CIGARETTE SMOKE-INDUCED PRO-INFLAMMATORY ALTERATIONS IN THE ENDOTHELIAL PHENOTYPE, THE PROTECTIVE EFFECT OF RESVERATROL

Dissertation

Zsuzsanna Orosz, M.D.

Semmelweis University
PhD School of Studies in Clinical Medicine

Supervisor: Zoltán Bártfai, M.D., Ph.D., Med.habil.

Scientific referees for the Ph. D. dissertation:
Zoltán Járai, M.D., Ph.D., Med.habil.
Zsuzsanna Csoma, M.D., Ph.D.

Committee at the Comprehensive Exam:
Chairman: Károly Cseh, M.D., Ph. D., D.Sc.
Members: Aladár Rónaszéki, M.D., Ph. D.
Zoltán Süttő, M.D., Ph. D.

Budapest
2011

TABLE OF CONTENTS

ABBREVIATIONS:

AA	arachidonic acid
Ang II	angiotensin II
AT1	angiotensin 1 receptor
ATP	adenosin triphosphate
BCECF	2',7' - Bis - (2 - carboxyethyl) - 5 - (and - 6) - carboxyfluorescein
CAEC	coronary arterial endothelial cell
CAT	catalase
CSE	cigarette smoke extract
CuSOD	kopper superoxide dismutase
CYP450	cytochrome P450
DCF	2',7'-dichlorodihydrofluorescein diacetate
DHE	dihydroethydine
DMSO	dimethyl sulfoxide
DNA	dinucleotide acid
DOCA	deoxycorticosterone acetate
DPI	diphenyleneiodonium
EB	ethidium bromide
ECM	extracellular matrix
EDRF	endothellium derived relaxing factor
eNOS	endothelial nitric oxide synthase
FCS	Fetal calf serum
GCH1	GTP cyclohydrolase
H_2O_2	hydrogen peroxide
HCAEC	human coronary arterial endothelial cell
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IHD	ischemic heart disease
I-κB	inhibitor of kappa B
IL-1β	interleukin-1beta
IL-6	interleukin 6
INDO	indomethacin
iNOS	inducible nitric oxide synthase

LDL	low density lipoprotein
L-NAME	N^{ω}-nitro-L-arginine-methyl-ester
MAP kinase	mitogen-activated protein *(*MAP*)* kinase
mM	millimol
MMP	matrix metalloproteinase
MnSOD	mangane superoxide dismutase
mRNA	messenger ribonucleotide acid
NAD(P)H	nicotinamide adenine dinucleotide phosphate
NF-κB:	nuclear factor kappa B
NO	nitrogen-monoxide
NOS	nitric oxide synthase
NOX	NAD(P)H oxidases
$O_2^{\cdot -}$	superoxide
OH-	hydroxil anion
ONOO-	peroxinitrite
PBS	phosphate buffer
pH	pondus Hidrogenii
phox	phagocyte oxidase
PMA	phorbol-12- myristate-13-acetate
QRT-PCR	real time quantitative polymerae chain reaction
ROS	reactive oxygen species
RPMI	Royal Park Memorial Institute culture medium
SIR1	sirtuin1
Sir2	sirtuin2
SNAP	S-nitrosopenicillamine
SNP	sodium nitroprusside
SOD	superoxide dismutase
THP-1	human acute monocytic leukemia cell line
TNF-α	tumor necrosis factor alpha
VCAM-1	vascular cell adhesion molecule-1
VSMC	vascular smooth muscle cell

1. INTRODUCTION

1.1. BIOLOGY OF CIGARETTE SMOKE

Cigarette smoking (CS) continues to be a major health hazard, and it contributes significantly to a cardiovascular morbidity and mortality. Cigarette smoking impacts all phases of atherosclerosis from endothelial dysfunction to acute clinical events, the latter being largely thrombotic. Both active and passive cigarette smoking exposure predispose to cardiovascular events [1].

The first cohort studies were published in the 1950s [2,3] that the death from coronary heart disease is more common in smokers than non smokers. Since that there are many reports demonstrating that cardiovascular disease is the most important cause of smoking related premature death [4]. The prospective investigation of British Doctors commenced in 1951 showed that half of excess mortality from smoking results from cardiovascular disease with approximately one third due solely to coronary heart disease [5]. Prospective studies in smokers have shown that the risk of death from coronary heart disease is almost doubled for both men and women [4] although there is evidence that the risk is greater for women. In the United States Manley [6] has estimated that one fifth of all heart-related deaths are due to cigarette smoking and the smoking alone doubles the risk of heart failure. Cigarette smokers are one and a half times more at risk of stroke than nonsmokers [4]. Cigarette smoking is an independent risk factor in the development of atherosclerotic lesions in intracranial internal carotid artery atherosclerosis [7]. In the Edinburgh Artery Study cigarette smoking was shown to have a direct effect on the risk of aortic aneurysm which was independent of atherosclerosis [8]. Smoking increases the risk of peripherial artery disease more than heart disease [10]. Increased tobacco use in humans and a smoking pattern which maximizes nicotine yield are also associated with and increased risk of peripherial artery disease [9].

Despite the strong epidemiological evidence linking cigarette smoking to the development of atherosclerosis, the mechanisms by which cigarette smoke causes diseases is poorly understood [10].

1.2. COMPOSITION OF CIGARETTE SMOKE

Cigarette smoke is separated into two phases: gaseous phase and particulate (tar) phase. The tar or particulate phase is defined a the material that is trapped when the smoke stream is passed through the Cambridge glass-fiber filter that retains 99.9% of all particulate material with a size >0.1 μm [11]. This part of cigarette smoke contains all the particulate phase of the smoke as well as the condensable part of the gas phase. Aldehydes, ketones, organic acid and alcohol are found in the particulate phase. The amount of tar from the smoke of one cigarette is between 3-40 mg depending on the burning and condensing conditions, the length of the cigarette, the use of a filter, porosity of paper, the content of tobacco, its weight and kind. The gas phase is the material that passes through the filter. It consists mainly of nitrogen, oxygen, carbon monoxide and carbon dioxide with traces of nitrogen oxide, ammonia, cyanides. Of all the well known constituents, nicotine, a component of the tar phase, is the addictive substance of cigarette smoke [9]. The particulate (tar) phase of cigarette smoke contains $>10^{17}$ free radicals/g, and the gas phase contains $>10^{15}$ free radicals/puff [11]. The radicals associated with the tar phase are long-lived (hours to months), whereas the radicals associated with the gas phase have a shorter lifespan (seconds) [9,11].

About 4000 compounds are generated by a lighted cigarette through many processes such as hydrogenation, pyrolysis, oxidation, decarboxylation, dehydration,... etc. [15-18]. In addition to these short-lived, highly reactive substances, previous studies have shown that aqueous cigarette tar extracts also contain pro-oxidant substances that have the potential to increase cellular production of ROS [1, 15, 16, 19-22]. The cigarette smoke produces generalized endothelial dysfunction in virtually every vascular bed [23-28], which is usually an indicator of an increased oxidative stress but the exact mechanism is not known.

Importantly, reactive oxygen species (ROS), including $O_2^{\cdot-}$ and hydrogen peroxide (H_2O_2) have been implicated in pro-atherogenic vascular phenotypic alterations [29-33], including induction of pro-inflammatory gene expression [34-40]. Although the effects of cigarette smoke on pro-inflammatory mechanisms in lung epithelium and circulating immunocytes have been extensively studied in the past [12], the possible link between

water soluble components of cigarette smoke, oxidative stress, expression of pro-inflammatory cytokines in intact blood vessels has not been well documented.

The major constituents which severely cause health hazards of the smokers are:

1. Nicotine and Tar in the particulate phase.

2. Carbon monoxide in the gas phase.

Main-stream smoke emerges into environment after it is drawn through the cigarette, filtered by smoker's own lungs, and then exhaled outside. Side-stream cigarette smoke is the smoke emitted from the burning end of the cigarette and enters directly into the environment. Many potentially toxic gas phase constituents are in higher concentration in side-stream smoke than in main-stream smoke [9] and nearly 85% of smoke in a room results from side-stream smoke. Mainstream cigarette smoke comprises 8% of tar and 92% gaseous components [11]. Side-stream smoke is generally diluted in a considerably larger volume of air. Thus, passive smokers are exposed to a quantitatively smaller and potentially qualitatively different smoke exposure than active smokers.

Passive smoking refers to the involuntary inhalation of tobacco smoke present in the air that people breath. Environmental tobacco smoke results of combination of sidestream smoke (85%) and a small faction of exhaled mainstream smoke (15%) from smokers [13]. A short-term increase in blood pressure was reported in passive smokers with acute exposure to cigarette smoke. Increase in carboxyhaemoglobin level, functional residual capacity, residual volume, heart rate together with eye irritation, nasal discharge and cough were also reported.

The first organs that come into contact with noxious agents are the oral and nasal cavities followed by upper respiratory tract and the lung. Hydrophobic compounds (the tar fraction of cigarette smoke) that precipitate in the oral cavity are swallowed and thereby reach the digestive system from the luminal side. Inhaled compounds are further “filtered” by precipitation on the surfaces of respiratory tract. Since hydrophobic agents,

like polycyclic aromatic hydrocarbons can diffuse across cellular membranes into the tissues, precipitate-contained chemicals can penetrate the mucosal linings and reach the circulation. The volatile, mainly hydrophilic, fraction reaches the alveoli and either with diffusion can across the lung-blood barrier or can retain in the lung. In the circulation the smoke chemicals can enter biochemical (albumin) or cellular (erythrocytes) transport systems, or can dissolve in serum [14]. Further cigarette smoke chemicals come into contact with detoxification systems (e.g. cytochrome P450 family, oxidant detoxification systems) [15]. The polycyclic aromatic hydrocarbons can be converted more dangerous and mutagenic agents by cytochrome P450.

1.3. ENDOTHELIAL FUNCTION AND DYSFUNCTION

Endothelial injury

Endothelial integrity and normal function are indispensable for the preservation of health. The estimated 1 to 6 $x10^{13}$ cells of the endothelial monolayer form an approximately 1 kg heavy organ in an adult human. Endothelial cells are playing central role in several physiological processes. They are responding to various substances, circulating hormones, antacoids, cytokins, drugs and physical and chemical stimuli (e.g. shear stress, pH). They are also synthesizing and releasing various factors modulating the vascular tone, permeability, blood flow, coagulation, thrombolysis, inflammation, angiogenesis and tissue repair.

The term of “endothelial dysfunction” was first described in the mid-eighties, following the major breakthrough by Furchgott and Zawadzki [16] who discovered that acetylcholine requires the presence of endothelial cells to relax underlying vascular smooth muscle cells. Before the factor released by acetylcholine was identified as nitric oxide (NO), it had already been observed that the endothelium dependent relaxation in the aorta of hypertensive rats [17] and hypercholesterolemic rabbits [18], was impaired. Similar observations were found in human coronary arteries of atherosclerotic patients [19], and it was suggested that this endothelial dysfunction could be an early marker of atherosclerosis.

Despite the strong epidemiological evidence linking cigarette smoking and cardiovascular disease, the pathomechanisms by which cigarette smoking acts and the components of smoke, responsible for these changes, remain poorly understood. Endothelial injury is considered to be a key initiating event in the pathogenesis of atherosclerosis [20,21], therefore it was reasonable to hypothesize that cigarette smoke, or some components of it, are responsible for damaging the endothelium. Components of smoke that gain access to the circulation will come in contact with blood and with the vascular endothelial cells that form a monolayer lining the vessels. These events could have significant implications for the initiation and development of atherosclerosis. Endothelial functions, including an increase in permeability and decreased nitric oxide (NO) production along with increased expression of adhesion molecules and adherence

of leukocytes to the vessel wall, have been shown to be impaired by risk factors for cardiovascular [22].

The underlying pathophysiology of atherosclerosis is attributed to endothelial injury induced by oxidative stress, which subsequently promotes atherogenesis via oxidation of low-density lipoproteins [23].

1.4. FORMATION AND ELIMINATION OF BIOLOGICALLY IMPORTANT FREE RADICALS

Reactive oxidant and free radical species play a major role in several cardiovascular disease pathogenesis. Two types of reactive species are produced in response to inflammation, ischaemic and reperfusion injury:

1. oxygen centered species (e.g. superoxide, hydrogen peroxide)
2. nitrogen-centered species (e.g. peroxynitrite).

Reactive oxygen species including superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl anion (OH-) are biologically important O_2 derivates that are increasingly recognized to be important in vascular biology. Oxidants exhibit a wide array of tissue-damaging cytotoxic effects, reacting and attacking a variety of biomolecules, including proteins, lipids and DNA.

Superoxide is generated from 1 electron reduction of molecular oxygen by various oxidases (Equation 1.) and it is the precursor of other ROS.

Equation 1. $O_2 + \text{electron} \rightarrow \cdot O_2^-$ (oxidase)

Superoxide has an unpaired electron, which makes it unstable. Superoxide is water soluble and membrane impermeable. Superoxide can be dismuted to H_2O_2 by superoxide dismutase (SOD) (Equation 2.). There are three forms of superoxide dismutase, cytosolic CuZnSOD, mitochondrial manganese SOD (MnSOD), and extracellular SOD (EC SOD) [24].

Equation 2. $O_2\cdot^- + O_2\cdot^- \rightarrow H_2O_2 + O_2$ (SOD)

H_2O_2 is a more stable molecule. H_2O_2 is lipid soluble, can go through cell membranes, and it has longer half life than $O\cdot_2^-$. H_2O_2 can be scavanged by catalase and glutathione peroxidase to form water. Hydrogen peroxide can also be reduced to generate the highly reactive $^{\cdot}OH$. Hydroxyl radical is extremely reactive and induces

molecular damage at the site of its formation Superoxide can react with nitric oxide (NO) to form peroxinitrite ($ONOO^-$) (Equation 3.).

Equation 3. $O_2{\cdot}^- + NO \rightarrow ONOO^-$

In physiological conditions the favored reaction of $O_2{\cdot}^-$ is the dismutation reaction yielding H_2O_2. However, when produced in excess, a significant amount of $O_2{\cdot}^-$ reacts with NO to produce $ONOO^-$, a reactive oxygen species.

The hydroxyl radical ($^{\cdot}OH^-$) is typically formed by oxidation of a reduced heavy metal ion (Fe^{++} or Cu^+, usually) by the hydrogen peroxide (Equation 4.):

Equation 4. $Fe^{++} + H_2O_2 \Rightarrow Fe^{+++} + {}^{\cdot}OH + :OH^-$ (Fenton reaction)

The last reaction, known as the Fenton Reaction, may be the most dangerous because it can occur in the cell nucleus and lead to DNA damage. The oxidized iron (Fe^{+++}) can then catalyze the Haber-Weiss Reaction between superoxide and hydrogen peroxide to produce more hydroxyl radicals (Equation 5.):

Equation 5. $O_2{\cdot}^- + H_2O_2 \Rightarrow O_2 + {}^{\cdot}OH- + :OH^-$ (Haber-Weiss reaction)

At neutral pH the Haber-Weiss reaction occurs only to a negligible extent when no metal ion is available to act as a catalyst.

In the vasculature, $O_2{\cdot}^-$, H_2O_2, NO, $OONO^-$, and ·OH- are all produced to varying degrees. These pro-oxidants are tightly regulated by anti-oxidants such as SOD, catalase, thioredoxin, glutathione, anti-oxidant vitamins, and other small molecules [25,26,27]. In vascular system, under normal conditions, the rate of ROS production is balanced by the rate of elimination, but under pathological conditions increased bioavailability of ROS can lead to oxidative stress. Oxidative stress and oxidative damage are involved in many cardiovascular diseases such as hypertension, atherosclerosis and diabetes. However, a mismatch between ROS formation and the ability to defend against them by antioxidants results in increased bioavailability of ROS leading to a state of oxidative stress [28-30].

1.5. SOURCE OF REACTIVE OXYGEN SPECIES IN VASCULAR CELLS

There are several enzymatic systems contributing to he generation of ROS in the vascular wall, including NAD(P)H oxidases (NOX), uncoupled nitric oxide synthase (NOS), xanthin oxidase, cytochrome P450, cyclooxigenase and mitochondrial electron transport chain.

Nitric oxide synthase:

Nitric oxide synthase (NOS), the enzyme primarily responsible for NO production, can also generate $O_2^{\cdot -}$ in conditions of substrate (arginine) or cofactor (tetrahydrobiopterin; BH_4) deficiency [31]. These findings have led to the concept of "NOS uncoupling", where the activity of the enzyme for NO production is decreased in association with an increase in NOS-dependent $O_2^{\cdot -}$ formation. eNOS uncoupling has been demonstrated in atherosclerosis, diabetes, hyperhomocysteinemia, and hypertension [32]. In hypertension, increased NAD(P)H oxidase-derived $O_2^{\cdot -}$leads to augmented ROS bioavailability, which causes oxidation of BH_4 and consequent uncoupling of eNOS, further contributing to ROS production. Gene transfer of GTP cyclohydrolase (GTPCH) I, the enzyme responsible for regenerating BH_4 [32], restored arterial GTPCH I activity and BH_4 levels, reduced ROS, and improved endothelium-dependent relaxation and NO release in DOCA-salt hypertensive rats, in which endothelial dysfunction results from NAD(P)H-dependent oxidant excess [33]. The potential role of uncoupling of NOS as a source of ROS in hypertension is also supported in human studies where increased endothelial $O_2^{\cdot -}$ production in vessels from diabetic and hypertensive patients is inhibited by sepiapterin, a precursor of BH_4 [34]. The relative importance of NOS- versus NAD(P)H oxidase-mediated $O_2^{\cdot -}$ generation in hypertension probably relates, in part, to the magnitude of endothelial dysfunction, since most conditions in which $O_2^{\cdot -}$ is derived from NOS are associated with marked endothelial dysfunction [27].

Other enzymatic sources capable of generating ROS in the vasculature are xanthine oxidase, cytochrome P450, mitochondrial respiratory chain enzymes, and phagocyte-derived myeloperoxidase [35]. However, the contribution of these enzymes to vascular generation of ROS is relatively minor compared with NAD(P)H oxidase.

1.6. ROLE OF NADPH OXIDASE

Vascular ROS are produced in endothelial, adventitial, and VSMCs [26,28] and derived predominantly from NAD(P)H oxidase, which is a multi-subunit enzyme [36], [37] that catalyzes the production of $O_2^{\cdot -}$ by the one electron reduction of oxygen using NAD(P)H as the electron donor: $2\ O_2 + NAD(P)H \rightarrow 2\ O_2^{\cdot -} + NAD(P)H + H^+$. The prototypical and best characterized NAD(P)H oxidase is that found in phagocytes (neutrophilic and eosinophilic granulocytes, monocytes, and macrophages [36,37]. Phagocytic NAD(P)H oxidase comprises at least five components: (phox for *PH*agocyte *OX*idase), p47phox, p67phox, p40phox, p22phox, and gp91phox [36,37]. Additional components include the small G proteins Rac2 (Rac1 in some cells) and Rap1A. In unstimulated cells, p40phox, p47phox, and p67phox exist in the cytosol, whereas p22phox and gp91phox are located in the membranes, where they occur as a heterodimeric flavoprotein, cytochrome b558. Upon cell stimulation, p47phox becomes phosphorylated and induces the formation of a protein complex by the cytosolic subunits, which then migrates to the membrane where it associates with cytochrome b558 to assemble the active oxidase. The active NADPH complex transfers electrons from the substrate to O_2 leading to $O_2^{\cdot -}$ generation [38]. Whereas phosphorylation of p47phox and p67phox is critically involved in the activation of NAD(P)H oxidase [38], phosphorylated p40phox is not essential for activation and has recently been reported to be a negative regulator of the oxidase.

It is now clearly established that NAD(P)H oxidase is also functionally important in non-phagocytic cells. In fact NAD(P)H oxidase is the primary source of $O_2^{\cdot -}$in the vasculature and is functionally active in all layers of the vessel wall: in the endothelium, the media, the adventitia , and in cultured VSMCs, fibroblasts, and endothelial cells [27-30]. Unlike phagocytic NAD(P)H oxidase, which is activated only upon stimulation and which generates $O_2^{\cdot -}$ in a burst-like manner extracellularly [38], vascular oxidases are constitutively active, preassembled, and produce $O_2^{\cdot -}$ intracellularly in a slow and sustained fashion and act as intracellular signaling molecules [37].

All of the phagocytic NAD(P)H oxidase subunits are expressed, to varying degrees, in vascular cells. In endothelial and adventitial cells p47phox, p67phox, p22phox, and

gp91phox are present [37]. The situation is more complex in VSMCs, where the major subunits are not always detected. Only p47phox and p22phox seem to be consistently expressed [37]. In rat aortic VSMCs, p22phox and p47phox, but not gp91phox, are present, whereas in human resistance arteries, all of the major subunits, including gp91phox, are expressed. Recent studies demonstrated that the newly discovered gp91phox (Nox2) homologs, Nox1, Nox4, and Nox5 (Nox for *N*AD(P)H *Ox*idase) are found in the vasculature. Nox1 mRNA is expressed in rat aortic VSMCs and may be a substitute for gp91phox in these cells [28]. Although initial studies suggested that Nox1 is a subunit-independent low capacity $O_2^{\cdot -}$-generating enzyme involved in the regulation of mitogenesis [39], recent data indicate that Nox1 requires p47phox and p67phox and that it is regulated by NoxO1 (Nox organizer 1) and NoxA1 (Nox activator 1) [39]. The exact role of NoxO1 and NoxA1 in vascular cells is currently unknown. Nox1 may be important in pathological processes as it is significantly upregulated in vascular injury [37]. Nox4 appears to be abundantly expressed in all vascular cell types [40] and may play an important role in constitutive production of $O_2^{\cdot -}$ in non-proliferating cells [40,41]. Nox4 is the major catalytic component of endothelial NAD(P)H oxidase. A unique p67phox homolog has also been identified, but it is not yet known whether this isoform is present in vascular cells. The functional significance of NAD(P)H oxidase subunit homologs in the vasculature is presently unclear and awaits further clarification.

