**INCREASED VASCULAR CONTRACTILITY IN ISOLATED
VESSELS FROM CIGARETTE SMOKING RATS IS MEDIATED BY
BASAL ENDOTHELIN RELEASE**

by

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ABSTRACT

The effect of chronic cigarette smoking on endothelin modulation of vascular contraction and CYP enzyme levels was studied in 20 male Sprague-Dawley rats. The animals were divided equally into smoking and non-smoking groups. The smoking group was exposed to 6 research cigarettes per rat per day 5 days a week for 16 weeks. The control group was sham smoked. Functional contractile studies were performed in aortas and carotid arteries to determine the regulation of vascular tone by basal release of endothelin. Liver samples were analyzed for CYP1A1 and CYP1A2 gene expression by RT-PCR. Plasma samples were assessed for endothelin-1 (ET-1) level by enzyme immunoassay (EIA). Treatment of aortas and carotid arteries with bosentan, the dual endothelin receptor antagonist, caused a significant reduction in constrictor responses of smoking rats, indicating increased regulation of tone by endothelin in smoker rats as compared to controls. There was also a greater expression of the cytochrome P450-liver enzymes (CYP1A1 and CYP1A2) in smoker rats. Body weight gain was also significantly decreased in smoker rats. We conclude that increased endothelin release in smoker rats contributes significantly to increased arterial tone and may therefore contribute to the cardiovascular pathophysiology associated with cigarette smoking, such as increased vascular muscularization, increased contraction, decreased dilation and possibly vasospasm.

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LIST OF SYMBOLS AND ABBREVIATIONS

$[Ca^{2+}]_i$	:	Intracellular calcium
©	:	Copyright
20-HETE	:	20-hydroxyeicosatetraenoic acid
ACh	:	Acetyl choline
AIx	:	Augmentation index
ANOVA	:	Analysis of variance
APC	:	Activated protein C
BH4	:	Tetrahydrobiopterin
BK	:	Bradykinin
BK-Channel	:	Ca^{+} -activated potassium channel
$CaCl_2$	:	Calcium chloride
CAD	:	Coronary artery disease
Calcr1	:	Calcitonin receptor-like
cDNA	:	Complementary deoxy ribonucleic acid
Cfh	:	Complement factor H
cGMP	:	Cyclic guanosine monophosphate
CO	:	Carbon monoxide
CO ₂	:	Carbon dioxide
COPD	:	Chronic obstructive pulmonary disease
CRP	:	C-reactive protein
CSE	:	Cigarette smoke extract
CVD	:	Cardiovascular disease
CYP	:	Cytochrome P450
DMSO	:	Dimethyl sulphoxide
DNA	:	Deoxy ribonucleic acid
dsDNA	:	Double stranded deoxyribonucleic acid
EDTA	:	Ethylene diamine tetraacetic acid

EIA	: Enzyme immunoassay
eNOS	: Endothelial nitric oxide synthase
EPCs	: Endothelial progenitor cells
Epxh2	: Epoxide hydrolase-2
ET	: Endothelin
ET-1	: Endothelin-1
ET-2	: Endothelin-2
ET-3	: Endothelin-3
ET-4	: Endothelin-4
ET _A	: Endothelin _A
ET _{B1}	: Endothelin _{B1}
ET _{B2}	: Endothelin _{B2}
ETS	: Environmental tobacco smoke
FMD	: Flow-mediated dilation
fmol	: Femto mole
GSH	: Glutathion
H ₂ O ₂	: Hydrogen peroxide
HDL	: High density lipoprotein
HUVECs	: Human umbilical vein endothelial cells
ICAM-1	: Intercellular adhesion molecule-1
IL-6	: Interleukin-6
IMT	: Intima-media thickness
JNK	: c-jun n-terminal kinase
K ⁺ -Channel	: Potassium channel
KCl	: Potassium chloride
KH ₂ PO ₄	: Potassium dihydrogen phosphate
LDL	: Low density lipoprotein
L-NAME	: L ^ω -nitro-l-arginine methyl ester
M	: Mole
MDA	: Malondialdehyde
MgSO ₄	: Magnesium sulphate

MMP	:	Matrix metalloproteinase
MMPs	:	Matrix metalloproteinases
mRNA	:	Messenger ribonucleic acid
NaCl	:	Sodium chloride
NaHCO ₃	:	Sodium bicarbonate
NCSS	:	Number cruncher statistical system
NO	:	Nitric oxide
° C	:	Degree Celsius
O ₂	:	Oxygen
O ₂ ⁻	:	Reactive oxygen species
ONOO ⁻	:	Peroxynitrite
PAA	:	Precipitating agent additive
PE	:	Phenylephrine
p ^H	:	Puissance de hydrogen
PH	:	Pulmonary hypertension
PKC	:	Protein kinase C
PASS	:	Power analysis and sample size
RNA	:	Ribonucleic acid
ROS	:	Reactive oxygen species
rRNA	:	Ribosomal ribonucleic acid
RT-PCR	:	Real-time polymerase chain reaction
SE	:	Standard error
SEM	:	Standard error of mean
SHS	:	Second hand smoke
SNP	:	Sodium nitroprusside
SOD	:	Superoxide dismutase
TF	:	Tissue factor
tPA	:	Tissue plasminogen activator
VCAM-1	:	Vascular cell adhesion molecule-1
Vit-C	:	Vitamin C
Vit-E	:	Vitamin D

VSMC : Vascular smooth muscle cell

VSMCs : Vascular smooth muscle cells

μM : Micromole

GLOSSARY

1. Antigen of atherogenesis:

Oxidized-LDL is considered as the antigen of atherogenesis. It has been suggested that immune responses are involved in atherogenesis. This possibility has been evaluated by analyzing immunocompetent cells in a murine model of the disease. Apolipoprotein E knockout (apoE $-/-$) mice are genetically hypercholesterolemic due to targeted disruption of the apolipoprotein E gene and develop severe atherosclerosis. Data obtained from experiments using such mice suggest that CD4⁺ T cells participate in the formation of atherosclerotic lesions in genetically hypercholesterolemic apoE $-/-$ mice, and that immune activation is part of the disease process. Thus it is speculated that a direct link may exist between cholesterol accumulation and T cell activation, possibly by autoimmune responses to modified lipoproteins.

2. Aortic aneurysm:

An aneurysm is a bulge in a blood vessel, much like a bulge on an over-inflated inner tube. Aneurysms are dangerous because they may burst. The aorta, the main artery leading away from the heart, can sometimes develop an aneurysm. Aortic aneurysms usually occur in the abdomen below the kidneys (abdominal aneurysm), but may occur in the chest cavity (thoracic aneurysm). This can happen if the wall of the aorta becomes weakened by build-ups of fatty deposits called plaque. This is called atherosclerosis. Aneurysms may also be due to cigarette smoking or to an inherited disease such as the Marfan syndrome.

3. Aortic systolic pressure:

Systolic pressure is the pressure of blood against the artery walls when the heart

has just finished contracting or pumping out blood. Aortic systolic pressure means the blood pressure produced at the root of the aorta. Measurement of central aortic systolic pressure has been taken directly, or estimated indirectly, and has been shown to be superior to brachial pressure in correlating with severity of existing disease and prediction of subsequent events. In most such studies, pressure has been recorded directly at cardiac catheterization or estimated from the carotid pressure or diameter waveform. Non-invasive methods also have been developed, whereby aortic systolic pressure can be generated from the radial pressure waveform using applanation tonometry and applying a generalized transfer function in a computerized process. Aortic systolic pressure has been shown to predict extent and severity of coronary atherosclerosis, independent of brachial pressure.

4. ApoE mice:

Mice homozygous for the ApoE $-/-$ (Apolipoprotein E knock out) mutation show a marked increase in total plasma cholesterol levels that are unaffected by age or sex. Fatty streaks in the proximal aorta are found at 3 months of age. The lesions increase with age and progress to lesions with less lipid but more elongated cells, typical of a more advanced stage of pre-atherosclerotic lesion.

5. Arterial compliance/vascular compliance:

Compliance is the ability of a blood vessel wall to expand and contract passively with changes in pressure. It is an important property of large arteries and veins. This ability of a vessel to distend with increasing transmural pressure (inside minus outside pressure) is quantified as vessel compliance (C), which is the change in volume (ΔV) divided by the change in pressure (ΔP). $C = \Delta V / \Delta P$.

6. **Arterial stiffness:**

It is the opposite function of compliance. Arterial stiffness increases at higher volumes and pressures.

7. **Atherosclerosis:**

Atherosclerosis is a disease affecting arterial blood vessels. It is commonly referred to as a "hardening" or "furring" of the arteries. It is caused by the formation of multiple plaques within the arteries. Pathologically, the atheromatous plaque is divided into three distinct components: (i) atheroma formation involves the nodular accumulation of a soft, flaky, yellowish material at the center of large plaques, composed of macrophages nearest the lumen of the artery, sometimes with (ii) underlying areas of cholesterol crystals, and possibly also (iii) calcification at the outer base of older/more advanced lesions. The following terms are similar, yet distinct, in both spelling and meaning, and can be easily confused: arteriosclerosis, arteriolosclerosis and atherosclerosis. Arteriosclerosis, is a general term describing any hardening (and loss of elasticity) of medium or large arteries (in Latin, Arterio meaning artery and sclerosis meaning hardening), arteriolosclerosis is arteriosclerosis mainly affecting the arterioles (small arteries), atherosclerosis is a hardening of an artery specifically due to an atheromatous plaque. Therefore, atherosclerosis is a form of arteriosclerosis.

8. **Atherothrombosis:**

Atherothrombosis, characterized by atherosclerotic lesion disruption with superimposed thrombus formation, is the major cause of acute coronary syndromes (ACS) and cardiovascular death. It is the leading cause of mortality in the industrialized world. Atherosclerosis is a diffuse process that starts early in

childhood and progresses asymptotically through adult life. Later in life, it is clinically manifested as coronary artery disease, stroke, transient ischaemic attack, and peripheral arterial disease.

9. **Augmentation Index (AIx):**

The augmentation index is defined as the proportion of central pulse pressure due to the late systolic peak, which is in turn attributed to the reflected pulse wave. The SphygmoCor system uses an empirically generated transfer function to calculate central pressure from the radial pulse waveform, which is measured by a hand-held tonometer. The same equipment can be used also at other superficial arterial sites, carotid and femoral, and by ECG-gating, the time for transmission of the arterial pulse wave between sites is calculated. This measurement, namely pulse wave velocity, is generally accepted as one of the valid estimates of arterial stiffness.

10. **Bad cholesterol:**

LDL is considered as “bad” cholesterol. When too much LDL cholesterol circulates in the blood, it can slowly build up in the inner walls of the arteries that supply the heart and brain. Together with other substances it can form plaque, a thick, hard deposit that can clog those arteries. This condition is known as atherosclerosis. If a clot forms and blocks a narrowed artery, it can cause a heart attack or stroke. The levels of HDL cholesterol and LDL cholesterol in the blood are measured to evaluate the risk of having a heart attack. LDL cholesterol of less than 100 mg/dL is the optimal level. Less than 130 mg/dL is near optimal for most people. A high LDL level (more than 160 mg/dL or 130 mg/dL or above if you have two or more risk factors for cardiovascular disease) reflects an increased risk of heart disease. That's why LDL cholesterol is often called "bad" cholesterol.

11. Calcr1:

A number of endothelial genes undergo dysregulation in response to environmental tobacco smoke. One of those genes is Calcr1 (calcitonin receptor-like). Calcr1 is part of the receptor complex for adrenomedullin (a vasodilator gene) and calcitonin gene-related peptide, thus associated with vasodilator function, which is impaired in cigarette smokers.

12. Cfh:

A number of endothelial genes undergo dysregulation in response to environmental tobacco smoke. One of those genes is Cfh (complement factor H, or adrenomedullin binding protein-1), which in addition to its established role in regulation of complement activation, has been shown to bind to the vasodilator adrenomedullin and modulate its effects, thus associated with vasodilator function, which is impaired in cigarette smokers.

13. CYP 1A1 and CYP1A2:

These are the genes that encode a member of the cytochrome P450 superfamily of enzymes. These are involved in lipid and drug metabolism. These are inhibited by fluoroquinolones and macrolides; induced by aromatic hydrocarbons, including the aromatic components of cigarette smoke. There are 3 main subtypes of CYP1A: M1, M2 and M3.

14. Cytochrome P450 enzymes:

Cytochrome P450 oxidase (abbreviated CYP for mammalian/plant and P450 for bacterial species) is a generic term for a large number of related, but distinct, oxidative enzymes important in animal, plant, and bacterial physiology. The cytochrome P450, a mixed-function monooxygenase, has about 500 amino acids

and a heme (hæm) group at the active site. Mammalian and plant cytochrome P450s use protein cofactors, cytochrome P450 reductase and cytochrome b5, and molecular oxygen (O₂) to function, while bacterial cytochrome P450s, like cytochrome P450 cam, use other protein cofactors to function. More than 6000 distinct cytochrome P450 sequences are known and officially named. In drug metabolism, cytochrome P450 is probably the most important element of Phase I metabolism in mammals.

15. **DNA-Ladder:**

A DNA ladder is a solution of DNA molecules of different lengths used in agarose gel electrophoresis. It is applied to an agarose gel as a reference to estimate the size of unknown DNA molecules. In addition it can be used to approximate the mass of a band by comparison to a special mass ladder. Different DNA ladders are commercially available depending on expected DNA length. The 1kb ladder with fragment ranging from about 0.5 kbp to 10 or 12 kbp and the 100 bp ladder with fragments ranging from 100 bp to just above 1000 bp are the most frequent. DNA ladders are often produced by a suitable restriction digest of a plasmid. There are special DNA ladders for supercoiled DNA and RNA.

