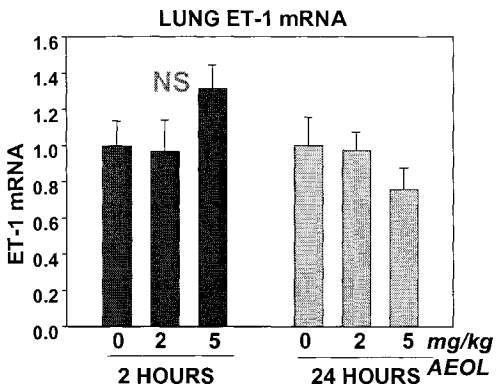
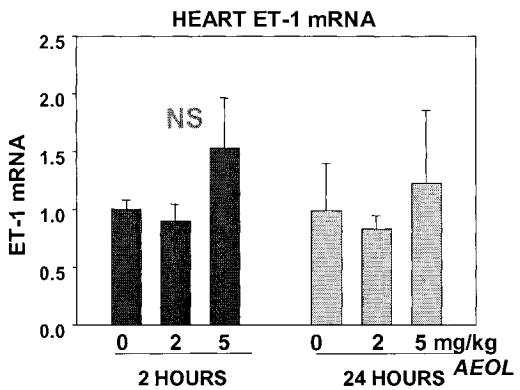
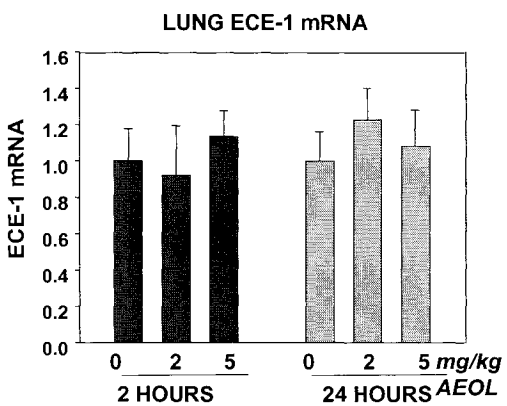

TOTAL PROTEIN (µg/ml)
700
600
500
400
300
200
100
0
NS
0
2
5
0
2
5mgAEOL 10150/kg
2hr post
24hr post

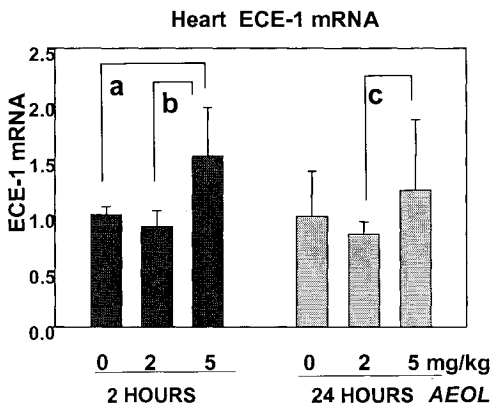
Figure 7. Lung and heart ET-1 & ECE-1 mRNA.

Mean±SE, n=6 animals/group. Results are expressed as fold change relative to the control samples. *Heart ECE-1: Two-way ANOVA, Time X dose factor interaction, p=0.006. Holm-Sidak:* ***a*** *within 2hours: 0 mg Vs 5 mg, P=0.036;* ***b*** *within 2 hours: 2 mg Vs 5 mg, p=0.05;* ***c*** *within 24 hours: 2 mg Vs 5 mg, p=0.029.*


LUNG ET-1 mRNA
ET-1 mRNA
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
NS
0 2 5 0 2 5 mg/kg
2 HOURS
24 HOURS AEOL


HEART ET-1 mRNA
ET-1 mRNA
2.5
2.0
1.5
1.0
0.5
0.0
NS
0 2 5 0 2 5 mg/kg
2 HOURS
24 HOURS AEOL


LUNG ECE-1 mRNA
ECE-1 mRNA
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 2 5 0 2 5 mg/kg
2 HOURS
24 HOURS AEOL

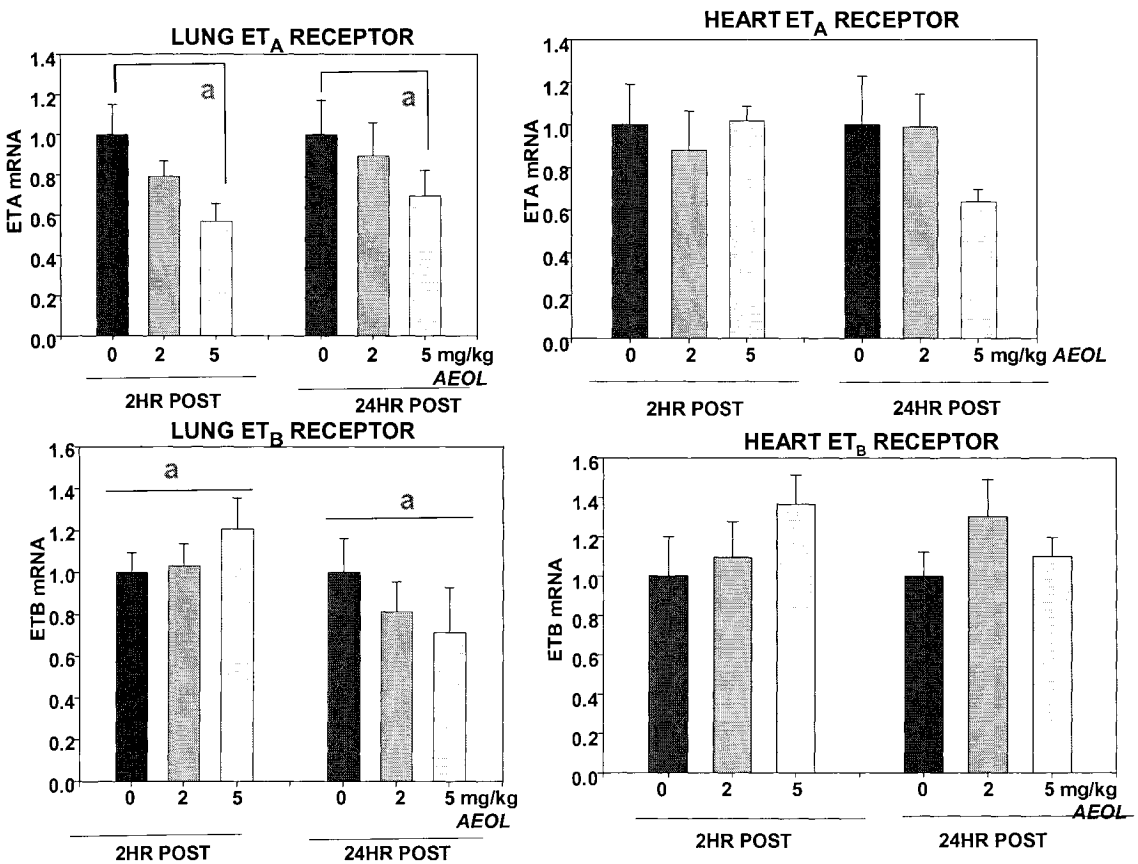

Heart ECE-1 mRNA
ECE-1 mRNA
2.5
2.0
1.5
1.0
0.5
0.0
a
b
c
0 2 5 0 2 5 mg/kg
2 HOURS
24 HOURS AEOL

ET_A and ET_B Receptor expression in Lung and Heart

Lung ET_A receptor expression was decreased significantly (*Two-way ANOVA: AEOL and time as factors. AEOL main effect, p=0.045, Holm-Sidak multiple comparison: 0 Vs 5 mg, p=0.014)* in the animals injected with 5 mg/kg body weight of the drug at 2 hours post exposure when compared to the animals injected with 0 and 2 mg/kg body weight of the drug. Significant changes were not noticed in the endothelin receptor expression in heart in the animals injected with different doses of the drug when compared to controls. Therefore, the results show that a dose of 2 mg/kg body weight of the drug does not alter the steady state mRNA expression levels of endothelin receptors (Figure 8).

Figure 8. Lung and heart ET_A & ET_B receptor mRNA.

Mean±SE. n=6 animals/group. Results are expressed as fold change relative to the control samples. *Lung ET_A: Two-way ANOVA, AEOL main effect, p=0.045. Holm-Sidak:* ***a*** *0 Vs 5 mg, p=0.014. Lung ET_B: Two-way ANOVA,* ***a*** *Time main effect, p=0.028.*

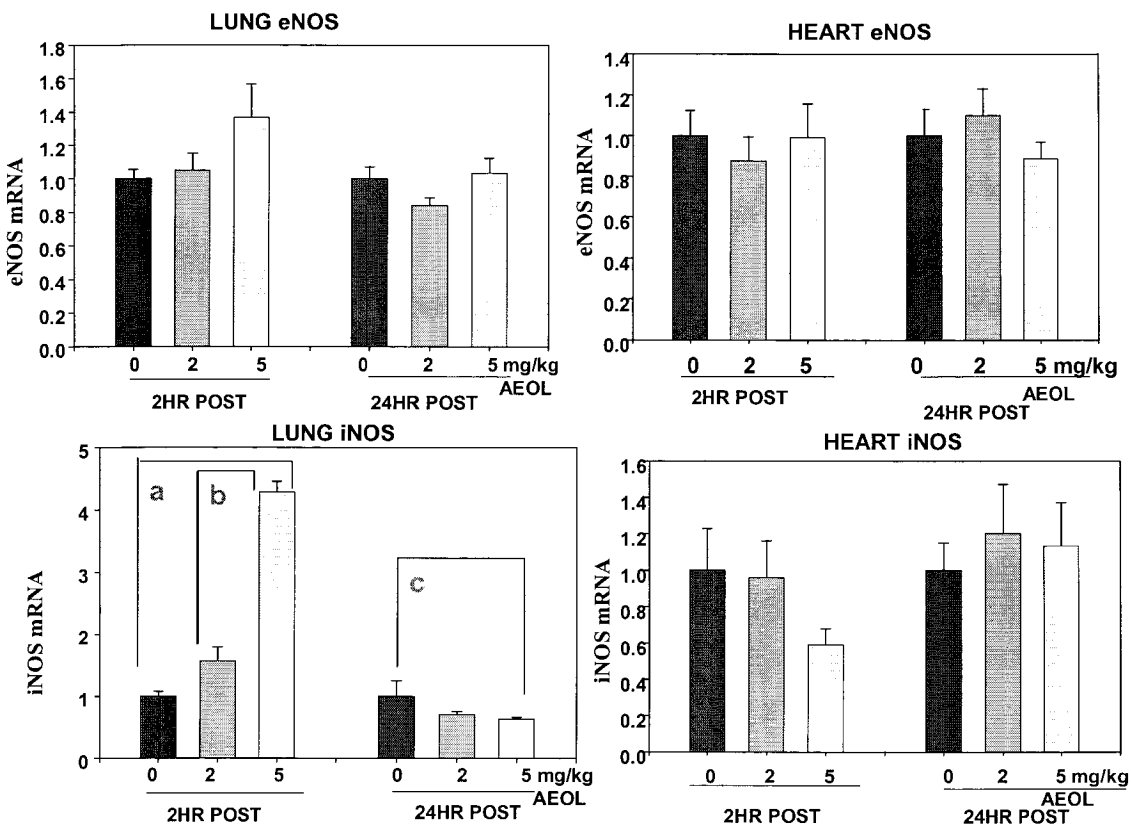

LUNG ET$_A$ RECEPTOR
ETA mRNA
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
a
0 2 5 0 2 5 mg/kg
AEOL
2HR POST
24HR POST
HEART ET$_A$ RECEPTOR
ETA mRNA
0 2 5 0 2 5 mg/kg AEOL
2HR POST
24HR POST
LUNG ET$_B$ RECEPTOR
ETB mRNA
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
a
0 2 5 0 2 5 mg/kg
AEOL
2HR POST
24HR POST
HEART ET$_B$ RECEPTOR
ETB mRNA
0 2 5 0 2 5 mg/kg
AEOL
2HR POST
24HR POST

3.1.2.3 eNOS and iNOS gene expression in Lungs and Heart

Nitric oxide, an essential molecule in the physiology of human body is produced by NOS. Endothelium derived NO is a potent endogenous vasodilator that contributes to the low pulmonary vascular tone and exerts an antiproliferative effects on smooth muscle cells (Barbera et al, 2001). iNOS is a mediator of unspecific host defense, central in the clearance of bacterial, viral, fungal and parasitic infections. However, excess production of NO appears to be linked to tissue damage and organ dysfunction (Lirk et al, 2002). In my study, lung iNOS expression has significantly increased at 5 mg/kg body weight of the drug after 2 hours and decreased after 24 hours of injection of the drug (*Two-way ANOVA, Time X Dose, $p<0.001$. Holm-Sidak: within 2hours, 0 Vs 5mg, $p<0.001$; 2mg Vs 5mg, $p<0.001$; within 24 hours, 0 Vs 5mg, $p=0.047$; within 2 and 5mg, 2 Vs 24 hours, $p<0.01$)* when compared to 0 and 2mg/kg body weight of the drug. Significant changes were not noticed in the lung eNOS and heart eNOS and iNOS expression at different dose levels of the drug (Figure 9).

Figure 9. Lung and heart eNOS & iNOS mRNA.