Vascular NAD(P)H oxidase is regulated by many humoral factors, including cytokines, growth factors, and vasoactive agents [37]. Physical factors, such as stretch, pulsatile strain, and shear stress also stimulate NAD(P)H oxidase activation. Of particular importance, with respect to hypertension, is Ang II, which stimulates activation of NAD(P)H oxidase, increases expression of NAD(P)H oxidase subunits, and induces ROS production in cultured VSMCs, endothelial cells, adventitial fibroblasts, and in intact arteries. Oxidase activation occurs acutely by stimulation of intracellular signaling molecules and chronically by upregulation of NAD(P)H oxidase subunits [37]. Interestingly ROS regulate AT_1 receptor gene expression, which in turn modulates ROS formation [42].

Platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), tumor necrosis factor (TNF)-α and thrombin also activate NAD(P)H oxidase in VSMCs [42].

Endothelin-1 increases NAD(P)H oxidase activity in human endothelial cells and, in intact vessels, this effect is mediated via ET_A receptors. Activators of peroxisome proliferator-activated receptors (PPARs), statins, and antihypertensive drugs, such as β-blockers, Ca^{2+} channel blockers, ACE inhibitors, and AT_1 receptor blockers, downregulate expression of oxidase subunits and decrease NAD(P)H oxidase activity [43]. These actions may have therapeutic potential in cardiovascular disease.

1.7. POSSIBLE ROLE OF H_2O_2 AND SUPEROXIDE IN VASCULAR TONE

Reactive oxygen species stimulate growth factor-like cellular responses, such as intracellular alkalinization, MAP kinase phosphorylation, tyrosine kinase activation, DNA synthesis, and increased expression of proto-oncogenes. During vascular damage in hypertension when oxidative stress is increased, redox-sensitive growth processes may lead to accelerated proliferation and hypertrophy, further contributing to vascular injury and remodeling [28]. In addition to growth-promoting actions, ROS induce apoptosis and differentiation under certain circumstances. This differential response appears to relate to the specific, the concentration of ROS, and the cellular localization of ROS [44]. At high concentrations (>100 μmol/l) H_2O_2 and peroxynitrite are pro-apoptotic and induce anoikis (cell detachment and shedding), whereas at lower concentrations they stimulate growth and differentiation [44].

Impaired endothelium-mediated vasodilation in hypertension and hypercholesterolemia has been linked to decreased NO bioavailability. This may be secondary to decreased synthesis of NO and/or to increased degradation of NO because of its interaction with $O_2^{\cdot -}$ to form $ONOO^-$ [45]. Peroxynitrite is a weak vasodilator compared to NO and has pro-inflammatory properties [46]. In experimental models of hypertension, hypercholesterolemia, and diabetes and in hypertensive patients, endothelial function is improved by anti-oxidant vitamins, probucol, SOD, or sepiapterin (a stable precursor of BH_4) [34].

Vasomotor tone may also be modulated through direct ROS effects. Reactive oxygen species appear to elicit both contraction and dilation, depending on the vascular bed and

type of species generated. Hydrogen peroxide causes vasodilation of pulmonary, coronary, and mesenteric arteries and has been considered to be an endothelium-derived relaxing factor [33] . In rat aorta, Ang II stimulates vasoconstriction via H_2O_2-dependent mechanisms [47], whereas in human and porcine vessels, acute vasoconstriction by Ang II is not mediated via ROS [48,49]. In aortic and mesenteric arteries from spontaneously hypertensive rat, redox-mediated contractile effects are enhanced [50]. Major factors underlying these differential vascular responses to activated oxygen metabolites could relate to the blood vessel studied, the presence or absence of the endothelium, the concentration and species of free radical studied, and the compartment in which $O_2^{\cdot -}$ or H_2O_2 predominate [51]. At present it is still unclear exactly what the functions of $O_2^{\cdot -}$ and H_2O_2 are with respect to vascular contraction/dilation in physiological and pathophysiological conditions.

1.8. POSSIBLE ROLE OF CIGARETTE SMOKE IN VASCULAR SYSTEM

Despite the strong epidemiological evidence that exists linking cigarette smoking and cardiovascular disease, the mechanisms by which cigarette smoking causes disease and the components of smoke responsible remain poorly understood. Endothelial injury is considered to be a key initiating event in the pathogenesis of atherosclerosis [52-54] and it has therefore seemed reasonable to hypothesize that cigarette smoke, or some components of it, may exert its effects by damaging the endothelium. Components of smoke that gain access to the circulation will come in contact with blood and with the vascular endothelial cells that form a monolayer lining the vessels. These cells are now known to have a crucial role in controlling the blood circulation and to be highly active metabolically [55,56]. Even minor disturbances to their normal functioning could have significant implications for the initiation and development of atherosclerosis. Endothelial functions, including an increase in permeability and decreased nitric oxide (NO) production along with increased expression of adhesion molecules and adherence of leukocytes to the vessel wall, have been shown to be impaired by risk factors for cardiovascular disease such as hypertension, hyperlipidaemia and hyperglycaemia [22].

Damaging effect of cigarette smoking on arteries

Direct evidence that cigarette smoking can result in atherosclerosis continues to be obtained from human investigations. Using serial quantitative coronary arteriography, Waters et al. [57] have shown that cigarette smoking accelerates coronary progression and new lesion formation. Interestingly, Lovastatin was shown to slow the progression of coronary atherosclerosis and prevent the development of new coronary lesions in smokers [58]. The relative thickness of the intima and media of an artery as measured by ultrasound can be used to provide an index of atherosclerosis.

Carotid artery atherosclerosis determined by ultrasound determination of plaque thickness, a measure of atherosclerosis, is accelerated depending on the level of cigarette use and this is independent of age, hypertension and diabetes [59]. Cigarette smoking has an adverse effect on the intimal medial ratio and stenosis of the carotid artery as measured by ultrasound [59-61]. Exposure to environmental tobacco smoke

(passive smoking), and cigarette smoking have been shown using the intimal/medial ratio as an index to cause progression of atherosclerosis in the carotid artery [62]. Cigarette smoking was shown to be an independent indicator of the severity of coronary and thoracic aortic atherosclerosis in men receiving coronary angiography and transoesophageal echocardiography [63]. Studies on the coronary arteries of trauma victims aged between 15 and 34 years of age suggest that intermediate atherosclerotic lesions progress rapidly into advanced lesions in smokers and that intima formerly having early lesions is replaced by intima with raised lesions [64]. This study also demonstrated that smoking was associated with more extensive fatty streaks and raised lesions in the abdominal aorta [65].

Radiological observations on women aged between 45 and 64 years showed a direct association between the development of calcified deposits, which have been shown to represent atherosclerotic change, in the abdominal aorta and the number of cigarettes smoked per day over a nine year period [66]. This study suggests that the rate of atherosclerotic change may be reduced by the cessation of smoking, but a residual effect appears to be present for a decade [66].

Cigarette smoking and endothelial modification

Direct evidence that cigarette smoking could result in endothelial injury was first obtained from morphological observations on the umbilical arteries from smoking mothers. The morphological alterations, which included the endothelium having an irregular appearance along with membrane disturbances evidenced by the formation of blebs or microvillous-like projections, were also associated with functional changes to the endothelium. In laboratory rats exposed to cigarette smoke Pittilo et al. [67] were able to demonstrate that the morphological alterations to the endothelium of the thoracic aorta were accompanied by reduced endothelial prostacyclin production as well as the adhesion of platelets to apparently intact cells. The ability of cigarette smoking to reduce endothelial prostacyclin production is well established [68].

Cigarette smoking and the components of smoke responsible for endothelial injury

Cigarette smoke is a complex mixture and only a few components have been examined in isolation to assess their effects on endothelial morphology and function. The effects of nicotine on both the morphology and prostacyclin production of rabbit and cultured human endothelial cells has been reviewed by Bull[69], Pittilo [10], and although deleterious effects result from exposure, these are much more limited than those observed with whole smoke [9,11]. Clinical studies support these findings in that observations on pipe smokers and users of transdermal nicotine devices suggest that components of smoke other than nicotine are the most important causes of acute cardiovascular events [70]. The effects of carbon monoxide exposure have also been reviewed [68] and are conflicting, with some reports providing evidence of morphological or functional alterations and others reporting no effects. Rats exposed to nicotine show an increased frequency of endothelial cell death which results in enhanced transendothelial leakage of macromolecules including low density lipoproteins [71]. Nicotine has been shown to enhance the release of platelet-derived growth factor by bovine aortic smooth muscle cells and is associated with alterations in the cytoskeleton [72]. Nicotine significantly stimulates DNA synthesis and endothelial cell proliferation in bovine pulmonary artery endothelial cells at concentrations lower than those obtained in the blood after smoking [73]. Data derived from epidemiological and animal studies suggest that carbon monoxide is not atherogenic [74]. However, exposure of cockerels to carbon monoxide does not have a discernible effect upon arteriosclerotic plaque development [75]. Bovine pulmonary artery endothelial cells exposed to carbon monoxide release higher concentrations of nitric oxide and it has been suggested that carbon monoxide causes oxidative stress through competition for intracellular binding sites which increase steady state levels of nitric oxide and the generation of peroxynitrite by the endothelium [76]. Carbon monoxide suppresses the production of endothelin-1 and platelet-derived growth factor B by endothelial cells [75]. There is experimental evidence with cockerels to indicate that exposure to environmental tobacco smoke at levels equivalent to those routinely encountered by people in smoke-filled environments is sufficient to promote arteriosclerotic plaque development [75,77,78]. In cholesterol-fed rabbits, passive smoking increases aortic and

pulmonary artery atherosclerosis in a dose-responsive way and is independent of changes in serum lipids [79,80]. One review of the literature concluded that passive smoking increases platelet activity, accelerates atherosclerotic lesion formation, and increases tissue damage following ischaemia or myocardial infarction [81]. However, it has been argued that the available experimental and epidemiological evidence to date does not support an association between environmental tobacco smoke exposure and an increased risk of heart disease [82,83].

Pittilo and Woolf [10] focused on the possible importance of free radicals as the mediator of cigarette smoke-induced endothelial damage for a number of reasons. Cigarette smoke has been known for many years to be a rich source of free radicals [84]. The scanning electron microscope changes seen in laboratory rats following smoke exposure [67], or cultured cells following exposure to plasma from humans after smoking , were similar to the changes seen in cardiac myocytes following free radical-induced lipid peroxidation [85,86]. Finally, there is a strong association between lipid peroxidation and endothelial cell injury [87]. Woolf et al. [56] and Pittilo [88] reviewed some of the supportive evidence and considered that free radical-induced lipid peroxidation might eventually be shown to be the mechanism responsible for the epidemiological relationship between cigarette smoking and atherosclerosis. It has been suggested that the high levels of lipid peroxidation and increased formation of reactive oxygen species within the vascular wall in atherosclerosis can overwhelm cellular antioxidant defence mechanisms and human oxidatively modified LDL-induced expression of antioxidant stress proteins in vascular cells [89].

On the contrary, studies showing no differences in free radical markers between smokers and nonsmokers suggest that any free radical activity generated from smoking is adequately scavenged. No significant differences in free radical markers were found between young adult smokers and nonsmoking volunteers [90]. Free radical activity due to cigarette smoking also appears to be adequately scavenged in young adults with diabetes who are free of significant macrovascular disease [91]. Furthermore, there is evidence to indicate that chemical modification of glutathione is a major damage mechanism of filtered cigarette smoke with free radical oxidations being less significant [92]. Despite these findings, there remains strong evidence to implicate free radicals as an important cause of cigarette smoke-induced endothelial injury. The morphological

alterations seen with cultured cells exposed to plasma obtained from volunteers after smoking [93] are associated with functional changes to the endothelium including activation of the hexosemonophosphate shunt, a sharp increase in the total glutathione content of the culture medium, release of angiotensin-converting enzyme from the cells and a decrease in the ability of the cells to produce ATP, all of which indicate both oxidative stress and cell injury [56].

Cigarette smoking is associated with dose-related and potentially reversible impairment of endothelium-dependent arterial dilation in asymptomatic young adults, consistent with endothelial dysfunction [94]. It augments endothelial-derived vaso relaxation but has no effect on endothelium-independent vasorelaxation [95]. It leads to a significant decrease in endothelium-dependent dilatation of the brachial artery [96]. It causes immediate constriction of proxymal and distal epicardial coronary arteries and an increase in coronary vessel tone despite an increase in myocardial oxygen demand [97], and cigarette smokers show an impairment in basal but not stimulated nitric oxide mediated vasodilation [98]. Cigarette smoking has been shown to affect endothelial nitric oxide synthase activity and protein levels [99]. It results in a decrease in exhaled nitric oxide in humans [100] suggesting that it may inhibit the enzyme nitric oxide synthase [101]. Cigarette smoke extracts cause an irreversible inhibition of nitric oxide synthase activity in pulmonary artery endothelial cells [102] and a reduction in constitutive nitric oxide synthase activity in the rat in a dose-dependent manner [103]. Conversely, other studies in the rat have shown that cigarette smoking results in an increase in nitric oxide synthase gene expression and protein production [104]. However, lower respiratory tract nitric oxide concentrations are increased following cigarette smoking [105] although, in humans, plasma and urinary levels of nitrate, a metabolite of inhaled nitric oxide, have been shown to be unchanged suggesting that nitric oxide is not absorbed from the inhaled smoke [106]. Inhibition of nitric oxide synthase activity may explain the impaired endothelium-independent vasodilation associated with cigarette smoking [102]. The decrease in endothelial nitric oxide synthase activity may also partly explain the high risk of pulmonary and vascular disease in cigarette smokers. Cigarette smoking affects nitric oxide-mediated coagulation of coronary artery tone which is associated with a decrease in the bioactivity of nitric oxide [107]. It has been shown to increase isoprostane levels and

reduce the generation of prostacyclin, L-arginine and L-citrulline in umbilical arteries and veins, and correlates with a direct vasoconstrictive effect [108].

Chronic smoking in the rat leads to age-independent moderate hypertension and a decrease in penile nitric oxide synthase activity [109]. It has been reported that exposure of cultured bovine pulmonary artery endothelial cells to carbon monoxide results in increased release of endothelial nitric oxide [110]. The acute cytotoxicity from carbon monoxide was owing to nitric oxide-derived oxidants. Incubation of isolated rabbit aortas with cigarette smoke extract inhibits endothelium-dependent relaxation in a dose-dependent manner [111]. If this incubation is carried out with free radical scavengers, attenuation of the inhibition occurs.

This suggests that free radicals in cigarette smoke extract induce the impairment of endothelium-dependent relaxation and this may be partly because of the suppression of nitric oxide production [111]. The same author has demonstrated, using cultured human endothelial cells, that cigarette smoke extract suppressed endothelial release of stable metabolites of nitric oxide and this was attenuated by free radical scavengers [111]. The superficial femoral veins from rabbits exposed to cigarette smoke have a significant decrease in endothelium-dependent relaxation in response to acetylcholine without smooth muscle injury [112]. Ascorbic acid protects rabbit arteries from cigarette smoke-induced endothelial injury by reducing the impairment of endothelium dependent acetylcholine relaxation caused by smoking, probably as a result of oxygen-free radicals [113].

It has been suggested that the presence of hypoxia and exogenous nitric oxide, which lead to endothelial dependent and independent vasorelaxation secondary to cigarette smoking, may serve to explain the apparent augmentation of endothelial-derived relaxation in the rat [95]. Cigarette smoking has been shown in the rat to increase aortic endothelial regeneration and serum levels of nitric oxide following balloon injury of the thoracic aorta [114]. Acute hypoxia causes pulmonary vessel constriction, and chronic hypoxia causes smooth muscle cell replication and extracellular matrix accumulation, resulting in vessel wall remodeling [76]. Oxidized low-density lipoprotein, hypoxia and pro-inflammatory cytokines induce haem oxygenase expression and activity in vascular endothelial and smooth muscle cells [115].

Cultured human umbilical endothelial cells from smokers convert significantly more LDL into an atherogenic form than do cells derived from nonsmokers [116]. The LDL modifications were strongly thiol-dependent and the enhanced superoxide production seen in the cells derived from smokers was dependent on the presence of cysteine in the medium [116]. There is also evidence that cigarette smoking results in endothelial dysfunction in hypercholesterolemic patients by enhancing the oxidation of LDL [117]. Cigarette smoke-modified low-density lipoprotein has been reported to impair endothelium-dependent relaxation in isolated rabbit arteries [118].

Cells derived from smokers have higher levels of intracellular glutathione than those from nonsmokers and it may be that stimulation of cysteine uptake by the cells rejecting the enhanced total glutathione content could account for the enhanced superoxide production, all of which may be relevant to the pathophysiology of smoking-related cardiovascular disease [116]. It has already been noted that there is evidence to indicate that chemical modification of glutathione is a major damage mechanism of filtered cigarette smoke with free radical oxidations being less significant [92]. Endothelial cell cGMP production is decreased in a dose-dependent manner following cigarette smoking and endothelial cell detachment is increased following smoking [119]. Externally added thiols protect endothelial cells from damage and it has been suggested that they may bind an unknown component of smoke to bring about this protection [119]. In humans, the endothelial dysfunction observed following cigarette smoking lasts for about one hour and is not attenuated with repeat exposure [120].

Cigarette smoking is associated with increased monocyte-endothelial cell adhesion in humans [121,122]. Studies on hamsters using intravital microscopy and scanning electron microscopy demonstrate that cigarette smoking results in leucocytes adhering in clusters to the aortic endothelium and that aggregates of leukocytes and platelets are formed [123,124]. There is evidence that phagocytes may employ myeloperoxidase-generated reactive nitrogen intermediates as a physiological pathway for initiating lipid peroxidation as well as forming biologically active lipid and sterol oxidation productions in vivo [125]. Increased generation of oxygen free radicals by polymorphonuclear leucocytes may be responsible for the enhanced risk of certain smoking-related diseases [126]. The promotion of neutrophil infiltration and free radical

production in rats exposed to cigarette smoke has been shown to contribute significantly to the development of experimental inflammatory bowel disease [127].

Cigarette smoking has been shown to increase re-endothelialization in the rat following large vessel injury and this is associated with an increase in serum nitric oxide levels [114]. There is evidence that cigarette smoke-induced cell proliferation in the pulmonary arterial vessels is partly mediated through stimulation of endothelin-A receptors [128]. Conversely, cigarette smoke represses angiogenesis in the rat [103]. Cigarette smoking, but not transdermal nicotine delivery, is associated with borderline increases in plasma endothelin-1 levels but these are restricted to the first 10 minutes after the onset of smoking [129].

In a study of monozygotic twins discordant for smoking [130] found that cigarette smoking was associated with an atherogenic lipid profile along with changes in platelets and white cells which might reflect endothelial cell damage. Cigarette smoking increases total serum cholesterol levels [131].

Smokers have mean serum levels that are higher than nonsmokers for total and low-density lipoprotein cholesterol and triglyceride whereas high-density lipoprotein cholesterol levels are lower [132]. There is evidence that cigarette smoking results in increased platelet consumption in human atherosclerotic vessels as well as the production of larger platelets which are more active [133]. Cigarette smoking causes marked inhibition of substance P-induced tissue plasminogen activator released in vivo in humans [134]. Serum from cigarette smokers contains higher levels of von Willebrand factor and was more cytotoxic to endothelial cells in vitro than serum from nonsmokers [135].

Cigarette smoke and endothelial dysfunction

The endothelial dysfunction is including several pathological conditions of the endothel, for examples: altered anticoagulant and anti-inflammatory properties of the endothelium, impaired modulation of vascular growth, and dysregulation of vascular remodeling [136]. In much of the literature this term has been used to refer to an impairment of endothelium-dependent vasorelaxation caused by a loss of nitric oxide (NO) bioactivity in the vessel wall. Endothelial dysfunction is also considered the first important clinical predictor of later development of atherosclerosis and its complications. Indeed, it has been shown that pathological conditions that are associated with an increased risk of coronary heart disease are characterized by a decreased NO synthesis/release interfering with endothelium dependent regulation of coronary circulation. A decline in NO bioavailability may be caused by decreased expression of endothelial cell NO synthase (eNOS) [137], a lack of substrate or cofactors for eNOS [138], alterations of cellular signaling such that eNOS is not appropriately activated [139], and accelerated NO degradation by ROS [53]. Even before it was known to be NO, early studies showed that the endothelium-derived relaxing factor (EDRF) could be inactivated by superoxide and stabilized by superoxide dismutase (SOD). Now the EDRF is known to be nitric oxide.

Previous studies provide the evidence that cigarette smoke can alter the vascular endothelium not only morphologically but functionally. Both nitric oxide and iron monoxide generated within the blood vessel wall are important cellular messengers involved in the regulation of vacular smooth muscle tone [89]. Carbon monoxide generated through haeme oxigenase inhibits mitogen-induced proliferation of vascular smooth muscle cells and there is evidence that endogenous carbon monoxide serves as a protective factor limiting the excessive vascular smooth muscle cell proliferation associated with vascular disease [140].

1.9. THE PROTECTIVE EFFECT OF RESVERATROL IN CARDIOVASCULAR SYSTEM

Resveratrol (3,4′,5 trihydroxystilbene), a naturally-occurring molecule known as a phytoalexin, is synthesized by plants in response to attacks by fungi, bacteria, or other injurious substances; it is also known to possess an array of cardioprotective effects. [141-143].

The carrier of this phytoalexine is rising up from the 80's as it was thought to play an important role in the "French paradox" as a result of a moderate wine consumption. The French paradox is an observation that the French people suffer a relatively low incidence of coronary heart disease, despite having a diet relatively rich in saturated fats [144,145]. However, some health researchers question the validity of this paradox; in 2008 it was found that high doses of resveratrol mimicked some of the benefits of caloric restriction, including reduced effects of aging in a mice study [146], which confirmed some earlier works showing a longer lifespan in yeast and flies by activating sirtuin2 (Sir2) a histone deacetylase [147].

Resveratrol can increase the expression of antioxidative enzymes including superoxide dismutase (SOD1–2), catalase and glutathione peroxidase (GPx). Our recent study demonstrated that resveratrol also reduces the expression of the NADPH oxidase NOX4 [148], also regulates NOX2, SOD3 and GTP cyclohydrolase 1 (GCH1) [149]. Previous studies have demonstrated that resveratrol stimulates endothelial NO production. Multiple mechanisms have been implicated in this action [143], including (1) the enhancement of eNOS expression [150,151], (2) the reduction of eNOS acetylation [152] and (3) the stimulation of eNOS phosphorylation at serine 1177 [153]. SIRT1 seems to be involved in resveratrol-induced eNOS upregulation and eNOS deacetylation.