16. **Elastic modulus:**

An elastic modulus, or modulus of elasticity, is the mathematical description of an object or substance's tendency to be deformed when a force is applied to it. The elastic modulus of an object is defined as the slope of its stress-strain curve: $\lambda = \text{stress}/\text{strain}$; where λ is the elastic modulus; stress is the force causing the deformation divided by the area to which the force is applied; and strain is the ratio of the change caused by the stress to the original state of the object. Because stress is measured in pascals and strain is a unitless ratio, the units of λ are therefore pascals as well. An alternative definition is that the elastic modulus

is the stress required to cause a sample of the material to double in length. This is not literally true for most materials because the value is far greater than the yield stress of the material or the point where elongation becomes nonlinear but some may find this definition more intuitive.

17. Endothelial dysfunction:

Endothelial dysfunction may be defined as decreased endothelium-dependent vascular relaxation or NO release, and decreased expression or activity of endothelial NO synthase.

18. Mainstream, sidestream and environmental tobacco smoke:

Cigarette smoke that is drawn through the tobacco into an active smoker's mouth is known as mainstream smoke. Sidestream cigarette smoke is the smoke emitted from the burning ends of the cigarette. Environmental tobacco smoke results from the combination of sidestream smoke (85%) and a small fraction of exhausted mainstream smoke (15%) from smokers.

19. Enzyme immunoassay:

An assay that uses an enzyme-bound antibody to detect antigen. The enzyme catalyzes a color reaction when exposed to substrate.

20. Epxh2:

A number of endothelial genes undergo dysregulation in response to environmental tobacco smoke. One of those genes is Epxh2 (epoxide hydrolase-2, or soluble epoxide hydrolase), which catalyzes the hydrolysis of the endogenous vasorelaxant epoxyeicosatrienoic acid (also known as endothelial-derived hyperpolarizing factor). Upregulation of Epxh2 with smoke exposure

suggests an important role in causing or exacerbating smoke exposure-related hypertension.

21. **Good cholesterol:**

HDL is considered as “good” cholesterol. About one-third to one-fourth of blood cholesterol is carried by high-density lipoprotein (HDL). HDL cholesterol is known as the "good" cholesterol because a high level of it seems to protect against heart attack. (Low HDL cholesterol levels [less than 40 mg/dL] increase the risk for heart disease.) Medical experts think that HDL tends to carry cholesterol away from the arteries and back to the liver and excreted from the body. Some experts believe that HDL removes excess cholesterol from plaque in arteries, thus slowing the buildup.

22. **Myogenic tone:**

Myogenic tone originates in the smooth muscle of blood vessels, particularly in small arteries and arterioles. When the lumen of a blood vessel is suddenly expanded, as occurs when intravascular pressure is suddenly increased, the smooth muscles respond by contracting. Conversely, a reduction in intravascular pressure results in smooth muscle relaxation and vasodilation. Electrophysiological studies have shown that vascular smooth muscle cells depolarize when stretched, leading to contraction. Stretching also increases the rate of smooth muscle pacemaker cells that spontaneously undergo depolarization and repolarization. Myogenic tone may play a role in autoregulation of blood flow and in reactive hyperemia. Myogenic behavior has not been clearly identified in all vascular beds, but it has been noted in the splanchnic and renal circulations, and may be present to a small degree in skeletal muscle.

23. Myograph:

The myograph (literal meaning 'muscle writer') is a device we use to measure the force generated by a contracting muscle. Myographs are examples of transducers that convert force into an electrical output that can be displayed on an oscilloscope or a computer monitor.

24. Polymerase chain reaction (PCR):

PCR is a molecular biology technique for enzymatically replicating DNA without using a living organism, such as *E. coli* or yeast. Like amplification using living organisms, the technique allows a small amount of the DNA molecule to be amplified exponentially. However, because it is an *in vitro* technique, it can be performed without restrictions on the form of DNA and it can be extensively modified to perform a wide array of genetic manipulations. PCR is commonly used in medical and biological research labs for a variety of tasks, such as the detection of hereditary diseases, the identification of genetic fingerprints, the diagnosis of infectious diseases, the cloning of genes, paternity testing, and DNA computing.

25. Pulse wave velocity:

Pulse wave velocity is a well-established technique for obtaining a measure of arterial stiffness between two locations in the arterial tree. The velocity of the pulse wave along an artery is dependent on the stiffness of that artery. Most commonly, pulse wave velocity is measured between the carotid and femoral peripheral artery sites in order to provide a measure of aortic stiffness. This aortic pulse wave velocity increases rapidly with age, typically doubling between the ages of 30 and 60. The SphygmoCor Pulse Wave Velocity Vx

System is sold as an add-on to Px System, and measures the velocity of the blood pressure waveform between any two superficial artery sites. It uses a single-lead ECG and then a tonometer to measure the pressure pulse waveform sequentially in the two peripheral artery sites (eg. carotid & femoral). When used in conjunction with the SphygmoCor Px System, the system provides a comprehensive assessment of the clinical significance of the arterial stiffness measured.

26. Reactive oxygen species:

ROS include oxygen ions, free radicals and peroxides both inorganic and organic. They are generally very small molecules and are highly reactive due to the presence of unpaired valence shell electrons. ROSs form as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling. However, during times of environmental stress ROS levels can increase dramatically which can result in significant damage to cell structures. This cumulates into a situation known as oxidative stress. Cells are normally able to defend themselves against ROS damage through the use of enzymes such as superoxide dismutases and catalases. Small molecule antioxidants such as ascorbic acid (vitamin-C), uric acid and glutathione also play important roles as cellular antioxidants.

27. RT-PCR (Real-time polymerase chain reaction):

In molecular biology, real-time polymerase chain reaction, also called quantitative real time polymerase chain reaction (QRT-PCR) or kinetic polymerase chain reaction, is a laboratory technique used to simultaneously quantify and amplify a specific part of a given DNA molecule. It is used to determine whether or not a specific sequence is present in the sample; and if it is present, the number of copies in the sample. It is the real-time version of quantitative polymerase chain reaction (Q-PCR), itself a modification of

polymerase chain reaction. The procedure follows the general pattern of polymerase chain reaction, but the DNA is quantified after each round of amplification; this is the "real-time" aspect of it. Although real-time quantitative polymerase chain reaction is often marketed as RT-PCR, it should not be confused with reverse transcription polymerase chain reaction, also known as RT-PCR.

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Special thanks are owed to my parents; who have first shown me the light of education and supported me throughout my years of education, both morally and financially.

To my parents

CO-AUTHORSHIP STATEMENT

The manuscript chapter of this thesis has co-authorship. Identification and design of the research work was done by me (Mohammad Mahmudur Rahman) along with my supervisor Professor Ismail Laher. The majority of the work described in the manuscript (experimental design, data collection, analysis of results, and the writing of the manuscript) was undertaken by myself, with some consultation with the authors whose names are included in the manuscript. Specifically, Dr. T.K.H. Chang carried out the work on the analysis of cytochrome P450 isotyping by RT-PCR. Others listed on the manuscript assisted with discussions and guidance related to specific aspects of the study.

1 INTRODUCTION

1.1 Introduction

Cigarette smoking is one of the most important preventable risk factors for cardiovascular disease at a global level (Pechacek et al., 2003). Regardless of gender, smokers have a three-fold increased risk of a heart attack. Even lower levels of exposure of only 8 to 10 cigarettes per day doubles the risk of heart attack in smokers, while smoking only one cigarette daily increases the risk by 6% (Teo et al., 2006). The current population of smokers throughout the globe is about 1.3 billion (Thun and da Costa e Silva, 2003). During the last century, 100 million people died worldwide as a result of tobacco-related conditions (Mackay and Ericksen, 2002) and it is expected that during this century, about 1 billion people will die of smoking-related diseases (Peto and Lopez, 2001). Cigarette smoking kills 47 000 Canadians each year through direct smoking or exposure to environmental tobacco smoke (Teo et al., 2006). Epidemiological surveys suggest that diseases of the heart and blood vessels account for over one-third of deaths in cigarette smokers (Leone et al., 1995). Active smoking is associated with an 80% increase of coronary artery disease (CAD) whereas passive smoking with a 30% increase of CAD (Glantz et al., 1991; Law et al., 1997). Thus both active and passive smoking are undoubtedly associated with cardiovascular diseases in both men and women although the particular component(s) of cigarette smoke or the specific mechanism(s) have not yet been fully elucidated. This review chapter focuses on cigarette smoke-induced

vasculopathy and the molecular mechanisms described so far (Figures 1.3). These data were obtained from *in vivo* and *in vitro* studies performed both in humans and experimental animals.

1.2 Structural and functional alterations of blood vessels induced by smoking

Clinical and experimental studies investigating cigarette smoke-induced alterations of the structure and function of blood vessels have utilized large conduit arteries, medium muscular arteries, resistance arteries and various sizes of veins, both in humans and animals. These investigations utilized exposure to acute or chronic cigarette smoke and /or nicotine, and even to chronic passive smoking. Cigarette smoke-induced structural alterations in the vasculature are summarized in Table 1.1.

1.2.1 Effects of smoking on conduit arteries and compliance vessels:

1.2.1.1 Human studies

Cigarette smoking alters the compliance and stiffening of arteries. Arterial compliance, the ability of a blood vessel wall to expand and contract passively with changes in pressure, is an important function of large arteries and veins. Acute cigarette smoking

causes a marked reduction in large elastic (Kool et al., 1993; Giannattasio et al., 1994), medium elastic (Kool et al., 1993) and small-sized (Marc et al., 2000) artery compliance accompanied by increases in blood pressure.

Cigarette smoking changes the flow properties of the blood and the stiffness of the arterial wall, which may explain the arterial damage observed in cigarette smoking hypertensive patients. Cigarette smoking is associated with higher levels of blood and plasma viscosity, hematocrit and pulse wave velocity (Levenson et al., 1987) all of which could damage the vessel. Cigarette smoking also increases the immunoreactivity of thromboplastin, an early marker of atherothrombosis (Matetzky et al., 2000).

Acute cigarette smoking reduces the distensibility of both medium-sized muscular arteries as well as large elastic arteries, thereby causing systemic artery stiffening. Adrenergic mechanisms are most likely responsible for alterations in arterial distensibility (Failla et al., 1997). Acute cigarette smoking increases arterial stiffness in large arteries in healthy young chronic smokers and nonsmokers with a higher aortic systolic blood pressure (Mahmud and Feely, 2003) but these changes are more prominent in chronic smokers (Kim et al., 2005). Chronic tobacco smoking is associated with endothelial dysfunction in arteries exhibiting increased stiffness as evidenced by a greater augmentation index (AIx) in subjects free of other cardiovascular risk factors (Rehill et al., 2006).

Several clinical and experimental studies demonstrate that smoking is associated with increases in the intima media thickness ratio (IMT) or arterial wall thickness in carotid

(Poredos et al., 1999; van den Berkmortel et al., 2000), femoral (van den Berkmortel et al., 2000) and brachial arteries (Esen et al., 2004) of both chronic and acute smokers. Resting blood flow and endothelium-dependent flow-mediated dilation (FMD) of brachial (Lekakis et al., 1997; Poredos et al., 1999; Tanriverdi et al., 2006) and epicardial (Zeicher et al., 1995) arteries are significantly impaired in smokers. Impairment of FMD and increased IMT are related to the duration and the number of cigarettes smoked. Thus smoking is associated with a dose- and/or time-related impairment of FMD and increased IMT of large human arteries. Increased wall thickness and impairment of endothelium-dependent dilation of arteries suggests that cigarette smoking distorts the vessel wall long before atherosclerosis is manifest. Most of these studies were performed in heterogeneous groups of elderly subjects predisposed to atherosclerotic disease or with other cardiovascular risk factors. However, a study performed in otherwise healthy middle-aged smokers demonstrated that cigarette smoking as a single cardiovascular risk factor causes wall thickening in carotid and femoral arteries (van den Berkmortel et al., 2000). This raises the possibility that cigarette smoking is a preventable cause of early atherosclerosis in otherwise healthy subjects.

The aorta acts as both a conduit and an elastic buffering chamber that modulates left ventricular output and coronary blood flow. Both active and passive smoking causes deterioration in the elastic properties of the aorta in humans (Stefanadis et al., 1998). A large epidemiological survey suggests that atherosclerotic disease is associated with a higher risk of abdominal aortic aneurysm, and that cigarette smoking increases the risk of aortic aneurysm independently of atherosclerosis (Lee et al., 1997). These effects could

be due to a loss of elastic properties of the abdominal aorta caused by smoking. The smoking of only one cigarette raises systolic and diastolic blood pressures, increases vascular resistance, impairs baroreflex activity and increases carotid artery wall tension in mild smokers (Arosino et al., 2006).

It is now established that acute and chronic smoking reduces peripheral blood flow and shear stress, contributing to an increased incidence of peripheral arterial disease in sedentary smokers. Studies designed to determine whether physical activity influences peripheral blood flow in chronic smokers demonstrated that basal femoral artery blood flow was ~50% higher in physically active smokers compared to that in sedentary smokers (Anton et al., 2006).

1.2.1.2 Animal studies

The terms acute, short-term and long-term are sometimes not very precise, but from different experimental (animal) models of cigarette smoking it is clear that exposure to one or more cigarettes for one time only is referred to as acute, whereas exposure to one or more cigarettes for few days to few weeks is referred to as short-term, and exposure to one or more cigarettes for few weeks to few months or even years is referred to as long-term or chronic smoking (Guo et al., 2006; Warner et al., 2006; Wright et al., 1997).