Mean±SE, n=6 animals/group. Results are expressed as fold change relative to the control samples. *Lung iNOS: Two-way ANOVA, Time X Dose, $p<0.001$. Holm-Sidak:* ***a*** *within 2 hours, 0 Vs 5 mg, $p<0.001$;* ***b*** *2 mg Vs 5 mg, $p<0.001$;* ***c*** *within 24 hours, 0 Vs 5 mg, $p=0.047$. within 2 and 5 mg, 2 Vs 24 hours, $p<0.01$.*

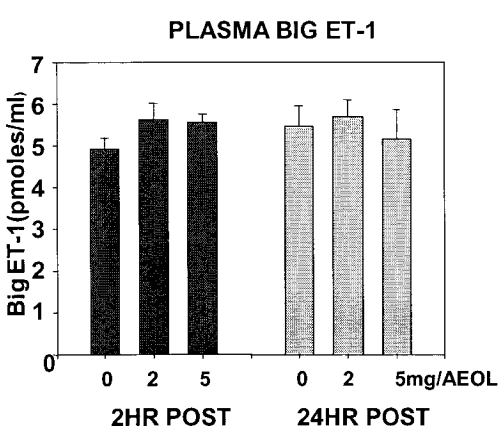

LUNG eNOS
eNOS mRNA
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 2 5 0 2 5 mg/kg
AEOL
2HR POST
24HR POST
HEART eNOS
eNOS mRNA
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 2 5 0 2 5 mg/kg
AEOL
2HR POST
24HR POST
LUNG iNOS
iNOS mRNA
5
4
3
2
1
0
a
b
c
0 2 5 0 2 5 mg/kg
AEOL
2HR POST
24HR POST
HEART iNOS
iNOS mRNA
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 2 5 0 2 5 mg/kg
AEOL
2HR POST
24HR POST

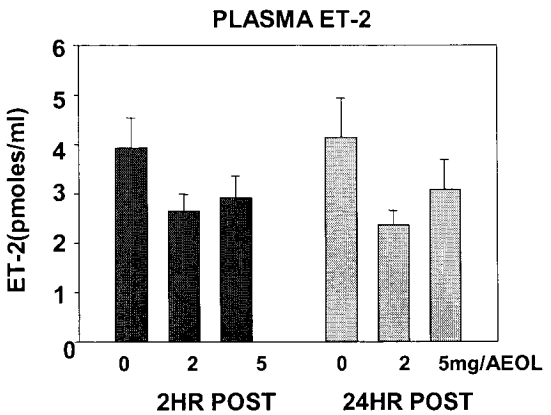
3.1.2.4 Plasma Endothelin

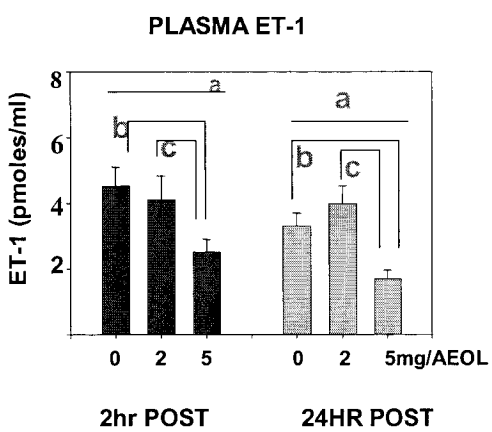
Endothelin-1 is the predominant isoform expressed in vasculature and the most potent vasoconstrictor currently known (Agapitov & Haynes, 2002). When plasma was analysed for peptide levels, the results indicate that there was no significant change in the Big ET-1 and ET-2 levels at different doses of the drug. But, Plasma ET-1 levels decreased significantly at 5 mg/kg body weight of the drug at both 2 and 24 hours post exposure (*Two-way ANOVA, time main effect, P=0.042. Holm-Sidak: 2 hr Vs 24 hr, p<0.05. AEOL main effect, p=0.001. Holm-Sidak: 0 Vs 5 mg, p<0.001; 2 mg Vs 5 mg, p<0.001)* whereas there was no change between 0 and 2 mg/kg body weight of the drug. Similarly plasma ET-3 levels decreased at 5 mg/kg body weight of the drug after 24 hours post exposure when compared to control and 2 mg/kg body weight of the drug. (*Two-way ANOVA, AEOL main effect, P=0.014, Holm-Sidak: 2 mg Vs 5 mg, P=0.004.).* At the dose of 5 mg/.kg body weight, the drug reduces plasma ET-1 (Figure 10).

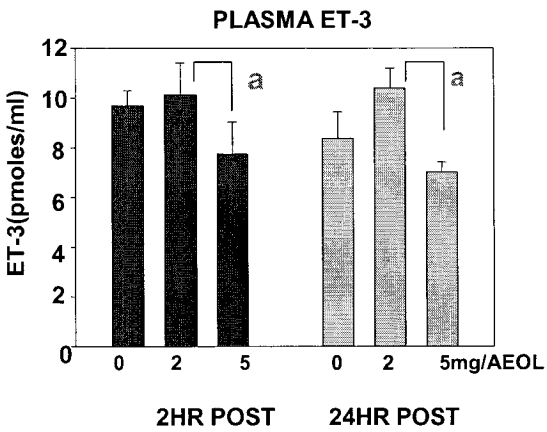
Figure 10. Levels of the bigET-1, ET-1, ET-2 and ET-3 peptide in plasma of animals treated with AEOL.

Mean±SE, n=6animals/group. *Plasma ET-1: Two-way ANOVA, time main effect, p=0.042. Holm-Sidak:* ***a*** *2 hour Vs 24 hour, p<0.05. AEOL main effect, p=0.001. Holm-Sidak:* ***b*** *0 Vs 5 mg, p<0.001;* ***c*** *2 mg Vs 5 mg, p<0.001. Plasma ET-3: Two-way ANOVA, AEOL main effect, P=0.014, Holm-Sidak:* ***a*** *2 mg Vs 5 mg, P=0.004.*


PLASMA BIG ET-1
Big ET-1(pmoles/ml)
0 1 2 3 4 5 6 7
0 2 5 0 2 5mg/AEOL
2HR POST
24HR POST


PLASMA ET-2
ET-2(pmoles/ml)
0 1 2 3 4 5 6
0 2 5 0 2 5mg/AEOL
2HR POST
24HR POST


PLASMA ET-1
ET-1 (pmoles/ml)
2 4 6 8
a
a
b
c
b
c
0 2 5 0 2 5mg/AEOL
2hr POST
24HR POST


PLASMA ET-3
ET-3(pmoles/ml)
0 2 4 6 8 10 12
a
a
0 2 5 0 2 5mg/AEOL
2HR POST
24HR POST

3.1.2.5 Plasma Isoprostanes

8-isoprostane is the stable end product of arachidonic acid oxidation generated by ROS attacks on membrane phospholipids (Waugh et al, 1996). Yura et al, 1999 have demonstrated that free-radical-generated F2-isoprostane stimulates DNA synthesis and endothelin-1 (ET-1) expression on endothelial cells. The results in my study indicate that plasma isoprostane levels have decreased significantly in the animals injected with 2mg/ kg body weight of the antioxidant drug at both 2 and 24 hours post exposure when compared to the control animals (*Two-way ANOVA, AEOL main effect, P=0.036. Holm-Sidak multiple comparison: 0 Vs 2 mg, p=0.011).* Significant changes were not noticed at 5mg/kg body weight of the drug when compared to control levels (Figure 11).

3.1.2.6 Plasma 3-Nitrotyrosine

Nitrotyrosine represents the stable end product of cell membrane protein-bound tyrosine nitration by peroxynitrite caused by increased NO (Mihm et al, 2001). In this study, the drug significantly reduced plasma 3-nitrotyrosine in the rats injected with 5mg/kg body weight of the drug and there seems to be dose dependent decrease in the plasma nitro tyrosine levels in the animals injected with the drug when compared to the control animals (*Two-way ANOVA, time point main effect, P=0.07; AEOL main effect, P=0.003, Holm-Sidak: 0 Vs5 mg, p<0.001; 2 mg Vs 5 mg, p=0.039)* after 2 and 24 hours after exposure (figure 12).

Figure 11. Plasma isoprostane

Mean±SE, n=6 animals/group. *Two-way ANOVA, AEOL main effect, P=0.036. Holm-Sidak:* ***a*** *0 Vs 2 mg, p=0.011.*

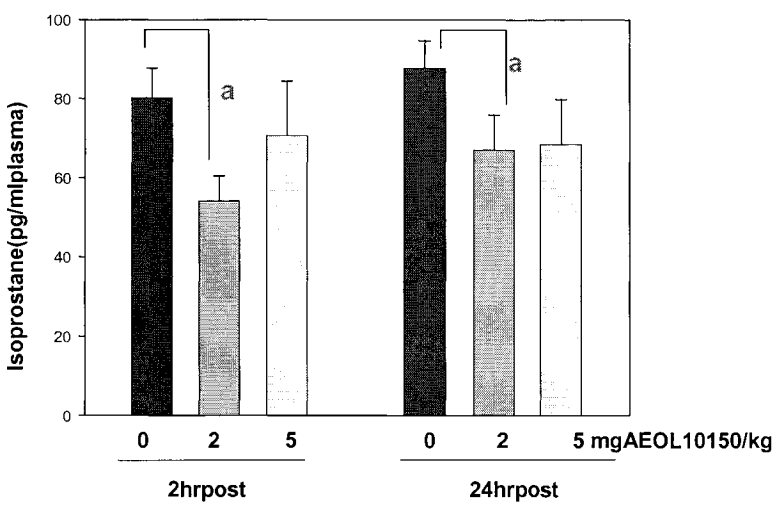
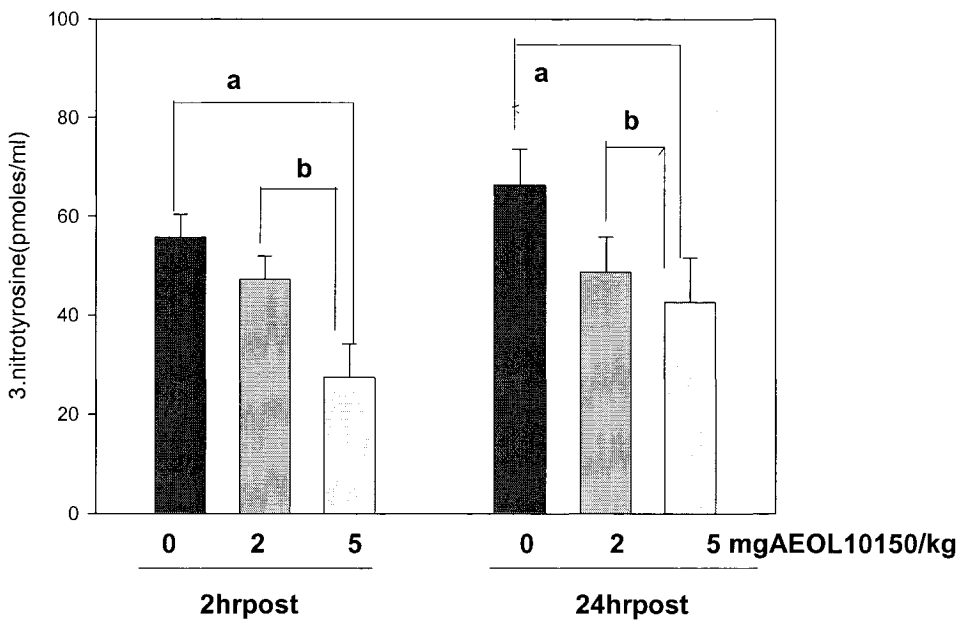

Isoprostane(pg/mlplasma)
100
80
60
40
20
0
a
a
0
2
5
0
2
5 mgAEOL10150/kg
2hrpost
24hrpost

Figure 12. Plasma 3-nitrotyrosine.

Mean+SE, n=6 animals/group. *Two-way ANOVA, time main effect, p=0.07; AEOL main effect, p=0.003,* ***a*** *0 Vs5 mg, p<0.001;* ***b*** *2 mg Vs 5 mg, p=0.039.*


100
80
60
40
20
0
3.nitrotyrosine(pmoles/ml)
a
b
a
b
0
2
5
0
2
5 mgAEOL10150/kg
2hrpost
24hrpost

3.1.3 Summary

A summary of the results of the dose response study is shown in Table 1 below (all arrows are $p<0.05$). From this study, it was found that:

1. The dose of 2mg/kg body weight of *AEOL 10150* does not alter plasma endothelin levels nor the ET-1, ECE-1, ET_A receptor, ET_B receptor, eNOS, iNOS expression in lungs and heart.

2. The dose of 2mg/kg body weight of the drug is sufficient to reduce oxidative stress, which is indicated by plasma isoprostane levels

3. *AEOL* decreases plasma 3-nitrotyrosine with significant decrease at the dose of 5mg/kg body weight of the drug when compared to the control levels.

Table 1. Dose response Study

	Lung			Heart			Plasma		
	0	2	5	0	2	5	0	2	5
2hr post			↓ETA ↑iNOS			↑ECE-1		↓IsoP ↓NT	↓ET-1 ↓ET-3 ↓NT
24hr post								↓IsoP ↓NT	↓ET-1 ↓ET-3 ↓NT

3.2 Particle exposure study

3.2.1 Specific aim

The aim of this study is to determine the effects of scavenging superoxide with *AEOL 10150* on the regulation of the pulmonary ET system genes and the circulating levels of ET in rats exposed to PM and ozone.

3.2.2 Results

3.2.2.1 ET-1 & ECE-1 gene expression in lungs and heart

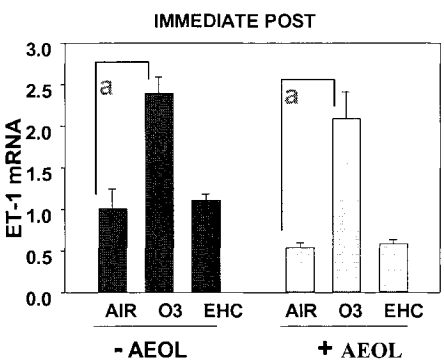
The results (Figure 13) indicate that expression of preproET-1 (*Two-way ANOVA, Exposure main effect, $p<0.001$; Holm-Sidak: ozone Vs air, $p<0.01$; ozone Vs EHC, $p<0.01$. AEOL main effect, $p<0.001$)* and ECE-1 (*Two-way ANOVA, Exposure main effect, $p=0.058$; AEOL main effect, $p=0.032$)* in lungs have increased immediately after exposure to Ozone. The increase in the expression was not reversed by the drug immediately. After 24 hours recovery in clean air, there is a trend showing an increase in the expression of preproET-1 due to ozone and particles (not significant), but the increase due to pollutants is reversed by the SODm (*Two-way ANOVA, AEOL, $p=0.027$*). Thomson et al (2004) have reported an increase in mRNA levels of prepro ET-1 two hours after exposure to air pollutants. There is no statistically significant change in the lung ECE-1 mRNA expression due to ozone and particles (Figure13).

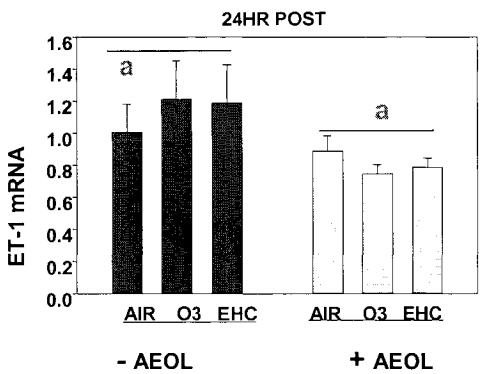
In the heart, the expression of preproET-1 (*Two-way ANOVA, Exposure main effect, $p=0.048$; Holm-sidak: ozone Vs air, $p=0.02$; EHC Vs air, $p=0.07$. AEOL main effect,*

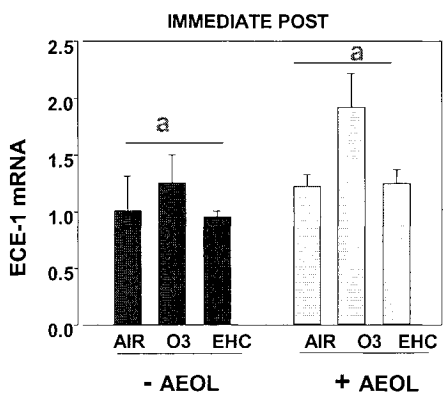
P=0.047) and ECE-1 (*Two-way ANOVA, Exposure main effect, P=0.023; Holm-sidak: ozone Vs air, p=0.008)* mRNA have increased immediately due to ozone and particle exposure (Figure14). The drug prevented the increase in the expresson of heart ET-1 immediately after ozone and particle exposure. The results show a trend in heart ET-1 and ECE-1 mRNA expression after 24 hours of exposure suggesting an increase in the expression due to ozone and particles and the drug might be preventing the increase caused by the pollutants but the changes are not statistically significant. Gong et al (1998) has demonstrated that O_3 exposure can increase myocardial work and impair pulmonary gas exchange to a degree that might be clinically important in persons with significant preexisting cardiovascular impairment, with or without concomitant lung disease.

Figure 13. Lung ET-1 & ECE-1 mRNA.