Controversially, some papers suggest the reexamination of the mechanism accounting for putative life-longevity effects of resveratrol since they queries its activating effect on Sir2 [154]. Despite of these contradictory data, many papers indicate a beneficial role on neurological, hepatic and cardiovascular systems and inflammatory processes [155] and emphasize its chemopreventive potential in inhibiting

cancer development by blocking the complex process of carcinogenesis at various stages of tumor initiation, promotion and progression.

Unfortunately, bioavailability of an *in vitro* investigated compound can dramatically determinate the biological effect *in vivo*. Resveratrol was shown to have a low bioavailability and rapid clearance from the plasma [156].

2. HYPOTHESIS AND AIMS OF STUDY

The cigarette smoke effect on possible functional and phenotypic changes of rat carotid arteries has not been investigated. On the basis of the aforementioned studies we hypothesized that water soluble components of cigarette smoke increase ROS generation in endothelial and/or smooth muscle cell which activate NF-κB and elicit the expression of pro-inflammatory mediators. To test this hypothesis, we characterized cigarette smoke-induced alterations in vascular $O_2^{\cdot -}$ and H_2O_2 production, endothelial NF-κB activation and expression of pro-inflammatory cytokines.

Specific aim 1: To determine whether the cigarette smoke increases the superoxide production. $O_2^{\cdot -}$ production was assessed by the lucigenin chemiluminescence and ethidium bromide fluorescence techniques.

Specific aim 2: To analyze the effect of cigarette smoke on the H_2O_2 production, we measured the H_2O_2 production with DCF or homovanillic acid fluorescence.

Specific aim 3: We tested whether cigarette smoke-induced pro-inflammatory changes in arterial phenotype: we used QRT-PCR, by which we characterized cigarette smoke-induced alterations in expression of pro- and anti-oxidant enzymes and pro-atherosclerotic genes (e.g. cytokines, chemokines) in carotid arteries.

Specific aim 4: To determine the NF-κB activity: The model predicts that CSE induces an increased generation $O_2^{\cdot -}$ by NAD(P)H oxidase, which scavenges vasodilator NO resulting in endothelial dysfunction. NAD(P)H oxidase-derived H_2O_2 (formed from $O_2^{\cdot -}$ via the action of SOD) activates the redox-sensitive transcription factor NF-κB up-regulating inflammatory gene expression. To prove the water soluble components of cigarette smoke promote pro-inflammatory phenotypic alterations in carotid arteries, we examined the NF-κB activity with dual luciferase assay.

Specific aim 5: To analyze whether the cigarette smoke extract alters rheology and shear forces at vascular surface which upregulates leukocyte adhesion molecules and

this may in part be responsible for the increased monocyte-endothelium cell adhesion we used the monocyte adhesion test on cigarette smoke treated vessels and cells.

Specific aim 6: Another set of experiments we analyzed whether the resveratrol protect against the TNF-α and IL-6 induced increased expression of adhesion molecules and resveratrol can inhibit TNF-α-induced NF-κB activation and monocyte adhesiveness.

3. MATERIALS AND METHODS

3.1. Animals and vessel isolation

Fourteen- to sixteen-week-old male Wistar rats (n = 20) were used. Protocols were approved by the Institutional Animal Care and Use Committee of New York Medical College and conformed to the current guidelines of the National Institutes of Health and the American Physiological Society for the use and care of laboratory animals. Animals were euthanized by a lethal injection of sodium pentobarbital and the carotid arteries were isolated and cleaned from the surrounding tissue as described [157,158].

3.2. Cigarette smoke exposure

The experimental group was exposed to the smoke of five commercial cigarettes (11 mg tar, 0.8 mg nicotine per cigarette) each day for a week according to the modified protocols of Meshi et al. [159], while the control group was not exposed to cigarette smoke.

3.3. Cigarette smoke extract preparation

Cigarette smoke extract (CSE; dissolved in DMSO, 40 mg/mL total particular matter, nicotine content: 6%; kept at -80 ^{o}C) was purchased from Murty Pharmaceuticals Inc. (Lexington, KY). From this stock solution working solutions (from 0.004 to 40 μg/mL final concentration) were prepared immediately before the experiments by dilution with physiological HEPES buffer. Assuming that cigarette smoke is extracted in the blood and equilibration occurs with the total blood volume, it is likely that plasma levels of water soluble components of cigarette smoke in smokers overlaps with the CSE concentrations used in the present study. Accordingly, the concentrations of nicotine in the CSE solutions used in these studies overlap with plasma levels of nicotine found in smokers.

3.4. Vessel culture and functional studies

On the 12-week, rats were anesthetized with intraperitoneal injection of sodium pentobarbital (50 mg/kg). Isolated carotid arteries were maintained in a stainless steel vessel culture chamber (Danish Myo Technology) under sterile conditions in F12

medium (GIBCO BRL) containing antibiotics (100 UI/L penicillin, 100 mg/L streptomycin) and supplemented with 5% FCS (Gibco/Invitrogen), as previously described [160,161]. Arteries were treated with CSE (0.004 to 40 µg/mL) or vehicle for 6 or 24 h in the absence or presence of inhibitors of signaling pathways, depending on the protocol. After the incubation period arterial segments were used for ROS measurements or were snap-frozen in liquid nitrogen for molecular biological processing. In other experiments endothelial function was assessed as previously described [162]. In brief, cultured arteries were cut into ring segments 2 mm in length and mounted on 40 µm stainless steel wires in the myographs chambers (Danish Myo Technology A/S, Inc., Atlanta, GA) containing Krebs buffer solution (118 mM NaCl, 4.7 mM KCl, 1.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.1 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 5.6 mM glucose; at 37°C; gassed with 95% air and 5% CO_2) for measurement of isometric tension. After an equilibration period of 1 hour during which a optimal passive tension of 0.5 g was applied to the rings (as determined from the vascular length-tension relationship), the vessels were contracted by phenylephrine (10^{-6} mol/L) and relaxations to acetylcholine (from 10^{-9} to 10^{-4} mol/L) and the NO donor S-nitrosopenicillamine (SNAP, from 10^{-9} to $3x10^{-5}$ mol/L mol/L) were obtained. In separate experiments vessels of control rats were incubated with the serum of cigarette smoke-exposed rats (for 6 h) or with nicotine (from 2.4 to 240 ng/mL, to match the nicotine concentration in the CSE used) and vascular ROS production was determined.

3.5. Measurement of vascular $O_2^{\cdot-}$ level: lucigenin chemiluminescence

$O_2^{\cdot-}$ production was assessed from vascular samples by the lucigenin chemiluminescence (5 µmol/L) method as we previously described [187, 192, 193]. In separate experiments, $O_2^{\cdot-}$ production of the carotid arteries pre-incubated with CSE (4 µg/mL, for 6 h) was determined in the absence and presence (pre-incubation: 1 hour) of diphenyleneiodonium (DPI, 10^{-5} mol/L, an inhibitor of flavoprotein-containing oxidases, including NAD(P)H oxidases), the $O_2^{\cdot-}$ scavengers SOD (200 U/mL) or Tiron (10 mmol/L), the cycloxygenase inhibitor indomethacin (10^{-5} mol/L), or N^{ω}-nitro-L-arginine-methyl-ester (L-NAME, $3x10^{-4}$ mol/L, an inhibitor of NO synthesis). In other experiments, superoxide production in CSE-treated aortic segments was also measured

using the same methods. NADPH oxidase activity was measured in vessel homogenates after the addition of 10^{-4} mol/L NADPH as reported [161].

3.6. Measurement of vascular $O_2^{\cdot-}$ level: ethidium bromide fluorescence

Hydroethidine, an oxidative fluorescent dye, was used to localize superoxide production in situ as we previously reported [161]. The living vessels pre-incubated with CSE (4 µg/mL, at 37 ^{o}C for 6 h) were incubated with hydroethidine (10^{-6} mol/L; at 37 ^{o}C for 60 min). Then the arteries were washed three times with warm buffer. Each experiment was performed in quadruplicates. *En face* preparations were imaged using Zeiss AxioCam Mrm camera mounted on a Zeiss Axiovert 200 fluorescence microscope (Carl Zeiss, Gottingen, Germany). In some experiments, confocal-like optical sections of the nuclei of endothelial and smooth muscle cells were captured using Zeiss Axioplan 2 microscope the equipped with the Zeiss ApoTome. All fields were selected by random movement of the microscope stage to another area within an intact luminal surface of the artery. Images were captured at x40 magnification and analyzed using the Zeiss Axionvision imaging software. Ten to fifteen entire fields per treatment group were analyzed with one image per field. The mean fluorescence intensities of each ethidium bromide (EB)–stained nuclei were measured in each view field.

3.7. Measurement of vascular H_2O_2 production

The cell-permeant oxidative fluorescent indicator dye C-H_2DCFDA (5 (and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate-acetyl ester, Invitrogen, Carlsbad CA) was used to assess H_2O_2 production in CSE-treated vessels according to the modified protocols of Miura et al [163]. C-H_2DCFDA is a 2’7’-dichlorofluorescein derivative that has longer retention within the cells. In brief, vessel segments were pre-incubated with CSE (0.04 to 40 µg/mL) then treated with C-H_2DCFDA (10^{-5} mol/L; at 37 ^{o}C for 60 min). In separate experiments vessels of control rats were incubated with the serum of cigarette smoke-exposed rats or with nicotine. In all experiments untreated arteries were used as controls. Then the arteries were washed three times. The endothelial layer of *en face* preparations were imaged as described above. Each experiment was performed in quadruplicates. Ten to fifteen entire fields per treatment group were analyzed with one image per field. The background-corrected mean

fluorescent intensities of each image were averaged. In some experiments vessels co-incubated with catalase were used as positive controls.

In addition, H_2O_2 production was also measured using the modified methods of Werner [164], after CSE treatment. Vessels were incubated with an assay mix consisting of homovanillic acid (HVA; 100 μmol/L), horseradish peroxidase (HRP, 5 U/mL) in Hepes-buffered salt solution (pH 7.5) at 37 ^{o}C for 1 h. The reaction was stopped with 80 μL/mL glycine solution (0.1 mol/L, pH 10, 0^{o}C). H_2O_2 -induced fluorescent product was assessed using a fluorimeter (excitation 321 nm, emission 421 nm) and the background-corrected fluorescent signal was normalized to tissue weight. Calibration curve was constructed using 0.01 to 100 μM H_2O_2 standards in assay mix (1 h at 37 ^{o}C) with or without catalase (200 U/mL).

3.8. Real-time quantitative PCR

QRT-PCR was used to elucidate the effect of smoking on the expression of inflammatory master cytokines (TNFα, IL-1β, IL-6) and iNOS in coronary arteries. These factors were shown to be associated with oxidative stress-related vascular inflammation and are considered to be early indicators of a pro-atherogenic microenvironment in the vascular wall. To elucidate the role of ROS and NAD(P)H oxidase in vascular inflammation, carotid arteries were treated with CSE in organoid culture with or without pre-treatment with PEG-catalase (200 U/mL), PEG-SOD (200 U/mL), apocynin ($3x10^{-4}$ mol/L, which inhibits NAD(P)H oxidases[187, 188]) or DPI. In separate experiments mRNA expression of the NAD(P)H oxidase catalytic subunit $gp91^{phox}$ was assessed in CSE-treated arteries. Total RNA from the arteries was isolated with Mini RNA Isolation Kit (Zymo Research, Orange, CA) and was reverse transcribed using Superscript II RT (Invitrogen) as described previously [161,165]. Real time RT-PCR technique was used to analyze mRNA expression using the Strategene MX3000, as reported [165,166]. Samples were run in triplicates. Efficiency of the PCR reaction was determined using dilution series of a standard vascular sample. Quantification was performed using the ΔΔCT method. The housekeeping gene β-actin was used for internal normalization. Oligonucleotides used for real-time QRT-PCR are listed in Table 1. Fidelity of the PCR reaction was determined by melting temperature analysis and visualization of product on a 2% agarose gel.

3.9. Transient transfection and luciferase assays

Effect of CSE on NF-κB activity in primary rat coronary arterial endothelial cells (CAECs) was tested by a reporter gene assay. CAECs were cultured as reported [166]. We used a NF-κB reporter comprised of an NF-κB response element upstream of firefly luciferase (NF-κB-Luc, Stratagene) and a renilla luciferase plasmid under the control of the CMV promoter (as an internal control). All transfections were performed with Novafector (Venn Nova LLC, Pompano Beach, Fl) following manufacturer protocols. Firefly and renilla luciferase activities were assessed after 42 h using the Dual Luciferase Reporter Assay Kit (Promega) and a luminometer. Pyrrolidine dithiocarbamate (10^{-5} mol/L), an inhibitor of NF-κB activation, was used as control. In other experiment the effect of resveratrol and TNF-α on NF-κB activity in HCAECs was tested by a reporter gene assay as described [166].

3.10. Monocyte adhesion assay

We measured adhesion of fluorescently labeled human monocytic (THP-1) cells to confluent monolayers of CAECs using a microplate-based assay as reported [166]. In brief, CAECs were grown to confluence in 96 well plates and were treated with increasing concentrations of CSE (incubation time: 6 h, at 37 ^{o}C) in the absence or presence (60 min pre-incubation) of apocynin, DPI or catalase. H_2O_2 and TNFα were used as positive controls. THP-1 cells were labeled with the fluorescent dye BCECF (5 μmol/L final concentration; Molecular Probes, Eugene. OR; in serum-free RPMI medium for 45 min at 37 °C). Then, cells were washed twice with pre-warmed (37°C) RPMI. PMA ((phorbol myristate acetate, 10^{-6} mol/L)-pretreated fluorescently labeled THP-1 cells (5 x 10^5 /well) were added the microplate wells containing confluent CAECs (medium removed; incubation time: 45 min, at 37 ^{o}C). Nonadherent THP-1 cells were removed by careful washing (three times with pre-warmed RPMI). Then, 200 μL of PBS was added to each well and fluorescence was measured using an Flx-800 (Bio-Tek Instruments) fluorescent plate reader (excitation : 485 nm; emission: 528 nm). Controls included measurement of total fluorescence of labeled cells before adhesion, controls for measuring autofluorescence of unlabeled cells, and measurement of monocyte adhesion to endothelial cell-free microplate wells.

In other experiments, monocyte enriched peripheral blood mononuclear cells (PBMC) were isolated from rats and BCECF-labeled mononuclear cell binding to the endothelium of carotid arteries and aortas pre-treated with CSE was determined as previously described [166]. Following treatment with CSE vessels were cut open (*en face*) and incubated with PMA (10^{-6} mol/L)-pretreated BCECF-loaded monocytes. After a 1 h incubation at 37 °C under, unbound monocytes were washed out. Bound monocytes were quantified by counting the cells under a fluorescent microscope.

In the resveratrol experiments, we measured adhesion of fluorescently labeled human monocytic (THP-1) cells to confluent monolayers of HCAECs using a microplate-based assay. In brief, HCAECs were grown to confluence in 96-well plates and were treated with TNF-α or IL-6 (0.1–10 ng/ml; incubation time: 2 h, at 37°C) in the absence or presence (60-min preincubation) of resveratrol (0.1–100 μmol/l) or pyrrolidine dithiocarbamate (10 μmol/l). THP-1 cells were labeled with the fluorescent dye calcein (5 μmol/l final concentration; Molecular Probes, Eugene, OR; in serum-free RPMI medium for 30 min at 37°C). Then cells were washed twice with prewarmed (37°C) RPMI. Phorbol myristate acetate (10^{-6} mol/l)-pretreated fluorescently labeled THP-1 cells (5 x 10^5/well) were added to the microplate wells containing confluent HCAECs (medium removed; incubation time: 120 min, at 37°C). Nonadherent THP-1 cells were removed by careful washing (three times with prewarmed RPMI). Then 200 μl of PBS were added to each well, and fluorescence was measured using an Flx-800 (Bio-Tek Instruments) fluorescent plate reader (excitation: 485 nm; emission: 528 nm). Controls included measurement of total fluorescence of labeled cells before adhesion, controls for measuring autofluorescence of unlabeled cells, and measurement of monocyte adhesion to endothelial cell-free microplate wells.

3.11. Vessel culture and conditions of electroporation

Square-wave electric pulses were delivered to the vessels with a cylindrical external electrode and an intraluminal electrode (1 cm long, 1-mm fixed distance between the electrodes; see Fig. 3C) by using an electric pulse generator (model CUY 201 BTX; Protech International, San Antonio, TX), and then the vessel segments were maintained in organoid culture for 24 h as described. The electric pulse was regulated as follows: voltage, 20 V; pulse-on time, 10 ms; interval time, 990 ms; and number of pulses, 10. These optimized parameters were determined in preliminary studies by measuring luciferase activity 1 day after electroporation of a CMV-driven renilla luciferase construct at various electrode voltages, pulse numbers, and pulse durations, according to the modified protocols of Matsumoto et al. [167] and Yamaoka et al. [168]. In separate experiments, electroporation with a pDsRed-Monomer vector (Clontech, Mountain View, CA) that expresses the red fluorescent DsRed-monomer fluorescent protein was used to assess the efficiency of the endothelial transfection by this method. Immunolabeling for -smooth muscle actin [169] was used to confirm the integrity of the vessel wall. Viability of endothelial and smooth muscle cells was assessed by measuring the magnitude of phenylephrine-induced contractions and acetylcholine-induced relaxations of vascular ring preparations, as described [166]. The concentration of the plasmid DNA solution used for transfection was adjusted to 1.5 µg/µl. After overnight culture, some vessels were pretreated with resveratrol (10 µmol/l, for 2 h). Then the aortas were incubated with TNF-α (10 ng/ml, for 4 h) and homogenized in 500 µl of dual luciferase assay lysis reagent (Promega). The firefly and renilla luciferase activities in homogenates of transfected aortas were measured by a procedure described previously [170].

3.12. Data analysis

Data were normalized to the respective control mean values. Data are mean±SEM Statistical analyses of data were performed by Student's *t* test or by 2-way ANOVA followed by the Tukey post hoc test, as appropriate. A value of $P<0.05$ was considered statistically significant.

4. RESULTS

4.1. Smoking-induced endothelial dysfunction

In vivo exposure of rats to cigarette smoke elicited impaired vascular relaxations to acetylcholine (Fig. 1A) and to SNAP (Fig. 1B), which were improved by apocynin treatment. *In vitro* CSE treatment tended to reduce NO-dependent relaxation of rat carotid arteries to acetylcholine (EC_{50}; Control: 3.0±0.8 x 10^{-6} mol/L, CSE: 5.0±1.3 x 10^{-6} mol/L, n.s.) and SNAP (EC_{50}; Control: 2.5±0.4 x 10^{-7} mol/L; 10.0±4.3 x 10^{-7} mol/L, n.s.), but the differences did not reach statistical significance.

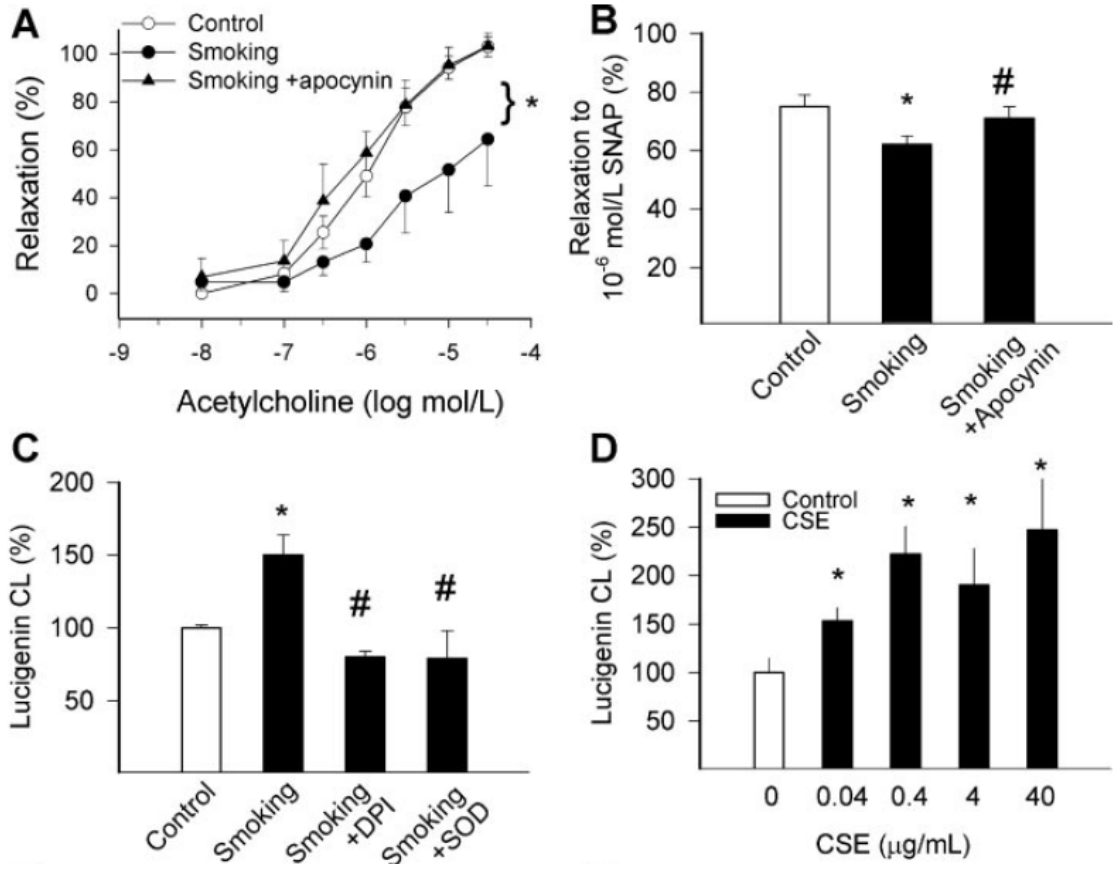

Figure 1. Relaxations to acetylcholine (**A**) and SNAP (**B**) in ring preparations of carotid arteries of control rats and rats exposed to cigarette smoke ("smoking", see Methods). The effect of pre-incubation with the NAD(P)H oxidase inhibitor apocyinin ($3x10^{-4}$ mol/L) is also shown. Data are mean ± S.E.M. (n=4-6 for each group). *p<0.05. **C:** $O_2^{\cdot-}$ generation in vessels of control rats and rats exposed to cigarette smoke. $O_2^{\cdot-}$ generation was determined by the lucigenin (5 µmol/L) chemiluminescence (CL) method in the absence and presence of the NAD(P)H oxidase inhibitor DPI (10^{-6} mol/L) or SOD (200 U/mL). Data are normalized to the mean value of the untreated control group. *P<0.05 (n=5-6 in each group). **D:** $O_2^{\cdot-}$ generation in rat carotid arterial segments treated with various concentrations of cigarette smoke extract (CSE). *P<0,05 (n=5-6 in each group).