In various animal models, chronic exposure to cigarette smoke or acute exposure to cigarette smoke extract (CSE) resulted in impaired endothelium-dependent vasodilation,

increased intimal thickening and/or hyperplasia, atherosclerotic plaques, aneurismal dilation and increased tone in the aorta as well as in other large and medium-sized arteries.

In pig pulmonary arteries, cigarette smoke extract caused a biphasic response in blood vessels with an intact endothelium: relaxation at lower concentrations and contraction at higher concentrations (Holden et al., 1990). Cigarette smoke releases endothelin, which at lower concentrations acts predominantly on ET_{B1} receptors localized on the endothelium producing NO and prostacyclin, resulting in vasorelaxation. The contraction was due to activation of thromboxane A₂ and PKC and to a direct effect of ET-1 on ET_A and ET_{B2} receptors located on the vascular smooth muscle. Both nicotine and cigarette smoke caused endothelium-dependent contraction of intrapulmonary arteries but the contractile response to CSE was greater than with nicotine alone (Holden et al., 1990). We recently reported that chronic exposure to mainstream smoke significantly increased arterial tone through ET-1-dependent mechanisms (Rahman et al., 2006). Chronic exposure to cigarette smoke (Jorge et al., 1995) and acute exposure to cigarette smoke-treated Krebs buffer (Raij et al., 2001) impaired endothelium-dependent vasorelaxation in rat and rabbit aortas (Ota et al., 1997).

Short-term (one week) exposure of Sprague Dawley rats and long-term (five weeks) exposure of apo E deficient mice increased intimal hyperplasia in the rat carotid (Petrik et al., 1995), produced wall thickening in rat pulmonary (He et al., 1991), and augmented thickening in mouse carotid (Tani et al., 2004) arteries, leading to the possibility that

cigarette smoke is a significant risk factor in developing arterial restenosis. Intimal thickening or hyperplasia can also lead to atherothrombotic diseases in smokers. Smoking is known to increase atherothrombotic plaques. Aortic root plaques of apo E deficient mice (genetically hypercholesterolemic) exposed to cigarette smoke had higher immunoreactivity for tissue factor, vascular cell adhesion molecule-1, and macrophages (all features relating to atherosclerosis) compared with non-smoking controls (Matetzky et al., 2000).

Cigarette smoking also increases the risk for developing abdominal aortic aneurysms. Long-term (12 weeks) exposure to cigarette smoke significantly increases (50% greater dilatation in smoking mice than the controls) the progression of aneurismal dilatation of the abdominal aorta of mice, while short-term (2 weeks) exposure was without effect on the initial development of abdominal aortic aneurysms (Buckley et al., 2004). The effects of cigarette smoking may be due to detrimental changes in elastic properties of the aorta.

1.2.2 Effects of smoking on small / resistance arteries:

1.2.2.1 Human studies

There are scant reports on the effects of cigarette smoking in human resistance arteries, reflecting the difficulty in obtaining these vessels for *in vitro* studies. However, a study investigating the effect of long-term smoking on coronary vasomotion and vasodilator

capacity in healthy smokers implicated abnormal endothelial function that was related to the duration of smoking (Campisi et al., 1998). In a more recent study, human middle cerebral arteries obtained at autopsy were incubated with lipid soluble smoke particles for 6 to 48 hours, causing the endothelium to swell and detach from its underlying structures. Lipid soluble smoke particles, but not nicotine, impaired endothelium-dependent dilatation (Zhang et al., 2006).

1.2.2.2 Animal studies

Chronic cigarette smoke in rats reduces coronary blood flow (Jorge et al., 1995) and chronic nicotine exposure decreased cerebral blood flow (Gerzanich et al., 2001). Chronic cigarette smoke increases pulmonary arterial pressure in guinea pigs (Wright et al., 1991) and coronary arterial pressure in mice (Guo et al., 2006). Lipid soluble smoke particles, but not nicotine, impairs endothelium-dependent dilatation in rat mesenteric arteries (Zhang et al., 2006) while the smoking of a single cigarette causes dysfunction of endothelium-dependent, but not endothelium-independent, vasodilation in rat cerebral arteries (Iida et al., 2006).

Smoking a single cigarette causes a biphasic response in the cerebral circulation of rats where there is an initial constriction that is followed by vasodilatation (Iida et al., 1998). Porcine coronary artery exposed to CSE also has a biphasic response (Murohara et al., 1994). The initial increase in tone is likely due to the breakdown of NO by superoxide anions derived from CSE or due to ET-1 effects on ET_A receptors of arterial smooth

muscle cells.

Among the different chemical constituents of cigarette smoke, nicotine is considered the most potent vasoconstrictor. Nicotine exposure potentiated norepinephrine-induced vasoconstriction in hamster cheek pouch arterioles (Mayhan et al., 1999), while the contractility of canine cerebral arteries was increased by nicotine through impairment of endothelial function and activation of PKC activity (Koide et al. 2005). Chronic nicotine exposure blunted NO-induced vasodilation (Gerzanich et al., 2001), suggesting vascular smooth muscle dysfunction as an additional component. This may be due to muscularization by ET-1, a potent mitogen. In a guinea pig model of chronic (up to 1 year) cigarette smoking, there was increased muscularization of the pulmonary arterioles producing pulmonary hypertension (Wright et al., 1991).

Chronic exposure of guinea pigs to cigarette smoke increased endothelin levels and vascular endothelial growth factor expression for the duration of the exposure period. It is likely that cigarette smoking triggers the production of endogenous factors that control long-term artery remodeling and vascular tone (Wright et al., 2004). This remodeling was also evident in rat mesenteric resistance arteries incubated with lipid soluble smoke particles where the arterial endothelium was swollen with loose attachment to underlying structures (Zhang et al., 2006). Cigarette smoking also increased the elastic modulus, wall thickness, and the wall thickness-to-radius ratio in mouse coronary arteries (Guo et al., 2006).

1.2.3 Effects of smoking on veins

The functional consequences of cigarette smoking on venous function have not been extensively studied in either humans or experimental animals. Smoking causes a thickening of the endothelial basal lamina (Higman et al., 1994) and impairs endothelium-dependent relaxation of saphenous veins (Higman et al., 1996; Freischlag et al., 1999). In humans, acute exposure of dorsal hand veins of healthy non-smokers to nicotine is associated with a blunted vasodilatory response to bradykinin, an endothelium-dependent vasodilator (Chalon et al., 2000; Sabha et al., 2000). Loss of endothelial viability due to increased apoptosis is another possible cause of smoking-induced impairment of vasodilation, as shown in isolated human umbilical vein endothelial cells (Wang et al., 2001).

1.3 Smoking and endothelial dysfunction

Cigarette smoking is a cardiovascular disease risk factor that is strongly associated with endothelial dysfunction (Verma and Anderson, 2002). An important manifestation of endothelial dysfunction is decreased endothelial-dependent vasodilatation due to reduced NO bioavailability that is possibly associated with a decreased expression or activity of endothelial NO synthase (eNOS). Endothelium-derived relaxation and/or production of endothelium-derived NO from the amino acid L-arginine by eNOS can be used as an indicator of endothelial function. However, the majority of clinical and experimental studies investigating cigarette smoke-induced endothelial dysfunction have monitored

impairment of endothelium-dependent vasorelaxation (Summarized in Table 2). Some studies report that cigarette smoke reduces eNOS expression and the associated NO production (McVeigh et al., 1996; Su et al., 1998; Wang et al., 2000; Barbera et al., 2001; Barua et al., 2001), while one study shows that whole cigarette smoke, but not nicotine itself, causes endothelial dysfunction (Zhang et al., 2006). However, it is important to stress that the majority of investigations demonstrate that both cigarette smoke and nicotine causing endothelial dysfunction (Table 1.2).

Impaired vasodilation due to reduced stimulated release or attenuated basal production of endothelium-derived nitric oxide is generally regarded as a useful marker of early changes in endothelial cells that can precede morphologic changes (Anggard E, 1994; Cohen et al., 1988). Long-term smokers without clinical evidence of vascular disease have abnormal endothelial function as manifested by a reduction in basal, but not stimulated, nitric oxide-mediated vasodilation (McVeigh et al., 1996). Acute smoking also produces a relatively brief period of endothelial dysfunction, while short-term smoking causes a substantial increase in blood pressure and heart rate along with increased circulating and locally released catecholamines (Lekakis et al., 1997). Both short-term smoking and nicotine chewing gum reduce endothelial-dependent vasodilation in the forearms of young habitual smokers (Sarabi and Lind, 2000). However, a more recent study suggests that lipid soluble smoke particles, but not nicotine, significantly impair endothelium-dependent dilatation in rat mesenteric and human middle cerebral arteries (Zhang et al., 2006).

There is also an isolated study showing that cigarette smoking paradoxically improves (by approximately 5-10%) endothelial-derived vasorelaxation in rat carotid arteries, possibly through hypoxia-induced production of carboxyhemoglobin (Nene et al., 1997).

A surprising finding, although an isolated one, is that smoking increases aortic endothelial regeneration after balloon injury as well as increasing serum nitric oxide levels in rats (Sarker et al., 1999), while a more recent study reports that cigarette smoke impedes the endothelial repair process in pig pulmonary artery cells (Su et al., 2004). In pig pulmonary artery cells, cigarette smoke extracts decreased monolayer wound repair, tube formation, cell migration and proliferation, suggesting that impaired angiogenesis may impede the repair process in the lungs of cigarette smokers and contribute to the altered structural remodeling observed in the lungs of patients with cigarette smoke-related COPD (Su et al., 2004). A new study by Michaud et al (2006) extended the findings of Su et al by demonstrating that endothelial progenitor cell (EPC) dysfunction could contribute to impaired blood vessel healing and growth in smokers. Cigarette smoking reduces the number of EPCs and impedes their differentiation and functional activity (Michaud et al., 2006).

Measuring circulating endothelial modulators can also monitor the interaction of cigarette smoking on endothelial function. For example, cigarette smoking decreases the levels of the antioxidants Vit-C and Vit-E, while it increases E-selectin, ICAM-1, VCAM-1 and ET-1 (Winkelmann et al., 2001; Ueno et al. 2006; Rahman et al, 2006).

1.4 Smoking and endothelin (ET)

The presence of a vasoconstrictor peptide secreted from vascular endothelium was first reported in 1985 (Hickey et al, 1985); thereafter, endothelin, one of the most potent endogenous vasoconstrictors known to date, was isolated from the supernatant of cultured porcine endothelial cells (Yanagisawa et al., 1988). Endothelin is 100 times more potent than norepinephrine and 10 times more potent than angiotensin II on a molar basis. Endothelin has important roles in the pathogenesis of cardiovascular disorders due to its powerful vasoconstrictor and mitogenic properties (Yanagisawa et al., 1988; and Naruse et al., 1994). Since the discovery of endothelin, four isoforms have been described: ET-1, ET-2, ET-3, and ET-4. These peptides are produced in a variety of tissues, including the vascular endothelium and smooth muscle cells, where they act as modulators of vasomotor tone, cell proliferation and hormone production (Luscher et al., 1992).

The major isoform of endothelin acting in the cardiovascular system is ET-1 which is produced by both endothelial cells and vascular smooth muscle cells (Levin et al., 1995). Stimuli for ET-1 release include hypoxia, shear stress, endotoxin, epinephrine, vasopressin, angiotensin II, thrombin, interleukin-1- β and cigarette smoking (Luscher et al., 1992; and Lee et al., 2001). ET-1 acts on three types of G-protein-coupled receptors: ET_A, ET_{B1} and ET_{B2} (Douglas et al., 1995). Of these receptors, ET_A and ET_{B2} receptors are expressed in vascular smooth muscle cells, while ET_{B1} is predominantly located in the vascular endothelium. Endothelin receptors on vascular smooth muscle cells mediate vasoconstriction while those on the endothelium modulate NO and prostacyclin release

(de Nucci et al., 1988; Sakurai et al., 1990; and Arai et al., 1990). Low concentrations of ET-1 act mainly on ET_{B1} receptors to cause vasodilation (de Nucci et al., 1988; Masaki et al., 1995).

Several reports implicate ET-1 activation in cardiovascular and pulmonary tissues as a result of cigarette smoking. Long-term smoking impairs endothelium-dependent vasodilator responses produced by low-concentrations of ET-1, whereas short-term smoking enhances ET-1-induced vasoconstriction in human brachial arteries (Kiowski et al., 1994).

Endothelin is a potent mitogen (Hirata et al., 1989) and can alter smooth muscle cell responses to other vasoactive agents (Thorin et al., 1998). Rabbit airway smooth muscle cell cultures exposed to cigarette smoke extract have increased proliferation via release of ET-1 and its associated autocrine mitogenic actions (Fang et al., 1997). Endothelin release also modulates the effects of acute cigarette smoking on cell proliferation in the rat airways and pulmonary arterial vessels, (Wright et al., 2001) in addition to mediating vasoconstriction in rat bronchioles (Wright et al., 1999) and rat carotid arteries (Rahman et al, 2006).