Mean±SE, n=4-6. Results are expressed as fold change relative to the air control samples. *Lung ET-1 immediate: Two-way ANOVA, Exposure main effect, P<0.001; Holm-Sidak:* ***a*** *ozone Vs air, p<0.001; ozone Vs EHC, p<0.001. AEOL main effect, p<0.001.Lung ET-1 24hour post: Two-way ANOVA,* ***a*** *AEOL main effect, p=0.027; Lung ECE-1 immediate: Two-way ANOVA,* ***a*** *AEOL main effect, p=0.032; Lung ECE-1 24hr post: Two-way ANOVA,* ***a*** *AEOL main effect, P=0.048;*


IMMEDIATE POST
ET-1 mRNA
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
AIR O3 EHC AIR O3 EHC
- AEOL
+ AEOL


24HR POST
ET-1 mRNA
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
a
AIR O3 EHC AIR O3 EHC
- AEOL
+ AEOL


IMMEDIATE POST
ECE-1 mRNA
2.5
2.0
1.5
1.0
0.5
0.0
a
a
AIR O3 EHC AIR O3 EHC
- AEOL
+ AEOL

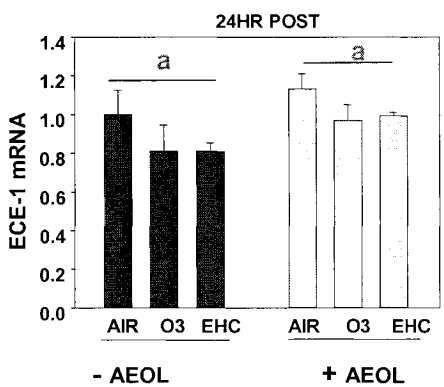
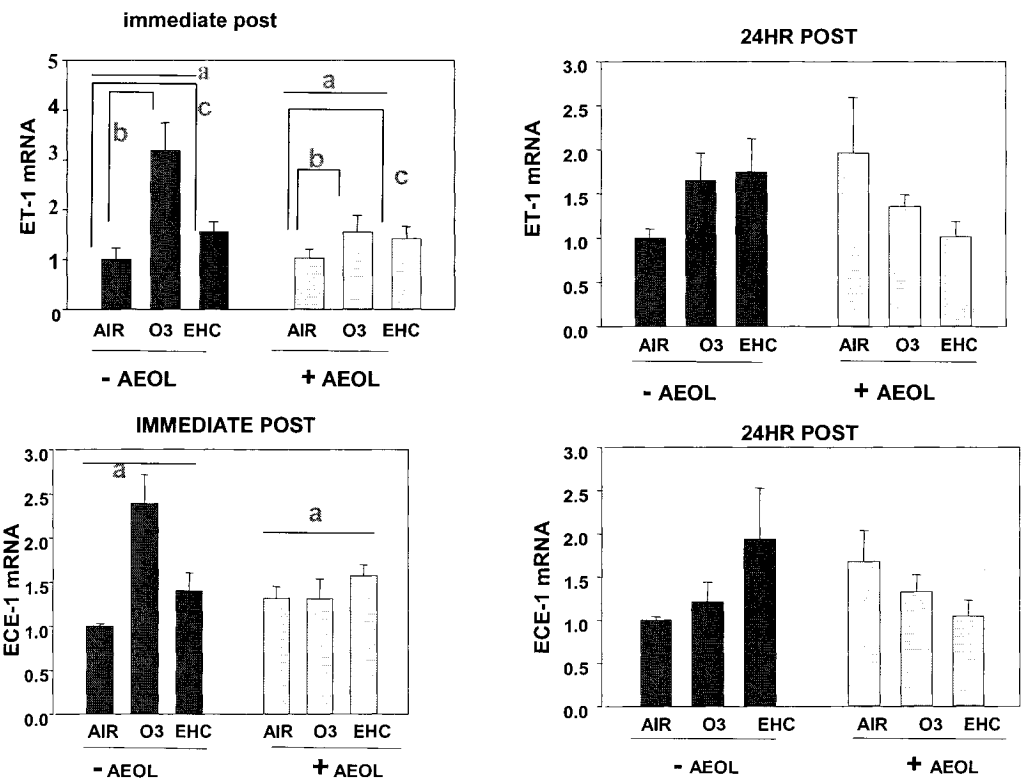

24HR POST
ECE-1 mRNA
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
a
AIR O3 EHC AIR O3 EHC
- AEOL
+ AEOL

Figure 14. Heart ET-1 & ECE-1 mRNA.

Mean±SE, n=4-*6 animals/group.* Results are expressed as fold change relative to the air control samples. *Heart ET-1 immediate: Two-way ANOVA, Exposure main effect,* ***a*** *p=0.048; Holm-sidak:* ***b*** *ozone Vs air, p=0.02;* ***c*** *EHC Vs air, p=0.07. AEOL main effect, p=0.047.Heart ECE-1 immediate: Two-way ANOVA,* ***a*** *Exposure main effect, p=0.023. Hold-Sidak: Ozone Vs air, p=0.008*

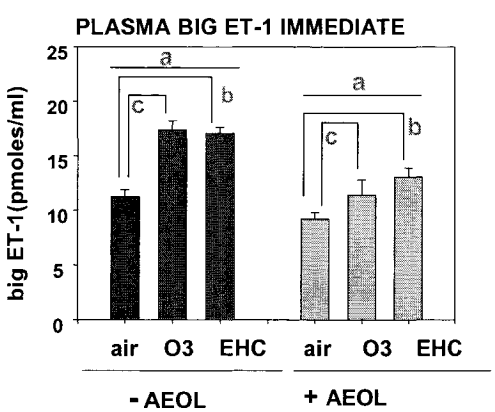

immediate post
ET-1 mRNA
5
4
3
2
1
0
a
b
c
a
b
c
AIR O3 EHC
AIR O3 EHC
- AEOL
+ AEOL
24HR POST
ET-1 mRNA
3.0
2.5
2.0
1.5
1.0
0.5
0.0
AIR O3 EHC
AIR O3 EHC
- AEOL
+ AEOL
IMMEDIATE POST
ECE-1 mRNA
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
AIR O3 EHC
AIR O3 EHC
- AEOL
+ AEOL
24HR POST
ECE-1 mRNA
3.0
2.5
2.0
1.5
1.0
0.5
0.0
AIR O3 EHC
AIR O3 EHC
- AEOL
+ AEOL

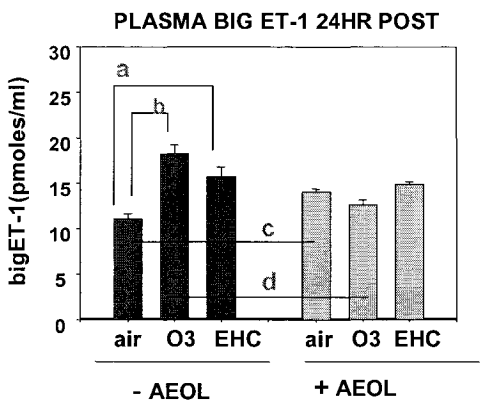
3.2.2.2 Plasma Endothelins

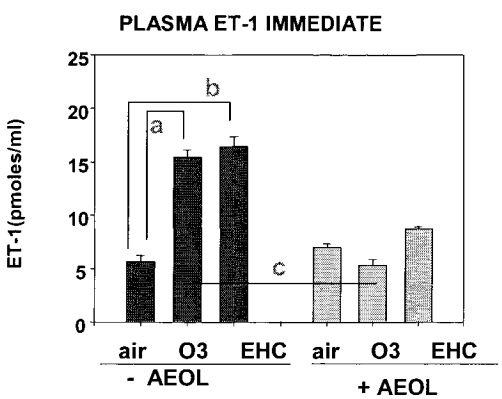
Plasma endothelins (bigET-1& ET-1) were increased immediately after exposure to ozone and particles ($p<0.01$). Pre-treatment of the animals with the SOD mimetic has prevented the increase due to air pollutants in the plasma levels (bigET-1: *AEOL X exposure* $p<0.079$; ET-1: *AEOL X exposure* $p<0.01$). After 24 hours recovery in clean air, plasma endothelin levels (bigET-1& ET-1) were increased in animals exposed to ozone or particles ($p<0.01$), an effect that was reversed by pre-treatment with the SOD mimetic (*AEOL X Exposure*, $p<0.01$) (Figure 15). Significant changes were not noticed in plasma ET-2 and ET-3 levels (Figure 16). Hirano et al (2003) suggested that the organic fraction of particulate materials in the urban air has a potency to cause oxidative stress to endothelial cells and may be implicated in cardiovascular diseases through functional changes of endothelial cells. Vincent et al (2001a) have reported an increase in plasma ET-1 in rats exposed to EHC-93 particles after 32 hours. Plasma endothelin-1 concentrations are elevated two-fold to fourfold in humans with moderate-to-severe congestive heart failure and closely correlate with the severity of this disorder, irrespective of the cause (Wei et al, 1994).

Figure 15. Plasma bigET-1 & ET-1.

Mean±SE, n=4-6 animals/group. Two-way ANOVA Big ET-1 immediate: ***a*** *AEOL main effect, p<0.001; Exposure main effect, p<0.001;* ***b*** *EHC Vs air, p<0.001;* ***c*** *ozone Vs air, p<0.001. Big ET-1 24hour: Exposure X AEOL, p<0.001: Within -AEOL,* ***a*** *ozone Vs air, P<0.001;* ***b*** *EHC Vs air, p<0.001; ozone Vs EHC, p=0.023.* ***c*** *Within air, + AEOL Vs - AEOL, P=0.021;* ***d*** *within ozone, 0 Vs 2, p<0.001. Plasma ET-1 immediate: Exposure Vs AEOL, p<0.001: Within -AEOL,* ***a*** *Ozone Vs air, p<0.001;* ***b*** *EHC Vs air, p<0.001; Within +AEOL, EHC Vs ozone, P=0.005;* ***c*** *within ozone, + AEOL Vs - AEOL, p<0.001. Plasma ET-1 24hr post: Exposure Vs AEOL, p<0.001. Within – AEOL,* ***a*** *ozone Vs air, p<0.001;* ***b*** *EHC Vs air, p<0.001;* ***c*** *within ozone, + AEOL Vs - AEOL, p<0.001;* ***d*** *within EHC, + AEOL Vs – AEOL, p< 0.001.*


PLASMA BIG ET-1 IMMEDIATE
big ET-1(pmoles/ml)
25
20
15
10
5
0
a
b
c
a
b
c
air O3 EHC air O3 EHC
- AEOL
+ AEOL


PLASMA BIG ET-1 24HR POST
bigET-1(pmoles/ml)
30
25
20
15
10
5
0
a
b
c
d
air O3 EHC air O3 EHC
- AEOL
+ AEOL


PLASMA ET-1 IMMEDIATE
ET-1(pmoles/ml)
25
20
15
10
5
0
a
b
c
air O3 EHC air O3 EHC
- AEOL
+ AEOL

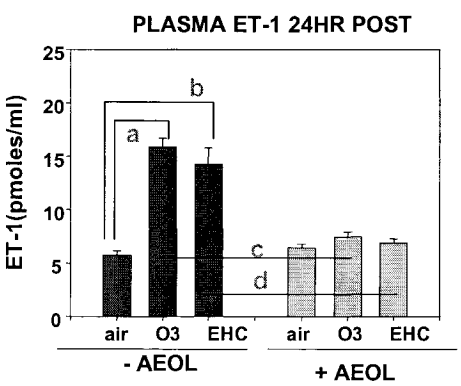
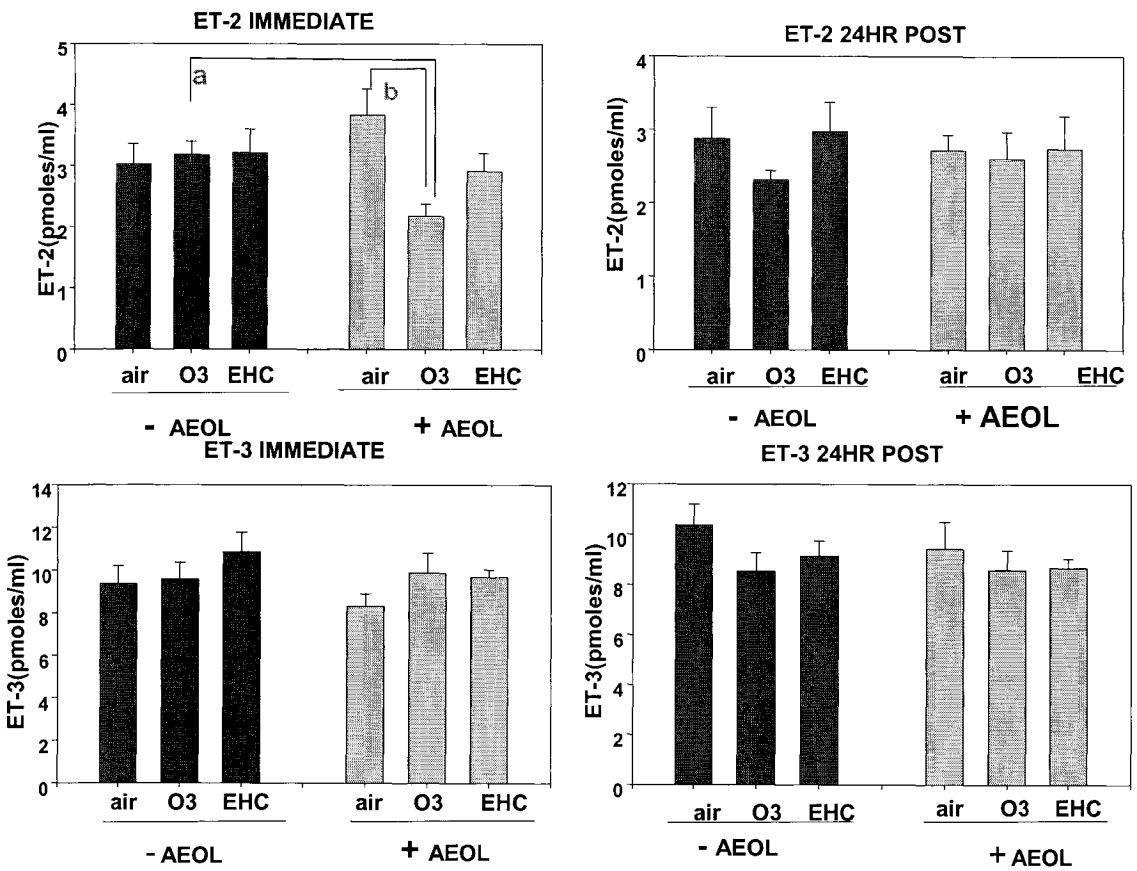

PLASMA ET-1 24HR POST
ET-1(pmoles/ml)
25
20
15
10
5
0
a
b
c
d
air O3 EHC air O3 EHC
- AEOL
+ AEOL

Figure 16. Plasma ET-2 & ET-3

Mean±SE, n=4-6 animals/group. *ET-2 immediate: Two-way ANOVA, exposure X AEOL, p=0.04; Holm-sidak:* ***a*** *within ozone, + AEOL Vs – AEOL, p=0.045;* ***b*** *within +AEOL, air Vs ozone, p=0.004.*


ET-2 IMMEDIATE
ET-2(pmoles/ml)
a
b
air O3 EHC air O3 EHC
- AEOL
+ AEOL
ET-2 24HR POST
ET-2(pmoles/ml)
air O3 EHC air O3 EHC
- AEOL
+ AEOL
ET-3 IMMEDIATE
ET-3(pmoles/ml)
air O3 EHC air O3 EHC
- AEOL
+ AEOL
ET-3 24HR POST
ET-3(pmoles/ml)
air O3 EHC air O3 EHC
- AEOL
+ AEOL

3.2.2.3 Plasma isoprostanes

Plasma isoprostane levels are increased in smokers, in hepatorenal syndrome and acute paracetamol intoxication and in scleroderma, all of which are pathophysiologic conditions in which oxidative stress is increased (Montuschi et al, 1999). Elevated systemic oxidative stress is considered risk factors for human CVD (Ridker, 1997) and has been associated with inflammatory processes and endothelial alterations (Kodavanti, 2002). In this study, plasma isoprostane levels increased due to ozone and particles and there was no effect of the drug immediately (*Two-way ANOVA with exposure and AEOL as factors: Exposure main effect, P=0.049. Holm-sidak: ozone Vs air, p=0.01*). After 24 hours, a trend of increase (not significant) can be seen in the plasma isoprostane levels and the drug has prevented the increase due to air pollutants (Figure 17). Montuschi et al (1999) have reported an increase in isoprostane levels in exhaled condensate of asthma patients where there is an evidence of imbalance of oxidants and antioxidants.