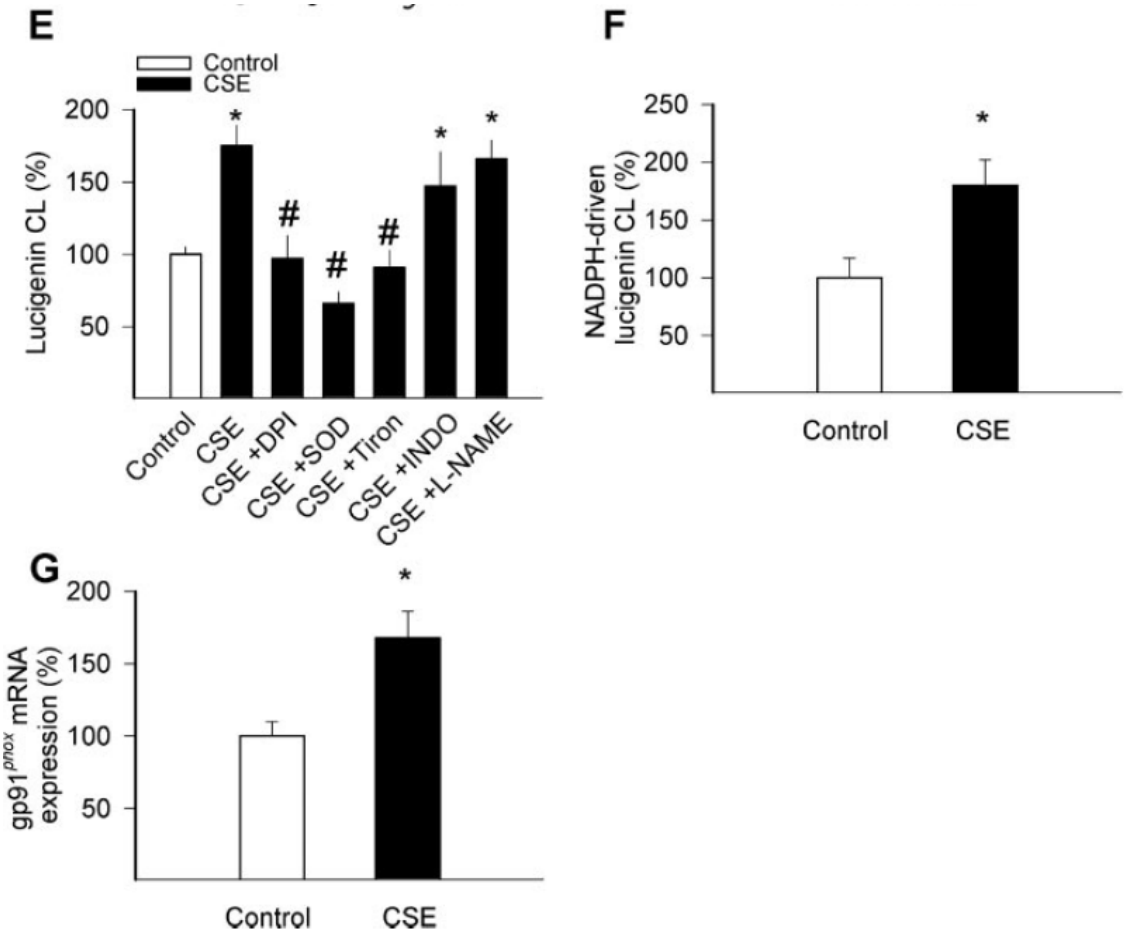

Figure 1. cont. E: $O_2^{\cdot -}$ generation in rat carotid arteries treated with CSE (4 µg/mL, for 6 hours) in the absence or presence of DPI (10^{-5} mol/L), SOD (200 U/mL), Tiron (10 mmol/L), indomethacin (INDO, 10^{-5} mol/L) and L-NAME (10^{-4} mol/L). Data are normalized to the mean value of the control group. *P<0.05 (n=8). **F:** NADPH (10^{-4} mol/L)-driven lucigenin CL in homogenates of vessels incubated overnight in the presence or absence of CSE (4 µg/mL). *P<0.05. **G:** mRNA expression of $gp91^{phox}$ in vessels incubated overnight in the presence or absence of CSE (4 µg/mL). *P<0.05.

4.2. Smoking and in vitro CSE exposure increase vascular $O_2^{\cdot -}$ and H_2O_2 production

In carotid arteries of cigarette smoke-exposed rats there was an increased SOD- and DPI-inhibitable lucigenin chemiluminescent signal indicating an increased NAD(P)H oxidase dependent $O_2^{\cdot -}$ generation (Fig. 1C). Also, in a dose-dependent manner CSE significantly increased $O_2^{\cdot -}$ production in the carotid arteries (Fig. 1D) and aortas (not shown). $O_2^{\cdot -}$ production in CSE treated vessels was significantly decreased by administration of DPI, Tiron or SOD, whereas it was unaffected by indomethacin, or L-NAME (Figure 1E). In CSE-treated vessels there was an increased NADPH-driven lucigenin CL (Fig. 1F) and an up-regulation of $gp91^{phox}$ mRNA (Fig. 1G).

Using the EB staining method, we found that in cross sections of carotid arteries of smoke-exposed rat the mean fluorescence intensity of endothelial and smooth muscle cell nuclei was significantly greater than that of control rats (Fig 2A). Representative fluorescent photomicrographs of EB-stained untreated and CSE treated carotid arteries (*en face* preparations) are shown in Figure 2B-C. In CSE treated vessels there was an intensive nuclear ethidium bromide staining, localized to the endothelial and smooth muscle cells, indicating that CSE promotes $O_2^{\cdot -}$ generation in both cell types (Fig. 2D).

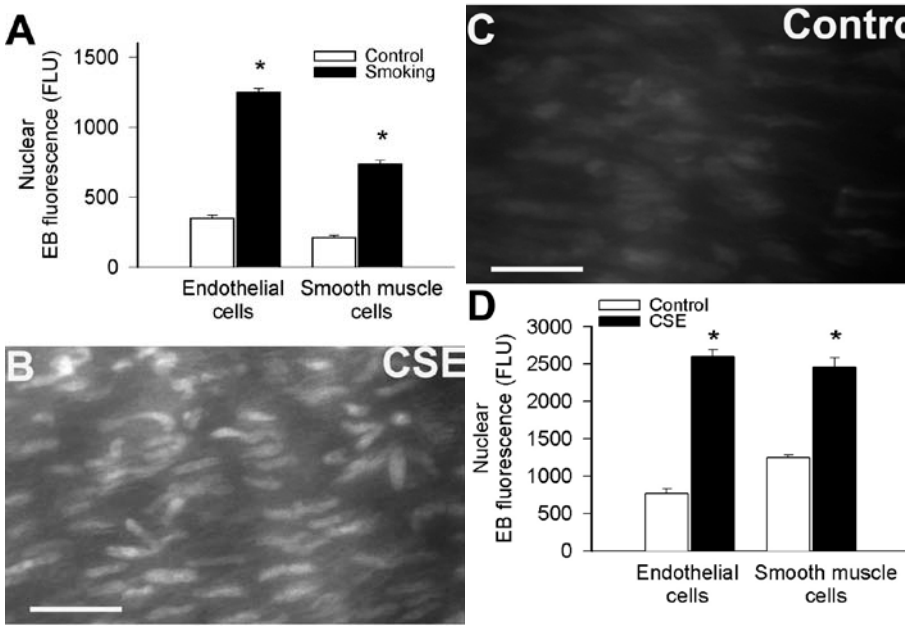

Figure 2. A: Nuclear ethidium bromide (EB) fluorescence in endothelial and smooth muscle cells in sections of carotid arteries of control rats and rats exposed to cigarette smoke. Vessels were incubated with the dye dihydroethidium, which produces a red nuclear fluorescence when oxidized to EB by $O_2^{\cdot -}$. **B:** Representative fluorescent photomicrographs of *en face* preparations of rat carotid arteries incubated with cigarette smoke extract (CSE; 4 µg/mL, for 6 h; Panel A) and untreated controls (**C**). On these compressed images the EB-stained elongated nuclei of vascular smooth muscle cells and the round nuclei of endothelial cells are visualized. Identical results were observed in 4 separate experiments. Scale bars: 10 µm. **D**: Summary graph showing the averaged fluorescence intensities of EB-stained nuclei of endothelial and smooth muscle cells (n=4 vessels in each group). Data are mean±S.E.M. *P<0,05

Exposure to CSE elicited substantial increases in vascular H_2O_2 generation as measured by both the DCF fluorescence (Fig. 3A) and HVA fluorescence (Fig. 3B) methods. Using exogenous H_2O_2 a calibration curve was constructed for HVA fluorescence, which was linear in the 10^{-7} to 10^{-4} mol/L range. DCF and HVA fluorescence could be inhibited by catalase showing the specificity of the signal (not shown). Incubation of vessels from control rats with the serum of cigarette smoke-exposed rats (for 6 h) significantly increased endothelial DCF fluorescence (Fig. 4A) and nuclear EB staining (not shown). Administration of nicotine did not result in significant increases in endothelial DCF fluorescence (Fig. 4B-C) and nuclear EB staining (not shown).

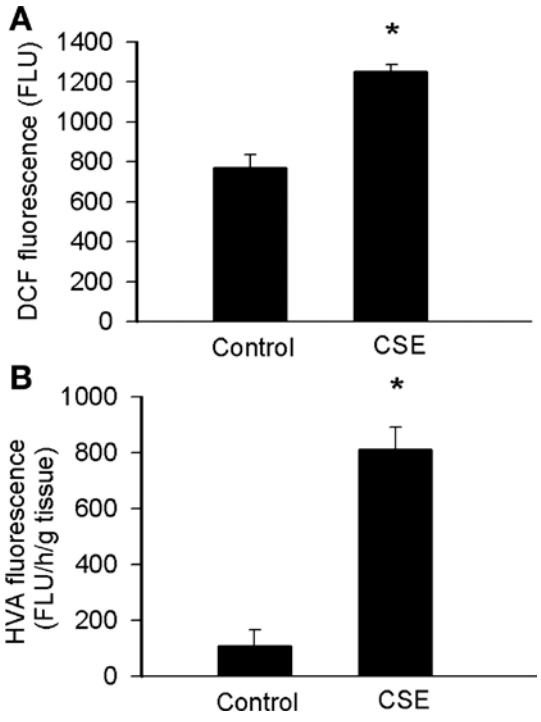

Figure 3. A: Cigarette smoke extract (CSE; 4 µg/mL) significantly increases H_2O_2 production in *en face* preparations of rat carotid arteries, as shown by the dichlorofluorescein (DCF) fluorescence method. Data are mean±S.E.M. *P<0,05. **B:** Demonstration of increased H_2O_2 production in rat carotid arteries exposed to CSE (4 µg/mL) using a homovanillic acid/horseradish peroxidase method. Results are expressed as arbitrary fluorescent units (mean ± S.E.M. n=3 for each group; *p<0.05 vs. untreated control).

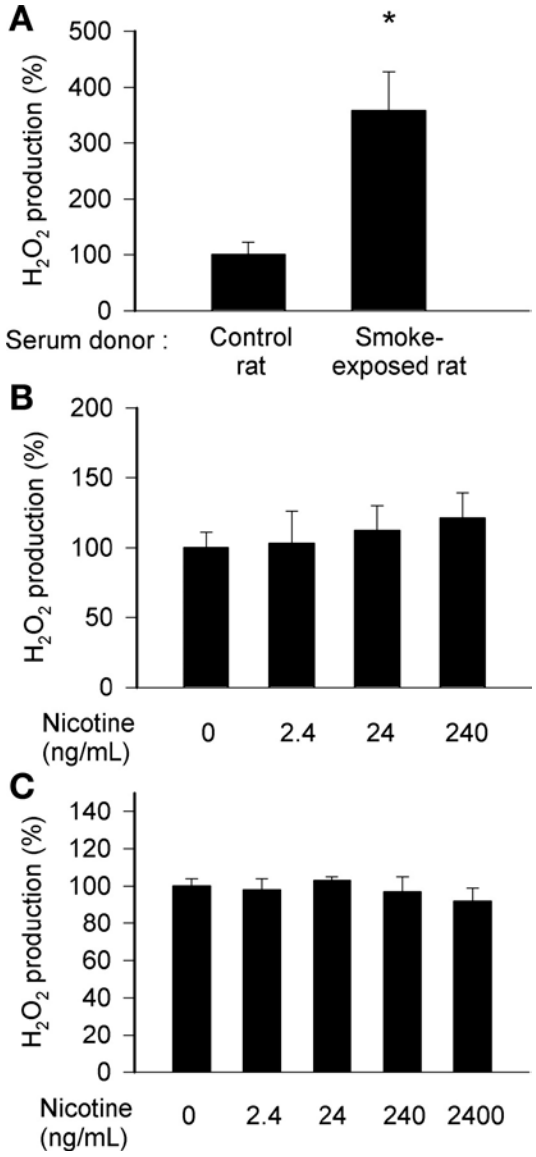

Figure 4. A: Incubation with serum from rats with cigarette smoke exposure (for 6 h) increases H_2O_2 production in *en face* preparations of rat carotid arteries, as shown by the dichlorofluorescein (DCF) fluorescence method. Data are mean±S.E.M. *P<0.05. **B:** H_2O_2 production in arteries treated with increasing doses of nicotine (DCF fluorescence method). Data are mean±S.E.M. *P<0.05. **C:** Results from plate reader-based measurements of DCF fluorescence in primary coronary arterial endothelial cells treated with increasing doses of nicotine. Data are mean±S.E.M. (n=8 for each group).

4.3. Smoking and in vitro CSE exposure up-regulate vascular expression of inflammatory markers

In coronary arteries of cigarette smoke-exposed rats mRNA expression of iNOS, TNF-α, IL-1β and IL-6 (Fig. 5A-D) and ICAM (not shown) significantly increased. Exposure of rat carotid arteries to increasing concentrations of CSE *in vitro* also elicited up-regulation of iNOS, TNF-α, IL-1β and IL-6 (Fig. 6A-D). The effect of CSE on ICAM-1 expression did not reach statistical significance (not shown). Expression of iNOS, TNF-α, IL-1β and IL-6 in CSE-treated vessels was significantly reduced by apocynin and PEG-catalase (Fig.6A-D). Similar results were obtained also with DPI.

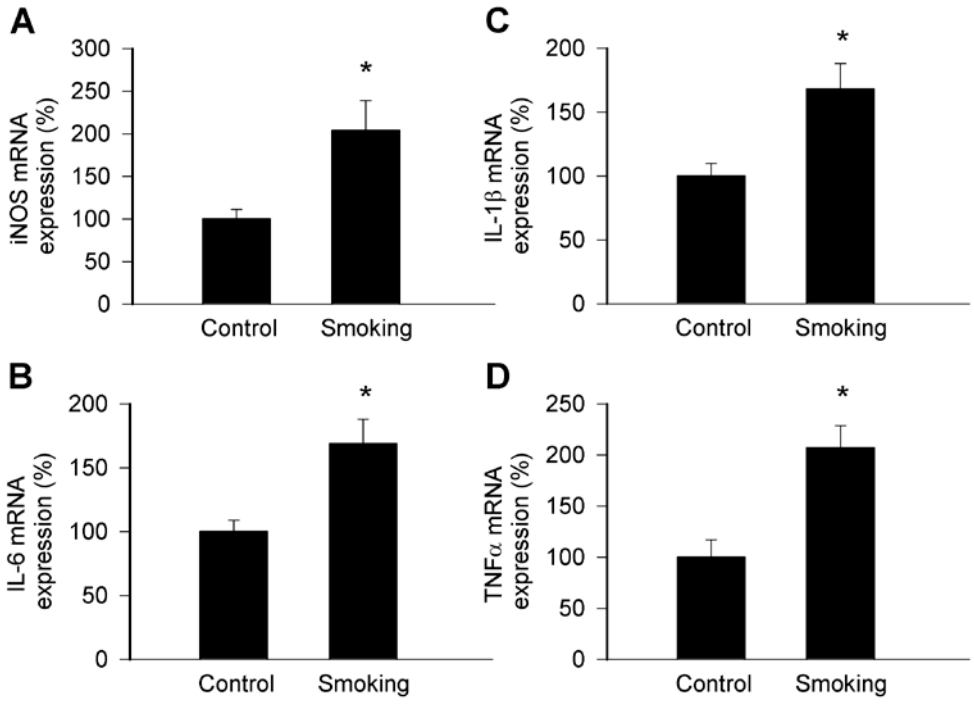

Figure 5.: Expression of iNOS (**A**), IL-6 (**B**), IL-1β (**C**) and TNF-α (**D**) in carotid arteries of control rats and rats exposed to cigarette smoke ("smoking", see Methods). Analysis of mRNA expression was performed by real-time QRT-PCR. β-actin was used for internal normalizations. Data are mean ± S.E.M. (n=5 for each group). **P*<0.05.

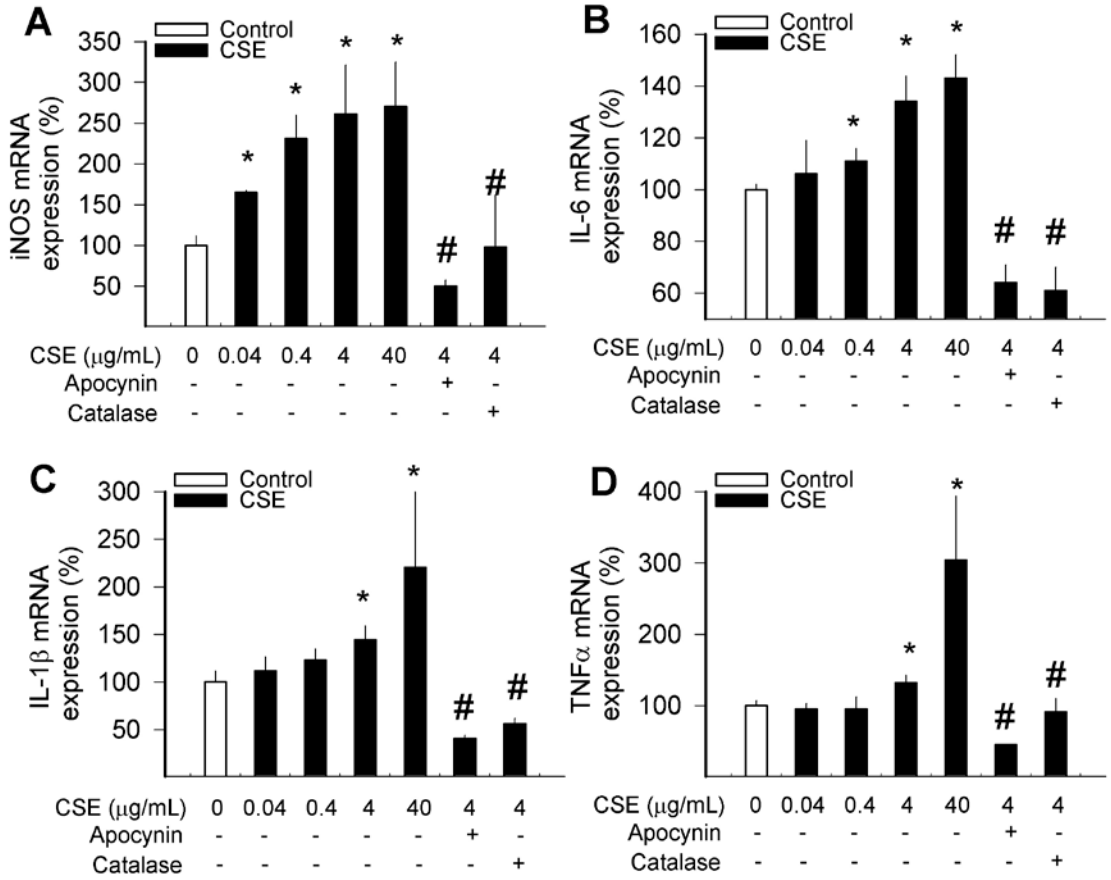

Figure 6. Effect of incubation with cigarette smoke extract (CSE; from 0.04 to 40 ng/mL, for 6 hours) on expression of iNOS (**A**), IL-6 (**B**), IL-1β (**C**) and TNF-α (**D**) in rat carotid arteries. Some vessels were pre-incubated with the NAD(P)H oxidase inhibitor apocynin ($3x10^{-4}$ mol/L) or catalase (200 U/mL). Analysis of mRNA expression was performed by real-time QRT-PCR. β-actin was used for internal normalizations. Data are mean ± S.E.M. (n=4-6 for each group). **P*<0.05.

4.4. Demonstration of CSE induced activation of NF-κB in endothelial cells

We demonstrated that CSE, in a concentration-dependent manner, significantly enhanced the transcriptional activity of NF-κB in CAECs (as indicated by an increase in the luciferase activity; Fig. 7A). Importantly, CSE induced NF-κB activity could be inhibited by catalase, DPI and apocynin (Fig. 7B), suggesting that NAD(P)H oxidase-derived H_2O_2 production plays a key role in CSE induced NF-κB activation in CAECs.