Acute cigarette smoke exposure (30 minutes) increases expression of ET-1 mRNA in rat hearts and lungs, while chronic exposure (6 months) did not alter the expression of ET-1 mRNA (Adachi et al., 2000). Related to this are findings that the gene expression and content of ET-1 in pulmonary artery endothelium is similar in smoker and control groups

(Barbera et al., 2001) and that ET-1 biosynthesis is similar in nonsmokers, light and heavy smokers (Barua et al., 2002). In contrast, Wright et al (2002) report an upregulation of ET-1 gene expression in pulmonary arteries 2 hours after smoke exposure. It is unclear why chronic cigarette smoking does not lead to increased ET-1 mRNA expression even though plasma levels of ET-1 are often increased (e.g. Rahman et al, 2006)

Although cigarette smoking causes the release of ET-1, it is unclear if this results from augmented ET-1 mRNA. The levels of ET-1 are raised in chronic smokers with hyperlipoproteinemia (Haak et al., 1994_a). Smoking-induced increases in plasma ET-1 are detected within 10 minutes of exposure to smoke (Haak et al., 1994; and Goerre et al., 1995). In addition to nicotine, other smoke components, such as CO or tar, may also be responsible for the increased plasma ET-1 levels in smokers (Goerre et al., 1995). Nicotine alone can stimulate ET-1 production in cultured human endothelial cells within 5 minutes of cigarette smoke exposure (Lee et al., 1999) and increase plasma ET-1 levels in nonsmokers within 15 minutes after chewing nicotine gum (Letizia et al, 1997). As a general rule, plasma ET-1 concentrations in human cigarette smokers are significantly elevated (Orem et al., 2001). The increases in ET-1 are persistent, as shown in a study of nearly 1500 chronic smokers with significantly increased raised plasma immunoreactive ET-1 (Hirai et al, 2004) Cigarette smoking-induced increases in plasma ET-1 may also lead to tissue hypoxemia and decreases in peripheral glucose utilization (Borissova et al., 2004).

In addition to stimulating ET-1 secretion by increasing its gene transcription (Adachi et al

2000) a recent study (Granstrom et al., 2006) also presents evidence that cigarette smoke increases ET_A and ET_B receptors in the smooth muscle cells of rat bronchial segments, possibly through increased translation rather than transcription.

Hashimoto et al (2004) reported that intraportal nicotine infusion in rats inhibits hepatic circulation through ET-1 and ETA and ETB receptors (Hashimoto et al., 2004). This inhibition of hepatic circulation may be due to endothelin-induced vasoconstriction and/or desensitization of vasodilatory mechanism. A subsequent study by Migneault et al (2005) reported that chronic increases in endothelin reduced the pulmonary vasodilator reserve in response to nitric oxide. These results suggest that chronic cigarette smoking causes chronic increases in plasma endothelin, which in turn causes desensitization of the vasodilatory mechanism and enhances the progression of atherosclerosis (Fig 1.1). *In vitro* studies suggest that the binding of ET-1 to its receptor activates protein kinase C (PKC) (Griendling et al., 1989; and Danthuluri et al., 1990). More specifically, ET-1 activates the α isoforms of PKC (PKC- α) in cultures of endothelial cells, thus deranging cellular integrity (Kuklin et al., 2005).

Nitric oxide (NO) produced by the vascular endothelium is a potent vasodilator with antiatherosclerotic properties. On the other hand ET-1, also produced by the vascular endothelium, is a potent vasoconstrictor having mitogenic and proliferative activity on vascular smooth muscle cells. In human athletes, acute exercise (for only 30 minutes) transiently increases plasma ET-1 levels (Maeda et al, 1997) but chronic exercise (6 weeks) significantly decreases the plasma ET-1 levels and increases production of NO

(Maeda et al., 2001), suggesting beneficial effects of exercise on the cardiovascular system. Cigarette smoking-induced increases in plasma ET-1 levels limit both exercise-induced vasodilation and the blood flow redistribution to active muscles (Doutreleau et al., 2004). A recent study designed to investigate whether physical activity influences peripheral blood flow in chronic smokers reports that basal femoral artery blood flow was ~50% higher in physically active smokers compared with sedentary smokers (Anton et al., 2006). Thus, regular exercise could help, at least to some extent, in delaying atherosclerosis and other vasculopathies by increasing NO production and decreasing ET-1 levels in chronic smokers.

1.5. Vasculopathy of passive smoking

Passive smoking, also known as second hand smoke (SHS) or environmental tobacco smoke (ETS), is a preventable risk factor for cardiovascular morbidity and mortality (Taylor et al., 1992; Zhu et al., 1994; and Kritiz et al., 1995). One report suggests that coronary disease mortality is increased by 20% in spouses of smokers, an effect that was ascribed to ETS (Steenland et al., 1996). Of the more than 200 000 yearly deaths due to myocardial infarction in the United States, it is estimated that between 35 000 to 40 000 patients die as result of ETS exposure (Scott et al., 1996), while another estimate is that of the nearly 480 000 smoke-related deaths that occur every year in the United States, about 53 000 are attributable to ETS (Glantz et al., 1991). A study of non-smoking female nurses concluded that regular exposure to passive smoking at home or work increased the risk of coronary heart disease (Kawachi et al., 1997), so that there can be up to a 25%

increase in the risk of coronary heart disease among nonsmokers (He et al., 1999). Even occasional ETS increases the risk of developing an acute coronary syndrome, especially when other risk factors are present (Panagiotakos et al., 2000). High levels of exposure to SHS of 22 hours or more per week can increase the risk of developing a heart attack by nearly 45% (Teo et al., 2006). These epidemiological findings are in keeping with the observation that passive smoking causes a substantial reduction of coronary flow velocity reserve in healthy nonsmokers (Otsuka et al., 2001), as well as leading to a deterioration of the elastic properties of human arteries (Stefanadis et al., 1998). Thus it is clear from environmental and experimental studies that passive smoking is no less harmful than active smoking, with both sharing similar mechanisms. In addition, the particle size present in ETS is smaller than that in the mainstream smoke (Kritz et al., 1995), causing the more dangerous smaller particles ($<2.5 \mu\text{M}$) to be deeply lodged in the bronchioles.

There are several proposed mechanisms by which passive smoking affects the cardiovascular system, including reducing oxygen carrying capacity of blood (Glantz et al., 1995), increasing platelet activation (Glantz et al., 1995), promoting endothelial damage (Celermajer et al., 1996) and accelerating atherosclerosis (Zhu et al., 1993). Passive smoking increases atherosclerosis in the cholesterol-fed rabbit (Zhu et al., 1993) and in cockerels (Penn et al., 1994). Animals exposed to ETS have increased infarct sizes after coronary artery ligation (Prentice et al., 1989; Zhu et al., 1994; Zhu et al., 1996), as well as increased numbers of plaques in aorta (Penn et al., 1993), with a greater atherosclerotic surface area (Zhu et al., 1993; Sun et al., 1994). In nearly 11 000 participant humans at risk for developing atherosclerosis, Howard et al (1998) found that

exposure to active smoke increased carotid intima-media thickness by 50%, while ETS increased this by 20%. Passive smoking causes a dose-related deterioration of endothelium-dependent vasodilation in healthy young adults, suggesting that this early arterial damage may be the starting point of atherosclerotic heart disease (Celermajer et al., 1996).

In utero and neonatal exposure to ETS causes vascular dysfunction in newborn rats. Aortic rings from newborn rats showed reduced maximal endothelium-dependent vasodilation to acetylcholine and also decreased sensitivity to the endothelium-independent vasodilator nitroglycerin, suggesting a detrimental effect of ETS on vascular smooth muscle function (Hutchison et al., 1998). In passive as well as active smokers, coronary arterial dysfunction can be confirmed by quantitative coronary angiography (Sumida et al., 1998). Passive smoking impairs endothelium-dependent relaxation of isolated rabbit thoracic aorta and coronary artery, with the impaired arterial relaxation possibly due to the increased degradation of released NO by superoxide anion derived from cigarette smoke (Torok et al., 2000). Direct evidence for production of free radicals by passive smoking is the raised level of plasma isoprostanes (a reliable marker of *in vivo* oxidative stress) following exposure to second hand smoke (Ahmadzadehfar et al., 2006).

1.6 Molecular mechanisms of vasculopathy induced by cigarette smoking

Cigarette smoking-induced vasculopathy involves different factors, ion channels, and cell signaling pathways (summarized in figure 1.3).

1.6.1 Oxidative Stress

Oxidative damage of cells can result from a combination of the following: 1) an increase in oxidant generation, 2) a decrease in antioxidant protection, or 3) a failure to repair oxidative damage. Oxidative damage is caused by reactive oxygen species (ROS), which includes, but is not limited to, superoxide, singlet oxygen, peroxynitrite or hydrogen peroxide. Under normal conditions, ROS are cleared from the cell by the action of superoxide dismutase (SOD), catalase, or glutathione (GSH) peroxidase. An important component of cell damage results from ROS-induced alterations of macromolecules such as polyunsaturated fatty acids in membrane lipids, essential proteins, and DNA. Most ROS originate from endogenous sources as by-products of normal and essential metabolic reactions, such as energy generation from mitochondria or the detoxification reactions involving hepatic cytochrome P-450 enzymes. Exogenous sources of ROS include exposure to cigarette smoke, environmental pollutants, excessive consumption of alcohol, exposure to ionizing radiation or asbestos, and bacterial, fungal or viral infections (Fiers et al., 1999; Nicholls and Budd, 2000; Hayes and McLellan, 1999).

Cigarette smoke is a rich source of free radicals (Pryor et al., 1993), there being two main categories: gas-phase radicals, which are only stable for a short duration (seconds), and tar-phase radicals, which are stable for hours to months (Smith et al., 2001; Pryor et al., 1993; and Pryor et al., 1998). These tar-phase radicals auto-oxidize to form $O_2^{\cdot -}$ and H_2O_2 in biological fluids (Pryor et al., 1993; and Pryor et al., 1998), and markedly affect NO bioavailability. Cigarette smoking increases vascular superoxide production, resulting in decreased NO bioavailability and increased production of cyclooxygenase-dependent and -independent vasoconstricting eicosanoids (Raij et al., 2001). Human coronary arterial endothelial cells incubated with smokers' serum have significantly lower NO production and eNOS activity but have increased eNOS expression, likely due to an increased production of H_2O_2 (Barua et al., 2003).

Anti-oxidants such as α -tocopherol and ascorbic acid exert differential protective effects on cigarette smoke extract-induced DNA damage and cell adhesion molecule expression in HUVECs (Chen et al., 2004). Cigarette smoke extract-induced inhibition of endothelial-dependent vascular relaxation is attenuated by free radical scavengers such as SOD, DMSO or by captopril, an angiotensin converting enzyme inhibitor. It is likely that captopril attenuates CSE-induced endothelial dysfunction partly through scavenging free radicals (Ota et al., 1997). Short-term exposure of bovine pulmonary arterial endothelial cells to cigarette smoke causes a large increase in superoxide ($O_2^{\cdot -}$) production while acrolin, a stable thiol-reactive agent found in cigarette smoke, increases $O_2^{\cdot -}$ production 5-fold. The effects of acrolin were prevented by prior inhibition of NADPH oxidase, suggesting that thiol-reactive stable compounds in cigarette smoke activate NADPH

oxidase and increase endothelial O_2^- production, thereby reducing NO bioavailability, resulting in endothelial dysfunction (Jaimes et al., 2004). The smoking-induced increase in oxidative stress can be monitored by measuring significantly higher levels of superoxide dismutase (SOD) and malondialdehyde (MDA) and lower levels of reduced glutathione (GSH) in subjects with normal coronary arteries; under these conditions, there is also a deterioration of coronary blood flow (Tanriverdi et al., 2006).

Tetrahydrobiopterin (BH4) is an essential cofactor for eNOS. Cigarette smoke-derived O_2^- reacts with NO giving rise to preoxynitrite (ONOO-), which depletes the BH4 reserve by oxidation (Heitzer et al., 2000). A deficiency of BH4 results in uncoupling of the eNOS enzyme (Figure 1.2), which then produces O_2^- instead of NO (Heitzer et al., 2000; and Vasquez-Vivar et al., 1998). Several studies using either *in vivo* or *in vitro* smoking models confirm that addition of exogenous BH4 will increase NO availability (Higman et al., 1996; Heitzer et al., 2000; and Heitzer et al., 2000; et al., 2000). These studies strongly suggest that free radical-mediated oxidative stress is central to the progression of atherosclerotic changes and vasomotor dysfunction in smokers (Kojda et al., 1999; and Nedeljkovic et al., 2003). Antioxidant agents have been found to either improve or reverse the atherosclerotic conditions in animal models of smoking or in human smokers (Heitzer et al., 2002; Barua et al., 2003; Fennessy et al., 2003)

1.6.2 eNOS expression and NO production

Low concentrations of extracts of cigarette smoke contract isolated porcine coronary

arteries by superoxide anion-mediated degradation of NO, while higher concentrations induce a cyclooxygenase-mediated vasodilation (Moruhara et al., 1994; Holden et al, 1990). In human saphenous veins taken from cigarette smokers, endothelium-dependent relaxation is inhibited due to a reduction in activity of eNOS related to an inadequate supply of the coenzyme tetrahydrobiopterin (Higman et al., 1996). Cigarette smoke decreases cGMP production in cultured endothelial cells (Nagy et al., 1997).

There are conflicting reports on the effects of acute smoking in the cerebral circulation. Smoking a single cigarette caused a biphasic effect on cerebrovascular tone: constriction of pial arterioles at 30 seconds, followed by dilation 5-10 minutes later. The vasodilation was likely due to a combination of nicotine-mediated sympathetic activation, NO production, and K^+ channel activation, while the constrictor response was partially due to thromboxane A2 release since the response was attenuated by seratrodist, a thromboxane A2 receptor antagonist (Iida et al., 1998).

