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

**Ph. D. thesis booklet**


## 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.Med.
Members: Aladár Rónaszéki, M.D., Ph. D.
Zoltán Süttő, M.D., Ph. D.

Budapest
2011

## 1. INTRODUCTION

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.

The first cohort studies were published in the 1950s, 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.

In the United States Manley 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. Cigarette smoking is an independent risk factor in the development of atherosclerotic lesions in intracranial internal carotid artery atherosclerosis. 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. Smoking increases the risk of peripherial artery disease more than heart disease. Increased tobacco use in humans and a smoking pattern which maximizes nicotine yield are also associated with and increased risk of peripherial artery disease.

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

## 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. 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. The particulate (tar) phase of cigarette smoke contains $>10^{17}$ free radicals/g, and the gas phase contains $>10^{15}$ free radicals/puff. About 4000 compounds are generated by a lighted cigarette through many processes such as hydrogenation, pyrolysis, oxidation, decarboxylation, dehydration,...etc.. 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. The cigarette smoke produces generalized endothelial dysfunction in virtually every vascular bed, 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, including induction of pro-inflammatory gene expression. Although the effects of cigarette smoke on pro-inflammatory mechanisms in lung epithelium and circulating immunocytes have been extensively studied in the past, 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 and nearly 85% of smoke in a room results from side-stream smoke. Mainstream cigarette smoke comprises 8% of tar and 92% gaseous components. 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. 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.

## ENDOTHELIAL FUNCTION AND DYSFUNCTION

Endothelial integrity and normal function are indispensable for the preservation of health. The term of "endothelial dysfunction" was first described in the mid-eighties, following the major breakthrough by Furchgott and Zawadzki who discovered that acetylcholine requires the presence of endothelial cells to relax underlying vascular smooth muscle cells.

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.

## 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 O2 derivates. Oxidants exhibit a wide array of tissue-damaging cytotoxic effects, reacting and attacking a variety of biomolecules. Superoxide is generated from 1 electron reduction of molecular oxygen by various oxidases and it is the precursor of other ROS. 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. $H_2O_2$ is a more stable molecule. $H_2O_2$ is lipid soluble, can go through cell membranes, and it has longer half life than $O_2^{\cdot-}$. $H_2O_2$ can be scavanged by catalase and glutathione peroxidase to form water. Hydrogen peroxide can also be reduced to generate the highly reactive $^{\cdot}$OH-. The hydroxyl radical ($^{\cdot}$OH) is typically formed by oxidation of a reduced heavy metal ion ($Fe^{++}$ or $Cu^{+}$, usually) by the hydrogen peroxide (Fenton reaction). In the vasculature $O_2^{\cdot-}$, $H_2O_2$, NO, $OONO^{-}$, and $\cdot OH$ 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.

## 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.

**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.

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. Resveratrol can increase the expression of antioxidative enzymes including superoxide dismutase (SOD1–2), catalase and glutathione peroxidase (GPx). 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.

## 2. HYPOTHESIS AND AIMS OF STUDY

The cigarette smoke effect on possible functional and phenotypic changes of rat carotid arteries have 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

### *Animals and vessel isolation*

Fourteen- to sixteen-week-old male Wistar rats (n = 20) were used, the carotid arteries and aortas were isolated and cleaned from the surrounding tissue.

### *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., while the control group was not exposed to cigarette smoke.

### *Cigarette smoke extract preparation*

Cigarette smoke extract (CSE; dissolved in DMSO, 40 mg/mL total particular matter, nicotine content: 6%; kept at -80 oC) 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.

### *Vessel culture and functional studies*

Isolated carotid arteries were maintained in a stainless steel vessel culture chamber (Danish Myo Technology) under sterile conditions. 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. The vessels were contracted by phenylephrine and relaxations to acetylcholine and the NO donor S-nitrosopenicillamine were obtained.

***Measurement of vascular O2.- level: lucigenin chemiluminescence***

$O_2^{\cdot-}$ production was assessed from vascular samples by the lucigenin chemiluminescence in the absence and presence (pre-incubation: 1 hour) of diphenyleneiodonium, SOD, Tiron, indomethacin, or Nω-nitro-L-arginine-methyl-ester.

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

Living vessels pre-incubated with CSE were incubated with hydroethidine. En face preparations were imaged using Zeiss AxioCam Mrm camera mounted on a Zeiss Axiovert 200 fluorescence microscope (Carl Zeiss, Gottingen, Germany). The mean fluorescence intensities of each ethidium bromide (EB)–stained nuclei were measured in each view field.