3.2.2.4 Plasma 3-nitrotyrosine

The reaction of NO and superoxide has been shown to yield peroxynitrite, a highly reactive oxidant species (Zingarelli B et al, 1997). Circulating levels of protein 3-nitrotyrosine may serve as a biomarker to assess atherosclerosis risk as well as to monitor the vasculoprotective action of the drugs (Radi R, 2004). In my study, (Figure 18) there is an increase in the levels of plasma nitrotyrosine due to ozone and particles immediately and 24hours post exposure and the drug abrogated the effects caused by pollutants (2 way *ANOVA*, Exposure Vs *AEOL*, $p<0.01$) (Figure 18).

Figure 17. Plasma isoprostane

Two way ANOVA with exposure and AEOL as factors: Exposure main effect, P=0.049. Holm-sidak multiple comparison: ozone Vs air, p=0.015.

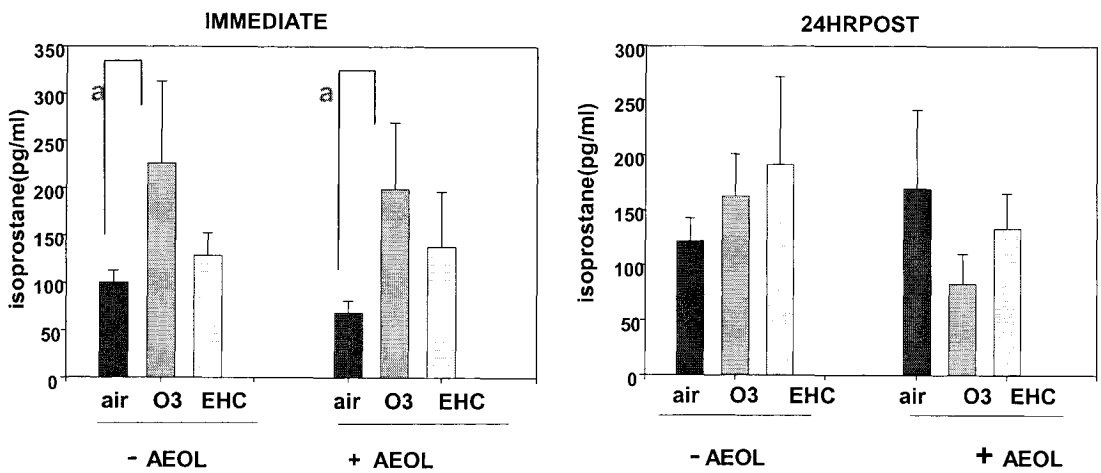
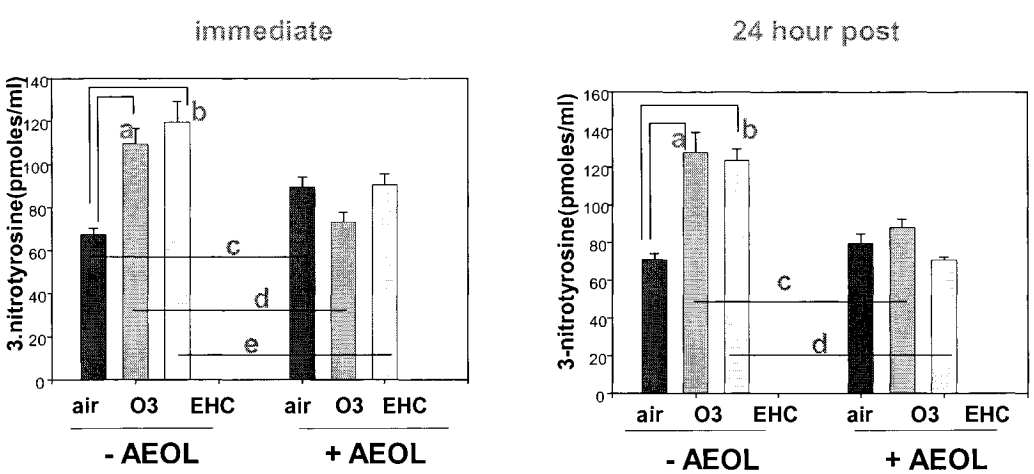

IMMEDIATE
isoprostane(pg/ml)
350
300
250
200
150
100
50
0
a
a
air
O3
EHC
air
O3
EHC
- AEOL
+ AEOL
24HRPOST
isoprostane(pg/ml)
300
250
200
150
100
50
0
air
O3
EHC
air
O3
EHC
- AEOL
+ AEOL

Figure 18. Plasma 3-nitrotyrosine

NT-immediate: *Two-way ANOVA exposure X AEOL, P<0.01; Holm-sidak multiple comparison: within -AEOL,* ***a*** *ozone Vs air, p<0.001;* ***b*** *EHC Vs air, p<0.001.* ***c*** *within air, + AEOL Vs- AEOL p<0.032,* ***d*** *within ozone and* ***e*** *EHC, + Vs - AEOL, p<0.001 and p<0.006 respectively.* NT-24 hour post: *Two-way ANOVA, exposure X AEOL, p<0.001; Holm-sidak multiple comparison: within -AEOL,* ***a*** *ozone Vs air, p<0.001;* ***b*** *EHC Vs air, p<0.001.* ***c*** *within ozone, + AEOL Vs - AEOL p<0.001,* ***d*** *within EHC, + Vs - AEOL, p<0.001.*


immediate
24 hour post
3.nitrotyrosine(pmoles/ml)
3-nitrotyrosine(pmoles/ml)
a
b
c
d
e
air
O3
EHC
- AEOL
+ AEOL

3.2.3 Summary

Tables 2 and 3 given below shows the summary of results of the lung and heart ET-1 and ECE-1 expression in particle exposure study (all arrows are $p<0.05$, otherwise indicated).

Table 2. Particle exposure study- LUNG

	Control			AEOL		
	Air	O3	EHC-93	Air	O3	EHC-93
Immediate		↑ ET-1,ECE-1			↑ET1,ECE-1	
24hr post		↑ ET-1 (NS)	↑ ET-1 (NS)			

Table 3. Particle exposure study-HEART

	Control			AEOL		
	Air	O3	EHC-93	Air	O3	EHC-93
Immediate		↑ET-1	↑ET-1		↑ET-1	↑ET-1 ↑ECE-1
24hr post		↑ET-1, ↑ECE-1 (NS)	↑ET-1, ↑ECE-1 (NS)			

Table 4 given below shows the summarized results of plasma endothelin, isoprostane and nitro tyrosine levels in particle exposure study (all arrows are $p<0.05$).

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Table 4. Particle exposure study-PLASMA

	Control			AEOL		
	Air	O3	EHC-93	Air	O3	EHC-93
Immediate		↑bET-1 ↑ET-1, ↑ IsoP,↑NT	↑bET-1, ET-1 ↑ NT		↑bET-1 ↑IsoP	↑bET-1
24hr post		↑bET-1,ET-1 ↑ NT	↑bET-1,ET-1 ↑ NT			

4 DISCUSSION

Air pollution PM exposure affects cardiac, pulmonary and vascular changes within healthy and susceptible individuals resulting in reports of increasing morbidity and mortality. Insight into the mechanisms by which air particulate pollution mediates its health effects is necessary to provide biological plausibility to epidemiological associations between PM exposure and adverse health effects. A surge of circulating endothelins after breathing air pollutants may account for acute cardiovascular symptoms and adverse events in susceptible individuals. Additional studies have shown that air pollutants induce an oxidative stress in the lungs. My objective was to block a potential superoxide surge during or after inhalation of pollutants with a superoxide dismutase (SOD) mimetic drug (*AEOL 10150*) in order to verify whether oxidative stress pathways play a role in the regulation of the endothelinergic system. Before considering using *AEOL 10150* in my studies on health impacts of air pollution, I have verified the impact of *AEOL 10150* on the baseline values of our critical toxicity endpoints. In this study endothelin expression is monitored as an indicator of endothelial dysfunction an acute effect of air pollutants. There is some evidence that *AEOL 10150* at high dose will have hypotensive effects, and we need to ensure that *AEOL 10150* at low dose will not modulate by itself the expression of the endothelin system, as this could constitute a confounding effect in the interpretation of an eventual protective effect of free radical scavengers in the pathway of induction of endothelins due to air pollutants. It could not be predicted from the literature what would be the effect of *AEOL 10150* on the expression of endothelin system genes.

Therefore, rats were treated with *AEOL 10150* to study the response of the endothelin system. The aim is to identify the highest safe dose of *AEOL 10150* that has no impact on the endothelin system such that the drug can be administered in subsequent studies of air pollution in order to block superoxide pathway. Warnholtz and Munzel (2000) have suggested that optimal dose of antioxidants must be titrated in order to improve rather than worsen vascular function. Tempol, a SODm treatment (10 or 100 mg/kg, i.v.) dose-dependently suppressed the ischemia/reperfusion-induced increments of renal ET-1 content (Sedeek et al, 2003).

4.1 Dose response study

4.1.1 Total protein in BAL

No significant changes were noticed in the total protein levels in the BAL at different dose levels of the drug indicating the absence of inflammation in rats injected with different doses of the drug.

4.1.2 ET-1 and ECE-1 gene expression in lungs and heart

In this study, heart ECE-1 expression was significantly increased two hours after injection of 5 mg *AEOL* /kg when compared to the control and 2mg/kg body weight of the drug. Goodwin et al (2002) have demonstrated an increase in heart ECE-1 mRNA expression after ischemia without a significant increase in prepro ET-1. Increased ECE-1 expression in heart may lead to increased tissue peptide levels. Endothelin is present in normal plasma, and its circulating and tissue concentrations are elevated in cardiovascular disease associated with endothelial dysfunction and coronary spasm (Lerman et al, 1995). Hypercholesterolemia

leads to endothelial dysfunction and is associated with increased ET levels in plasma and tissue. In addition, the increased release of ET-1 stimulates the synthesis of transforming growth factor-ß1, basic fibroblast growth factor, epiregulin, platelet-derived growth factor, and various adhesion molecules implicated in atherogenesis (Luscher and Barton, 2000). Chronic ET-1 stimulation can result in myocardial fibrosis and hypertrophy and vascular fibrosis with extracellular matrix proliferation (Rich and Mclaughlin, 2003). Khan et al (2002) have shown that an extract of red wine polyphenols with antioxidant properties causes a concentration-dependent inhibition of endothelin-1 synthesis in cultured bovine aortic endothelial cells. Several cardiovascular drugs have been considered as antioxidant supplements when used in patients suffering from different cardiovascular diseases. Lopez-Ongil et al (2000) have shown that superoxide inhibits ECE by ejecting zinc from the enzyme suggesting that superoxide play an important role in regulating the biology of blood vessels. In this study the antioxidant drug at the dose of 5 mg/kg body weight, increases ECE-1 expression in the heart and this might lead to increased tissue peptide levels. In failing hearts, an activation of the ET system has been found; myocardial tissue levels of ET-1 are increased along with increased density of ET receptors mainly in the form of ETA because of upregulation in the myocardium (Ito et al, 1993).

4.1.3 ETA and ETB receptor expression in Lung and Heart

In this study, lung ET_A receptor expression has decreased in animals injected with 5 mg/kg of the drug after 2 hours when compared to control and 2 mg/kg of the drug and ET_B receptor expression has increased (not significant) at the dose of 5 mg/kg of the drug.

Binding of ET-1 to either ET_A or ET_B receptors on vascular smooth muscle cells leads to contraction, whereas activation of ET_B receptors located on endothelial cells induces relaxation (Hirata et al, 1995). ET-1 was described to exert a bi-directional effect by either enhancing NO production via ET_B receptors located in endothelial cells or blunting its effect via ET_A receptors prevalently located in the vascular smooth muscle cells. (Lee et al, 2004).

4.1.4 eNOS and iNOS gene expression in Lungs and Heart

Results suggest that lung eNOS expression might be increased (not significant) at 5mg/kg body weight of the drug after 2hours when compared to 0 and 2 mg/kg body weight of the drug. Also, lung iNOS expression has significantly increased significantly at 5mg/kg body weight of the drug after 2 hours when compared to 0 and 2 mg/kg body weight of the drug.

The endothelium plays an important role in the regulation of blood pressure, and the lung, with its large vascular bed, is the main source of various components of blood pressure regulating systems. One of the compounds is NO, a vasodilating compound that is synthesized in the lung by endothelial nitric oxide synthase (eNOS) (Adnot et al, 1995). Since NO and ET-1 are vascular tension regulators secreted by endothelial cells, the expression of NOS genes were studied to see if *AEOL 10150* influence the regulation of NO. In addition to reducing the bioavailability of NO, ROS may also be involved in the regulation of the expression of eNOS, the enzyme that generates NO in the endothelium. To maintain an adequate eNOS activity and therefore the ability of endothelium to produce NO, it is essential to preserve the expression of eNOS enzyme (Farre and Casado, 2001). As a result of induction of iNOS, large amounts of the free radical NO might be formed, causing tissue damage. NO is an effective vasorelaxing compound, counterbalancing the effects of

endothelins and angiotensin II, but at high concentrations it contributes to endothelial damage. Endothelial dysfunction results in an imbalance between mediators that are involved in the regulation of blood pressure (Ulrich et al, 2002). Nitric oxide produced by iNOS results in a fall in blood pressure, a decrease in peripheral vascular resistance, and a hyporesponsiveness of arteries and veins to endogenous and exogenous vasoconstrictors (Szabo, 1995).

4.1.5 Plasma endothelins

In this study, plasma ET-1 decreased significantly in animals injected with 5 mg/kg of the drug after 2 and 24 hours when compared to control and animals with 2 mg/kg body weight of the drug. Ross et al (2002) have shown that 5 mg/kg of *AEOL 10150* injected subcutaneously caused hypotensive effect in rats. A dose of 0.5mg/kg body weight of *AEOL 10150* given intravenously to rats has been shown to cause hypotension by stimulation of histamine release, which is a well-known vasodilator. But, no such effect was noticed in rats when the drug (4.5 micrograms/kg) was given via intra-cerebro-ventricular route (Sheng et al, 2002) or when the drug (5mg/kg) was administered intratracheally (Smith et al, 2002).