The immunohistochemical expression of eNOS (in pulmonary arterial endothelium) and eNOS protein content (in lung tissue) is lower in smokers (Barbera et al., 2001). In humans, the post partum placenta of smokers has lower eNOS protein levels (Wang et al., 2000). Incubation of pulmonary artery endothelial cells with cigarette smoke extracts decreases eNOS mRNA, protein, and eNOS activity (Su et al., 1998). Thus, cigarette smoking is associated with reduced expression of eNOS, protein content and eNOS activity in arteries and this diminished synthesis of NO contributes to the alterations in the structure and endothelial function of blood vessels. In mice exposed to the long-term (16

weeks) effects of cigarette smoke, there is a reduction of eNOS protein expression in the femoral and carotid arteries (Guo et al., 2006).

There are also discrepant findings related to eNOS expression changes due to cigarette smoking, where rats exposed to cigarette smoke showed increased eNOS mRNA (after 2 days) and protein (after 7 days) in the whole lung (Wright et al., 1999). Likewise, in a study of healthy smokers, there is reduced endothelium-dependent vasodilation, NO generation, and eNOS activity in spite of increased eNOS protein expression (Barua et al., 2001).

Cigarette smoking also has additional effects on vascular excitation-contraction coupling. Cigarette smoking causes an upregulation of voltage-gated Ca^{2+} channels and a downregulation of Ca^{2+} -activated K^+ (BK) channels in rat cerebral arteries, effects that will attenuate NO-mediated dilation (Gerzanich et al., 2001). In canine basilar arteries nicotine potentiates contractile response through PKC activation (Koide et al., 2005).

1.6.3 Smoking and development of atherosclerosis

Cigarette smoking promotes the initiation and progression of atherosclerosis by inhibiting vasodilation, increasing vasoconstriction, stabilizing thrombus, initiating inflammation, and modifying lipid profiles. Cigarette smoking increases superoxide ($\text{O}_2^{\cdot-}$) production (Raij et al., 2001; Barua et al., 2003) and $\text{O}_2^{\cdot-}$ inactivates the primary vasodilator nitric oxide (NO) (Wei et al., 1985; Rubanyi et al., 1986), thereby producing endothelial

dysfunction by reducing NO bioavailability. Levels of superoxides are increased in atherosclerotic vessels (Miller et al., 1998; Hathaway et al., 2002) and inactivation of NO by superoxide plays a key role in endothelial dysfunction of atherosclerotic arteries (Heistad, 2006).

Increased vasoconstriction is another risk factor for atherosclerosis. Increased endothelin production induced by cigarette smoking (Haak et al., 1994; Rahman et al., 2006) leads to vasoconstriction, thereby helping initiation and progression of atherosclerosis.

Cigarette smoking helps in stabilization of thrombin at the site of arterial injury. In normal arteries, activated protein C (APC) is a potent anticoagulant (Esmon and Owen, 1981; Dahlback et al., 2005) which inhibits propagation of thrombus to the arterioles. Cigarette smoking decreases activated protein C levels (Farnandez et al., 2002), thereby allowing propagation of thrombus and thus helping initiation and formation of atherosclerosis down the artery.

Cigarette smoking promotes atherosclerosis by affecting the lipid profile of smokers. Smokers have higher serum cholesterol, triglyceride and LDL levels but lower HDL levels (Sinha et al., 1995; Craig et al., 1989). Cigarette smoke stimulates the release of catecholamine by the adrenal cortex (Andersson et al., 1993), leading to the increased serum concentrations of free fatty acid (FFA) observed in smokers; FFA is a well-known stimulant of hepatic secretion of VLDL and hence triglyceride; and HDL vary inversely with VLDL concentrations in serum (Craig et al., 1989). Cigarette smoking also increases

oxidative modification of plasma LDL (Frei et al., 1991). Circulatory products of lipid peroxidation and autoantibody titers to oxidized LDL (ox-LDL) are significantly increased in smokers (Heitzer et al., 1996). This is why ox-LDL is considered as an antigen for atherosclerosis development (Hansson et al., 1996).

The inflammatory response is an essential component in the initiation and propagation of atherosclerosis. Levels of multiple inflammatory markers including C-reactive protein, interleukin-6, and tumor necrosis factor alpha are increased in cigarette smokers (Tracy et al., 1997; Bermudez et al., 2002; Mendall et al., 1997). Proinflammatory cytokines, e.g., VCAM-1, ICAM-1, E-selectin levels are also increased in cigarette smokers indicating increased leukocyte-endothelial cell interaction producing inflammation (Bermudez et al., 2002; Mazzone et al., 2001). Genetic predisposition has also been found to influence the initiation and progression of atherosclerosis in smokers (Wang et al., 2000; Wang et al., 2002).

1.6.4 Smoking and matrix metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases, which are capable of degrading many extracellular matrix proteins, but they are also capable of processing a number of bioactive molecules. The MMPs play an important role in tissue remodeling associated with various physiological and pathological processes such as morphogenesis, angiogenesis, tissue repair etc. MMP activity is correlated with vascular smooth muscle cell migration and proliferation after vascular injury (Bendeck et al., 1994; Zempo et al.,

1994; Southgate et al., 1996) and inhibition of MMP activity suppresses VSMC proliferation (Bendeck et al., 1994; Zempo et al., 1996). Leukocytes, endothelial cells and VSMCs are the principal sources of MMPs in the vasculature (Chase et al., 2003) and cigarette smoking may activate these vascular sources of MMPs by increasing vascular reactive oxygen species (ROS) and vascular inflammation (Perlstein and Lee, 2006).

Excessive extracellular matrix breakdown is a major determinant of aortic expansion and aneurysm formation (Shah, 1997) and there is much evidence supporting a strong association between cigarette smoking and development of aneurysms (MacSweeney et al., 1994; Alcorn et al., 1996; Chang et al. 1997; Lee et al., 1997; Brown et al., 1999; Vardulaki et al., 2000; Lindholt et al. 2001). The association of smoking with aneurismal subarachnoid hemorrhage also involves MMPs such as MMP-9 which is markedly increased in intracranial aneurysm (Kim et al., 1997). Cigarette smoke induces chronic inflammation and the increased local production of MMPs results in pulmonary emphysema (Shapiro, 1995; Shapiro, 2000; Shapiro, 2002) and aortic aneurysms in smokers (Koch et al., 1990; Thompson et al., 1996) through the degradation of the extracellular matrix, accelerated destructive remodeling of elastin, collagen and other structural proteins (Buckley et al., 2004).

The effects of cigarette smoking as a source of ROS and the associated decreases in NO level in smokers' vasculature have been described above. Increased ROS (Galis et al., 1998) and decreased NO (Chen et al., 2004) induce MMP transcription, thus establishing a central role of cigarette smoking in MMP transcription. ROS activates the latent pro-

forms of MMPs (Rajagopalan et al., 1996) and NO inhibits MMP activation (Phillips et al., 2004). Moreover, plasmin activates MMPs, and smoking is associated with increased plasminogen activator levels (Lindholt et al., 2003). On the contrary, it has also been counter-intuitively reported that ROS induces tissue inhibitor of MMPs activity (Li et al., 2004). Thus the role of MMP in smoking-related vascular disease is complex and still not clear. Additional data on the role of MMPs and vascular damage may provide important clues to the therapeutic approach for vascular disease among smokers.

1.6.5 Smoking and cell signaling pathways

The gaseous components of cigarette smoke destroy or inactivate the thiol-containing guanylate cyclase in endothelial cells, making it possible that externally added thiols (e.g., glutathione, GSH etc.) may be able to protect endothelial cells by binding to unknown components of cigarette smoke (Nagy et al., 1997). Cigarette smoke and its formaldehyde components are able to attenuate the release of NO by reducing receptor-activated increases in endothelial Ca^{2+} (Mazak et al., 2002).

1.6.6 Smoking and Apoptosis

Cigarette smoke activates caspase-3 to induce apoptosis of human umbilical vein endothelial cells (Wang et al., 2001). Stimulation of c-jun n-terminal kinase (JNK), or stress-activated protein kinase, is important in the cellular response to environmental

stress and pro-inflammatory cytokines. The JNK pathway is activated when cells are exposed to noxious stimuli, and is also involved in cell differentiation and apoptosis (Kuo et al, 2005). Consistent with this is the observation that cigarette smoke-induced apoptosis of vascular endothelial cells occurs through the JNK pathway that is activated, at least partially, by oxidative stress. Cigarette smoke-induced activation of JNK phosphorylation and endothelial cell injuries are both inhibited by superoxide dismutase and catalase (Hoshino et al., 2005).

1.6.7 Smoking, gene modulation and vascular immune modulation

Treatment of human primary arterial endothelial cells with nicotine at concentrations similar to those in the blood of smokers results in increased mRNA levels of eNOS, angiotensin-1 converting enzyme, tissue type plasminogen activator, plasminogen activator inhibitor-1, von Willebrand factor, and vascular cell adhesion molecule-1. Thus nicotine alters the expression of a number of endothelial genes whose products play major roles in regulating vascular tone and thrombogenicity (Zhang et al., 2001).

Cigarette smoking dysregulates a number of endothelial genes, some with known or potential relevance to vasoregulation, including soluble epoxide hydrolase or epoxide hydrolase-2 (Epxh2), complement factor H (Cfh) or adrenomedullin binding protein-1, and calcitonin receptor-like (Calcrl). Epxh2 catalyzes the hydrolysis of the endothelial-derived hyperpolarizing factor epoxyeicosatrienoic acid (Imig et al., 2002). Cfh binds to the vasodilator adrenomedullin and modulates its effects (Zhou et al., 2002), and Calcrl is

part of the receptor complex for adrenomedullin and calcitonin gene-related peptide (Hay et al., 2002). Endothelial cells collected from tobacco smoke-exposed mouse aortas show a more than three-fold upregulation of complement factor H, calcitonin receptor-like, and soluble epoxide hydrolase; thus these changes may contribute to the hypertensive response during cigarette smoking (Maresh et al., 2005). In apoE deficient mice that have been fed a Western diet and exposed to cigarette smoke for 5 weeks, carotid artery cuffing produced increased intimal thickening (Tani et al, 2004). Immune modulation by cigarette smoke, as characterized by aberrant antibody responses to oxidized LDL and reduced lymphotoxin β mRNA expression, was associated with increased intimal thickening (Tani et al., 2004).

1.6.8 Smoking, tissue factors and cell injury

Effective endogenous fibrinolysis requires rapid release of tissue plasminogen activator (tPA) from the vascular endothelium. Cigarette smoking inhibits tPA release in human smokers, thus increasing the risk of atherothrombosis in smokers through a reduction in fibrinolytic capacity (Newby et al., 1999). Chronic exposure to cigarette smoke both in apoE deficient mice and human subjects increases tissue factor (TF) expression and thrombogenicity, representing additional mechanisms for the increased risk of atherothrombotic events in smokers (Matetzky et al., 2000). Long-term exposure to cigarette smoke leads to increased experimental aneurismal dilatation of mouse abdominal aorta, possibly caused by smoking-induced inhibition of connective tissue

repair processes within the aortic wall (Buckley et al., 2004).

1.7 Hypothesis and specific objectives

Based on the above-mentioned evidence, the central hypothesis of my thesis work is that increased endothelin (ET-1) and decreased NO bioavailability contribute to increased vascular contractility in cigarette smoking rats.

The following objectives are addressed experimentally:

- To examine the effect of smoking on vascular contractility.
- To evaluate basal NO levels.
- To examine stimulated NO production.
- To determine the effect of smoking on liver enzyme gene expression (CYP 1A1, 1A2).
- To measure plasma ET-1 levels.
- To evaluate the effect of smoking on body weight.

1.8 Tables

Table 1. 1 Cigarette smoke-induced structural alterations in the vasculature.

Blood vessel	Structural alteration	Species	Mode of exposure	Reference
<u>Active smoking:</u>				
Aorta	Aneurysm	Human	Chronic Smoking	Lee et al., 1997
Aorta	↓Elastic properties	Human	Chronic Smoking	Stefanadis et al., 1998
Aorta	Aneurysm	Mouse	Chronic Smoking	Buckley et al., 2004
Aorta root	↑ atherosclerotic plaques	Mouse	Chronic Smoking	Matetzky et al., 2000
Brachial a.	↑ IMT	Human	Chronic Smoking	Esen et al., 2004
Carotid a.	↑ IMT	Human	Chronic Smoking	Poredos et al., 1999
Carotid a.	↑ atherosclerotic plaques	Human	Chronic Smoking	Matetzky et al., 2000
Carotid a.	↑ wall thickening	Human	Chronic Smoking	van den Bergmortel et al., 2000
Carotid a.	Intimal hyperplasia	Rat	Chronic Smoking	Petric et al., 1995
Carotid a.	↑ intimal thickening	Mouse	Chronic Smoking	Tani et al., 2004
Cerebral a.	Swollen and loose endothelium	Human	Acute smoking	Zhang et al., 2006
Coronary a.	↑ wall thickening	Mouse	Chronic Smoking	Guo et al., 2006
Epicardial a.	↑ wall thickening & atherosclerosis	Human	Chronic Smoking	Zeiber et al., 1995
Femoral a.	↑ wall thickening	Human	Chronic Smoking	van den Bergmortel et al., 2000
Mesenteric a.	Swollen endothelium	Rat	Acute smoking	Zhang et al., 2006
Pulmonary a.	↑ wall thickening	Rat	Chronic Smoking	He et al., 1991
Pulmonary al.	↑ muscularization	Guineapig	Chronic Smoking	Wright et al., 1991
Saphenous v.	Thickened endothelium	Human	Chronic Smoking	Higman et al., 1994
<u>Passive smoking:</u>				
Aorta	Atherosclerosis	Rabbit	Chronic Smoking	Zhu et al., 1993
Aorta	Atherosclerosis	Rabbit	Chronic Smoking	Sun et al., 1994

Blood vessel	Structural alteration	Species	Mode of exposure	Reference
Aorta	Deterioration of elastic properties	Human	Chronic Smoking	Stefanadis et al., 1998
Aorta	Atherosclerotic plaques	Cockerel	Chronic Smoking	Penn et al., 1993
Brachial a.	Endothelial cell damage	Human	Chronic Smoking	Celermajer et al., 1996
Carotid a.	↑ IMT	Human	Chronic Smoking	Howard et al., 1998
Pulmonary a.	Atherosclerosis	Rabbit	Chronic Smoking	Zhu et al., 1993
Pulmonary a.	Atherosclerosis	Rabbit	Chronic Smoking	Sun et al., 1994

IMT=intima-media thickness, al.= arteriole, a.= artery, v.= vein, ↑ =increased, ↓=damage.