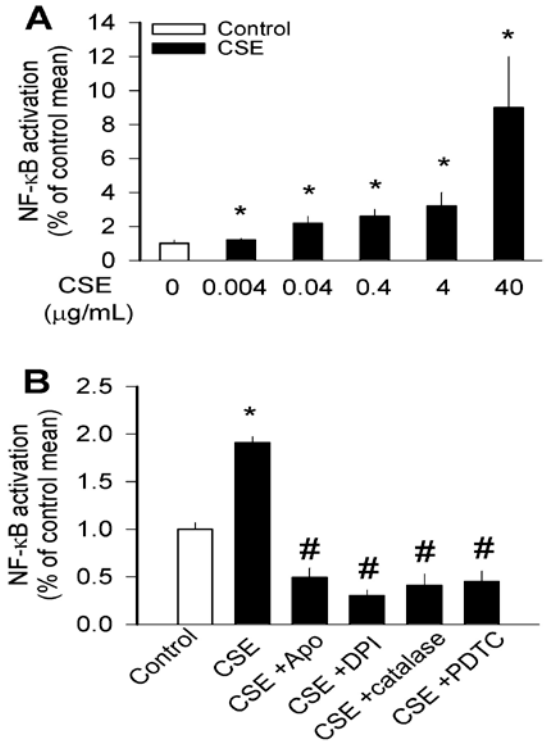

Figure 7. A: Reporter gene assay showing the effect of increasing concentrations of cigarette smoke extract (CSE) on NF-κB reporter activity in primary coronary arterial endothelial cells (CAECs). Endothelial cells were transiently co-transfected with NF-κB-driven firefly luciferase and CMV-driven ranilla luciferase constructs followed by CSE stimulation. Cells were then lysed and subjected to luciferase activity assay. After normalization relative luciferase activity was obtained from four to seven independent transfections. *p<0.05. **B:** The effects of pre-treatment with NAD(P)H oxidase inhibitors ($3x10^{-4}$ mol/L apocynin [APO], 10^{-5} DPI) or catalase (200 U/mL) on CSE-induced NF-κB reporter activity in CAECs. The NF-κB inhibitor PDTC was used as control. (Data are mean ± S.E.M. *p<0.05. vs. untreated; #P<0.05 vs. CSE only).

4.5. Smoking and in vitro CSE exposure enhance monocyte edhesion to the endothelium

Adhesiveness of activated THP-1 monocytic cells to the endothelial surface of carotid arteries of smoke-exposed rats was significantly increased (Fig. 8A). In vitro exposure of cultured carotid arteries and aortas to CSE also increased monocyte adhesiveness the endothelium (Fig. 8B). Incubation of CAECs with CSE also resulted in dose-dependent increases in the adhesion of THP-1 cells (Fig. 8C), which could be inhibited by apocynin, DPI or catalase (Fig. 8C). Increased monocyte adhesiveness induced by H_2O_2 and TNFα are shown as positive controls in Fig. 8C.

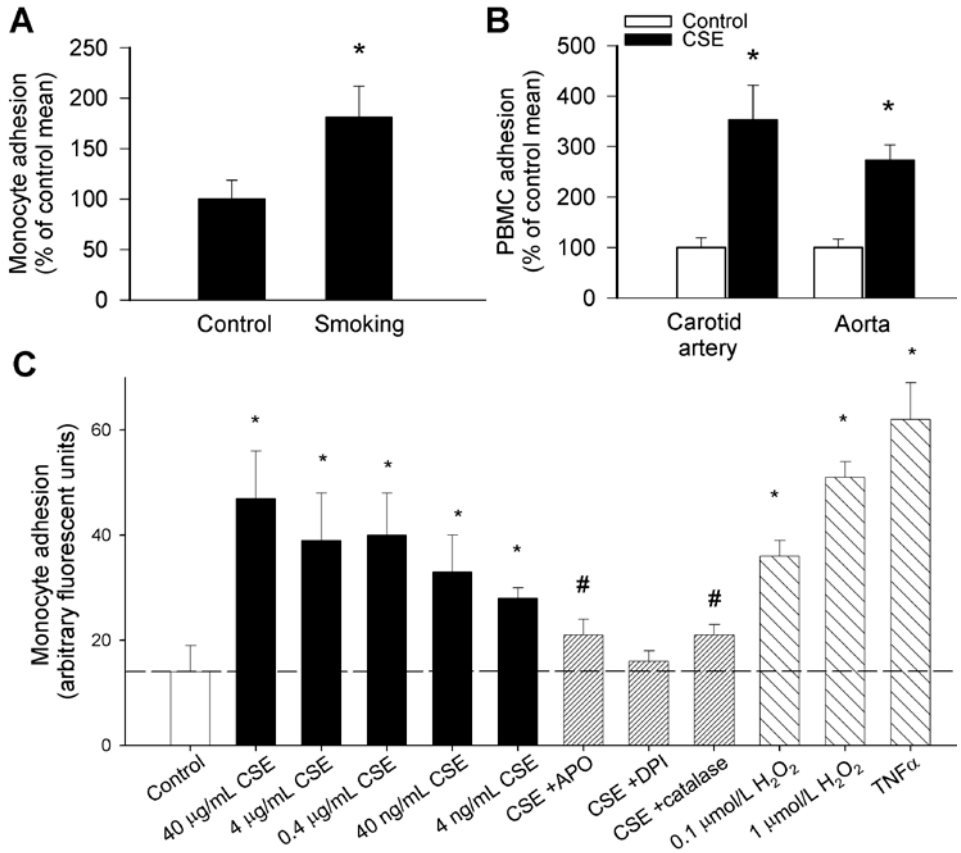

Figure 8. A: Results of monocyte adhesion assay (see Methods). Adhesion of fluorescently labeled PMA-stimulated THP-1 monocytes to the endothelial surface was significantly increased in carotid arteries of rats with *in vivo* exposure to cigarette smoke ("smoking"). Data are mean ± S.E.M. *p<0.05. vs. control. **B:** Treatment of carotid arteries and aortas with cigarette smoke extract (CSE) significantly increased adhesion of fluorescently labeled PMA-stimulated monocyte enriched peripheral blood mononuclear cells (PBMC). The effects of CSE were also assessed after pretreatment with the NAD(P)H oxidase inhibitor apocynin ($3x10^{-4}$ mol/L) and DPI (10^{-5} mol/L) or catalase (200 U/mL). Data are mean ± S.E.M. *p<0.05. vs. control. **C:** Treatment of primary coronary arterial endothelial cells with increasing concentrations of CSE significantly increased the adhesion of fluorescently labeled PMA-stimulated monocytes. The effects of pre-treatment with the NAD(P)H oxidase inhibitors DPI and apocynin or the H_2O_2 scavenger catalase on CSE-induced monocyte adhesion are shown. TNFα (10 ng/mL) and H_2O_2 were used as positive control. Data are mean ± S.E.M. *p<0.05. vs. control. #p<0.05 vs. CSE alone.

4.6. Resveratrol inhibits TNF-α, IL-6, and H_2O_2-induced increases in monocyte adhesiveness to HCAECs

TNF-α and IL-6 significantly increased monocyte adherence to cultured HCAECs in a concentration-dependent manner (Fig. 9. A and B). Pretreatment of the vessels with increasing concentrations of resveratrol reduced or prevented monocyte adhesion induced by both cytokines (Fig. 9. A and B). TNF-α-induced increases in monocyte adhesiveness were significantly reduced by pretreatment of HCAECs with apocynin or SOD plus catalase (Fig. 10A). Administration of H_2O_2 also substantially increased monocyte adherence to HCAECs in all concentrations studied (Fig. 10B), and this effect was also attenuated by resveratrol (Fig. 10B).

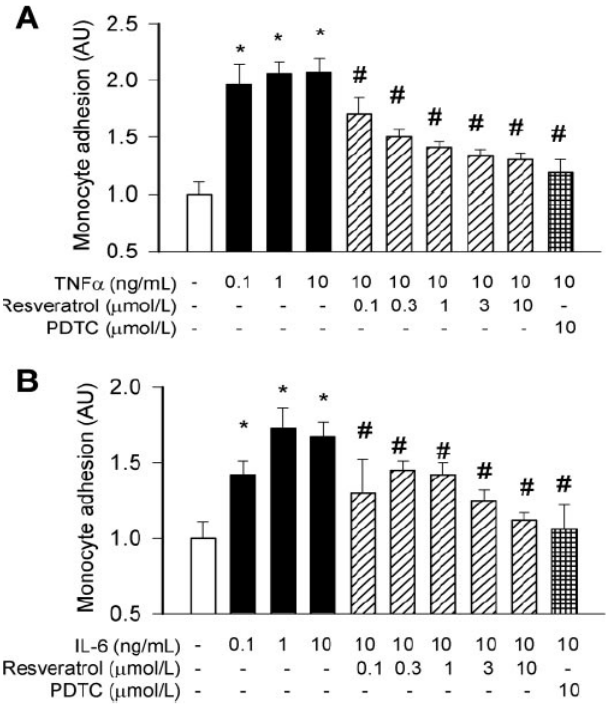

Figure 9. Treatment (2 h) of primary human coronary arterial endothelial cells (HCAECs) with increasing concentrations of TNF-α (**A**) and IL-6 (**B**) significantly increased the adhesion of fluorescently labeled phorbol myristate acetate-stimulated THP-1 monocytic cells. The effects of 10 ng/ml TNF-α and IL-6 were also assessed after pretreatment with increasing concentrations of resveratrol or an inhibitor of NF-κB [10 μmol/l pyrrolidine dithiocarbamate (PDTC)]. Values are means ± SE (n = 8 for each group). *P < 0.05 vs. untreated control. #P < 0.05 vs. cytokine treatment alone.

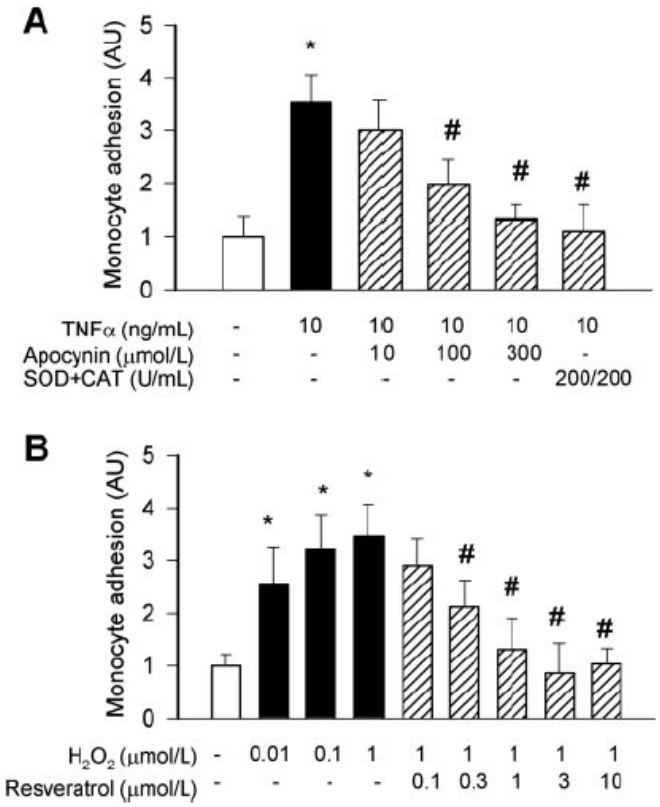

Figure 10. A: pretreatment of primary HCAECs with apocynin or the free-radical scavenger SOD plus catalase (CAT) significantly inhibited adhesion of fluorescently labeled phorbol myristate acetate-stimulated THP-1 monocytic cells induced by 10 ng/ml TNF-α. **B**: treatment of HCAECs with increasing concentrations of H_2O_2 significantly increased monocyte adhesion, which was inhibited by resveratrol. Values are means ± SE (n = 6–8 for each group). *P < 0.05 vs. untreated control. #P < 0.05 vs. cytokine treatment alone.

4.7. Resveratrol inhibits cytokine-induced NF-κB activation in HCAECs

To determine the effect of resveratrol on TNF-α-induced NF-κB activation, we transiently transfected HCAECs with a NF-κB-driven reporter gene construct and then pretreated the cells with resveratrol followed by stimulation with TNF-α (10 ng/ml, for 2 h). A significant increase in luciferase activity over the vector control was noted upon stimulation with TNF-α in the absence of resveratrol (Fig. 11A). Pretreatment of HCAECs (for 1 h) with resveratrol prevented TNF-α-induced NF-B activation in a concentration-dependent manner (Fig. 11A). IL-1 and IL-6 also activated NF-κB in HCAECs, and these effects were also significantly attenuated by resveratrol (Fig. 11E).

4.8. Resveratrol inhibits NF-κB activation in cultured aortas

First, we determined the optimal conditions for electroporation of reporter gene constructs. We found that luciferase activity increased in proportion to the voltage up to 20 V. However, at higher voltages, vascular luciferase activity was decreased. Acetylcholine-induced relaxations were unaltered up to 30 V (Fig. 11E); however, higher voltages resulted in a significant impairment of endothelium-dependent relaxations (not shown). The luciferase activity increased in proportion to pulse-on time and reached its maximum at 10 ms. Using a red fluorescent protein construct, we demonstrated that, using these voltage settings, the vascular endothelium can be effectively transfected (Fig. 11D). In cultured aortic segments transfected with the NF-κB reporter construct, TNF-α elicited significant increases in luciferase activity (Fig. 11B). TNF-α-induced increases in NF-κB activity were abolished by pretreatment with resveratrol (Fig. 11B).

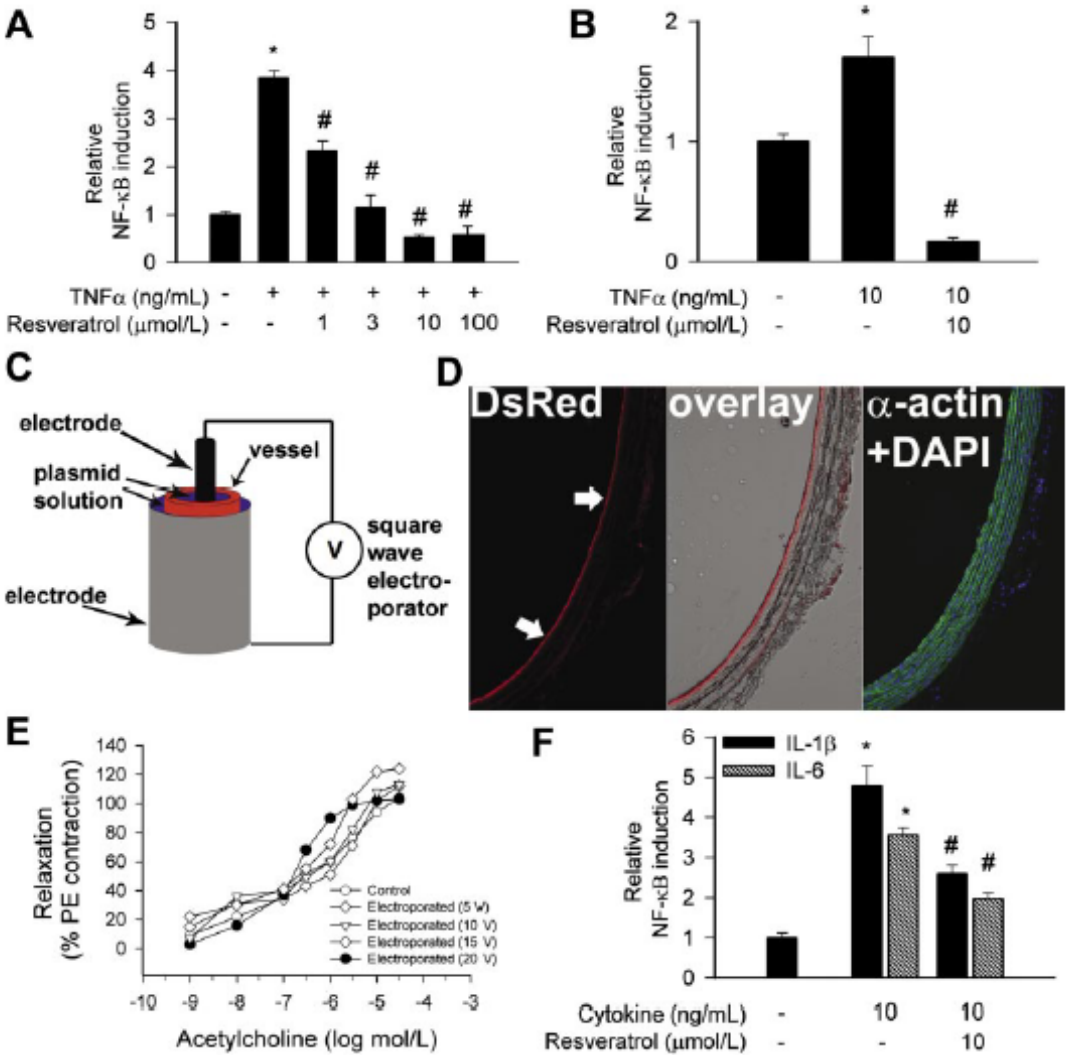

Figure 11. A: reporter gene assay showing the effects of TNF-α (10 ng/ml) on NF-κB reporter activity in cultured primary HCAECs in the absence and presence of resveratrol. Cells were transiently cotransfected with NF-κB-driven firefly luciferase and cytomegalovirus-driven renilla luciferase constructs followed by TNF-α stimulation. Cells were then lysed and subjected to luciferase activity assay. After normalization, relative luciferase activity was obtained from six independent transfections. In control experiments, PDTC was used to inhibit NF- κB activity. Values are means ± SE. *P < 0.05 vs. control; #P < 0.05 vs. without resveratrol. **B**: resveratrol inhibits the effect of TNF-α (10 ng/ml) on NF-κB reporter activity in rat aortas electroporated with the reporter gene construct. Values are means ± SE. *P < 0.05 vs. control; #P < 0.05 vs. without resveratrol. **C**: schematic representation of the

electroporation method for the transfer of genes into the vascular endothelium. Luminal space of the vessel segments was filled with DNA solution, and electric pulses were delivered to the vessel wall using an intraluminal electrode. **D left**: representative fluorescent image showing the endothelial expression of DsRed (red fluorescence; arrows) in a rat aorta segment electroporated with the pDsRed-Monomer Vector. Middle: overlay of red fluorescent image and bright field image. **D right**: immunolabeling for α-smooth muscle actin (green fluorescence) shows that there is no tissue necrosis in the electroporated vessel. 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI: blue) was used for nuclear staining. **E**: acetylcholine-induced relaxation in cultured (24 h) segments of the same rat aorta electroporated with different voltage settings. **F**: reporter gene assay showing the effects of resveratrol on IL-1β and IL-6 (10 ng/ml)-induced NF- κB reporter activity in HCAECs. PE, phenylephrine. Values are means ± SE. *P < 0.05 vs. control; #P < 0.05 vs. without resveratrol.

5. DISCUSSION

The cigarette smoke exposure elicits significant endothelial dysfunction in rat carotid arteries, which could be reversed by inhibition of the NAD(P)H oxidase (Fig. 1A-B). This finding accords with the increased NAD(P)H oxidase-dependent $O_2^{\cdot -}$ generation in these vessels (Fig. 1C). It is likely that water soluble components of cigarette smoke are directly responsible for the activation of the vascular NAD(P)H oxidase, because exposure of isolated arteries to CSE *in vitro*, in the absence of activated leukocytes, elicited significant $O_2^{\cdot -}$ production in a concentration-dependent manner (Fig. 1D). The primary source of CSE-induced $O_2^{\cdot -}$ generation seems to be the NAD(P)H oxidase (Fig. 1E,F), supporting the ex vivo observations (Fig. 1A-C). Accordingly, CSE seems to increase the expression of $gp91^{phox}$ in rat arteries (Fig. 1G) and in human pulmonary artery endothelial cells gp91 docking sequence-tat peptide (similar to apocynin) was reported to inhibit CSE-induced $O_2^{\cdot -}$ generation [171].

Dihydroethidine imaging revealed that both endothelial cells and vascular smooth muscle cells exhibit an up-regulated $O_2^{\cdot -}$ generation in vessels of cigarette smoke-exposed animals (Fig. 2A). Similarly, CSE challenge elicited oxidative stress in both cell types (Fig. 2B-D), mimicking the effects of in vivo exposure to cigarette smoke. Studies support the idea that CSE in vitro may induce NAD(P)H oxidase(s) in other cell types as well [171,172]. Previously we have shown that NAD(P)H oxidase(s) are abundantly expressed in rat arteries and an increased NAD(P)H oxidase activity is responsible for enhanced endothelial $O_2^{\cdot -}$ production in pathophysiological conditions such as aging, hyperhomocysteinemia [169] and hypertension [160]. NAD(P)H oxidase represent a common pathway eliciting endothelial dysfunction, thus it is logical to hypothesize that smoking will aggravate vascular injury in these pathophysiological conditions. We have shown previously that PKC-dependent phosphorylation of the $p47^{phox}$ subunit is crucial to the regulation of vascular NAD(P)H oxidase activity [173]. One can hypothesize that cigarette smoke particulate constituents activate protein kinase C, which leads to the increased vascular oxidative stress. It should be noted that in addition of the NAD(P)H oxidase, other cellular sources (such as xanthine oxidase, cytochrome P_{450} and mitochondrial sources) can also produce significant amounts of $O_2^{\cdot -}$, however, the role of these enzymes in CSE-induced oxidative stress is not well

understood (our data suggest that cyclooxygenase and eNOS does not play a major role in CSE-induced oxidative stress (Fig 1D).

We would like to point out that in the present study in addition to apocynin (which inhibits the activation of NAD(P)H oxidase by inhibiting the association of the cytoplasmic regulatory subunits and the membrane-bound oxidase subunits) we have also used the non-specific inhibitor DPI (which inhibits flavin-containing enzymes, including NAD(P)H oxidase and other enzyme systems as well). Because SOD enzymes catalyze the removal of $O_2^{\cdot -}$ with a rate constant of $2x10^9$ $mol^{-1} \cdot L \cdot s^{-1}$, it is likely that a significant portion of $O_2^{\cdot -}$ is dismutated, increasing also H_2O_2 levels. Indeed, in CSE-treated vessels there was a substantially increased H_2O_2 production (Fig. 3).