The effects of ET-1 on heart are multiple, and are more or less a function of the plasma level of ET-1. With normal ET-1 plasma levels, it exerts a positive inotropic effect and elevated plasma levels of ET-1 result in a decline in cardiac output (Khan, 2005). In this study, at the dose of 5mg/kg body weight the drug reduces plasma ET-1 and this might lead to changes in cardiac function. Overall, in this study, heart ECE-1 expression has increased at 5mg/kg body weight, which might lead to increased tissue peptide levels and heart ET_A receptor expression has decreased in the animals injected with 5mg/kg body weight of the drug. The results also suggest that there might be an autocrine feedback mechanism modulating

vasoconstriction in response to reduced plasma endothelin by the drug at 5mg/kg body weight by stimulation of nitric oxide synthases. Overall, there is no significant change in the endothelin system gene expression in the animals injected with 2mg/kg body weight of the drug when compared to control levels.

4.1.6 Plasma isoprostanes

Many oxidative *footprints* (products damaged by ROS) are thought to be the result of nonenzymatic reactions between reactive oxygen species and organic molecules, such as proteins, lipids, or DNA. For instance, ROS can react with nitrogen species and then tyrosine to form nitrotyrosine. ROS react with lipids to liberate isoprostanes (Bowler & Crapo 2002b). Measurement of F2-isoprostanes is the most reliable approach to assess oxidative stress in vivo, providing an important tool to explore the role of oxidative stress in the pathogenesis of human disease (Montuschi et al, 2004) and it might provide a sensitive biochemical basis in dose-finding studies with antioxidants (Robert & Morrow, 2000). The results indicate that plasma isoprostane levels have decreased significantly in the animals injected with 2 mg/ kg body weight of the drug at both 2 and 24 hours post exposure when compared to the control animals. Significant changes were not noticed at 5 mg/kg body weight of the drug when compared to control levels. Consistent with this study, Day et al (1999) have shown that metalloporhyrins are potent inhibitors of lipid peroxidation.

4.1.7 Plasma 3-nitrotyrosine

Nitrotyrosine (NT), a marker of protein nitration by peroxynitrite, has been found in inflammatory cells, airway epithelium, and vascular endothelium of human lung

allotransplants (de Andrade, 2000). In this study, SODm, *AEOL 10150* at the dose of 5 mg/kg significantly reduced the plasma nitrotyrosine levels and marginally reduced (not significant) the nitrotyrosine levels at the dose of 2 mg/kg in plasma when compared to the control animals at both 2 and 24 hr post exposure.

4.1.8 Conclusion

There was an altered expression in lung ET-1, heart ECE-1, lung ET_A, lung eNOS & lung iNOS mRNA and changes in the plasma ET-1 levels at the dose of 5 mg/kg body weight of the drug and no significant impact at the dose of 2 mg/kg body weight of the drug in the endothelin system when compared to the control levels. So, a dose of 2 mg/kg body weight of the drug was selected for the next phase of this study to see if oxidative stress pathway controls the induction of endothelin system due to air pollution.

4.2 Particle exposure study

4.2.1 ET-1 & ECE-1 gene expression in lungs and heart

In this study, the results indicate that expression of preproET-1 and ECE-1 in lungs have increased 2.5 and 1.25 fold respectively immediately after exposure to Ozone. The increase of prepro ET-1 and ECE-1 mRNA expression measured immediately after the exposure to ozone was not prevented by pre-treatment with SODm. After 24 hours recovery in clean air, there is an increase in the expression of preproET-1 due to ozone and particles (not significant), but the increase due to pollutants is reversed by the SODm. In the heart, the expressions of preproET-1 and ECE-1 mRNA have increased immediately (2-2.5 fold) due to ozone exposure. After 24 hours recovery in clean air, expression of prepro ET-1 mRNA and ECE-1 might have increased due to ozone and particles, and the drug has brought down

the increase due to air pollutants. Lung oxidants increased immediately upon exposure to concentrated ambient particles, significant oxidative stress in the heart was observed only after a 1-h lag phase (Gurgueira et al, 2002) suggesting that lung cells signal the heart of the presence of an oxidant insult probably via either nervous or systemic mediators (Gonzalez-Flecha, 2004). Increasing evidence suggests that ROS such as superoxide anion, hydrogen peroxide, and hydroxyl radical are produced by a variety of cell types and may modulate physiological and pathophysiological processes (Schnackenberg, 2002) and they also have direct vasocontractile effects on several vessels such as rat aortas (Peters et al, 2000). Oxidative stress is known to stimulate renal ET-1 production at a stage of its gene expression (Hughes, 1996). Moreover, some antioxidative agents could suppress the ET-1 production in renal and vascular endothelial cells (Ohkita et al, 2002). Other studies have indicated that increasing oxidant stress reduces ET-1 production by endothelial cells (Michael et al, 1997; Saito et al, 1998). Patients with a variety of inflammatory lung diseases, including the acute respiratory distress syndrome, have evidence for increased ET-1 production and reactive oxygen species generation in their lungs (Langleben et al, 1993). Kahler et al (2001) and Ruef et al (2001) have shown that oxidative stress increases ET-1 synthesis in human smooth muscle cells. Gurgueira et al (2002) have shown that inhalation of ambient air particles, but not control inert particles, rapidly increases the steady-state concentrations of oxidants and an increase in the activity of several antioxidant enzymes in both heart and lung. In my study, ozone and particles might have increased ET-1 and ECE-1 expression due to oxidative stress and the antioxidant drug might have reversed the effect.

4.2.2 Plasma endothelins

In this study, plasma endothelins (bigET-1& ET-1) were increased immediately after exposure to ozone and particles. Pre-treatment of the animals with the SOD mimetic has prevented the ET-1 increase due to air pollutants in the plasma levels. After 24 hours recovery in clean air, plasma endothelin levels (bigET-1& ET-1) were increased in animals exposed to ozone or particles, an effect that was reversed by pre-treatment with the SOD mimetic. Significant changes were not noticed in plasma ET-2 and ET-3 levels. Bouthillier et al (1998) reported an increase in immunoreactive ET-1 in plasma after 20 hours in rats exposed to particles alone or in combination of ozone. Yu-Chen Lei (2005) has demonstrated an increase in peripheral blood ET-1 and oxidative stress marker in diabetic rats after PM exposure. Hirano et al (2003) suggested that the organic fraction of particulate materials in the urban air has a potency to cause oxidative stress to endothelial cells and may be implicated in CVDs through functional changes of endothelial cells. Because CVD represents the number one cause exhibiting hypertensive disorder during older age, the risk of PM health effects can be enormous in this sector of population (Kodavanti et al, 2002). The plasma levels of ET-1 and its precursor Big ET-1 did not strictly correlate with the mRNA levels of prepro ET-1 and ECE-1 indicating that the production of the peptide may be regulated by the drug at the level of post-transcriptional or even at the post-translational processing. The drug may be upregulating ET_B receptors resulting in binding of ET-1 (clearance) thereby decreasing circulating levels of ET-1 rather than decreasing the production *de novo*. Loffler et al (1993) have shown that blockade of ET_B receptor increases circulating level of ET-1 within 15 minutes. Also, the enzymes involved in the conversion of prepro ET-1 to the mature peptide may operate in an oxidant dependent mechanism. In the

presence of the antioxidant *AEOL 10150*, matrix metalloproteases and chymases may be upregulated and might result in the formation of $ET_{1-31, 32}$ peptides resulting in lower levels of plasma ET_{1-21}.

In this study, there was an approximately 150% increase in the plasma ET-1 levels immediately and 24 hours post exposure to pollutants and the drug completely reversed the increased levels caused by pollutants. Endothelin elicits a sustained pressor response when administered intravenously, which suggests its importance in blood pressure maintanence and generation of vasospasm (Tomoh Masaki, 2005). In the clinical setting, only plasma concentrations of ET-1 can be measured; these concentrations are affected by the production, clearance, and breakdown of ET-1. In atherosclerosis, myocardial infarction, pulmonary hypertension, heart failure, and renal failure, ET-1 levels are elevated in tissue and plasma. In many cardiovascular diseases, increased plasma ET-1 levels are a marker of ET activation (Luscher &Barton, 2000). Despite overlapping ranges of plasma ET-1 between healthy and sick human individuals, a 25% increase in plasma ET-1 has high predictive value for chronic heart failure (Galatius-Jensen et al, 1994) and a decrease of ET-1 on the order of 20% in chronic heart failure patients was associated with improvement of symptoms, leading investigators to postulate a direct role of ET-1 in the pathophysiology (Tsutamoto et al, 1995). A link between free radical generation and vasoactive mediator production has been identified (Boulanger, 1999). Xia et al (2003) have shown that antioxidant therapy with an herb extract, *Salvia miltiorrhiza* decreases plasma endothelin-1 and thromboxane B2 after cardiopulmonary bypass in patients with congenital heart disease. Lee et al (2004) have suggested that sesamin which has been proved to be antihypertensive and antioxidative, induces nitric oxide and decreases ET-1 production. Sedeek et al (2003) have reported that

the increase in arterial pressure in response to endothelin-1 was completely abolished by the SODm, Tempol. Elevated circulating levels of ET-1 have been observed in patients with the acute respiratory distress syndrome (Druml et al, 1993), a condition associated with increased H_2O_2 production by the lung (Sznajder, 1989). Plasma ET-1 levels in these patients average five- to eightfold higher than in control subjects. Animal models of acute lung injury associated with increased oxidant production, such as ischemia-reperfusion (Okada et al, 1995), also have elevated ET-1 levels. Pulmonary ischemia followed by reperfusion increases circulating ET-1, ET-1 mRNA in pulmonary tissue (Okada et al, 1995) and ET-1 release from the lung (Vemulapalli et al, 1992). Kang (2002) has shown that serum total ET concentrations were significantly elevated in both myocardial infarct rats and sham-operated controls in response to PM exposure. Klot et al (2005) have shown that air pollution is associated with increased risk of hospital cardiac readmissions of myocardial infarction survivors in 5 European cities. Interestingly, increased plasma endothelin levels predict mortality one year after acute myocardial infarction (Omland et al 1994).

Several studies support the hypothesis that smoking causes endothelial dysfunction through elevation of ET-1 and oxidative damage of nitric oxide (Haak et al., 1994) by a large number of free radicals known to exist in smoke, both in gas and tar phase (Church and Pryor, 1985). In contrast, antioxidant supplementation in long-term smokers leads to improvement of endothelial function and supports the suggestion that smoking increases oxidative stress (Raitakari et al, 2000). Papamichael et al (2004) have demonstrated that red wine's antioxidants counteract acute endothelial dysfunction caused by cigarette smoking in healthy non-smokers. Wine polyphenols have antioxidant properties and favorably influence

endothelial function, in particular by stimulating nitric oxide mediated vasodilation and inhibiting the endothelin-1 pathway (Caimi et al, 2004).

4.2.3 Plasma isoprostanes

In this study, plasma isoprostane levels were increased due to ozone ($p<0.05$) and particles (NS) at both 2 and 24 hours post exposure. There was no significant effect of the drug. After 24 hours, the drug might have prevented the increase due to air pollutants but the changes were not statistically significant. It has been shown by others that exposure to ambient $PM_{2.5}$ for 2 hours doubles ROS generation in the hearts and lungs of rats (Gurgueira et al, 2002). This may occur in response to a variety of transition metals or free radical components known to exist within $PM_{2.5}$ as a result of atmospheric chemical reactions (Dellinger et al, 2001). Personal exposure to ambient concentrations of $PM_{2.5}$ is also associated with increased levels of markers of lipid and protein oxidation in human blood (Sorensen et al, 2003). Evidence indicates that systemic oxidative stress does occur in groups at risk from the adverse effects of PM (Donaldson et al, 2001). Kodavanti et al (2000) have shown that spontaneously hypertensive rats had higher basal levels of oxidative stress in BAL and upon challenge with residual oil fly ash, there was a greater injury and an attenuated antioxidant response. Dietrich et al (2003) have shown that daily antioxidant supplementation (especially with Vitamin C) decreases F2-isoprostanes in passive smokers. Oxidative inactivation of NO by superoxide has been proposed as a plausible explanation for endothelial dysfunction (Harrison, 1997). Oxidation of NO by O_2 results in the formation of peroxynitrite, which could initiate lipid peroxidation or play a role in the oxidation of lipoproteins (White et al, 1994).

4.2.4 Plasma 3-nitrotyrosine

In this study, the catalytic antioxidant *AEOL 10150* prevented the nitrotyrosine increase caused by the pollutants, presumably by preventing peroxynitrite formation. Effects of endothelin-1 are partly counterbalanced by vasodilatory influences of endothelial nitric oxide (NO; Vanhoutte 2000). Endothelial NO synthase produces NO, which traverses the extracellular space to induce smooth muscle relaxation in the vessel wall. One ROS that can be produced in the presence of certain pollutant components is superoxide, which can react with NO to form the potent oxidant peroxynitrite. Peroxynitrite is likely involved in lipid peroxidation (O'Donnell and Freeman 2001). Therefore, an additional potential mechanism whereby pollutant components can increase BP includes superoxide-mediated inhibition of the actions of NO in inducing vasodilatation. Peroxynitrite has been shown to act as a nitrating agent leading to the appearance of nitrotyrosine residues in proteins, a marker of peroxynitrite-mediated tissue injury (Ischiropoulos, 1998). SOD has been reported to inhibit peroxynitrite production by epithelial cells treated with cigarette smoke (Muller et al, 1997). Similarly, macrophages from rats pretreated with an SOD mimetic and challenged with carrageenan were unable to generate peroxynitrite (Salvemini et al, 2001).

4.2.5 Conclusion

The oxidative stress mediated by PM may arise from direct generation of ROS from the surface of particles, soluble compounds such as transition metals or organic compounds, altered function of mitochondria, or NADPH-oxidase in endothelial cells and inflammatory cells capable of generating ROS and RNS (Risom et al, 2005). $PM_{2.5}$ inhalation has been shown to induce systemic inflammation and cytokine production, possibly related to free radical activity of components in PM (Li et al, 1996). In turn, these have the capacity to

enhance vascular ET expression by direct mechanisms or via activation of oxidative stress pathways (Levin, 1995). Ruidavets et al, 2005 have demonstrated that short-term O_3 exposure within a period of 1 to 2 days is related to acute coronary events in middle-aged adults without heart disease, whereas NO2 and SO2 are not. Ozone mediates a pulmonary inflammatory response via oxidative stress (Kelly, 2003) and Samet et al (2001) have reported that dietary antioxidants protect against O_3- induced pulmonary function decrements in humans. Roberts et al (2003) have shown that pretreatment of rats with dimethythiourea before intratracheal instillation of residual oil fly ash inhibited ROFA-induced pulmonary inflammation, cytotoxicity, ERK MAPK activation and cytokine gene expression, demonstrating that oxidative stress is critical to lung injury induced by air pollution particles.