Table 1. 2 Cigarette smoke-induced impairment of endothelium-dependent vasodilation.

Vessel	Exposure	Species	Reference
<u>Active smoking:</u>			
Aorta	Chronic smoking	Rat	Jorge et al., 1995
Aorta	Acute smoking	Rat	Raij et al., 2001
Aorta	Acute smoking	Rabbit	Ota et al., 1997
Brachial a.	Acute smoking	Human	Lekakis et al., 1995
Brachial a.	Chronic smoking	Human	Tanriverdi et al., 2006
Brachial a.	Chronic smoking	Human	McVeigh et al., 1996
Brachial a.	Acute smoking	Human	Lekakis et al., 1997
Brachial a.	Acute smoking	Human	Sarabi and Lind, 2000
Brachial a.	Acute nicotine	Human	Sarabi and Lind, 2000
Cerebral a.	Acute smoking	Rat	Iida et al., 2006
Dorsal hand v.	Acute nicotine	Human	Chalon et al., 2000
Dorsal hand v.	Acute nicotine	Human	Sabha et al., 2000
Epicardial a.	Chronic smoking	Human	Zeiber et al., 1995
Femoral a.	Chronic smoking	Rabbit	Freischlag et al., 1999
Cerebral a	Acute smoking	Human	Zhang et al., 2006
Mesenteric a.	Acute smoking	Rat	Zhang et al., 2006
Saphenous v.	Chronic smoking	Human	Higman et al., 1996
<u>Passive smoking:</u>			
Aorta	Chronic smoking	Rat	Hutchison et al., 1998
Aorta	Chronic smoking	Rabbit	Torok et al., 2000
Brachial a.	Chronic smoking	Human	Celermajer et al., 1996
Coronary a.	Chronic smoking	Human	Sumida et al., 1998
Coronary a.	Chronic smoking	Human	Otsuka et al., 2001

1.9 Figures

Figure 1.1 Smoking induced release of endothelin (ET-1) activates a number of events that leads to dysfunction of the cardiovascular system.

Figure 1. 2 Uncoupling of eNOS leads to increased oxidative stress. eNOS= endothelial nitric oxide synthase, ONOO⁻ = Peroxynitrite, NO = nitric oxide, O₂^{•-} = superoxide anion, ↑ = increase/ upregulation, ↓ = decrease/downregulation, BH4= tetrahydrobiopterin.

Figure 1. 3 Possible mechanisms of cigarette smoke-induced vasculopathy. cGMP= cyclic guanosin monophosphate, eNOS= endothelial nitric oxide synthase, TXA₂= thromboxane A₂, PKC= protein kinase C, ET-1= endothelin-1, NO= nitric oxide, O₂⁻ = superoxide anion, JNK= c-Jun N-terminal Kinase, tPA = tissue plasminogern activator, TF = tissue factor, EDRF = endothelial derived relaxing factor, EDR = endothelial derived relaxation, ↑ = increase/upregulation, ↓ = decrease/downregulation.

1.10 References

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2 MANUSCRIPT CHAPTER¹

INCREASED VASCULAR CONTRACTILITY IN ISOLATED VESSELS FROM CIGARETTE SMOKING RATS IS MEDIATED BY BASAL ENDOTHELIN RELEASE

2.1 Introduction

Cigarette smoking is a major risk factor in the etiology of cardiovascular diseases (Raij et al., 2001). For example, about 30% of Canadians smoke regularly (Survey: Health Canada, 1994) and more than 16 000 smoking related deaths occur in Canada each year (Illing et al., 1991), while in the United States cigarette smoking is responsible for more than 200 deaths daily (Czermin and Waldherr, 2003). It is estimated that cigarette smoking accounts for about 76% of all peripheral vascular disease (Cole et al., 1993) and overshadows all other major risk factors, including diabetes, obesity, hypertension, blood-lipid abnormalities and blood clotting disorders (Castledon et al., 1981).

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It is estimated that when cigarettes are smoked, more than 4 000 different chemicals are formed, and are either inhaled through the cigarette as mainstream smoke or from the air as side stream or secondhand smoke (Jonas et al., 1992). The many smoke constituents cause cardiovascular and cerebrovascular diseases. All tissues and organs are susceptible to the harmful effects of cigarette smoking, with organs of the pulmonary and cardiovascular system being particularly vulnerable (Sin and Mann, 2003). Cigarette smoking presents a particularly important risk factor for atherosclerosis and hypertension by virtue of increases in plasma levels of oxidized lipids (McBride, 1992).

Cigarette smoking consistently impairs endothelial-dependent vasodilation in humans (Poredos et al., 1999; Ueda et al., 2000; Campisi et al., 1998; Campisi et al., 1999) and animals (Nene et al., 1997; Ota et al., 1997; Mayhan, 1999; Mayhan and Patel, 1997). What is less clear is the vascular effect of cigarette smoking on the release of endothelin, a potent vasoconstrictor and mitogen. Endothelin mediates the effects of acute cigarette smoke exposure on cell proliferation of rat airways and pulmonary arterial vessels (Wright et al. 2001). Following chronic cigarette smoking, increases in endothelin levels may play an important role in the pathogenesis of atherosclerosis (Haak et al., 1994). It is thought that in addition to the nicotine present in cigarette smoke, other components such as, carbon monoxide and tar, can also increase plasma endothelin levels (Goerre et al., 1995).

Cigarette smoking raises plasma levels of endothelin in humans (Haak et al., 1994; Goerre et al., 1995) and rats (Wright et al., 2001), possibly by causing an up-regulation of

gene expression of endothelin synthetic enzymes (Wright et al., 2002). However, there are no reports examining the role of endothelin release on vascular contractile activity in an animal model of cigarette smoking. In this study, we show for the first time that increased vascular constriction caused by cigarette smoking may in part be due to augmented release and or activity of endogenous endothelin as recorded by significantly increased plasma ET-1 levels in smoking rats in addition to a greater inhibition of arterial contractility by bosentan. Significantly increased plasma ET-1 levels in smoking rats also strengthened this finding. In our study, we measured mRNA expression of CYP1A1 and CYP1A2 to confirm that our smoking protocol produced functional alterations and also to find out any link between these cytochrome enzymes and ET-1-mediated vasoconstriction.

2.2 Methods and materials

2.2.1 Animals

Twenty Sprague–Dawley rats (weighing 35–40 g) were purchased from the UBC Lab Animal Center and randomly divided into smoking and non-smoking, control groups. All protocols were approved by the Animal Care Committee of the University of British Columbia (Appendix A). The rats used in this study were housed in a temperature- and

humidity-controlled environment with a 12-hour light–dark cycle and had free access to food and water. Body weights were recorded on a weekly basis for the duration of this study.

2.2.2 Cigarette smoke exposure

After 1 week of acclimatization, rats in the S group were exposed to cigarette smoke by a standard cigarette-smoking machine designed by Hogg (Simani et al., 1974). Rats were exposed to 6 University of Kentucky 1R3F research grade cigarettes (Appendix B) per rat per day for 5 days a week for 16 weeks. This level of exposure leads to carboxy-hemoglobin levels of ~5% (Churg et al., 1987), levels that are similar to those occurring in human smokers (Prignot, 1987). The smoking machine utilized a nose-only chamber where the eyes were protected and the machine allowed for 20 ml puffs to be drawn from each cigarette. The rate of smoking was standardized at 10 puffs per cigarette and delivered at a rate of 1 puff per minute with a cycle of 15 seconds of smoke and 45 of air per minute. The rats were given a 10-minute rest period between cigarettes. Group C was sham-smoked.

2.2.3 Vessel contraction studies

After 16 weeks of cigarette smoke exposure, rats were injected with sodium pentobarbital (30 mg/kg) and heparin (500 U/kg.) by the intraperitoneal route. After loss of all reflexes, rats were decapitated and the thoracic aortas and the carotid arteries were collected from each animal and placed in ice-cold (4 °C) physiological salt saline (PSS) until use.

2.2.3.1. Wire myograph

The aortas and carotid arteries were cleaned of connective and adipose tissue and cut into 2-mm rings, with special care being taken to not damage the endothelium. The aortic and carotid arterial rings were mounted on tungsten wires (diameter 40 µm) and placed in separate wire myograph chambers (Multi Myograph Model 610 M, Danish Myotech, Aarhus, Denmark). The chambers had a 5-ml capacity and were filled with physiological salt solution (PSS) maintained at 37 °C and continuously aerated with a gas mixture containing 95% O₂ and 5% CO₂. During the equilibration, the resting tension was gradually increased to 10 mN for aortas and 7 mN for carotid arteries, so that the vessels remained stable at this optimal basal tension for 60 min, during which time the bath solution was changed at 15-minute intervals. The optimal basal tensions were established from preliminary length-active tension curve. The force generated reported in our work is

the force generated in addition to the baseline tension applied to the vessels.

To both aorta and carotid artery preparations phenylephrine (10^{-9} M to 10^{-5} M) was added in a cumulative manner to generate contraction-response curves. After refreshing the PSS several times to re-establish baseline tensions, the vessels were incubated with 10 μ M bosentan for 30 min. A concentration-response curve to phenylephrine (10^{-9} M to 10^{-5} M) was generated again. To investigate the effects of N^o-nitro-L-arginine methylester (L-NAME), aortic rings were pre-treated with this compound (L-NAME, 10 μ M) for 30 min. Similar to the study with bosentan; concentration-response curves to phenylephrine (10^{-9} M to 10^{-5} M) were generated before and after incubation with L-NAME.

2.2.4. CYP1A1 and CYP1A2 gene expression measured by reverse transcription and real-time polymerase chain reaction (RT-PCR)

Liver samples were collected (500-700mg) in small plastic tubes, snap frozen in liquid nitrogen and kept at -80°C until further use. Total cellular RNA was isolated using Trizol reagent (Chang et al., 2006), and total RNA concentration was determined using the RiboGreen RNA Quantitation Kit (Jones et al., 1998). Reverse transcription was conducted with Superscript II reverse transcriptase (Chang *et al.*, 2006) and total cDNA

concentration was determined using the PicoGreen dsDNA Quantitation Kit (Singer et al., 1997). The sequences of the forward and reverse primers used to amplify CYP1A1 cDNA (GenBank accession number X00469) were 5'-CTG-GTT-CTG-GAT-ACC-CAG-CTG-3' and 5'-CCT-AGG-GTT-GGT-TAC-CAG-G-3', respectively (Borlak and Thum, 2001). The sequences of the forward and reverse primers used to amplify CYP1A2 cDNA (GenBank accession number K02422) were 5'-TCC-CTC-AGG-AGA-AGA-TTG-T-3' and 5'-ACC-TGC-CAC-TGG-TTT-ATG-3', respectively (Ma et al., 2003). The sequences of the forward and reverse primers used to amplify the cDNA of 18s rRNA (GenBank accession number V01270) were 5'-CTT-TGG-TCG-CTC-GCT-CCT-C-3' and 5'-CTG-ACC-GGG-TTG-GTT-TTG-AT-3', respectively (Proudnikov et al., 2003). PCR was performed using a real-time DNA thermal cycler (Light Cycler, Roche Diagnostics, Mannheim, Germany). Each 20 μ l PCR reaction mixture contained 1 $\times$ PCR buffer (20 mM Tris-HCl, pH 8.4, and 50 mM KCl), 1 unit Platinum *Taq* DNA polymerase, 3 mM magnesium chloride, 10 ng of total cDNA, 200 μ M deoxynucleoside-5'-triphosphate mix, 0.2 μ M each of the forward and reverse primers, 0.25 mg/ml bovine serum albumin, and 2 μ l of a 3.3 $\times$ SYBR green I solution. The amplification conditions were: a) 95°C for 1 s, 56°C for 6 s, and 72°C for 14 s (CYP1A1); b) 95°C for 1 s, 53°C for 6 s, and 72°C for 15 s (CYP1A2); and c) 95°C for 1 s, 61°C for 6 s, and 72°C for 10 s (18s rRNA). In all cases, the initial denaturation was performed at 95°C for 5 min. Calibration curves were constructed with known amounts of the respective purified cDNA. The level of CYP1A1 and CYP1A2 gene expression was normalized to that of 18s rRNA.

2.2.5. Quantitative assay of plasma ET-1 levels

EDTA-plasma samples from non-smoking and smoking rats were precipitated before determination of ET-1 levels by enzyme immunoassay (EIA) according to the manufacturer's instructions (Biomedica, distributed by Medicorp, Montreal, Quebec, Canada). The detailed methodology of ET-1 measurement is presented in Appendix C.