The component(s) of CSE that activate NAD(P)H oxidase at present are unknown. Although nicotine may impair endothelium-mediated vasodilation in microvessels [174], it could not mimic the effect of serum from cigarette smoke-exposed rats (Fig. 4A) or CSE (Fig. 3) on endothelial ROS production in our experiments (Fig. 4B, C)[175].

A study suggested that acrolein, a thiol-reactive α, β-unsaturated aldehyde that is abundantly present in cigarette smoke, is a potent inducer of NAD(P)H oxidase-derived $O_2^{\cdot -}$ generation in pulmonary arterial endothelial cells [171]. Other components of cigarette tar extracts that may promote ROS generation include semiquinones, hydroquinones, and quinines [176,177], acroleine-related α, ß-unsaturated aldehydes, such as crotonaldehyde, α,ß-unsaturated ketones, and a number of saturated aldehydes [178-180]. Because of their stability and water solubility, acrolein and other related compounds are likely to reach vascular beds remote from the primary site of exposure and, possibly, induce the production of ROS.

The next finding in this study was that *in vivo* exposure to cigarette smoke provokes an increase in the expression of pro-inflammatory cytokines (including IL-6, TNFα and IL-1β) and cytokine-sensitive inflammatory mediators (iNOS) in the vascular wall (Fig. 5). Importantly, these pro-inflammatory phenotypic alterations could also be mimicked by *in vitro* CSE challenge (Fig. 6). Studies suggest that exposure of cultured human endothelial cells to CSE or serum from smokers also results in pro-inflammatory gene expression [52,55,121,175,181,182]}. Atherosclerosis is a chronic inflammatory disease and pathological and epidemiological evidence suggest that pro-inflammatory cytokines

play a central role orchestrating the patholological processes underlying the development of the atherosclerotic plaque. Our findings are of great significance showing that cigarette smoke components are able to elicit a pro-atherogenic microenvironment in the vascular wall in the absence of circulating factors and immunocytes. Previously we have shown that vascular expression of pro-inflammatory cytokines and iNOS are frequently up-regulated in conditions associated with increased H_2O_2 production, such as hyperhomocysteinemia [169], aging [183] and hypertension [170]. To elucidate the possible link between cigarette smoke, production of ROS and pro-inflammatory cytokine expression we pharmacologically inhibited NAD(P)H oxidase and administered antioxidants to scavenge H_2O_2 and $O_2^{\cdot-}$. We found that both apocynin and catalase (but not SOD) prevented CSE-induced up-regulation of pro-inflammatory mediators, suggesting a central role for NAD(P)H oxidase-derived H_2O_2 in the process (Fig. 6).

We found that CSE can significantly increase NF-κB activation in endothelial cells (Fig. 7). The findings that apocynin and catalase were able to prevent CSE-induced activation of NF-κB [182] in endothelial cells (Fig. 7) provide strong evidence that NAD(P)H oxidase-derived H_2O_2 promotes vascular inflammation via NF-κB. This view is in line with our recent results showing that exogenous H_2O_2 is a very potent activator of NF-κB in endothelial cells [170].

Previous studies suggested that even moderate cigarette smoking leads to activation of circulating monocytes and their increased adhesion to the endothelium [184]. We found that both *in vivo* exposure of rats to cigarette smoke and *in vitro* incubation of vessels with CSE enhance the adhesive capacity of the vascular endothelial cells as well. (Fig. 8A-B). The role of water soluble components of cigarette smoke is supported by the findings that serum collected from smokers increases endothelial expression of endothelial adhesion molecules, including ICAM-1 [23]. Our results support the view that NAD(P)H oxidase-derived H_2O_2 plays a central role in endothelial activation induced by constituents of cigarette smoke. These findings agree with the results that increasing plasma vitamin C concentrations in smokers by oral supplementation decreased monocyte adhesion to values found in nonsmokers [185].

Water soluble components of cigarette smoke increase NAD(P)H-oxidase derived H_2O_2 generation in endothelial and smooth muscle cell, which induce a pro-inflammatory

vascular phenotype likely via mechanisms that involve NF-κB activation (Fig. 8D). We propose that cigarette smoking-induced oxidative stress and vascular inflammation will support atherosclerotic plaque formation in the carotid arteries increasing the morbidity of stroke.

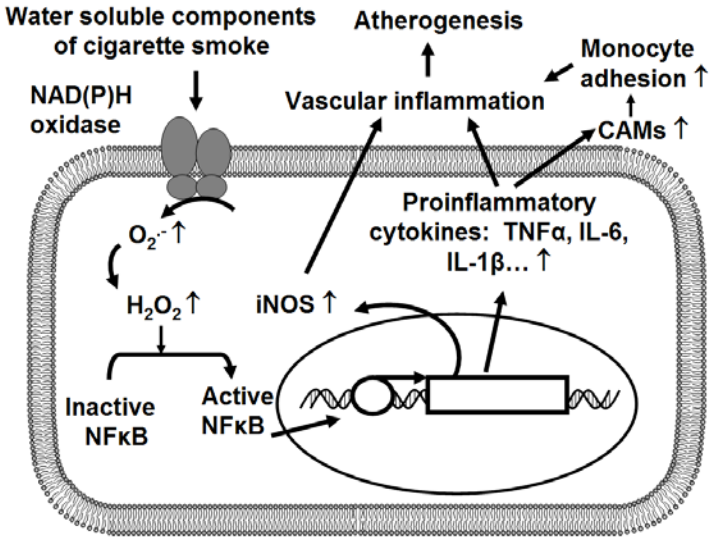

Figure 12: Proposed scheme for the mechanisms by which water soluble components of cigarette smoke promote pro-inflammatory phenotypic alterations in carotid arteries. The model predicts that CSE induces an increased generation $O_2^{\cdot-}$ by NAD(P)H oxidase, which scavenges vasodilator NO resulting in endothelial dysfunction. NAD(P)H oxidase-derived H_2O_2 (formed from $O_2^{\cdot-}$ via the action of SOD) activates the redox-sensitive transcription factor NF-κB up-regulating inflammatory gene expression. The resulting pro-inflammatory phenotype of carotid arteries will likely increase monocyte recruitment to the vascular wall and promote the development of atherosclerosis, especially if other risk factors (e.g. hypertension, hypercholesterolemia, hyperhomocysteinemia) are also present.

It was shown that the polyphenol, resveratrol has several beneficial effect on vascular health. The mechanism of action of resveratrol is not completely understood [186,187]. Resveratrol was reported to block the phosphorylation of p65 subunit of NF-κB inhibiting the nuclear translocation of NF-κB [186]. It is to be seen whether antioxidant action of resveratrol (e.g., scavenging of H_2O_2) contributes to its inhibitory effects on

NF-κB activation [188]. Interestingly, resveratrol does not inhibit TNF-α-dependent phosphorylation and degradation of I-κB [186].

We have shown that TNF-α-induced increased monocyte adhesiveness to HCAECs is NF-κB dependent, and it can be inhibited by resveratrol (Fig. 9A). IL-6 also elicited endothelial activation, and this effect also could be attenuated by resveratrol (Fig. 9B). It is significant that resveratrol also attenuated H_2O_2-induced monocyte adhesion to HCAECs in a similar concentration range (Fig. 10B). The findings that TNFα-induced monocyte adhesiveness was also attenuated both by inhibition of NAD(P)H oxidase and by reactive oxygen species scavengers (Fig. 10A) suggest that NAD(P)H oxidase-derived H_2O_2 plays a central role in endothelial activation. Indeed, previous studies by us and others demonstrated that TNF-α activates NAD(P)H oxidase(s) [189,190] in endothelial cells.

In addition TNF-α-induced NF-κB activation in HCAECs is inhibited by pretreatment with resveratrol (Fig. 11A). We confirmed that resveratrol was effective against TNF-α -induced NF-κB activation in intact blood vessels as well (Fig. 11B). Besides TNF-α, NF-κB can also be activated by other proinflammatory cytokines in many cell types. Accordingly, we found that IL-6 and IL-1 also activated NF-κB in HCAECs and that resveratrol effectively inhibited the activation of NF-κB induced by both cytokines (Fig. 11E). These results suggest that resveratrol may act at a step in which all of these agents converge in the signal transduction pathway leading to NF-κB activation. Importantly, we have evidence that administration of H_2O_2 activates NF-κB in coronary arterial endothelial cells [170,190]. In line with this finding, previously we demonstrated that TNFα -induced endothelial NF-κB activation can also be prevented by catalase and NAD(P)H inhibitors [166,190]. Taken together, these results suggest that NAD(P)H oxidase-derived H_2O_2 mediates cytokine-induced activation of NF-κB and that resveratrol interferes with this process. Submicromolar levels of resveratrol are sufficient to suppress cytokine-induced, NF-κB -dependent cellular responses. It is also important to note that many studies showed that other polyphenols are likely to exert resveratrol-like biological actions and are also present in the Mediterranean diet and red wines [191-193]. Obviously, further studies are required to define the potential beneficial health effects of resveratrol in chronically cigarette smoking patients.

6. CONCLUSION

1. We determined that in vivo cigarette smoke exposure and in vitro incubation with CSE increased NAD(P)H oxidase-dependent $O_2^{\cdot -}$ generation in the vessels by the lucigenin chemiluminescence and ethidium bromide fluorescence techniques. It is likely that water soluble components of cigarette smoke are directly responsible for the activation of the vascular NAD(P)H oxidase, because exposure of isolated arteries to CSE in vitro, in the absence of activated leukocytes, elicited significant $O_2^{\cdot -}$ production in a concentration-dependent manner. The primary source of CSE-induced $O_2^{\cdot -}$ generation seems to be the NAD(P)H oxidase.

2. We have shown that water soluble components of cigarette smoke increase NAD(P)H-oxidase derived H_2O_2 generation in endothelial and smooth muscle cell.

3. In vivo exposure to cigarette smoke provokes an increase in the expression of pro-inflammatory cytokines (including IL-6, TNFα and IL-1β) and cytokine-sensitive inflammatory mediators (iNOS) in the vascular wall. These pro-inflammatory phenotypic alterations could also be mimicked by in vitro CSE challenge.

4. Cigarette smoke extract can significantly increase NF-κB activation in endothelial cells. The findings that apocynin and catalase were able to prevent CSE-induced activation of NF-κB in endothelial cells provide strong evidence that NAD(P)H oxidase-derived H_2O_2 promotes vascular inflammation via NF-κB.

5. Both in vivo exposure of rats to cigarette smoke and in vitro incubation of vessels with CSE enhance the adhesive capacity of the vascular endothelial cells as well.

6. We have shown that TNF-α-induced increased monocyte adhesiveness to HCAECs is NF-κB dependent, and it can be inhibited by resveratrol. IL-6 also elicited endothelial activation, and this effect also could be attenuated by resveratrol. It is significant that resveratrol also attenuated H_2O_2-induced monocyte adhesion to

HCAECs in a similar concentration range. The findings that TNFα-induced monocyte adhesiveness was also attenuated both by inhibition of NAD(P)H oxidase and by reactive oxygen species scavengers suggest that NAD(P)H oxidase-derived H_2O_2 plays a central role in endothelial activation.

We confirmed that resveratrol was effective against TNF-α-induced NF-κB activation in intact blood vessels as well. Besides TNF-α, NF-κB can also be activated by other proinflammatory cytokines in many cell types. Accordingly, we found that IL-6 and IL-1 also activated NF-κB in HCAECs and that resveratrol effectively inhibited the activation of NF-κB induced by both cytokines.

7. SUMMARY

Although the cardiovascular morbidity and mortality induced by cigarette smoking exceeds the numbers of lung cancer related death, the molecular basis of smoking-induced vascular injury remains unclear. Epidemiological studies suggest that Mediterranean diets rich in resveratrol are associated with reduced risk of vascular diseases. To test the link between cigarette smoke, oxidative stress and vascular inflammation, rats were exposed to cigarette smoke of 5 cigarettes per day (for one week) or isolated arteries were exposed to cigarette smoke extract (CSE) in organoid culture. Carotid arteries isolated from control rats and cigarette smoke exposed rats NO-mediated flow-induced dilatation was measured by videomicroscopy. Arterial $O_2^{\cdot -}$ was measured by lucigenin chemiluminenscence and ethidium bromide fluorescence method. Arterial H_2O_2 production was measured by C-H_2DCFDA fluorescent method and modified method of Werner. The expression of proinflammatory cytokines and iNOS were assessed by quantitative RT-PCR. In separate experiments we evaluated whether resveratrol inhibits TNF-α-induced signal transduction in human coronary arterial endothelial cells (HCAECs). Cigarette smoke impaired acetylcholine-induced relaxations of carotid arteries, which could be improved by the inhibition of NAD(P)H oxidase. Lucigenin chemiluminescence measurements and dihydroethidine staining showed that both smoking and *in vitro* CSE exposure significantly increased vascular $O_2^{\cdot -}$ production. CSE also increased vascular H_2O_2 production. Vascular mRNA expression of the pro-inflammatory cytokines and iNOS was significantly increased by both smoking and CSE exposure, which could be prevented by inhibition of NAD(P)H. In cultured endothelial cells CSE elicited NF-κB activation and increased monocyte adhesiveness, which were prevented by apocynin and catalase. In TNF-α -treated HCAECs, resveratrol (in the submicromolar range) significantly attenuated expression of NF-κB-dependent inflammatory markers like iNOS. All these changes suggest that water soluble components of cigarette smoke (that are likely to be present in the bloodstream *in vivo* in smokers) elicit a pro-inflammatory shift in vascular phenotype and activate the vascular NAD(P)H oxidase. NAD(P)H oxidase-derived H_2O_2 activates NF-κB leading to pro-inflammatory alterations in vascular phenotype, which likely promotes development of atherosclerosis, especially if other risk factors are also present. Resveratrol at nutritionally relevant concentrations inhibits TNF-α-induced NF-κB activation and inflammatory gene expression and attenuates endothelial activation.

ÖSSZEFOGLALÓ

A cigarettafüst által okozott kardiovaszkuláris megbetegedések és halálozás aránya meghaladja a dohányzás okozta tüdőrák mortalitását, azonban a dohányzás okozta vaszkuláris károsodások molekuláris mechanizmusa pontosan még mindig nem ismert pontosan. Epidemiológiai megfigyelések alapján a rezveratrolban gazdag mediterrán diéta csökkenti a vaszkuláris megbetegések rizikóját. A cigarettafüst, az oxidatív stressz és a vaszkuláris gyulladás közötti kapcsolat tisztázására patkányok voltak cigarettafüstnek kitéve (egy hétig), valamint izolált patkány artériákat kezeltünk emelkedő koncentrációban vízben szolubilis cigarettafüst kivonattal (CSE) szervkultúrában. A karotisz artériákban a NO-mediált áramlás indukálta tágulást video mikroszkóppal mértük meg. Az artériákban termelődött $O_2^{\cdot-}$-ot lucigenin kemilumineszcencia és etidium bromide fluoreszcencia segítségével detektáltuk. Az artériás H_2O_2 produkciót C-H_2DCFDA fluoreszcencia változás és módosított Werner reakció segítségével mértük le. A gyulladásos citokinek expresszióját valamint az iNOS-t kvantitatív RT-PCR segítségével mértük. A transzkripciós faktor NF-κB aktivitását cigarettafüst kivonattal előkezelt endotél sejteken, valamint rezveratrol hatására tranziens transzkripcióval detektáltuk. Az endotélsejtek felszínén a cigarettafüst hatására megjelenő adhéziós molekulákat monocita adhéziós teszt segítségével mutattuk ki. Lucigenin kemilumineszcencia mérés során és a dihidroetidin festéssel azt tapasztaltuk, hogy mind a dohányzás mind az in vitro cigarettafüst kivonat hatására szignifikánsan emelkedett az erekben a $O_2^{\cdot-}$ termelés, valamint a H_2O_2 produkció. A dohányzás, valamint a cigarettafüst kivonat hatására az erekben a gyulladásos citokinek és az iNOS mRNS termelődése szignifikánsan növekedett, ezt a NAD(P)H oxidáz gátlásával megelőztük. Endotél sejtkultúrában a cigarettafüst extraktum megnövelte az NF-κB aktivációt és a monocita adhéziót, melyeket apocyninnel és katalázzal gátolni tudtunk. A TNF-α-val előkezelt HCAEC sejtekben a rezveratrol szignifikánsan csökkentette az NF-κB-dependens gyulladásos markerek expresszióját, gátolta az aktivált THP-1 sejtek adhézióját, a rezveratrol előkezelés szintén megelőzi a H_2O_2 függő endotél aktivációt. Riporter gén vizsgálatot alkalmazva úgy találtuk, hogy humán koronária arteriális endotél sejtekben TNF-α szignifikánsan növelte az NF-κB aktivitását, amelyet a rezveratrol gátolt. A cigarettafüst vízben oldékony összetevői gyulladásos folyamatot indítanak be az érrendszerben és aktiválják az ott található NAD(P)H oxidázt. A NAD(P)H oxidáz eredetű H_2O_2 aktiválja az NF-κB-ét, ez vezet az érgyulladáshoz, amely az érszűkület kialakulásában játszik szerepet. A rezveratrol gátolja a TNF-α indukálta NF-κB aktivációt és ezáltal a gyulladásos génexpresszót is, ezáltal csökkenti az endotélsejtek aktivációját.

8. REFERENCES

1. Ambrose JA, Barua RS: The pathophysiology of cigarette smoking and cardiovascular disease: An update. J Am Coll Cardiol 2004;43:1731-1737.
2. Doll R, Hill AB: Lung cancer and other causes of death in relation to smoking; a second report on the mortality of british doctors. Br Med J 1956;2:1071-1081.
3. Hammond EC, Horn D: Landmark article march 15, 1958: Smoking and death rates--report on forty-four months of follow-up of 187,783 men. By e. Cuyler hammond and daniel horn. Jama 1984;251:2840-2853.
4. Fowler G: James mackenzie lecture 1992. The indians' revenge. Br J Gen Pract 1993;43:78-81.
5. Doll R, Peto R: Mortality in relation to smoking: 20 years' observations on male british doctors. Br Med J 1976;2:1525-1536.
6. Manley AF: Cardiovascular implications of smoking: The surgeon general's point of view. J Health Care Poor Underserved 1997;8:303-310.
7. Ingall TJ, Homer D, Baker HL, Jr., Kottke BA, O'Fallon WM, Whisnant JP: Predictors of intracranial carotid artery atherosclerosis. Duration of cigarette smoking and hypertension are more powerful than serum lipid levels. Arch Neurol 1991;48:687-691.
8. Lee AJ, Fowkes FG, Carson MN, Leng GC, Allan PL: Smoking, atherosclerosis and risk of abdominal aortic aneurysm. Eur Heart J 1997;18:671-676.
9. Powell JT: Vascular damage from smoking: Disease mechanisms at the arterial wall. Vasc Med 1998;3:21-28.
10. Pittilo RM: Cigarette smoking and endothelial injury: A review. Adv Exp Med Biol 1990;273:61-78.
11. Pryor WA, Stone K: Oxidants in cigarette smoke. Radicals, hydrogen peroxide, peroxynitrate, and peroxynitrite. Ann N Y Acad Sci 1993;686:12-27; discussion 27-18.
12. Vlahos R, Bozinovski S, Jones JE, Powell J, Gras J, Lilja A, Hansen MJ, Gualano RC, Irving L, Anderson GP: Differential protease, innate immunity, and nf-kappab induction profiles during lung inflammation induced by subchronic cigarette smoke exposure in mice. Am J Physiol Lung Cell Mol Physiol 2006;290:L931-945.

13. Taylor AE, Johnson DC, Kazemi H: Environmental tobacco smoke and cardiovascular disease. A position paper from the council on cardiopulmonary and critical care, american heart association. Circulation 1992;86:699-702.

14. Bernhard D, Moser C, Backovic A, Wick G: Cigarette smoke - an aging accelerator? Exp Gerontol 2006

15. Beckman JS, Koppenol WH: Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and ugly. Am J Physiol 1996;271:C1424-1437.

16. Furchgott RF, Zawadzki JV: The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 1980;288:373-376.

17. Lockette W, Otsuka Y, Carretero O: The loss of endothelium-dependent vascular relaxation in hypertension. Hypertension 1986;8:II61-66.

18. Ibengwe JK, Suzuki H: Changes in mechanical responses of vascular smooth muscles to acetylcholine, noradrenaline and high-potassium solution in hypercholesterolemic rabbits. Br J Pharmacol 1986;87:395-402.

19. Ludmer PL, Selwyn AP, Shook TL, Wayne RR, Mudge GH, Alexander RW, Ganz P: Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. N Engl J Med 1986;315:1046-1051.

20. Ross R: The pathogenesis of atherosclerosis--an update. N Engl J Med 1986;314:488-500.

21. Ross R: The pathogenesis of atherosclerosis: A perspective for the 1990s. Nature 1993;362:801-809.

22. Haller H: Endothelial function. General considerations. Drugs 1997;53 Suppl 1:1-10.

23. Yamaguchi Y, Matsuno S, Kagota S, Haginaka J, Kunitomo M: Oxidants in cigarette smoke extract modify low-density lipoprotein in the plasma and facilitate atherogenesis in the aorta of watanabe heritable hyperlipidemic rabbits. Atherosclerosis 2001;156:109-117.

24. Benov LT, Beyer WF, Jr., Stevens RD, Fridovich I: Purification and characterization of the cu,zn sod from escherichia coli. Free Radic Biol Med 1996;21:117-121.