It is possible that the acute systemic inflammation and oxidative stress are responsible for triggering endothelial dysfunction leading to vasoconstriction (Bonetti, 2003). Alternatively increased production of endothelins may play a role in the acute vasoconstriction (Vincent et al, 2001a). In this study, I have shown that there is a dose effect of the drug on the endothelin system and the drug at a dose of 2 mg/kg body weight injected subcutaneously prevented the increase in peptide levels in rats caused by pollutants probably by scavenging peroxynitrite. From my study, it is not clear if *AEOL* prevents the rise in plasma ET-1 by abrogating the upregulation of prepro ET-1 in the lungs. However, the results of gene expression studies in lungs after 24 hours post exposure reveal that overall, *AEOL* treated animals had lowered the expression of ET-1 (*AEOL* main effect, $p<0.05$). The results indicate that the SOD mimetic *AEOL 10150*, at the dose injected subcutaneously in the rats,

does not affect the circulating endothelin levels in air control animals, but prevents the pollutant-dependent changes in plasma endothelin levels. This supports the hypothesis that air pollutants alter the endothelinergic system through an oxidative stress pathway and implicates the superoxide anion as a key intermediate. It is possible that acute effects of air pollution may be through endothelial dysfunction, and that ET-1 dependent regulation may be prevented by dietary antioxidants. For example, it is known that consumption of red wine reduces circulating ET-1 levels (Corder et al, 2001). Additionally, as enhanced plasma ET-1 levels are reported in several pathological situations, such as heart failure or hypertension, perhaps pharmacological control of endogenous ET-1 levels by *AEOL 10150* could have therapeutic benefits.

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BIBLIOGRAPHY

Adnot S., Raffestin B., Eddahibi S. 1995. NO in the lung. Respir Physiol. Aug; 101(2):109-20. Review.

Agapitov A V., Haynes W G. 2002. Role of endothelin in cardiovascular disease. J Renin Angiotensin Aldosterone Syst. Mar;3(1):1-15. Review.

Alexander R W. 1998 Atherosclerosis as disease of redox-sensitive genes. Trans Am Clin Climatol Assoc.; 109:129-45; discussion 145-6.

Barbera J A., Peinado V I., Santos S., Ramirez J., Roca J., Rodriguez-Roisin R., 2001. Reduced expression of endothelial nitric oxide synthase in pulmonary arteries of smokers.Am J Respir Crit Care Med. Aug 15; 164(4):709-13.

Barry B E., Miller F J, and J. D. Crapo. 1985. Effects of inhalation of 0.12 and 0.25 parts per million ozone on the proximal alveolar region of juvenile and adult rats. *Lab. Invest.* 53: 692-704).

Barton M., Haudenschild C C., d'Uscio L V., Shaw S., Munter K., Luscher T F. 1998. Endothelin ETA receptor blockade restores NO-mediated endothelial function and inhibits atherosclerosis in apolipoprotein E-deficient mice. Proc Natl Acad Sci U S A. Nov 24; 95(24):14367-72.

Batalha J R., Saldiva P H., Clarke R W., Coull B A,, Stearns R C,, Lawrence J,, Murthy G G., Koutrakis P., Godleski J,J. 2002. Concentrated ambient air particles induce vasoconstriction of small pulmonary arteries in rats. Environ Health Perspect. Dec; 110(12):1191-7.

Beckman J S., Beckman T W., Chen J., Marshall P A., Freeman B A 1990. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci U S A. Feb; 87(4):1620-4.

Berger R and Pacher R. 2003. The role of the endothelin system in myocardial infarction--new therapeutic targets?Eur Heart J. Feb;24(4):294-6.

Best P J and Lerman A. 2000; Endothelin in cardiovascular disease: from atherosclerosis to heart failure. J Cardiovasc Pharmacol. 35(4 Suppl 2):S61-63. Review.

Bhatnagar A. 2004. Cardiovascular pathophysiology of environmental pollutants. Am J Physiol Heart Circ Physiol. Feb; 286(2):H479-85.

Blais V., Fugere M., Denault J B., Klarskov K., Day R., Leduc R. 2002. Processing of proendothelin-1 by members of the subtilisin-like pro-protein convertase family.FEBS Lett. Jul 31; 524(1-3):43-8.

Bonetti P O., Lerman L O, Lerman A. 2003. Endothelial dysfunction: a marker of atherosclerotic risk. *Arterioscler Thromb Vasc Biol.* 23: 168–175.

Boulanger CM, Lüscher TF. Hirudin and nitrates inhibit the thrombin-induced release of endothelin from the intact porcine aorta. *Circ Res.* 1991;68:1768–1772.

Boulanger C M. 1999. Secondary endothelial dysfunction: hypertension and heart failure. J Mol Cell Cardiol. Jan;31(1):39-49. Review.

Bouthillier L., Vincent R., Goegan P., Adamson I.Y., Bjarnason S., Stewart, M., Guenette J., Potvin M., Kumarathasan P. 1998. Acute effects of inhaled urban particles and ozone: lung morphology, macrophage activity, and plasma endothelin-1. Am.J. Pathol. 153, 1873–1884.

Bowler R P and Crapo J D. 2002a.Oxidative stress in airways: is there a role for extracellular superoxide dismutase? Am J Respir Crit Care Med. Dec 15; 166(12 Pt 2): S38-43.Review.

Bowler R P andCrapo J D. 2002b. Oxidative stress in allergic respiratory diseases. J Allergy Clin Immunol. Sep; 110(3): 349-56. Review.

Bowler R P., Arcaroli J., Abraham E., Patel M., Chang LY, Crapo JD. 2003. Evidence for extracellular superoxide dismutase as a mediator of hemorrhage-induced lung injury. Am J Physiol Lung Cell Mol Physiol. Apr; 284(4): L680-7. Epub 2003 Jan 10.

Bowler R P., Sheng H., Engchild J J., Pearlstein R D, Warner D S., Crapo J D.. 2002. A Catalytic Antioxidant (AEOL 10150) attenuates expression of inflammatory genes in stroke. Free Radical Biology and medicine, Vol.33, No.8, pp 1141-1152.

Brook R D., Brook J R., Urch B., Vincent R., Rajagopalan S., Silverman F. 2002. Inhalation of fine particulate air pollution and ozone causes acute arterial vasoconstriction in healthy adults. Circulation. Apr 2;105(13):1534-6.

Brunner F., du Toit E F., Opie L H. 1992. Endothelin release during ischaemia and reperfusion of isolated perfused rat hearts. J Mol Cell Cardiol.. Nov; 24(11):1291-305.

Caimi G., Carollo C., Lo Presti R. 2004 Chronic renal failure: oxidative stress, endothelial dysfunction and wine.Clin Nephrol. Nov; 62(5):331-5.

Calderon-Garciduenas L., Mora-Tiscareno A., Fordham L A., Valencia-Salazar G., Chung C J., Rodriguez-Alcaraz A., Paredes R., Variakojis D., Villarreal-Calderon A., Flores-Camacho L., Antunez-Solis A., Henriquez-Roldan C., Hazucha M J. 2004. Respiratory damage in

children exposed to urban pollution. Caramori G and A Papi Oxidants and asthma Thorax, Feb 59: 170,.Pediatr Pulmonol.,2003 Aug;36(2):148-61.

Caramori G and A Papi. 2004. Oxidants and asthma Thorax, Feb; 59: 170.

Chance B., Sies H., Boveris A. 1979. Hydroperoxide metabolism in mammalian organs. Physiol Rev. Jul; 59(3):527-605.

Church D F and Pryor W A.1985. Free-radical chemistry of cigarette smoke and its toxicological implications. Environ Health Perspect. Dec; 64:111-26.

Corder R, Carrier M, Khan N, et al. Cytokine regulation of endothelin-1 release from bovine aortic endothelial cells. *J Cardiovasc Pharmacol.* 1995; 26(suppl 3):S56–S58.

Corder R, Douthwaite JA, Lees DM, Khan NQ, Viseu Dos Santos AC, Wood EG, Carrier MJ. 2001. Endothelin-1 synthesis reduced by red wine. Nature. Dec 20-27; 414(6866): 863-4.

Costa D L and Dreher K L. 1997.Bioavailable transition metals in particulate matter mediate cardiopulmonary injury in healthy and compromised animal models. Environ Health Perspect. Sep;105 Suppl 5:1053-60.

Cuzzocrea S., Dennis P., Riley., Achille P., Caputi., and Salvemini D. 2001. Antioxidant Therapy: A New Pharmacological Approach in Shock, Inflammation, and Ischemia/Reperfusion Injury Pharmacol. Rev., Mar; 53: 135.

D' Andrade J., john p. crow, liliana viera., c. bruce alexander, k. randall young, david c. mcgiffin, george l. zorn., sha zhu, sadis matalon, and robert m. jackson Protein Nitration, Metabolites of Reactive Nitrogen Species, and Inflammation in Lung AllograftsAm. J. Respir. Crit. Care Med., Jun 2000; 161: 2035.

D'Orléans-Juste P., Plante, M., Honore, J. C., Carrier, E., and Labonte. 2003. J.Synthesis and degradation of endothelin-1. *Can. J. Physiol. Pharmacol.* 81, 503–510.

Day B J., Batinic-Haberle I., Crapo J D. 1999. Metalloporphyrins are potent inhibitors of lipid peroxidation. Free Radic Biol Med. Mar; 26(5-6):730-6.

Day B J and Crapo J D. 1996. A metalloporphyrin superoxide dismutase mimetic protects against paraquat-induced lung injury in vivo.Toxicol Appl Pharmacol. Sep; 140(1):94-100.

Deby C and Goutier R. 1990. New perspectives on the biochemistry of superoxide anion and the efficiency of superoxide dismutases.Biochem Pharmacol. Feb 1; 39(3):399-405. Review.

Dellinger B., Pryor W A , Cueto R.. 2001. Role of free radicals in the toxicity of airborne fine particulate matter. *Chem Res Toxicol.*; 14: 1371–1377.

Dietrich M., Block G., Benowitz N L., Morrow J D, Hudes M., Jacob P 3rd, Norkus E P., Packer L. 2003. Vitamin C supplementation decreases oxidative stress biomarker f2-isoprostanes in plasma of nonsmokers exposed to environmental tobacco smoke. Nutr Cancer. 45(2):176-84.

Dockery D W. 2001 Epidemiologic evidence of cardiovascular effects of particulate air pollution.Environ Health Perspect. Aug; 109 Suppl 4:483-6. Review.

Donaldson K., Stone V., Seaton A., MacNee W. 2001 Ambient particle inhalation and the cardiovascular system: potential mechanisms.Environ Health Perspect. Aug; 109 Suppl 4:523.

Dröge W. 2002. Free Radicals in the Physiological Control of Cell Function Physiol Rev, 82: 47.

Druml, W., H. Steltzer, W. Waldhèusl, K. Lenz, A. Hammerle, H. Vierhapper, S. Gasic, and O. F. Wagner. 1993. Endothelin-1 in adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* 148: 1169-1173.

Farré A L and Casado S. 2001.Heart Failure, Redox Alterations, and Endothelial Dysfunction Hypertension,; 38: 1400.

Fridovich I. 1978. The biology of oxygen radicals. Science. Sep 8; 201(4359):875-80

Folkesson H G., Westrom B. R., Pierzynowski S. G., and Karlsson B. W. 1991. Lung to blood passage of different-sized molecules during lung inflammation in the ratH J Appl Physiol,; 71: 1106.

Galatius-Jensen S., Wroblewski H., Emmeluth C., Bie P., Haunsø S., Kastrup J: 1994. Plasma endothelin-1 in chronic heart failure: a predictor of cardiac death? Circulation, 90: I-379.

Galley H F and Webster N R. 2004. Physiology of the endothelium.. Br J Anaesth. 93(1):105-13. Epub 2004 Apr 30. Review.

Gilmour P S., Brown D M., Lindsay T G., Beswick P H., MacNee W., Donaldson K. 1996. Adverse health effects of PM10 particles: involvement of iron in generation of hydroxyl radical. Occup Environ Med. Dec; 53(12):817-22.

Goldberg, M. S., Burnett, R. T., Bailar, J. C., III, Tamblyn, R., Ernst, P, Flegel, K., Brook, J., Bonvalot, Y., Singh, R., Valois, M. F., *et al.* . 2001. Identification of persons with cardiorespiratory conditions who are at risk of dying from the acute effects of ambient air particles. *Environ.Health Perspect*, 109(Suppl. 4), 487–494.

Gong H jr., richard wong., radha j. sarma., william s. linn., emmett d., Sullivan., deborah a. shamoo., karen r. Anderson., and shankar b. prasad 1998.Cardiovascular Effects of Ozone Exposure in Human Volunteers Am. J. Respir. Crit. Care Med., Volume 158, Number 2, August 538-546.

Goodwin A T., Khan M A., Chester A H., Amrani M.,Yacoub M H. 2002.Up-regulation of endothelin-converting-enzyme mRNA expression following cardioplegic arrest.Clin Sci (Lond). Aug; 103 Suppl 48:206S-209S.

Gonzalez-Flecha B. 2004.Oxidant mechanisms in response to ambient air particles. Mol Aspects Med. Feb-Apr;25(1-2):169-82. Review.

Griendling K K., Minieri C A., Ollerenshaw J D., Alexander R W. 1994. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. Circ Res. Jun; 74(6):1141-8.

Griendling, K. K., and Harrison, D. G. 1999. *Circ. Res.* 85, 562-563.

Gryparis A., Forsberg B., Katsouyanni K., Analitis A., Touloumi G., Schwartz J., Samoli E., Medina S., Anderson H R., Niciu E M., Wichmann H E., Kriz B., Kosnik M.., Skorkovsky J., Vonk J M., Dortbudak Z. 2004. Acute effects of ozone on mortality from the "air pollution and health: a European approach" project.Am J Respir Crit Care Med. 15; 170(10):1080-7. Epub Jul 28.

Guarnieri C., Flamigni F., Caldarera C M. 1980.Role of oxygen in the cellular damage induced by re-oxygenation of hypoxic heart. J Mol Cell Cardiol. Aug; 12(8):797-808.

Gurgueira, S.A., Lawrence, J., Coull, B., Krishnamurthy, G.G., González-Flecha, B. 2002. Rapid increases in the steady-state concentration of reactive oxygen species in the lungs and heart after particulate air pollution inhalation. *Environ. Health Perspect.*, 110 pp. 749-755.

Haak P, Jungmann E, Raab C, et al. Elevated endothelin-1 levels after cigarette smoking. Metabolism 1994;43:267-269.

Hahn AW, Resink TJ, Scott-Burden T, et al. Stimulation of endothelin mRNA and secretion in rat vascular smooth muscle cells: a novel autocrine function. *Cell Regul.* 1990;1:649–659.

Harrison D G. 1997.Cellular and molecular mechanisms of endothelial cell dysfunction. J Clin Invest. 100(9):2153-7. Review.

Haynes W G., Webb D J. 1998. Endothelin as a regulator of cardiovascular function in health and disease.J Hypertens. Aug; 16(8):1081-98.

Hearse D J and Tosaki A. 1987.Reperfusion-induced arrhythmias and free radicals: studies in the rat heart with DMPO. J Cardiovasc Pharmacology. Jun; 9(6):641-50.

Heunks L M A., Dekhuijzen P N. 2000. Respiratory muscle function and free radicals: from cell to COPD. Thorax.Aug; 55(8):704-16.Review.