2.2.6. Solutions and chemicals

The composition of the PSS was (in mM): NaCl 119, KCl 4.7, KH_2PO_4 1.18, NaHCO_3 24, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.17, CaCl_2 1.6, glucose 5.5 and EDTA 0.026; the pH was adjusted at 7.4. For 80 mM KCl buffer the NaCl in PSS was substituted on an equal molar basis by KCl. All electrolytes, glucose, acetylcholine, L-NAME, and phenylephrine were purchased from Sigma while bosentan was a gift from Actelion Ltd., Switzerland.

2.2.7. Statistical analysis

All results are expressed as mean $\pm$ S.E. of n experiments. Data were analyzed with NCSS 2000 and PASS 2000 software using analysis of variance (ANOVA) and/or

repeated-measures ANOVA with Bonferroni's multiple comparison tests when appropriate. Graphpad Prism, version 3.02, was used to calculate concentration-response of drugs and also to perform unpaired *t*-test comparison for plasma ET-1 levels between control and smoking groups. The results of statistical tests were considered statistically significant at $P < 0.05$.

2.3 Results

2.3.1 Effects of cigarette smoking on body weight gain

Body weight gain of the smoking group was significantly reduced within 7 weeks of cigarette smoke exposure (308.66 ± 7.85 vs. 353.86 ± 7.65 for smoking and control groups, respectively, $P < 0.01$). At the 7th week, the average body weight of the smoking group was 12.77% less than the control group and this difference increased to 23.64% at the end of 16th week (458.91 ± 12.91 vs. 600.96 ± 10.09 for smoking and control group, respectively, $P < 0.0001$) (Figure 2.1).

2.3.2. Vasomotor response of aorta

The maximal contraction in response to adrenergic receptor stimulation with phenylephrine in smoker rat aortas markedly exceeded that of control rat aortas (Figure 2.2). The inhibition of NO production with L-NAME (10 μ M) resulted in a nearly three-fold increase in the maximal contraction in control aortas and 10–15% increase in smoker aortas (Figure 2.3). An augmented sensitivity to PE of smoker rat aortas is demonstrated in Figure 2.2. Pretreatment of smoker and control aortas with L-NAME resulted in leftward shifts of the concentration-response curve while at the same time increasing the maximal response to phenylephrine (in smoker rats), indicating decreased basal nitric oxide production in smoker rats (Figure 2.3).

The endothelium produces vasoconstrictors such as endothelin (Yanagisawa et al., 1988 and Masaki, 1998) and we examined the effect of cigarette smoking on endogenous endothelin-mediated modulation of vascular contractility. For this, phenylephrine-induced concentration-response curves obtained in the absence and presence of bosentan were compared.

2.3.3 Vasomotor response of the carotid artery

Carotid arteries from smoker rats generated greater contraction in response to adrenergic receptor stimulation with phenylephrine than those from control rats (Figure 2.4). Incubation with bosentan caused a greater reduction in phenylephrine induced contraction in smoker rat carotid arteries than in carotid arteries obtained from control rats, suggesting a greater role for endothelin release under basal conditions in the cigarette smoking groups.

2.3.4 Liver enzyme CYP 1A1 and CYP 1A2 gene expression

Liver samples were analyzed for CYP1A1 and CYP1A2 gene expression by real-time PCR and the data normalized to 18s rRNA (Figure 2.5). Based on real-time PCR, CYP1A1 and CYP1A2 gene expression was 25.13-fold and 13.95-fold greater in the smoking group compared to the control group, and the increases were significant for CYP 1A1 ($P < 0.001$) as well as for CYP 1A2 ($P < 0.05$) (Table 2.1).

2.3.5 Plasma ET-1 levels

Plasma ET-1 levels in smoking rats were 2.825 ± 0.070 fmol/ml, a value significantly higher ($P < 0.0001$) than that in non-smoking control rats (0.7906 ± 0.058 fmol/ml) (Figure 2.6).

2.4 Discussion

2.4.1 Vasoconstriction study

This study demonstrates vascular dysfunction in cigarette smoking rats. This dysfunction is manifested by an increased contractile response of vascular smooth muscle to α -adrenergic stimulation and a decreased regulation of vascular tone by reduced release of basal NO level in the aorta of smoker rats. The reduced body weight gain and increased mRNA expression of CYP1A1 and CYP1A2 in the smoker rats confirm systemic effects to cigarette smoke in these rats.

Cigarette smoking directly elevates plasma leptin concentrations (Nicklas et al., 1999); leptin stimulates smooth muscle cell proliferation and migration (Oda et al., 2001 and Goetz et al., 2002) and vascular wall calcification (Parhami et al., 2001). The increased leptin stimulation of vascular smooth muscle cells leads to cell proliferation and migration, and may contribute, at least in part, to the increased vascular contractility in the smoker rats.

Increased vascular tone in the cigarette smoker rats may be secondary to both an abnormal response of smooth muscle and endothelial dysfunction. Decreased levels of NO bioavailability in smoker rats vasculature may be due to reduced NO synthesis in the endothelium and/or to enhanced NO breakdown. The increased reactive oxygen species produced in smoker rats (Church et al., 1985; and Chow et al., 1998) could scavenge NO (Clark et al. 2005) and thus indirectly lead to augmented vasoconstriction.

Vascular contraction to α -adrenergic-activated contractile response can be mediated by the endothelium-dependent vascular production of endothelin (Arikawa et al., 2001) or angiotensin II (Lemos, 2002; Iida et al., 1998). Thus, the increased vasoconstriction in the smoker rats may not be due only to decreased basal NO but could also be the result of enhanced sensitization of smooth muscle to adrenergic stimulation produced by the endogenous release of endothelin (Henrion and Laher, 1993). Our results from the quantitative assay of plasma levels of endothelin also strengthened this hypothesis.

Smoking is known to increase oxidative stress (Heitzer et al., 2000; Vasquez et al., 1998) and reactive oxygen species induce NO breakdown (Nagy et al., 1997; Ota et al., 1997; Su et al., 1998; Higman et al., 1996) while also increasing contractile prostaglandin production (Yang et al., 2002). Thus, in smoker rats the effect of increased endothelin release at rest coupled with a reduced basal production of NO would lead to increased vascular tone. Other effects of reactive oxygen species such as altering Ca^{2+} extrusion (Liu et al., 2002; Grover et al., 1999), increasing intracellular calcium ($[\text{Ca}^{2+}]_i$) (Li et al., 1999) and reducing K^+ channel activity (Iida et al., 1998) can also increase vascular

reactivity.

2.4.2 Effect of chronic smoking on CYP1A1 and CYP1A2 gene expression

Tobacco smoke is a mixture of numerous cytochrome P450 substrates, inducers and inhibitors, which can modify cytochrome P450s expression (Czekaj et al., 2005). In our study, we measured CYP1A1 and CYP1A2 gene expression in smoking and control rats to confirm that our smoking protocol produced functional changes, and we found significantly increased expressions of both CYP1A1 and CYP1A2 mRNA. Previous studies have reported that short-term exposure to tobacco smoke-induced both CYP1A1 and CYP1A2 expression (Kawamoto et al., 1993), but that longer exposures (up to 8 weeks) predominantly induced CYP1A1 levels (Wardlaw et al., 1998). Another study has shown that CYP1A1 was preferentially induced by short- and long-term (up to 16 weeks) tobacco smoke exposure (Mori et al., 2003). We exposed the rats for 16 weeks to cigarette smoke and determined that the CYP1A1 mRNA expression was increased 25-fold while CYP1A2 mRNA expression was 13-fold higher. The expressions of cigarette smoke-induced liver CYP1A1 and CYP1A2 levels are similar to previous findings (Kawamoto et al. 1993; Wardlaw et al., 1998; and Czekaj et al., 2005). Hercule et al (2000) linked ET-1 to renal production of CYP-arachidonic acid metabolites in the rat isolated kidney; namely, ET-1 released 20-HETE (20-hydroxyeicosatetraenoic acid) associated with renal vasoconstriction. They also found that both ET_A and ET_B receptors mediate ET-1 vasoconstriction and that 20-HETE production linked to both receptors

makes a major contribution to ET-1-induced renal arteriolar vasoconstriction in the rat. Thus, increase level of P450 enzymes are linked with increased ET-1 levels as well as increased vasoconstriction.

2.4.3. Effects of smoking on plasma ET-1 levels

ET-1 is the most potent and abundant endogenous vasoconstrictor produced by both the vascular endothelial and smooth muscle cells (Levin, 1995). Only limited data are available on the effects of cigarette smoke or its components on ET-1 levels in the plasma or tissue of human or animal model. Two previous studies performed in human volunteers reported a rise in plasma ET-1 level as a transitory phenomenon (Haak et al, 1994 and Goerre et al, 1995). Results from one of those studies also suggests that the increase in the level of ET-1, a powerful vasoconstrictor and mitogen, may play an important role in the atherogenesis arising from smoking (Haak et al., 1994). Plasma ET-1 concentrations in patients undergoing coronary angiography who were cigarette smokers were found higher than those of patients who were no-smokers (Orem et al, 2001). The increase in plasma ET-1 levels was accompanied by a significant increase in systolic blood pressure in human subjects suggesting the role of the most potent vasoconstrictor - endothelin (Borissova et al, 2004). In another study, it was found that ET-1 increased arterial blood pressure significantly (mean increase 8%) in smokers (Ottosson-Seeberger et al, 1997). In our study, we found significant increases in plasma ET-1 in chronic cigarette smoking rats ($P < 0.0001$), which supports our findings of increased vascular contractility in the

aorta and carotid arteries of cigarette smoking rats. However, we believe that the most likely explanation for our findings resides in ET-1-induced augmentation of reactive oxygen species production in endothelial cells and smooth muscle cells, which in turn contributes to a reduction in the bioavailability of NO (Wedgwood et al, 2001; Li et al, 2003; Callera et al, 2003) resulting in increased vascular contractility.

2.4.4 Effects of smoking on body weight gain

Nicotine receptors are present in the appetite-regulating areas of the brain, particularly in the arcuate nucleus of the hypothalamus where nicotine mediates decreases in food intake thereby reducing body weight gain; however, the mechanism by which cigarette smoking or nicotine administration suppresses appetite is not well described (Chen et al, 2005). Cigarette smoking significantly decreases food intake, body weight and fat mass (Chen et al, 2005), and decreases appetite by inhibiting the activity of orexigenic peptides and stimulating the activity of anorexigenic peptides. Cigarette smoking increases plasma levels of catecholamines (Cryer et al., 1976), which in turn reduces plasma leptin levels (Caruli et al., 1999; Fritsche et al., 1998), and reduces food intake (Wellman et al., 2000). Thus, our finding of decreased weight gain caused by cigarette smoking is consistent with the multitudinous effects of plasma nicotine on food intake and energy expenditure.

2.5 Conclusion

In conclusion, this study demonstrates that cigarette smoking induces a pronounced increase of vascular smooth muscle contractility that is associated with reduced basal NO release and augmented endothelin release. These effects were observed in a systemic artery (aorta) as well as a main feeder artery to the cerebral circulation (external carotid artery), suggesting important effects in the mediation of both peripheral and central vascular dysfunction in cigarette smokers.

2.6 Tables

Table 2. 1 Relative mRNA expression (normalized to 18s rRNA).

	Mean $\pm$ SEM	
	CYP 1A1 mRNA	CYP 1A2 mRNA
Control	0.0000363 $\pm$ 0.0000135	0.12666666 $\pm$ 0.0166666
Smoking	0.0009125 $\pm$ 0.0001057	1.7666667 $\pm$ 0.5886236
Fold increase	25.13	13.95
<i>P</i> -value (<i>t</i> -test)	0.001	0.05

2.7 Figures

Figure 2. 1 Effect of cigarette smoking on body weight gain. Body weight gain was significantly ($p<0.0001$) reduced in the smoking rats starting from week 7 through week 16. [* = Significant difference ($p<0.0001$) in body weight gain in smokers versus control rats].

Figure 2. 2 Contractile response of rat aortae. PE-induced contractions in the absence and presence of bosentan in control and smoking rats. ($\square$ =control before bosentan, Δ = control after bosentan, $\blacksquare$ =smoking before bosentan, $\blacktriangle$ = smoking after bosentan). [* = significant difference ($P<0.001$) before and after bosentan treatment in smoking rat aortae. $\dagger$ = significant difference ($P<0.001$) between smoking and control rat aortae before bosentan treatment).

Figure 2. 3 Contractile response of rat aorta. a. PE-induced contractions in the presence and absence of L-NAME in control rat aortae. b. PE-induced contractions in the presence and absence of L-NAME in smoking rat aortae. ($\square$ = Control before L-NAME, $\circ$ = Control after L-NAME, $\blacktriangle$ = Smoking before L-NAME, $\blacksquare$ = Smoking after L-NAME). [* = Significant difference ($P<0.001$) in PE-response before and after L-NAME incubation].

Figure 2. 4 Contractile response of rat carotid arteries. PE-induced contractions in the absence and presence of bosentan in control and smoking rats. (Δ =control before bosentan, $\square$ = control after bosentan, $\blacktriangle$ =smoking before bosentan, $\blacksquare$ = smoking after bosentan). [$\dagger$ = Significant ($P<0.001$) difference in PE-induced contraction between smoking and control rat before bosentan treatment; * = significant ($P<0.001$) difference in PE-induced contraction in smoking rat aortae before bosentan treatment.]

Figure 2. 5 The gel presents results of PCR obtained via thermal block cyclor. (L=100bp DNA ladder, C1 –C4=PCR fragments of controls, S1-S5 =PCR fragments of smoking).

Figure 2. 6 Plasma levels of ET-1 in non-smoking control (n = 10) and smoking rats (n = 10). White bar represents control group, and the solid bar represents smoking group. [* = Significance for unpaired t test comparison is shown ($P < 0.0001$)].