25. Stralin P, Karlsson K, Johansson BO, Marklund SL: The interstitium of the human arterial wall contains very large amounts of extracellular superoxide dismutase. Arterioscler Thromb Vasc Biol 1995;15:2032-2036.
26. Halliwell B: Antioxidant defence mechanisms: From the beginning to the end (of the beginning). Free Radic Res 1999;31:261-272.
27. Channon KM: Oxidative stress and coronary plaque stability. Arterioscler Thromb Vasc Biol 2002;22:1751-1752.
28. Griendling KK, Sorescu D, Ushio-Fukai M: Nad(p)h oxidase: Role in cardiovascular biology and disease. Circ Res 2000;86:494-501.
29. Landmesser U, Spiekermann S, Dikalov S, Tatge H, Wilke R, Kohler C, Harrison DG, Hornig B, Drexler H: Vascular oxidative stress and endothelial dysfunction in patients with chronic heart failure: Role of xanthine-oxidase and extracellular superoxide dismutase. Circulation 2002;106:3073-3078.
30. Zalba G, San Jose G, Moreno MU, Fortuno MA, Fortuno A, Beaumont FJ, Diez J: Oxidative stress in arterial hypertension: Role of nad(p)h oxidase. Hypertension 2001;38:1395-1399.
31. Cosentino F, Rubattu S, Savoia C, Venturelli V, Pagannonne E, Volpe M: Endothelial dysfunction and stroke. J Cardiovasc Pharmacol 2001;38 Suppl 2:S75-78.
32. Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM, Mitch WE, Harrison DG: Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. J Clin Invest 2003;111:1201-1209.
33. Somers MJ, Mavromatis K, Galis ZS, Harrison DG: Vascular superoxide production and vasomotor function in hypertension induced by deoxycorticosterone acetate-salt. Circulation 2000;101:1722-1728.
34. Bagi Z, Koller A: Lack of nitric oxide mediation of flow-dependent arteriolar dilation in type i diabetes is restored by sepiapterin. J Vasc Res 2003;40:47-57.
35. Taniyama Y, Griendling KK: Reactive oxygen species in the vasculature: Molecular and cellular mechanisms. Hypertension 2003;42:1075-1081.
36. Jones SA, O'Donnell VB, Wood JD, Broughton JP, Hughes EJ, Jones OT: Expression of phagocyte nadph oxidase components in human endothelial cells. Am J Physiol 1996;271:H1626-1634.

37. Lassegue B, Clempus RE: Vascular nad(p)h oxidases: Specific features, expression, and regulation. Am J Physiol Regul Integr Comp Physiol 2003;285:R277-297.

38. Babior BM: The activity of leukocyte nadph oxidase: Regulation by p47phox cysteine and serine residues. Antioxid Redox Signal 2002;4:35-38.

39. Banfi B, Clark RA, Steger K, Krause KH: Two novel proteins activate superoxide generation by the nadph oxidase nox1. J Biol Chem 2003;278:3510-3513.

40. Wingler K, Wunsch S, Kreutz R, Rothermund L, Paul M, Schmidt HH: Upregulation of the vascular nad(p)h-oxidase isoforms nox1 and nox4 by the renin-angiotensin system in vitro and in vivo. Free Radic Biol Med 2001;31:1456-1464.

41. Lassegue B, Sorescu D, Szocs K, Yin Q, Akers M, Zhang Y, Grant SL, Lambeth JD, Griendling KK: Novel gp91(phox) homologues in vascular smooth muscle cells : Nox1 mediates angiotensin ii-induced superoxide formation and redox- sensitive signaling pathways. Circ Res 2001;88:888-894.

42. Marumo T, Schini-Kerth VB, Brandes RP, Busse R: Glucocorticoids inhibit superoxide anion production and p22 phox mrna expression in human aortic smooth muscle cells. Hypertension 1998;32:1083-1088.

43. Dandona P, Karne R, Ghanim H, Hamouda W, Aljada A, Magsino CH, Jr.: Carvedilol inhibits reactive oxygen species generation by leukocytes and oxidative damage to amino acids. Circulation 2000;101:122-124.

44. Deshpande NN, Sorescu D, Seshiah P, Ushio-Fukai M, Akers M, Yin Q, Griendling KK: Mechanism of hydrogen peroxide-induced cell cycle arrest in vascular smooth muscle. Antioxid Redox Signal 2002;4:845-854.

45. Tschudi MR, Barton M, Bersinger NA, Moreau P, Cosentino F, Noll G, Malinski T, Luscher TF: Effect of age on kinetics of nitric oxide release in rat aorta and pulmonary artery. J Clin Invest 1996;98:899-905.

46. Szabo C: Multiple pathways of peroxynitrite cytotoxicity. Toxicol Lett 2003;140-141:105-112.

47. Torrecillas G, Boyano-Adanez MC, Medina J, Parra T, Griera M, Lopez-Ongil S, Arilla E, Rodriguez-Puyol M, Rodriguez-Puyol D: The role of hydrogen peroxide in the contractile response to angiotensin ii. Mol Pharmacol 2001;59:104-112.

48. Schuijt MP, Tom B, de Vries R, Saxena PR, Sluiter W, van Kats JP, Danser AH: Superoxide does not mediate the acute vasoconstrictor effects of angiotensin ii: A study in human and porcine arteries. J Hypertens 2003;21:2335-2344.

49. Touyz RM, Chen X, Tabet F, Yao G, He G, Quinn MT, Pagano PJ, Schiffrin EL: Expression of a functionally active gp91phox-containing neutrophil-type nad(p)h oxidase in smooth muscle cells from human resistance arteries: Regulation by angiotensin ii. Circ Res 2002;90:1205-1213.

50. Gao YJ, Lee RM: Hydrogen peroxide induces a greater contraction in mesenteric arteries of spontaneously hypertensive rats through thromboxane a(2) production. Br J Pharmacol 2001;134:1639-1646.

51. Touyz RM, Schiffrin EL: Increased generation of superoxide by angiotensin ii in smooth muscle cells from resistance arteries of hypertensive patients: Role of phospholipase d-dependent nad(p)h oxidase-sensitive pathways. J Hypertens 2001;19:1245-1254.

52. Wang H, Ye Y, Zhu M, Cho C: Increased interleukin-8 expression by cigarette smoke extract in endothelial cells. Environmental Toxicology and Pharmacology 2000;9:19-23.

53. Harrison D, Griendling KK, Landmesser U, Hornig B, Drexler H: Role of oxidative stress in atherosclerosis. Am J Cardiol 2003;91:7A-11A.

54. Harrison DG, Cai H, Landmesser U, Griendling KK: Interactions of angiotensin ii with nad(p)h oxidase, oxidant stress and cardiovascular disease. J Renin Angiotensin Aldosterone Syst 2003;4:51-61.

55. Nordskog BK, Blixt AD, Zieske AW, Hellmann GM: Mmp-1 polymorphic expression in aortic endothelial cells: Possible role in lesion development in smokers and nonsmokers. Cardiovasc Toxicol 2004;4:75-83.

56. Noronha-Dutra AA, Epperlein MM, Woolf N: Effect of cigarette smoking on cultured human endothelial cells. Cardiovasc Res 1993;27:774-778.

57. Waters D, Lesperance J, Gladstone P, Boccuzzi SJ, Cook T, Hudgin R, Krip G, Higginson L: Effects of cigarette smoking on the angiographic evolution of coronary atherosclerosis. A canadian coronary atherosclerosis intervention trial (ccait) substudy. Ccait study group. Circulation 1996;94:614-621.

58. Luscher TF, Tanner FC, Noll G: Lipids and endothelial function: Effects of lipid-lowering and other therapeutic interventions. Curr Opin Lipidol 1996;7:234-240.

59. Dempsey RJ, Moore RW: Amount of smoking independently predicts carotid artery atherosclerosis severity. Stroke 1992;23:693-696.

60. Howard G, Burke GL, Szklo M, Tell GS, Eckfeldt J, Evans G, Heiss G: Active and passive smoking are associated with increased carotid wall thickness. The atherosclerosis risk in communities study. Arch Intern Med 1994;154:1277-1282.

61. Diez-Roux AV, Nieto FJ, Comstock GW, Howard G, Szklo M: The relationship of active and passive smoking to carotid atherosclerosis 12-14 years later. Prev Med 1995;24:48-55.

62. Howard G, Wagenknecht LE, Burke GL, Diez-Roux A, Evans GW, McGovern P, Nieto FJ, Tell GS: Cigarette smoking and progression of atherosclerosis: The atherosclerosis risk in communities (aric) study. Jama 1998;279:119-124.

63. Inoue T, Oku K, Kimoto K, Takao M, Nomoto J, Handa K, Kono S, Arakawa K: Relationship of cigarette smoking to the severity of coronary and thoracic aortic atherosclerosis. Cardiology 1995;86:374-379.

64. Zieske AW, Takei H, Fallon KB, Strong JP: Smoking and atherosclerosis in youth. Atherosclerosis 1999;144:403-408.

65. McGill HC, Jr., McMahan CA, Malcom GT, Oalmann MC, Strong JP: Effects of serum lipoproteins and smoking on atherosclerosis in young men and women. The pday research group. Pathobiological determinants of atherosclerosis in youth. Arterioscler Thromb Vasc Biol 1997;17:95-106.

66. Witteman JC, Grobbee DE, Valkenburg HA, van Hemert AM, Stijnen T, Hofman A: Cigarette smoking and the development and progression of aortic atherosclerosis. A 9-year population-based follow-up study in women. Circulation 1993;88:2156-2162.

67. Pittilo RM, Mackie IJ, Rowles PM, Machin SJ, Woolf N: Effects of cigarette smoking on the ultrastructure of rat thoracic aorta and its ability to produce prostacyclin. Thromb Haemost 1982;48:173-176.

68. Pittilo RM, Bull HA, Gulati S, Rowles PM, Blow CM, Machin SJ, Woolf N: Nicotine and cigarette smoking: Effects on the ultrastructure of aortic endothelium. Int J Exp Pathol 1990;71:573-586.

69. Bull HA, Pittilo RM, Woolf N, Machin SJ: The effect of nicotine on human endothelial cell release of prostaglandins and ultrastructure. Br J Exp Pathol 1988;69:413-421.

70. Benowitz NL, Gourlay SG: Cardiovascular toxicity of nicotine: Implications for nicotine replacement therapy. J Am Coll Cardiol 1997;29:1422-1431.

71. Lin L, Nasjletti A: Prostanoid-mediated vascular contraction in normotensive and hypertensive rats. Eur J Pharmacol 1992;220:49-53.

72. Cucina A, Sapienza P, Borrelli V, Corvino V, Foresi G, Randone B, Cavallaro A, Santoro-D'Angelo L: Nicotine reorganizes cytoskeleton of vascular endothelial cell through platelet-derived growth factor bb. J Surg Res 2000;92:233-238.

73. Villablanca AC: Nicotine stimulates DNA synthesis and proliferation in vascular endothelial cells in vitro. J Appl Physiol 1998;84:2089-2098.

74. Smith CJ, Steichen TJ: The atherogenic potential of carbon monoxide. Atherosclerosis 1993;99:137-149.

75. Penn A, Currie J, Snyder C: Inhalation of carbon monoxide does not accelerate arteriosclerosis in cockerels. Eur J Pharmacol 1992;228:155-164.

76. Kourembanas S, Morita T, Liu Y, Christou H: Mechanisms by which oxygen regulates gene expression and cell-cell interaction in the vasculature. Kidney Int 1997;51:438-443.

77. Penn A, Chen LC, Snyder CA: Inhalation of steady-state sidestream smoke from one cigarette promotes arteriosclerotic plaque development. Circulation 1994;90:1363-1367.

78. Penn A, Snyder CA: Inhalation of sidestream cigarette smoke accelerates development of arteriosclerotic plaques. Circulation 1993;88:1820-1825.

79. Sun D, Huang A, Koller A, Kaley G: Short-term daily exercise activity enhances endothelial no synthesis in skeletal muscle arterioles of rats. J Appl Physiol 1994;76:2241-2247.

80. Zhu BQ, Sun YP, Sievers RE, Isenberg WM, Glantz SA, Parmley WW: Passive smoking increases experimental atherosclerosis in cholesterol-fed rabbits. J Am Coll Cardiol 1993;21:225-232.

81. Glantz SA, Parmley WW: Passive smoking and heart disease. Mechanisms and risk. Jama 1995;273:1047-1053.

82. Lee CK, Klopp RG, Weindruch R, Prolla TA: Gene expression profile of aging and its retardation by caloric restriction. Science 1999;285:1390-1393.

83. Denson KW: Setting the tobacco record straight? Lancet 2000;356:337.

84. Lyons MJ, Gibson JF, Ingram DJ: Free-radicals produced in cigarette smoke. Nature 1958;181:1003-1004.

85. Noronha-Dutra AA, Steen EM: Lipid peroxidation as a mechanism of injury in cardiac myocytes. Lab Invest 1982;47:346-353.

86. Noronha-Dutra AA, Steen EM, Woolf N: The early changes induced by isoproterenol in the endocardium and adjacent myocardium. Am J Pathol 1984;114:231-239.

87. Hennig B, Chow CK: Lipid peroxidation and endothelial cell injury: Implications in atherosclerosis. Free Radic Biol Med 1988;4:99-106.

88. Jeremy JY, Mikhailidis DP, Pittilo RM: Cigarette smoking and cardiovascular disease. J R Soc Health 1995;115:289-295.

89. Siow RC, Sato H, Leake DS, Ishii T, Bannai S, Mann GE: Induction of antioxidant stress proteins in vascular endothelial and smooth muscle cells: Protective action of vitamin c against atherogenic lipoproteins. Free Radic Res 1999;31:309-318.

90. Leonard MB, Lawton K, Watson ID, MacFarlane I: Free radical activity in young adult cigarette smokers. J Clin Pathol 1995;48:385-387.

91. Leonard MB, Lawton K, Watson ID, Patrick A, Walker A, MacFarlane I: Cigarette smoking and free radical activity in young adults with insulin-dependent diabetes. Diabet Med 1995;12:46-50.

92. Maranzana A, Mehlhorn RJ: Loss of glutathione, ascorbate recycling, and free radical scavenging in human erythrocytes exposed to filtered cigarette smoke. Arch Biochem Biophys 1998;350:169-182.

93. Pittilo RM, Clarke JM, Harris D, Mackie IJ, Rowles PM, Machin SJ, Woolf N: Cigarette smoking and platelet adhesion. Br J Haematol 1984;58:627-632.

94. Celermajer DS, Sorensen K, Ryalls M, Robinson J, Thomas O, Leonard JV, Deanfield JE: Impaired endothelial function occurs in the systemic arteries of children with homozygous homocystinuria but not in their heterozygous parents. J Am Coll Cardiol 1993;22:854-858.

95. Nene S, Gelabert H, Moore W, Quinones-Baldrich W, Santibanez-Gallerani A, Ignarro L: Cigarette smoking increases endothelial-derived vasorelaxation in the rat carotid artery in a dose-dependent manner. J Surg Res 1997;71:101-106.

96. Lekakis J, Papamichael C, Vemmos C, Nanas J, Kontoyannis D, Stamatelopoulos S, Moulopoulos S: Effect of acute cigarette smoking on endothelium-dependent brachial artery dilatation in healthy individuals. Am J Cardiol 1997;79:529-531.

97. Quillen JE, Rossen JD, Oskarsson HJ, Minor RL, Jr., Lopez AG, Winniford MD: Acute effect of cigarette smoking on the coronary circulation: Constriction of epicardial and resistance vessels. J Am Coll Cardiol 1993;22:642-647.

98. McVeigh GE, Lemay L, Morgan D, Cohn JN: Effects of long-term cigarette smoking on endothelium-dependent responses in humans. Am J Cardiol 1996;78:668-672.

99. Wang XL: Cigarette smoking, DNA variants in endothelial nitric oxide synthase gene and vascular disease. Contrib Nephrol 2000;130:53-67.

100. Verleden GM, Dupont LJ, Verpeut AC, Demedts MG: The effect of cigarette smoking on exhaled nitric oxide in mild steroid-naive asthmatics. Chest 1999;116:59-64.

101. Kharitonov SA, Robbins RA, Yates D, Keatings V, Barnes PJ: Acute and chronic effects of cigarette smoking on exhaled nitric oxide. Am J Respir Crit Care Med 1995;152:609-612.

102. Su Y, Han W, Giraldo C, De Li Y, Block ER: Effect of cigarette smoke extract on nitric oxide synthase in pulmonary artery endothelial cells. Am J Respir Cell Mol Biol 1998;19:819-825.

103. Ma L, Chow JY, Liu ES, Cho CH: Cigarette smoke and its extract delays ulcer healing and reduces nitric oxide synthase activity and angiogenesis in rat stomach. Clin Exp Pharmacol Physiol 1999;26:828-829.

104. Wright JL, Dai J, Zay K, Price K, Gilks CB, Churg A: Effects of cigarette smoke on nitric oxide synthase expression in the rat lung. Lab Invest 1999;79:975-983.

105. Chambers DC, Tunnicliffe WS, Ayres JG: Acute inhalation of cigarette smoke increases lower respiratory tract nitric oxide concentrations. Thorax 1998;53:677-679.

106. Rangemark C, Wennmalm A: Smoke-derived nitric oxide and vascular prostacyclin are unable to counteract the platelet effect of increased thromboxane formation in healthy female smokers. Clin Physiol 1996;16:301-315.

107. Kugiyama K, Yasue H, Ohgushi M, Motoyama T, Kawano H, Inobe Y, Hirashima O, Sugiyama S: Deficiency in nitric oxide bioactivity in epicardial coronary arteries of cigarette smokers. J Am Coll Cardiol 1996;28:1161-1167.

108. Obwegeser R, Oguogho A, Ulm M, Berghammer P, Sinzinger H: Maternal cigarette smoking increases f2-isoprostanes and reduces prostacyclin and nitric oxide in umbilical vessels. Prostaglandins Other Lipid Mediat 1999;57:269-279.

109. Xie Y, Garban H, Ng C, Rajfer J, Gonzalez-Cadavid NF: Effect of long-term passive smoking on erectile function and penile nitric oxide synthase in the rat. J Urol 1997;157:1121-1126.

110. Thom SR, Xu YA, Ischiropoulos H: Vascular endothelial cells generate peroxynitrite in response to carbon monoxide exposure. Chem Res Toxicol 1997;10:1023-1031.

111. Ota Y, Kugiyama K, Sugiyama S, Ohgushi M, Matsumura T, Doi H, Ogata N, Oka H, Yasue H: Impairment of endothelium-dependent relaxation of rabbit aortas by cigarette smoke extract--role of free radicals and attenuation by captopril. Atherosclerosis 1997;131:195-202.

112. Freischlag JA, Johnson D, Farooq MM, Doty J, Cambria RA, Seabrook GR, Towne JB: Cigarette smoke impairs endothelium-dependent relaxation in rabbit superficial femoral veins. J Surg Res 1999;81:77-80.

113. Mays BW, Freischlag JA, Eginton MT, Cambria RA, Seabrook GR, Towne JB: Ascorbic acid prevents cigarette smoke injury to endothelium-dependent arterial relaxation. J Surg Res 1999;84:35-39.

114. Sarkar R, Gelabert HA, Mohiuddin KR, Thakor DK, Santibanez-Gallerani AS: Effect of cigarette smoke on endothelial regeneration in vivo and nitric oxide levels. J Surg Res 1999;82:43-47.

115. Siow RC, Sato H, Mann GE: Heme oxygenase-carbon monoxide signalling pathway in atherosclerosis: Anti-atherogenic actions of bilirubin and carbon monoxide? Cardiovasc Res 1999;41:385-394.

116. Pech-Amsellem MA, Myara I, Storogenko M, Demuth K, Proust A, Moatti N: Enhanced modifications of low-density lipoproteins (ldl) by endothelial cells from smokers: A possible mechanism of smoking-related atherosclerosis. Cardiovasc Res 1996;31:975-983.

117. Heitzer T, Just H, Munzel T: Antioxidant vitamin c improves endothelial dysfunction in chronic smokers. Circulation 1996;94:6-9.

118. Kagota S, Yamaguchi Y, Shinozuka K, Kwon YM, Kunitomo M: Cigarette smoke-modified low density lipoprotein impairs endothelium-dependent relaxation in isolated rabbit arteries. Gen Pharmacol 1996;27:477-481.

119. Nagy J, Demaster EG, Wittmann I, Shultz P, Raij L: Induction of endothelial cell injury by cigarette smoke. Endothelium 1997;5:251-263.

120. Lekakis J, Papamichael C, Vemmos C, Stamatelopoulos K, Voutsas A, Stamatelopoulos S: Effects of acute cigarette smoking on endothelium-dependent arterial dilatation in normal subjects. Am J Cardiol 1998;81:1225-1228.

121. Adams MR, Jessup W, Celermajer DS: Cigarette smoking is associated with increased human monocyte adhesion to endothelial cells: Reversibility with oral l-arginine but not vitamin c. J Am Coll Cardiol 1997;29:491-497.

122. Dovgan PS, Edwards JD, Zhan X, Wilde M, Agrawal DK: Cigarette smoking increases monocyte adherence to cultured endothelial cell monolayer. Biochem Biophys Res Commun 1994;203:929-934.

123. Lehr HA, Kress E, Menger MD, Friedl HP, Hubner C, Arfors KE, Messmer K: Cigarette smoke elicits leukocyte adhesion to endothelium in hamsters: Inhibition by cuzn-sod. Free Radic Biol Med 1993;14:573-581.

124. Lehr HA, Frei B, Arfors KE: Vitamin c prevents cigarette smoke-induced leukocyte aggregation and adhesion to endothelium in vivo. Proc Natl Acad Sci U S A 1994;91:7688-7692.

125. Schmitt D, Shen Z, Zhang R, Colles SM, Wu W, Salomon RG, Chen Y, Chisolm GM, Hazen SL: Leukocytes utilize myeloperoxidase-generated nitrating intermediates as physiological catalysts for the generation of biologically active oxidized lipids and sterols in serum. Biochemistry 1999;38:16904-16915.

126. Kalra J, Chaudhary AK, Prasad K: Increased production of oxygen free radicals in cigarette smokers. Int J Exp Pathol 1991;72:1-7.