Highsmith R F. 1998. In: Endothelins: molecular biology, physiology, and pathology, Humana Press, Totowa, N.J. pp 9.

Hirano S., Furuyama A., Koike E., Kobayashi T. 2003.Oxidative-stress potency of organic extracts of diesel exhaust and urban fine particles in rat heart microvessel endothelial cells. Toxicology. May 3; 187(2-3):161-70.

Hirata Y., Emori T., Eguchi S., et al. 1993.Endothelin receptor subtype B mediates synthesis of nitric oxide by cultured bovine endothelial cells. *J Clin Invest.* 91:1367–1373.

Hosoda K., Nakao K., Hiroshi-Arai, Suga S., Ogawa Y., Mukoyama M., Shirakami G., Saito Y., Nakanishi S., Imura H. 1991.Cloning and expression of human endothelin-1 receptor cDNA. FEBS Lett. Aug 5; 287(1-2):23-6.

Housset B. 1994. [Free radicals and respiratory pathology] C R Seances Soc Biol Fil. 188(4):321-33.Review.

Hughes A K., Stricklett P K., Padilla E., Kohan D. 1996. E.Effect of reactive oxygen species on endothelin-1 production by human mesangial cells.Kidney Int. Jan; 49(1):181-9.

Ischiropoulos H. 1998. Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species.Arch Biochem Biophys. Aug 1; 356(1):1-11. Review.

Ito H., Hirata Y., Adachi S., Tanaka M., Tsujino M., Koike A., Nogami A., Murumo F., Hiroe M. 1993.Endothelin-1 is an autocrine/paracrine factor in the mechanism of angiotensin II-induced hypertrophy in cultured rat cardiomyocytes.J Clin Invest. Jul; 92(1):398-403.

Jimenez L A., Thompson J., Brown D A., Rahman I., Antonicelli F., Duffin R., Drost E M., Hay R T., Donaldson K., MacNee W. 2000. Activation of NF-kappaB by PM(10) occurs via an iron-mediated mechanism in the absence of IkappaB degradation. Toxicol Appl Pharmacol. Jul 15;166(2):101-10.

Kahler J., Ewert A., Weckmuller J., Stobbe S., Mittmann C., Koster R., Paul M., Meinertz T., Munzel T. 2001.Oxidative stress increases Endothelin-1 synthesis in human coronary artery smooth muscle cells. Journal of Cardiovascular Pharmacology, Vol.38:49-57.

Kang Y J, Li Y., Zhou Z., Roberts A M., Cai L., Myers S R., Wang L., Schuchke D A. 2002. Elevation of serum endothelins and cardiotoxicity induced by particulate matter (PM2.5) in rats with acute myocardial infarction.Cardiovasc Toxicol.;2(4):253-61.

Kelly F J. 2003. Oxidative stress: its role in air pollution and adverse health effects. *Occup Environ Med.* 60: 612–616.

Khan I A. 2005. Role of endothelin-1 in acute myocardial infarction.Chest. 127(5):1474-6.

Kinney, P. L., Nilsen D. M., Lippmann M., Brescia M., Gordon T., McGovern T., Fawal H. El., Devlin R. B., and. Rom W. N. 1996. Biomarkers of lung inflammation in recreational joggers exposed to ozone. *Am. J. Respir. Crit. Care Med.* 154: 1430-1435.

Kinnula V L andCrapo J D. 2003. Superoxide dismutases in the lung and human lung diseases. Am J Respir Crit Care Med. Jun 15; 167(12):1600-19. Review.

Klot S V., Peters A., Aalto P., Bellander T., Berglind N., D'Ippoliti D., Elosua R., Hormann A., Kulmala M., Lanki T., Lowel H., Pekkanen J., Picciotto S., Sunyer J., Forastiere F. 2005. Health Effects of Particles on Susceptible Subpopulations (HEAPSS) Study Group Ambient air pollution is associated with increased risk of hospital cardiac readmissions of myocardial infarction survivors in five European cities. Circulation. 112(20):3073-9.

Kodavanti, U.P., Schladweiler, M.C., Ledbetter, A.D., Watkinson, W.P., Campen, M.J., Winsett, D.W., Richards, J.R., Crissman, K.M., Hatch, G.E. and Costa, D.L., 2000.The spontaneously hypertensive rat as a model of human cardiovascular disease: evidence of exacerbated cardiopulmonary injury and oxidative stress from inhaled emission particulate matter. *Toxicol. Appl. Pharmacol.*, 164, pp. 250–263.

Kodavanti U P., Schladweiler M C., Ledbetter A D., Hauser R., Christiani D C., McGee J., Richards J R., Costa D L. 2002.Temporal association between pulmonary and systemic effects of particulate matter in healthy and cardiovascular compromised rats.J Toxicol Environ Health A. Oct 25; 65(20):1545-69.

Kumarathasan P., Goegan P., Vincent R. (2001). An Automated High-Perfomance Liquid Chromatography Flourescence Method for the Analyses of Endothelins in Plasma Samples. Analytical Biochemistry 299, 37-44.

Kumarathasan P., Vincent R, Goegan P, Bjarnason S, Guenette J. 2002. Alteration in aromatic hydroxylation and lipid oxidation status in the lungs of rats exposed to ozone. Toxicology Mechanisms and methods, 12:195-210.

Kumarathasan P., Renaud Vincent and Susantha Mohottalage. 2003a. Biomarker analysis in toxicology of air pollutants. Recent Research Developments in Analytical Biochemistry, Vol. 3, 187-201.

Kumarathasan P and Vincent R. 2003b.New approach to the simultaneous analysis of catecholamines and tyrosines in biological fluid. Journal of chromatography A, 987, 349-358.

Kuntzen C., Gulberg V., Gerbes A L. 2005. Use of a mixed endothelin receptor antagonist in portopulmonary hypertension: a safe and effective therapy? Gastroenterology. 128(1):164-8.

Kunzli N., Kaiser R., Medina S., Studnicka M., Chanel O., Filliger P., Herry M., Horak F Jr., Puybonnieux-Texier V., Quenel P., Schneider J., Seethaler R., Vergnaud J C., Sommer H.. 2000. Public-health impact of outdoor and traffic-related air pollution: a European assessment.Lancet. Sep 2; 356(9232):795-801.

Langleben, D., M. DeMarchie, Laporta D.,. Spanier A. H., Schlesinger R. D., and Stewart D. J.1993. Endothelin-1 in acute lung injury and the adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* 148: 1646-1650.

Lee C C., Chen P R., Lin S., Tsai S C., Wang B W., Chen W W., Tsai C E., Shyu K G. 2004. Sesamin induces nitric oxide and decreases endothelin-1 production in HUVECs: possible implications for its antihypertensive effect. J Hypertens. Dec; 22(12):2329-3.

Lerman A., Holmes D J., Bell M R., et al. 1995. Endothelin in coronary endothelial dysfunction and early atherosclerosis in humans. *Circulation.* 92:2426–2431.

Levin E R. 1995. Endothelins.N Engl J Med. Aug 10; 333(6):356-63.

Levin ER. Endothelins as cardiovascular peptides.Am J Nephrol. 1996; 16(3):246-51. Review.

Li, N.. Jawed Alam., M. Indira Venkatesan., Arantza Eiguren-Fernandez., Debra Schmitz., Emma Di Stefano., Ndaisha Slaughter., Erin Killeen., Xiaorong Wang., Aaron Huang., Meiying Wang., Antonio H. Miguel., Arthur Cho., Constantinos Sioutas., and Andre E. 2004. NelNrf2 Is a Key Transcription Factor That Regulates Antioxidant Defense in Macrophages and Epithelial Cells: Protecting against the Proinflammatory and Oxidizing Effects of Diesel Exhaust Chemicals J. Immunol., Sep 173: 3467 – 3481.

Li X Y., Gilmour P S., Donaldson K., MacNee W. 1996. Free radical activity and pro-inflammatory effects of particulate air pollution (PM10) in vivo and in vitro.Thorax. Dec; 51(12):1216-22.

Lippmann, M. 1993. Health effects of tropospheric ozone: review of recent research findings and their implications to air quality standards. *J. Expos. Anal. Environ. Epidemiol.* 3: 103-129.

Lirk P., Hoffmann G., Rieder J. 2002. Inducible nitric oxide synthase--time for reappraisal. Curr Drug Targets Inflamm Allergy. Mar; 1(1):89-108. Review.

Livak K J and Schmittgen T D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta DeltaC(T)) Method.Methods. Dec; 25(4):402-8.

Llesuy S., Milei J., Picone V., Gonzalez Flecha B., Beigelman R., Boveris A. 1995. Effect of vitamins A and E on ischemia-reperfusion damage in rabbit heart. Mol Cell. Biochem. Apr 12; 145(1):45-51.

Loffler B M., Breu V., Clozel M. 1993. Effect of different endothelin receptor antagonists and of the novel non-peptide antagonist Ro 46-2005 on endothelin levels in rat plasma. FEBS. Lett. Oct 25; 333(1-2):108-10.

López-Ongil' S., Veronica Senchak., Marta Saura., Carlos Zaragoza., Michael Ames., Barbara Ballermann., Manuel Rodríguez-Puyol., Diego Rodríguez-Puyol., and Charles J. Lowenstein. 2000. Superoxide Regulation of Endothelin-converting Enzyme J. Biol. Chem., Vol. 275, Issue 34, 26423-26427.

Luscher T F., Barton M. 2000. Endothelins and endothelin receptor antagonists: therapeutic considerations for a novel class of cardiovascular drugs. Circulation. Nov 7; 102(19):2434-40.

Ma, X.L., Gao, F., Nelson, A.H., Lopez, B.L., Christopher, T.A., Yue, T.L. and Barone, F.C., 2001. Oxidative inactivation of nitric oxide and endothelial dysfunction in stroke-prone spontaneous hypertensive rats. *J. Pharmacol. Exp. Ther.* 298, pp. 879–885.

Madamanchi N R., Hakim Z S., Runge M S. 2005.Oxidative stress in atherogenesis and arterial thrombosis: the disconnect between cellular studies and clinical outcomes.J Thromb Haemost Feb; 3(2):254-67.Review.

Matsumoto H., Suzuki N., Onda H., Fujino M. 1989. Abundance of endothelin-3 in rat intestine, pituitary gland and brain.Biochem Biophys Res Commun. Oct 16; 164(1):74-80.

Matsuura A, Yamochi W, Hirata K, et al. Stimulatory interaction between vascular endothelial growth factor and endothelin-1 on each gene expression. *Hypertension.* 1998;32:89–95.

Michael J R., Markewitz B A., Kohan D E. 1997. Oxidant stress regulates basal endothelin-1 production by cultured rat pulmonary endothelial cells. Am J Physiol. Oct; 273(4 Pt 1):L768-74.

Mihm M J., Coyle C M., Schanbacher B L., Weinstein D M., Bauer J A. 2001. Peroxynitrite induced nitration and inactivation of myofibrillar creatine kinase in experimental heart failure. Cardiovasc Res. Mar; 49(4):798-807.

Miller FJ, Graham JA. Toxicol Lett. 1988 Dec; 44(3):231-46. Research needs and advances in inhalation dosimetry identified through the use of mathematical dosimetry models of ozone. Health Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711.

Monge J C. 1998; Neurohormonal markers of clinical outcome in cardiovascular disease: is endothelin the best one? J Cardiovasc Pharmacol. 32 Suppl 2:S36-42.

Montuschi P., Corradi M., Ciabattoni G., Nightingale J., Kharitonov S A., Barnes P J. 1999. Increased 8-isoprostane, a marker of oxidative stress, in exhaled condensate of asthma patients. Am J Respir Crit Care Med. Jul; 160(1):216-20.

Montuschi P., Barnes P J., Roberts L J. 2004. Isoprostanes: markers and mediators of oxidative stress. FASEB J. Dec; 18(15):1791-800. Review.

Muller T., Haussmann H J., Schepers G. 1997. Evidence for peroxynitrite as an oxidative stress-inducing compound of aqueous cigarette smoke fractions.Carcinogenesis. Feb; 18(2):295-301.

Nel A. 2005. Atmosphere Air pollution-related illness: effects of particles. Science. May 6; 308(5723):804-6. Erratum in: Science. 2005 Aug 26; 309(5739):1326.

Nussdorfer G G., Rossi G P., Malendowicz L K., Mazzocchi G. 1999. Autocrine-paracrine endothelin system in the physiology and pathology of steroid-secreting tissues.Pharmacol Rev. Sep;51(3):403-38. Review.

O'Donnell V B., Freeman B A. 2001. Interactions between nitric oxide and lipid oxidation pathways: implications for vascular disease.Circ Res. Jan 19; 88(1):12-21. Review.

Ogawa Y., Nakao K., Arai H., Nakagawa O., Hosoda K., Suga S., Nakanishi S., Imura H. 1991. Molecular cloning of a non-isopeptide-selective human endothelin receptor. Biochem Biophys Res Commun. Jul 15; 178(1):248-55.

Ohkita M., Takaoka M., Kobayashi Y., Itoh E., Uemachi H., Matsumura Y. 2002. Involvement of proteasome in endothelin-1 production in cultured vascular endothelial cells. Jpn J Pharmacol. Feb; 88(2):197-205.

Okada, M., Yamashita C., Okada M., and Okada K. 1995. Contribution of endothelin-1 to warm ischemia/reperfusion injury of the rat lung.*Am. J. Respir. Crit. Care Med.*152: 2105-2110, [Abstract].

Omland T., Lie R T., Aakvaag A., Aarsland T., Dickstein K.1994. Plasma endothelin determination as a prognostic indicator of 1-year mortality after acute myocardial infarction.Circulation. Apr; 89(4):1573-9.

Ozaki S., Ohwaki K., Ihara M., et al. 1995. ETB-mediated regulation of extracellular levels of endothelin-1 in cultured endothelial cells. *Biochem Biophys Res Commun.* 209:483–489.

Papamichael C., Karatzis E., Karatzi K., Aznaouridis K., Papaioannou T., Protogerou A., Stamatelopoulos K., Zampelas A., Lekakis J., Mavrikakis M. . 2004. Red wine's antioxidants counteract acute endothelial dysfunction caused by cigarette smoking in healthy nonsmokers.Am Heart JFeb;147(2):E5.

Palmer R M J., Rees D D , Ashton D S., Moncada S. 1988. L-Arginineis the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. Biochem BiophysRes Commun 153:1251-1256.

Patel M and Day B J, 1999. Metalloporphyrin class of therapeutic catalytic antioxidants. TiPS. 20:359.

Peters, A., Frohlich, M., Doring, A., 2001. Particulate air pollution is Sorensen, M., Daneshvar, B., Hansen, M., Dragsted, L.O., Hertel, O., Knudsen, L., Loft, S., 2003.Personal PM2.5 exposure and markers of oxidative stress in blood. Environ. Health Perspect. 111, 161–166.

Pope C A 3rd., Burnett R T., Thun M J., Calle E E., Krewski D., Ito K., Thurston G D. 2002. Lung cancer, cardiopulmonary mortality, and long-term exposure to fine particulate air pollution. JAMA. Mar 6; 287(9):1132-41.