2.8 References

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3 CONCLUDING CHAPTER

GENERAL DISCUSSION AND CONCLUSIONS

3.1 General discussion

This study demonstrates vascular dysfunction in cigarette smoking rats with a pronounced increase of vascular smooth muscle contractility that is related to reduced basal NO release and augmented endothelin release. The reduced body weight gain and increased mRNA expression of CYP1A1 and CYP1A2 in the smoker rats confirm systemic effects to cigarette smoke in these rats.

In this study we did not find impairment of endothelium-dependent vasorelaxation either in conduit arteries (data not shown) or in resistance arteries (Appendix D), although we found significant reduction in basal levels of NO. There are different pathways of vasorelaxation: NO-mediated relaxation, EDHF-mediated relaxation, prostacyclin-mediated relaxation etc. Thus, NO is mainly but not exclusively responsible for the vasorelaxation (Vequad et al., 1999). In rat gracilis muscle arterioles NO was only partially involved in the dilation. In the same study, prostacyclin was demonstrated to be responsible for the remaining component of the dilation obtained in the absence of NO synthesis (Koller et al., 1994). Prostacyclin biosynthesis is elevated in cigarette smokers (McAdam et al., 2005) and it is an effective vasodilator (Wet et al., 2004).

Another significant mediator of vasorelaxation is carbon monoxide (CO), which is a potent inducer of hypoxia. Hypoxia will produce endothelial-derived vasorelaxation.

CO can induce relaxation of vascular tissues with different diameters in different animal models (Wang et al., 1997; Wang, 1998). Exogenously applied CO also induced a concentration-dependent relaxation of rat-tail artery precontracted with phenylephrine. Like NO, CO also stimulates cyclic guanosine monophosphate (cGMP)-mediated pathways. The activation of soluble guanylate cyclase (sGC) results in an increase in cGMP level. In vascular smooth muscle, increased cGMP production subsequently induces relaxation by lowering intracellular calcium concentration. CO also modulates K^+ channels resulting in hyperpolarization, which causes vasorelaxation by inactivating voltage-dependent calcium channels (Wang et al., 1997; Wang, 1998). Rats exposed to cigarette smoke have shown significantly higher amounts of carboxyhemoglobin relative to the controls (Renne et al., 2006; Gentry-Nielsen et al., 2004; Nene et al., 1997). Similarly, human smokers have also shown increased carboxyhemoglobin levels (Hart et al., 2006; Rickert et al., 1981). This increased carboxyhemoglobin level may produce hypoxia which will ultimately produce endothelium-derived vasorelaxation.

Another contributing factor for endothelium-dependent vasorelaxation is ET-1 itself. ET-1 acts on three types of G-protein-coupled receptors: ET_A , ET_{B1} and ET_{B2} (Douglas et al., 1995). Among these three receptors, ET_A and ET_{B2} are expressed in vascular smooth muscle cells, and ET_{B1} predominantly in the vascular endothelium; the former two are responsible for vasoconstriction while the last one for vasodilation by releasing NO and

prostacyclin (de Nucci et al., 1988; Sakurai et al., 1990; and Arai et al., 1990). At low concentrations ET-1 mainly acts on the ET_{B1} receptors, thus producing vasodilation (Masaki et al., 1995).

There is evidence that smoking in rats is associated with an increase in serum NO level (Sarkar et al., 1999) that could have a relaxing effect. However, in this study we found decreased basal NO but we did not measure the level of NO in serum or tissue. It would be interesting to measure the NO level directly in the bath solution while doing the functional study in vascular tissue.

3.2 Comments on strengths and weaknesses of the thesis research

The strength of the research lies in the fact that by using a rat model, we basically investigated the effects of cigarette smoking as an independent risk factor of cardiovascular disease or disease condition as rats are free from the risk of development of hyperlipidemia or dyslipidemia and atherosclerosis.

The weakness of this study is that we failed to show significant impairment of endothelium-dependent vasodilation both in conduit and resistance arteries of smoker rats (Appendix D, Figure 4.1 and 4.2). In this study, one of the objectives was to examine the endothelium-dependent vasorelaxation by acetylcholine-induced NO production. Experiments addressing this aim failed to show reduction in stimulated NO production in the smoker rats although there was a reduction in basal NO. This could be due to the

species variation. It is known that rats are resistant to development of atherosclerosis due to high HDL (“good” cholesterol) and low LDL (“bad” cholesterol) (Chapman et al., 1980). This special characteristic of rat may, in part, lead to the failure of showing reduced stimulated NO in smoker rats. So, rat may not be a good model to investigate cigarette smoke-induced endothelial dysfunction. As an alternative model of cigarette smoking, one could use transgenic mice, for example, ApoE deficient mice that readily develop atherosclerosis and this could be good model for investigating endothelial dysfunction.

3.3 Evaluation of current knowledge and proposals for new ideas related to the field of study

Cigarette smoking is associated with increased levels of ET-1, which has been implicated in the pathogenesis of COPD, asthma and PH (Hay, 1999; Nikolaou et al., 2003). It is estimated that more than 80% patients with COPD have been cigarette smokers (Peto et al., 2001) and that more than 50% of smokers have evidence of COPD (Lundback et al., 2003). It is reported that 91% of patients with severe COPD have PH (Scharf et al., 2002). Plasma levels of ET-1 are increased both in patients with severe COPD (Channick et al., 2004) and PH (Yamakami et al., 1997; Moore et al., 2004). ET-1 plays an important role in increasing pulmonary vascular resistance (Vizza et al., 2006). There is also evidence that ET_A and ET_B receptor expression is increased in the pulmonary arteries of patients with COPD and PH (Davie et al., 2002). Hypertension in COPD is a result of direct cigarette smoke-mediated effects on the vasculature by ET-1 and that interference with

ET-1 production and activity may be beneficial (Wright et al., 2006).

We found endothelin-1 (ET-1)-induced vasoconstriction in cigarette smoker rats that can be used as the basis for therapeutic target. For example, in smokers with chronic obstructive pulmonary disease (COPD), asthma or pulmonary hypertension (PH) we can use an ET-1 blocker or an ET-1 antagonist to ameliorate these disease conditions.

In the treatment of pulmonary arterial hypertension the newly added drugs are ET-1 receptor antagonists. For example, bosentan, a mixed ET_A and ET_B receptor antagonist, improves exercise tolerance and survival in PAH (Rubin et al., 2002). Investigators can focus on developing new drugs, which could selectively block ET-1 receptor subtypes (e.g., ET_A or ET_{B2}) or directly inhibit production and/or release of the constrictor peptide ET-1, thus ameliorating these disease conditions.

3.4 Discussion of any potential applications of the research findings

Cigarette smoking results in increased levels of ET-1, a peptide released from vascular endothelium throughout the body, including the corpus cavernosum, which causes smooth muscle constriction. There is a physiological role of ET-1 in the control of erectile function and it may play a role in detumescence (McVary, 2006; Andersson, 2003; Morano, 2003; Becker et al., 2001); in smokers (Khan et al., 1998) or in patients with COPD (Koseoglu et al., 2005) the situation is worse. A body of evidence shows that there is a link between cigarette smoking, ET-1 and an increased risk of erectile dysfunction (McVary, 2006; Andersson, 2003; Morano, 2003). ET-1 antagonists may be beneficial in the treatment of erectile dysfunction in smokers. Many men with erectile dysfunction do not respond to oral sildenafil or alprostadil injection (Goldenberg, 1998). This group of people may benefit from ET-1 antagonists.

3.5 Comments on future research

For further research, an important therapeutic question to address is whether an oral ET-1 antagonist can improve vascular dysfunction in smokers. There are many unanswered questions relating to endothelin and cigarette smoking. Acute smoking was found to increase ET-1 mRNA but chronic smoking did not (Adachi et al., 2000). Further research should be addressed to clarify this issue. We used bosentan, a dual endothelin receptor blocker, to examine the endothelin-induced augmented vasoconstriction. It would be interesting to use specific receptor blockers and explore the difference in contractile response of the vessels from non-smokers and cigarette smokers to α -adrenergic stimulation.

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Appendix B

Research cigarette specification

Introduction

During the late 1960's when increased emphasis was being placed on smoking and health research, it was determined that a cigarette that could be used as a research standard should be developed. In 1968, the Scientific Advisory Board of the Council of Tobacco Research requested the Industry Technical Committee to oversee the production of such a cigarette. The University of Kentucky's Tobacco and Health Research Program provided the organizational structure for developing such a cigarette. This reference cigarette served as an international standard for research purposes. The reference cigarette is useful for scientists involved in many aspects of research, and it provides a basis for comparing data that have been collected in different laboratories. The Kentucky Tobacco Research and Development Center produces different reference cigarettes (e.g., 1R3F, 2R4F, 1R5F etc) that vary in nicotine level and other ingredients. We used 1R3F research cigarette that contains the highest nicotine level.

Composition of the 1R3F research cigarette:

1R3F-the Kentucky Tobacco Research & Development Center equivalent to the experimental blend specified and used by the National Cancer Institute as their standard for experimental work. The reconstituted tobacco sheet portion of this blend was manufactured using the Schweitzer Process.

Flue-cured	*32.54%
Burley	20.04%
Turkish	11.09%
Maryland	1.06%
Reconstituted Sheet	27.17%
Invert Sugar	5.30%
Glycerine	2.80%

* Wet weight basis

Smoke analyses by FTC method

Cigarette	TPM mg/cig	FTC Tar mg/cig	Nicotine mg/cig	Water mg/cig	Puff count/cig	CO mg/cig	NOX mg/cig
1R3F	18.10	15.0	1.16	1.88	8.60	17.20	0.27
1R5F	2.08	1.67	0.16	0.30	7.18	2.95	0.11
2R4F	11.70	9.70	0.85	1.12	9.20	13.00	0.22

TPM=Total Particulate Matter, FTC=Federal Trade Commission, Cig= cigarette, CO=

Carbon Monoxide, NOX= Nitrogen Oxide.

Appendix C

Details of methodology for ET-1 measurement

Measurement of endothelin in EDTA-plasma samples after precipitation:

Freshly collected EDTA-plasma sample from smoker and nonsmoker rats was put on ice immediately and centrifuged within one hour. Samples were stored at -80° C until the assay was done. As lipemic and hemolytic plasma samples may give erroneous results, these kinds of samples were not assayed. Samples were mixed well before assaying. Duplicates for all values were used.

Step1: Precipitation of plasma samples

1. To pipette 1 ml sample in a polypropylene tube.
2. To add 1.5 ml of diluted PAA (Precipitating Agent Additive). Mixed well on a vortex mixer.
3. To cool sample to 4° C and to centrifuged for 20 min at 3000X g at 4° C.
4. To transfer supernatant into another polypropylene tube.
5. To dry all supernatants of the samples in a stream of nitrogen overnight.

Step 2: Performance of the assay

1. Dried samples were re-dissolved in 500 μ l of assay buffer, mixed well and spin down insoluble substances.
2. Using the diluted endothelin stock were prepared serial dilutions with the PB-buffer down to approximately 0.6 fmol/ml. PB-buffer was used as a zero standard.
3. Proceeded with assay protocol.

Assay Protocol:

1. All reagents and samples were equilibrated at room temperature (18-26°C) before use in the assay.
2. Positions were marked for BLANK/STD (Standards)/SAMPLE/CTRL (Control) on the supplied protocol sheet.
3. Microtiter strips were taken out of the alubag, taking a minimum of one well as Blank.
4. Added a 50 μ l STD/SAMPLE/CTRL (Standard, white cap/Sample/Control, yellow cap) in duplicate into respective well, except blank.
5. Added a 200 μ l AB (Detection antibody, green cap) into each well, except blank, swirl gently.
6. Covered tightly and incubate at room temperature (18-26°C) overnight (16-24 hours).

7. Aspirated and washed wells 5x with 300 μ l diluted WASHBUF (Wash buffer), removed remaining WASHBUF by hitting plate against paper towel after the latest wash.
8. Added a 200 μ l CONJ (Conjugate) into each well.
9. Covered tightly and incubate 1 hour at room temperature.
10. Aspirated and washed wells 5x with 300 μ l diluted WASHBUF (Wash buffer), removed remaining WASHBUF by hitting plate against paper towel after the last wash.
11. Added a 200 μ l SUB (Substrate) into each well.
12. Incubated for 30 minutes at room temperature (18-26°C) in the dark.
13. Added a 50 μ l STOP (Stop solution) into each well, shaken well.
14. Measured absorbance immediately at 450 nm.

Calculations of results:

The blank extinction was subtracted from all other values. The Standard curve was constructed from the Standard values using MS Excel program. Respective dilution factors were taken into consideration.

Plasma levels (fmol/ml) of endothelin-1 (ET-1) in control and smoking rats:

Control (n=10)	Smoking (n=10)
1.20542441	6.061024611
2.258915118	5.369161226
1.253139126	6.207935711
1.307132094	5.592667002
2.165996986	6.003264691
1.467855349	4.791562029
1.648669011	5.8814666
1.255650427	5.306378704
1.624811652	5.932948267
1.600000000	5.344048217

Graph Pad Prism was used to analyze the data.

Appendix D

Micro vessel study by pressure myograph

Figure 4. 1 Lack of difference both in endothelium-dependent (Bradykinin=BK) and – independent (Sodium Nitro Prusside=SNP) vasodilatation between control and smoking rat middle cerebral artery (MCA).

Figure 4. 2 Lack of difference in endothelium-dependent (ACh) vasodilatation between control and smoking rat septal coronary artery.