127. Guo X, Wang WP, Ko JK, Cho CH: Involvement of neutrophils and free radicals in the potentiating effects of passive cigarette smoking on inflammatory bowel disease in rats. Gastroenterology 1999;117:884-892.
128. Dadmanesh F, Wright JL: Endothelin-a receptor antagonist bq-610 blocks cigarette smoke-induced mitogenesis in rat airways and vessels. Am J Physiol 1997;272:L614-618.
129. Goerre S, Staehli C, Shaw S, Luscher TF: Effect of cigarette smoking and nicotine on plasma endothelin-1 levels. J Cardiovasc Pharmacol 1995;26 Suppl 3:S236-238.
130. Calori G, D'Angelo A, Della Valle P, Ruotolo G, Ferini-Strambi L, Giusti C, Errera A, Gallus G: The effect of cigarette-smoking on cardiovascular risk factors: A study of monozygotic twins discordant for smoking. Thromb Haemost 1996;75:14-18.
131. Jorge PA, Ozaki MR, Almeida EA: Endothelial dysfunction in coronary vessels and thoracic aorta of rats exposed to cigarette smoke. Clin Exp Pharmacol Physiol 1995;22:410-413.
132. Tang J, Muir J, Lancaster T, Jones L, Fowler G: Health profiles of current and former smokers and lifelong abstainers. Oxcheck study group. Oxford and collaborators health check. J R Coll Physicians Lond 1997;31:304-309.
133. Kario K, Matsuo T, Nakao K: Cigarette smoking increases the mean platelet volume in elderly patients with risk factors for atherosclerosis. Clin Lab Haematol 1992;14:281-287.
134. Newby DE, Wright RA, Labinjoh C, Ludlam CA, Fox KA, Boon NA, Webb DJ: Endothelial dysfunction, impaired endogenous fibrinolysis, and cigarette smoking: A mechanism for arterial thrombosis and myocardial infarction. Circulation 1999;99:1411-1415.
135. Blann A: Von willebrand factor antigen, atherothrombotic mechanisms and smoking. J Intern Med 1993;233:425.
136. Gimbrone MA, Jr.: Vascular endothelium: An integrator of pathophysiologic stimuli in atherosclerosis. Am J Cardiol 1995;75:67B-70B.
137. Wilcox JN, Subramanian RR, Sundell CL, Tracey WR, Pollock JS, Harrison DG, Marsden PA: Expression of multiple isoforms of nitric oxide synthase in normal and atherosclerotic vessels. Arterioscler Thromb Vasc Biol 1997;17:2479-2488.

138. Pou S, Pou WS, Bredt DS, Snyder SH, Rosen GM: Generation of superoxide by purified brain nitric oxide synthase. J Biol Chem 1992;267:24173-24176.
139. Shimokawa H, Flavahan NA, Vanhoutte PM: Loss of endothelial pertussis toxin-sensitive g protein function in atherosclerotic porcine coronary arteries. Circulation 1991;83:652-660.
140. Togane Y, Morita T, Suematsu M, Ishimura Y, Yamazaki JI, Katayama S: Protective roles of endogenous carbon monoxide in neointimal development elicited by arterial injury. Am J Physiol Heart Circ Physiol 2000;278:H623-632.
141. Bradamante S, Barenghi L, Villa A: Cardiovascular protective effects of resveratrol. Cardiovasc Drug Rev 2004;22:169-188.
142. Baur JA, Sinclair DA: Therapeutic potential of resveratrol: The in vivo evidence. Nat Rev Drug Discov 2006;5:493-506.
143. Li H, Forstermann U: Resveratrol: A multifunctional compound improving endothelial function. Editorial to: "Resveratrol supplementation gender independently improves endothelial reactivity and suppresses superoxide production in healthy rats" By s. Soylemez et al. Cardiovasc Drugs Ther 2009;23:425-429.
144. Simini B: Serge renaud: From french paradox to cretan miracle. Lancet 2000;355:48.
145. de Lorgeril M, Salen P, Paillard F, Laporte F, Boucher F, de Leiris J: Mediterranean diet and the french paradox: Two distinct biogeographic concepts for one consolidated scientific theory on the role of nutrition in coronary heart disease. Cardiovasc Res 2002;54:503-515.
146. Pearson GJ, Olson KL, Panich NE, Majumdar SR, Tsuyuki RT, Gilchrist DM, Damani A, Francis GA: Maintenance of improved lipid levels following attendance at a cardiovascular risk reduction clinic: A 10-year experience. Vasc Health Risk Manag 2008;4:1127-1135.
147. Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, Zipkin RE, Chung P, Kisielewski A, Zhang LL, Scherer B, Sinclair DA: Small molecule activators of sirtuins extend saccharomyces cerevisiae lifespan. Nature 2003;425:191-196.
148. Spanier G, Xu H, Xia N, Tobias S, Deng S, Wojnowski L, Forstermann U, Li H: Resveratrol reduces endothelial oxidative stress by modulating the gene expression of

superoxide dismutase 1 (sod1), glutathione peroxidase 1 (gpx1) and nadph oxidase subunit (nox4). J Physiol Pharmacol 2009;60 Suppl 4:111-116.

149. Xia N, Daiber A, Habermeier A, Closs EI, Thum T, Spanier G, Lu Q, Oelze M, Torzewski M, Lackner KJ, Munzel T, Forstermann U, Li H: Resveratrol reverses endothelial nitric-oxide synthase uncoupling in apolipoprotein e knockout mice. J Pharmacol Exp Ther;335:149-154.

150. Wallerath T, Deckert G, Ternes T, Anderson H, Li H, Witte K, Forstermann U: Resveratrol, a polyphenolic phytoalexin present in red wine, enhances expression and activity of endothelial nitric oxide synthase. Circulation 2002;106:1652-1658.

151. Csiszar A, Labinskyy N, Pinto JT, Ballabh P, Zhang H, Losonczy G, Pearson K, de Cabo R, Pacher P, Zhang C, Ungvari Z: Resveratrol induces mitochondrial biogenesis in endothelial cells. Am J Physiol Heart Circ Physiol 2009;297:H13-20.

152. Mattagajasingh I, Kim CS, Naqvi A, Yamamori T, Hoffman TA, Jung SB, DeRicco J, Kasuno K, Irani K: Sirt1 promotes endothelium-dependent vascular relaxation by activating endothelial nitric oxide synthase. Proc Natl Acad Sci U S A 2007;104:14855-14860.

153. Klinge CM, Blankenship KA, Risinger KE, Bhatnagar S, Noisin EL, Sumanasekera WK, Zhao L, Brey DM, Keynton RS: Resveratrol and estradiol rapidly activate mapk signaling through estrogen receptors alpha and beta in endothelial cells. J Biol Chem 2005;280:7460-7468.

154. Kaeberlein M, McDonagh T, Heltweg B, Hixon J, Westman EA, Caldwell SD, Napper A, Curtis R, DiStefano PS, Fields S, Bedalov A, Kennedy BK: Substrate-specific activation of sirtuins by resveratrol. J Biol Chem 2005;280:17038-17045.

155. Zou S, Carey JR, Liedo P, Ingram DK, Muller HG, Wang JL, Yao F, Yu B, Zhou A: The prolongevity effect of resveratrol depends on dietary composition and calorie intake in a tephritid fruit fly. Exp Gerontol 2009;44:472-476.

156. de la Lastra CA, Villegas I: Resveratrol as an anti-inflammatory and anti-aging agent: Mechanisms and clinical implications. Mol Nutr Food Res 2005;49:405-430.

157. Ungvari Z, Csiszar A, Kaminski PM, Wolin MS, Koller A: High intravascular pressure increases superoxide production by nad(p)h oxidase in skeletal muscle arteries. Faseb J 2003.

158. Ungvari Z, Csiszar A, Kaminski PM, Wolin MS, Koller A: Chronic high pressure-induced arterial oxidative stress: Involvement of protein kinase c-dependent nad(p)h oxidase and local renin-angiotensin system. Am J Pathol 2004;165:219-226.

159. Meshi B, Vitalis TZ, Ionescu D, Elliott WM, Liu C, Wang XD, Hayashi S, Hogg JC: Emphysematous lung destruction by cigarette smoke. The effects of latent adenoviral infection on the lung inflammatory response. Am J Respir Cell Mol Biol 2002;26:52-57.

160. Ungvari Z, Csiszar A, Huang A, Kaminski PM, Wolin MS, Koller A: High pressure induces superoxide production in isolated arteries via protein kinase c-dependent activation of nad(p)h oxidase. Circulation 2003;108:1253-1258.

161. Csiszar A, Ungvari Z, Edwards JG, Kaminski PM, Wolin MS, Koller A, Kaley G: Aging-induced phenotypic changes and oxidative stress impair coronary arteriolar function. Circ Res 2002;90:1159-1166.

162. Gupte SA, Arshad M, Viola S, Kaminski PM, Ungvari Z, Rabbani G, Koller A, Wolin MS: Pentose phosphate pathway coordinates multiple redox-controlled relaxing mechanisms in bovine coronary arteries. Am J Physiol Heart Circ Physiol 2003;285:H2316-2326.

163. Miura H, Bosnjak JJ, Ning G, Saito T, Miura M, Gutterman DD: Role for hydrogen peroxide in flow-induced dilation of human coronary arterioles. Circ Res 2003;92:e31-40.

164. Werner E: Determination of cellular h2o2 production. Sci STKE 2003;2003:PL3.

165. Csiszar A, Ungvari Z, Koller A, Edwards JG, Kaley G: Proinflammatory phenotype of coronary arteries promotes endothelial apoptosis in aging. Physiol Genomics 2004;17:21-30.

166. Csiszar A, Ahmad M, Smith KE, Labinskyy N, Gao Q, Kaley G, Edwards JG, Wolin MS, Ungvari Z: Bone morphogenetic protein-2 induces pro-inflammatory endothelial phenotype. Am J Pathol 2006;

167. Matsumoto T, Komori K, Shoji T, Kuma S, Kume M, Yamaoka T, Mori E, Furuyama T, Yonemitsu Y, Sugimachi K: Successful and optimized in vivo gene transfer to rabbit carotid artery mediated by electronic pulse. Gene Ther 2001;8:1174-1179.

168. Yamaoka T, Yonemitsu Y, Komori K, Baba H, Matsumoto T, Onohara T, Maehara Y: Ex vivo electroporation as a potent new strategy for nonviral gene transfer into autologous vein grafts. Am J Physiol Heart Circ Physiol 2005;289:H1865-1872.
169. Ungvari Z, Csiszar A, Edwards JG, Kaminski PM, Wolin MS, Kaley G, Koller A: Increased superoxide production in coronary arteries in hyperhomocysteinemia: Role of tumor necrosis factor-alpha, nad(p)h oxidase, and inducible nitric oxide synthase. Arterioscler Thromb Vasc Biol 2003;23:418-424.
170. Csiszar A, Smith KE, Koller A, Kaley G, Edwards JG, Ungvari Z: Regulation of bone morphogenetic protein-2 expression in endothelial cells: Role of nuclear factor-kappab activation by tumor necrosis factor-alpha, h2o2, and high intravascular pressure. Circulation 2005;111:2364-2372.
171. Jaimes EA, DeMaster EG, Tian RX, Raij L: Stable compounds of cigarette smoke induce endothelial superoxide anion production via nadph oxidase activation. Arterioscler Thromb Vasc Biol 2004;24:1031-1036.
172. Raij L, DeMaster EG, Jaimes EA: Cigarette smoke-induced endothelium dysfunction: Role of superoxide anion. J Hypertens 2001;19:891-897.
173. Li JM, Mullen AM, Yun S, Wientjes F, Brouns GY, Thrasher AJ, Shah AM: Essential role of the nadph oxidase subunit p47(phox) in endothelial cell superoxide production in response to phorbol ester and tumor necrosis factor-alpha. Circ Res 2002;90:143-150.
174. Mayhan WG, Sharpe GM: Superoxide dismutase restores endothelium-dependent arteriolar dilatation during acute infusion of nicotine. J Appl Physiol 1998;85:1292-1298.
175. Orosz Z, Csiszar A, Labinskyy N, Smith K, Kaminski PM, Ferdinandy P, Wolin MS, Rivera A, Ungvari Z: Cigarette smoke-induced proinflammatory alterations in the endothelial phenotype: Role of nad(p)h oxidase activation. Am J Physiol Heart Circ Physiol 2007;292:H130-139.
176. Pryor WA, Stone K, Zang LY, Bermudez E: Fractionation of aqueous cigarette tar extracts: Fractions that contain the tar radical cause DNA damage. Chem Res Toxicol 1998;11:441-448.
177. Zang LY, Stone K, Pryor WA: Detection of free radicals in aqueous extracts of cigarette tar by electron spin resonance. Free Radic Biol Med 1995;19:161-167.

178. Bock FG, Swain AP, Stedman RL: Carcinogenesis assay of subfractions of cigarette smoke condensate prepared by solvent-solvent separation of the neutral fraction. J Natl Cancer Inst 1972;49:477-483.
179. Schmeltz I, Stills CD, Chamberlain WJ, Stedman RL: Analysis of cigarette smoke fraction by combined gas chromatography--infrared spectrophotometry. Anal Chem 1965;37:1614-1616.
180. Swain AP, Cooper JE, Stedman RL: Large-scale fractionation of cigarette smoke condensate for chemical and biologic investigations. Cancer Res 1969;29:579-583.
181. Nordskog BK, Blixt AD, Morgan WT, Fields WR, Hellmann GM: Matrix-degrading and pro-inflammatory changes in human vascular endothelial cells exposed to cigarette smoke condensate. Cardiovasc Toxicol 2003;3:101-117.
182. Shen Y, Rattan V, Sultana C, Kalra VK: Cigarette smoke condensate-induced adhesion molecule expression and transendothelial migration of monocytes. Am J Physiol 1996;270:H1624-1633.
183. Csiszar A, Ungvari Z, Koller A, Edwards JG, Kaley G: Aging-induced proinflammatory shift in cytokine expression profile in rat coronary arteries. Faseb J 2003;17:1183-1185.
184. Bergmann S, Siekmeier R, Mix C, Jaross W: Even moderate cigarette smoking influences the pattern of circulating monocytes and the concentration of sicam-1. Respir Physiol 1998;114:269-275.
185. Weber C, Erl W, Weber K, Weber PC: Increased adhesiveness of isolated monocytes to endothelium is prevented by vitamin c intake in smokers. Circulation 1996;93:1488-1492.
186. Manna SK, Mukhopadhyay A, Aggarwal BB: Resveratrol suppresses tnf-induced activation of nuclear transcription factors nf-kappa b, activator protein-1, and apoptosis: Potential role of reactive oxygen intermediates and lipid peroxidation. J Immunol 2000;164:6509-6519.
187. Pellegatta F, Bertelli AA, Staels B, Duhem C, Fulgenzi A, Ferrero ME: Different short- and long-term effects of resveratrol on nuclear factor-kappab phosphorylation and nuclear appearance in human endothelial cells. Am J Clin Nutr 2003;77:1220-1228.

188. Labinskyy N, Csiszar A, Veress G, Stef G, Pacher P, Oroszi G, Wu J, Ungvari Z: Vascular dysfunction in aging: Potential effects of resveratrol, an anti-inflammatory phytoestrogen. Curr Med Chem 2006;13:989-996.

189. De Keulenaer GW, Alexander RW, Ushio-Fukai M, Ishizaka N, Griendling KK: Tumour necrosis factor alpha activates a p22phox-based nadh oxidase in vascular smooth muscle. Biochem J 1998; 329: 653-657.

190. Csiszar A, Smith K, Labinskyy N, Orosz Z, Rivera A, Ungvari Z: Resveratrol attenuates tnf-alpha-induced activation of coronary arterial endothelial cells: Role of nf-kappab inhibition. Am J Physiol Heart Circ Physiol 2006; 291: H1694-1699.

191. Ashikawa K, Majumdar S, Banerjee S, Bharti AC, Shishodia S, Aggarwal BB: Piceatannol inhibits tnf-induced nf-kappab activation and nf-kappab-mediated gene expression through suppression of ikappabalpha kinase and p65 phosphorylation. J Immunol 2002; 169: 6490-6497.

192. Reiterer G, Toborek M, Hennig B: Quercetin protects against linoleic acid-induced porcine endothelial cell dysfunction. J Nutr 2004; 134: 771-775.

193. Wung BS, Hsu MC, Wu CC, Hsieh CW: Resveratrol suppresses il-6-induced icam-1 gene expression in endothelial cells: Effects on the inhibition of stat3 phosphorylation. Life Sci 2005; 78: 389-397.

9. LIST OF PUBLICATIONS

This work is based on the following articles:

1. Orosz Z, Csiszar A, Labinskyy N, Smith K, Kaminski PM, Ferdinandy P, Wolin MS, Rivera A, Ungvari Z.: Cigarette smoke-induced proinflammatory alterations in the endothelial phenotype: role of NAD(P)H oxidase activation. *Am J Physiol Heart Circ Physiol.* 2007 Jan; 292(1): H130-9. **IF*: 3,973**

2. Ungvari Z, Orosz Z, Rivera A, Labinskyy N, Xiangmin Z, Olson S, Podlutsky A, Csiszar A.: Resveratrol increases vascular oxidative stress resistance. *Am J Physiol Heart Circ Physiol.* 2007 May;292(5):H2417-24. **IF*: 3,973**

3. Csiszar A, Smith K, Labinskyy N, Orosz Z, Rivera A, Ungvari Z.: Resveratrol attenuates TNF-alpha-induced activation of coronary arterial endothelial cells: role of NF-kappaB inhibition. *Am J Physiol Heart Circ Physiol*. 2006 Oct;291(4):H1694-9. **IF*: 3,724**

Further publications

Magyar P., Orosz Zs.: Severe acute respiratory syndrome (SARS*) Háziorvos Továbbképző Szemle* 2003: 8, 452-454. **IF*: 0**

M. Csikós , Z. Orosz, G. Bottlik , H. Szöcs , Z. Szalai, Z. Rozgonyi, J. Hársing , É. Török, L. Bruckner-Tuderman, A. Horváth and S. Kárpáti: Dystrophic epidermolysis bullosa complicated by cutaneous squamosus cell carcinoma and pulmonary and renal amyloidosis *Clinical and Experimental Dermatology*, 2003:28(2); 163-166. **IF*: 1,223**

Ágoston M., Örsi F., Fehér E., Hagymási K., Orosz Zs. Blázovics A., Fehér J., Vereckei A. Silymarin and Vitamin E reduce amiodarone-induced lysosomal phospholipidosis in rats. *Toxicology* 2003 190(3): 231-241 **IF*: 2,061**

Vajda E., Egri G., Ivaskevics K., Orosz Zs., Bártfai Z., Géczi I., Lantos Á., Nagy A., Magyar P.: Primer pulmonary pseudolymphomatosis. *Medicina Thoracalis* 2003 *:* 56, 208-211 **IF*: 0**

Orosz Zs., Vajda E., Sápi Z., Nagy A., Lantos Á., Appel J.: Desmoplasticus malignus mesothelioma esete. *Medicina Thoracalis* 2004, 57: 62-67 **IF*: 0**

Németh Gy., Orosz Zs., Samir H.A.R., Nagy A., Mészáros Zs., Ivaskevics K., Lantos Á.: Lövési sérülés következtében kialakult autológ mellkasi léptranszplantáció. *Medicina Thoracalis* 2004, 57: 33-35 **IF*: 0**

Prónai L., Schandl L., Orosz Zs., Magyar P. and Tulassay Z.: Lower Prevalence of Helicobacter pylori Infection in Patients With Inflammatory Bowel Disease But Not With Chronic Obstructive Pulmonary Disease – Antibiotic Use in the History Does Not Play a Significant Role. *Helicobacter* 2004 9(3): 278 - 283 **IF*: 2,313**

Vajda E., Appel J., Egri G., Sápi Z., Orosz Zs., Nagy A., Géczi I., Lantos Á., Magyar P.: Endobronchial granularcell (Abrikossoff-) tumor. *Medicina Thoracalis* 2004, 57: 27-32 **IF*: 0**

Csiszar A, Labinskyy N, Orosz Z, Ungvari Z.: Altered mitochondrial energy metabolism may play a role in vascular aging. *Med Hypotheses*. 2006;67(4):904-8. **IF*: 1,299**

Labinskyy N, Csiszar A, Orosz Z, Smith K, Rivera A, Buffenstein R, Ungvari Z.: Comparison of endothelial function, O2-* and H2O2 production, and vascular oxidative stress resistance between the longest-living rodent, the naked mole rat, and mice.
Am J Physiol Heart Circ Physiol. 2006 Dec;291(6):H2698-704. **IF*: 3,724**

Csiszar A, Labinskyy N, Smith K, Rivera A, Orosz Z, Ungvari Z. **:**Vasculoprotective effects of anti-tumor necrosis factor-alpha treatment in aging
Am J Pathol. 2007 Jan;170(1):388-98 **IF*: 5,487**

Csiszar A, Labinskyy N, Smith KE, Rivera A, Bakker EN, Jo H, Gardner J, Orosz Z, Ungvari Z.: Downregulation of bone morphogenetic protein 4 expression in coronary arterial endothelial cells: role of shear stress and the cAMP/protein kinase A pathway.
Arterioscler Thromb Vasc Biol. 2007 Apr;27(4):776-82. **IF*: 7,221**

Ungvari Z, Orosz Z, Labinskyy N, Rivera A, Xiangmin Z, Smith K, Csiszar A.: Increased mitochondrial H2O2 production promotes endothelial NF-kappaB activation in aged rat arteries.
Am J Physiol Heart Circ Physiol. 2007 Jul; 293(1):H37-47. **IF*: 3,973**

Csiszar A, Labinskyy N, Orosz Z, Xiangmin Z, BuffensteinR, Ungvari Z.: Vascular aging in the longest-living rodent, the naked mole rat.
Am J Physiol Heart Circ Physiol. 2007 Aug;293(2):H919-27. **IF*: 3,973**

10. ACKNOWLEDGMENTS

I would like to thank Dr. Zoltan Bartfai for being my program director. I thank to my advisors Dr. Zoltan Ungvari and Dr. Anna Csiszár for sponsoring the studies and for their thoughtful discussions, insight and guidance throughout the course of this thesis, arriving at this point would not have been possible without their support.

I would like to acknowledge Prof. Gabor Kaley for his support with my research work in the Department of Physiology at the New York Medical College, Dr. Anna Csiszar for her help in adapting molecular biology methods to study vascular preparations. Much of the staff of Dr Ungvari's laboratory have been involved in furthering my scientific goals and I would like to especially thank Dr. Nazar Labinskyy for our collaboration.

I have been fortunate enough to work under later Prof. Pal Magyar's guidance in the Department of Pulmonology of Semmelweis University, I thank to him for convincing me to take the scientific road.

A special thanks to my mother, Mrs. Bela Orosz, to my father, Mr. Bela Orosz and to my brother, Andras Orosz, for their love and moral support, which were always the source of my strength.