Quyyumi A A. 1998. Effects of aspirin on endothelial dysfunction in atherosclerosis. Am J Cardiol. Nov 19; 82(10A):31S-33S. Review.

Radi R. 2004. Nitric oxide, oxidants, and protein tyrosine nitration PNAS. 101: 4003 - 4008.

Radil R, Beckman J S, Bush K M, and Freeman B A. 1991. Peroxynitrite Oxidation of Sulfhydryls-the cytotoxic potential of superoxide and nitric oxide. The journal o f biological chemistry. 266(7):4244-4250.

Raitakari O T., Adams M R., McCredie R J., Griffiths K A., Stocker R., Celermajer D S. 2000. Oral vitamin C and endothelial function in smokers: short-term improvement, but no sustained beneficial effect. J Am Coll Cardiol. May; 35(6):1616-21.

Rich S and McLaughlin V V. 2003. Endothelin receptor blockers in cardiovascular disease.Circulation. Nov 4; 108(18):2184-90.

Ridker P M. 1997. Intrinsic fibrinolytic capacity and systemic inflammation: novel risk factors for arterial thrombotic disease. Haemostasis.; 27 Suppl 1:2-11.Review.

Risom L., Moller P., Loft S. 2005. Oxidative stress-induced DNA damage by particulate air pollution. Mutat Res. 592(1-2):119-37.

Roberts L J and Morrow J D. 2000. Measurement of F (2)-isoprostanes as an index of oxidative stress in vivo. Free Radic Biol Med. Feb 15; 28(4):505-13. Review.

Roberts E S., Richards J H., Jaskot R., Dreher K L.2003. Oxidative stress mediates air pollution particle-induced acute lung injury and molecular pathology. Inhal Toxicol. Nov; 15(13):1327-46.

Rodeheffer R J., Lerman A., Heublein D M., Burnett J C Jr. 1992. Increased plasma concentrations of endothelin in congestive heart failure in humans. Mayo Clin Proc. Aug; 67(8):719-24.

Ross A D., Sheng, Warner D S., Piantadosi C A., Haberle I B., Day B J., Crapo J D. 2002. Hemodynamic effects of metalloporphyrin catalytic antioxidants: Structure-activity relationships and species specificity. Free Radical Biology and Medicine, Vol.33, No.12, pp.1657-1669.

Rubanyi G M and Polokoff M A 1994. Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. Pharmacol Rev. Sep; 46(3):325-415. Review.

Ruef J., Moser M., Kubler W., Bode C. 2001. Induction of endothelin-1 expression by oxidative stress in vascular smooth muscle cells. Cardiovasc Pathol. Nov-Dec; 10(6):311-5.

Ruidavets J B., Cournot M., Cassadou S., Giroux M., Meybeck M., Ferrieres J. 2005. Ozone air pollution is associated with acute myocardial infarction.Circulation. Feb 8; 111(5):563-9.

Russell F D and Davenport A P Secretory pathways in endothelin synthesis British Journal of Pharmacology (1999) 126, 391-398.

Saito T., Itoh H., Chun T., Igaki T., Mori Y., Yamashita J., Doi K., Tanaka T., Inoue M., Masatsugu K., Fukunaga Y., Sawada N., Tojo K., Saito Y., Hosoya T., Nakao K. 1998. Oxidative stress suppresses the endothelial secretion of endothelin.J Cardiovasc Pharmacol. 31 Suppl 1:S345-7.

Salvemini D., Mazzon E., Dugo L., Riley D P., Serraino I., Caputi A P., Cuzzocrea S. 2001. Pharmacological manipulation of the inflammatory cascade by the superoxide dismutase mimetic, M40403.Br J Pharmacol. Feb; 132(4):815-27.

Samet J M., Dominici F., Curriero F C., Coursac I., Zeger S L. 2000. Fine particulate air pollution and mortality in 20 U.S. cities, 1987-1994. N Engl J Med. Dec 14; 343(24):1742-9.

Samet J M., Hatch G E., Horstman D., Steck-Scott S., Arab L., Bromberg P A., Levine M., McDonnell W F., Devlin R B. 2001. Effect of antioxidant supplementation on ozone-induced lung injury in human subjects. Am J Respir Crit Care Med. Sep 1; 164(5):819-25.

Schiffrin E L. 2001. A critical review of the role of endothelial factors in the pathogenesis of hypertension.J Cardiovasc Pharmacol. Nov; 38 Suppl 2:S3-6.

Schins R.P., Lightbody J.H.,. Borm P.J., Shi T., Donaldson K., and V. Stone. 2004.Inflammatory effects of coarse and fine particulate matter in relation to chemical and biological constituents, *Toxicol. Appl. Pharmacol.* 195 pp. 1–11.

Schnackenberg C G. 2002. Oxygen radicals in cardiovascular-renal disease. Curr Opin Pharmacol. Apr; 2(2):121-5.Review.

Schwarting A, Schlaak J, Lotz J, et al. Endothelin-1 modulates the expression of adhesion molecules on fibroblast-like synovial cells (FLS). *Scand J Rheumatol.* 1996;25:246–256.

Schwartz J. 1999. Air pollution and hospital admissions for heart disease in eight U.S. counties. Epidemiology. Jan; 10(1):17-22.

Seaton A., MacNee W., Donaldson K., Godden D. 1995. Particulate air pollution and acute health effects.Lancet. Jan 21; 345(8943):176-8.

Sedeek M H., Llinas M T., Drummond H., Fortepiani L., Abram S R., Alexander B T., Reckelhoff J F., Granger J P. 2003.Role of reactive oxygen species in endothelin-induced hypertension. Hypertension. Oct; 42(4):806-10. Epub 2003 Jul 21.

Sheng H., Enghild J J., Bowler R., Patel M., Batinic-Haberle I., Calvi C L., Day B J., Pearlstein R D, Crapo J D., Warner D S. 2002. Effects of metalloporphyrin catalytic antioxidants in experimental brain ischemia. Free Radic Biol Med. Oct 1; 33(7):947-61.

Shore S., Kobzik L., Long N C., Skornik W., Van Staden C J., Boulet L., Rodger I W., Pon D J. 1995. Increased airway responsiveness to inhaled methacholine in a rat model of chronic bronchitis. Am J Respir Crit Care Med. Jun; 151(6):1931-8.

Silbajoris R., Ghio A J., Samet J M., Jaskot R., Dreher K L., Brighton L E. 2000. In vivo and in vitro correlation of pulmonary MAP kinase activation following metallic exposure. Inhal Toxicol. Jun; 12(6):453-68.

Smith K R., Uyeminami D L., Kodavanti U P., Crapo J D., Chang L Y., Pinkerton K E. 2002. Inhibition of Tobacco Smoke-Induced Lung Inflammation by a Catalytic Antioxidant. Free Radical Biology and Medicine. Vol.33, No.8, pp.1106-1114.

Sorensen M., Daneshvar B., Hansen M., et al. 2003. Personal PM2.5 exposure and markers of oxidative stress in blood. *Environ Health Perspect.* 111: 161–166.

Spieker L E., Noll G., Luscher T F. 2001.Therapeutic potential for endothelin receptor antagonists in cardiovascular disorders.Am J Cardiovasc Drugs.1(4):293-303.

Stamler J S., Singel D J., Loscalzo J. 1992. Biochemistry of nitric oxide and its redox-activated forms. Science. Dec 18;258(5090):1898-902.

Steinbeck M J., Khan A U., Karnovsky M J.1993. Extracellular production of singlet oxygen by stimulated macrophages quantified using 9, 10-diphenylanthracene and perylene in a polystyrene film.J Biol Chem. Jul 25; 268(21):15649-54.

Szabo C. 1995. Alterations in nitric oxide production in various forms of circulatory shock. New Horiz. Feb; 3(1):2-32. Review.

Sznajder, J. I., A. Fraiman, J. B. Hall, W. Sanders, G. Schmidt, G. Crawford, A. Nahum, P. Factor, and L. D. 1989. Wood. Increased hydrogen peroxide in the expired breath of patients with acute hypoxemic respiratory failure. *Chest* 96: 606-612, [Abstract].

Tao F., Gonzalez-Flecha B., Kobzik L. 2003. Reactive oxygen species in pulmonary inflammation by ambient particulates. Free Radic Biol Med. Aug 15; 35(4):327-40. Review.

Tarpey M M and Fridovich I. 2001. Methods of Detection of Vascular Reactive Species: Nitric Oxide, Superoxide, Hydrogen Peroxide, and Peroxynitrite. Circ Res 89:224-236.

Tellez-Rojo, M. M., Romieu, I., Ruiz-Velasco, S., Lezana, M. A., and Hernandez-Avila, M. M. 2000. Daily respiratory mortality and PM10 pollution in Mexico City: Importance of considering place of death. *Eur. Respir. J.* **16,** 391–396.

Thomson E., Goegan P., Kumarathasan P., Vincent R. 2004. Air pollutants increase gene expression of the vasoconstrictor endothelin-1 in the lungs.Biochim Biophys Acta. May 24; 1689(1):75-82.

Thomson E., Kumarathasan P., Goegan P., Aubin R A., Vincent R. 2005. Differential regulation of the lung endothelin system by urban particulate matter and ozone.Toxicol Sci. Nov; 88(1):103-13.

Tomoh Masaki. 1993. Endothelins: homeostatic and compensatory actions in the circulatory and endocrine systems Endocr. Rev., Jun 14: 256.

Tsutamoto T., Hisanaga T., Fukai D., Wada A., Maeda Y., Maeda K., Kinoshita M. 1995. Prognostic value of plasma soluble intercellular adhesion molecule-1 and endothelin-1 concentration in patients with chronic congestive heart failure. Am J Cardiol. Oct 15; 76(11):803-8.

Turko I V and Murad F. 2002. Protein nitration in cardiovascular diseases. Pharmacol Rev 54:619-634.

Tzeng, H.P., Yang, R.S., Ueng, T.H., Lin-Shiau, S.Y. and Liu, S.H., 2003. Motorcycle exhaust particulates enhance vasoconstriction in organ culture of rat aortas and involve reactive oxygen species. *Toxicol. Sci.* 75, pp. 66–73.

Ulrich, M.M., Alink, G.M., Kumarathasan, P., Vincent, R., Boere, A.J., Cassee, F.R..2002. Health effects and time course of particulate matter on the cardiopulmonary system in rats with lung in.ammation. J. Toxicol. Environ. Health A 65, 1571–1595.

Vanhoutte P M. 2000. Say NO to ET.J Auton Nerv Syst. Jul 3; 81(1-3):271-7. Review.

Vemulapalli, S., M. Rivelli, P. J. S. Chiu, M. delPrado, and J. A. Hey. 1992. Phosphoramidon abolishes the increases in endothelin-1 release induced by ischemia-hypoxia in isolated perfused guinea pig lungs. *J. Pharmacol. Exp. Ther.* 262: 1062-1069.

Vincent R., Vu D., Hatch G., Poon R., Dreher K., Guenette J., Bjarnason S., Potvin M., Norwood J., McMullen E. 1996. Sensitivity of lungs of aging Fischer 344 rats to ozone: assessment by bronchoalveolar lavage. Am J Physiol. Oct;271(4 Pt 1):L555-65.

Vincent, R., Bjarnason, S. G., Adamson, I. Y., Hedgecock, C., Kumarathasan, P., Guenette, J., Potvin, M., Goegan, P., and Bouthillier, L. 1997a. Acute pulmonary toxicity of urban particulate matter and ozone. *Am. J. Pathol.* **151,** 1563–1570.

Vincent, R., Kumarathasan, P., Goegan, P., Bjarnason, S.G., Guenette, J., Berube, D., Adamson, I.Y., Desjardins, S., Burnett, R.T., Miller, F.J., Battistini, B., 2001a. Inhalation toxicology of urban ambient particulate matter: acute cardiovascular effects in rats. Res. Rep. Health. Eff. Inst.(104), 5–54 discussion 55-62.

Vincent R., Kumarathasan P., Mukherjee B., Gravel C., Bjarnason S., Urch B., Speck M., Brook J., Tarlo S., Zimmerman., Silverman F. 2001b. Exposure to urban particles (PM2.5) causes elevations of the plasma vasopeptides endothelin (ET)-1 and ET-3 in humans. American journal of Respiratory and Critical Care Medicine 163:A313.

Waugh R J and Murphy R C. 1996. Mass spectrometric analysis of four regioisomers of F2-isoprostanes formed by free radical oxidation of arachidonic acid. J Am Soc Mass Spectrom 7:490-499.

Warner T D. 1999.Relationships between the endothelin and nitric oxide pathways. Clin Exp Pharmacol Physiol. Mar; 26(3):247-52.

Warnholtz A and Munzel T. 2000. Why do antioxidants fail to provide clinical benefit? Curr Control Trials Cardiovasc Med.1(1):38-40.

Wei C M., Lerman A., Rodeheffer R J., McGregor C G., Brandt R R., Wright S, Heublein D M., Kao P C., Edwards W D., Burnett J C Jr. 1994. Endothelin in human congestive heart failure.Circulation. Apr; 89(4):1580-6.

White C R., Brock T A., Chang L Y., Crapo J., Briscoe P., Ku D., Bradley W A., Gianturco S H., Gore J., Freeman B A, et al. 1994. Superoxide and peroxynitrite in atherosclerosis.Proc Natl Acad Sci U S A. Feb 1;91(3):1044-8.

Xia Z., Gu J., Ansley D M., Xia F., Yu J. 2003. Antioxidant therapy with Salvia miltiorrhiza decreases plasma endothelin-1 and thromboxane B2 after cardiopulmonary bypass in patients with congenital heart disease.J Thorac Cardiovasc Surg. Nov; 126(5):1404-10.

Xiao G G., Nel A E., Loo J A. 2005. Nitrotyrosine-modified proteins and oxidative stress induced by diesel exhaust particles. Electrophoresis. Jan; 26(1):280-92.

Yanagisawa M., Kurihara H., Kimura S., Goto K., Masaki T.1988.A novel peptide vasoconstrictor, endothelin, is produced by vascular endothelium and modulates smooth muscle Ca2+ channels.J Hypertens Suppl. Dec;6(4):S188-91.

Young I S and Woodside J V. 2001. Antioxidants in health and disease.J Clin Pathol. Mar; 54(3):176-86. Review.

Yu-Chen Leia., Jing-Shiang Hwangb., Chang-Chuan Chana., Chung-Te Leec., Tsun-Jen Cheng. 2005.Enhanced oxidative stress and endothelial dysfunction in streptozotocin-diabetic rats exposed to fine particles Environmental Research 99.335–343.

Yura T., Fukunaga M., Khan R., Nassar G N., Badr K F., Montero. 1999.A Free-radical-generated F2-isoprostane stimulates cell proliferation and endothelin-1 expression on endothelial cells. Kidney Int. Aug; 56(2):471-8.

Zingarelli B., Day B J., Crapo J D., Salzman A L., Szabo C. 1997. The potential role of peroxynitrite in the vascular contractile and cellular energetic failure in endotoxic shock.Br J Pharmacol. Jan; 120(2):259-67.

Zouridakis E G., Schwartzman R., Garcia-Moll X., Cox I D., Fredericks S., Holt DW., Kaski J C. 2001. Increased plasma endothelin levels in angina patients with rapid coronary artery disease progression.Eur Heart J. Sep; 22(17):1578-84.