***Measurement of vascular H2O2 production***

The cell-permeant oxidative fluorescent indicator dye C-H2DCFDA (5 (and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate-acetyl ester, Invitrogen, Carlsbad CA) was used to assess H2O2 production in CSE-treated vessels according to the modified protocols of Miura et al. In addition, H2O2 production was also measured using the modified methods of Werner.

***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. The carotid arteries were treated with CSE in organoid culture with or without pre-treatment with PEG-catalase, PEG-SOD, apocynin, or DPI. In separate experiments mRNA expression of the NAD(P)H oxidase catalytic subunit gp91phox 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).

***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.

***Monocyte adhesion assay.***

We measured adhesion of fluorescently labeled human monocytic (THP-1) cells to confluent monolayers of HCAECs using a microplate-based assay.

***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) 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. The luciferase activities were measured 1 day after electroporation of a CMV-driven renilla luciferase construct at various electrode voltages, pulse numbers, and pulse durations.

## 4. RESULTS

### Smoking-induced endothelial dysfunction

*In vivo* exposure of rats to cigarette smoke elicited impaired vascular relaxations to acetylcholine and to SNAP, which were improved by apocynin treatment. *In vitro* CSE treatment tended to reduce NO-dependent relaxation of rat carotid arteries to acetylcholine, but the differences did not reach statistical significance.

### 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. Also, in a dose-dependent manner CSE significantly increased $O_2^{\cdot-}$ production in the carotid arteries and aortas. $O_2^{\cdot-}$ production in CSE treated vessels was significantly decreased by administration of DPI, Tiron or SOD, whereas it was unaffected by indomethacin, or L-NAME.

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.

Exposure to CSE elicited substantial increases in vascular $H_2O_2$ generation as measured by both the DCF fluorescence and HVA fluorescence 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 and nuclear EB staining. Administration of nicotine did not result in significant increases in endothelial DCF fluorescence and nuclear EB staining.

**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, TNFα, IL-1β and IL-6 and ICAM significantly increased. Exposure of rat carotid arteries to increasing concentrations of CSE *in vitro* also elicited up-regulation of iNOS, TNFα, IL-1β and IL-6. The effect of CSE on ICAM-1 expression did not reach statistical significance. Expression of iNOS, TNFα, IL-1β and IL-6 in CSE-treated vessels was significantly reduced by apocynin and PEG-catalase. Similar results were obtained also with DPI.

**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). Importantly, CSE induced NF-κB activity could be inhibited by catalase, DPI and apocynin, suggesting that NAD(P)H oxidase-derived $H_2O_2$ production plays a key role in CSE induced NF-κB activation in CAECs.

**Smoking and in vitro CSE exposure enhance monocyte adhesion 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. Incubation of CAECs with CSE also resulted in dose-

dependent increases in the adhesion of THP-1 cells, which could be inhibited by apocynin, DPI or catalase .

**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. Pretreatment of the vessels with increasing concentrations of resveratrol reduced or prevented monocyte adhesion induced by both cytokines. TNF-α-induced increases in monocyte adhesiveness were significantly reduced by pretreatment of HCAECs with apocynin or SOD plus catalase. Administration of $H_2O_2$ also substantially increased monocyte adherence to HCAECs in all concentrations studied, and this effect was also attenuated by resveratrol.

**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 . Pretreatment of HCAECs (for 1 h) with resveratrol prevented TNF-α -induced NF-κB activation in a concentration-dependent manner . IL-1 and IL-6 also activated NF-κB in HCAECs, and these effects were also significantly attenuated by resveratrol.

**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. Using a red fluorescent protein construct, we demonstrated that, using these voltage settings, the vascular endothelium can be effectively transfected . In cultured aortic segments transfected with the NF-κB reporter construct, TNF-α elicited significant increases in luciferase activity. TNF-α-induced increases in NF-κB activity were abolished by pretreatment with 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. This finding accords with the increased NAD(P)H oxidase-dependent $O_2^{\cdot-}$ generation in these vessels. 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, supporting the ex vivo observations. Accordingly, CSE seems to increase the expression of $gp91^{phox}$ in rat arteries. 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. Similarly, CSE challenge elicited oxidative stress in both cell types. 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).

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

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. Importantly, these pro-inflammatory phenotypic alterations could also be mimicked by *in vitro* CSE challenge.

Recent studies suggest that exposure of cultured human endothelial cells to CSE or serum from smokers also results in pro-inflammatory gene expression.

At the end we found that CSE 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.

There are more major findings in the resveratrol study. First, 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 second important finding is that TNF-α -induced NF-κB activation in HCAECs is inhibited by pretreatment with resveratrol. 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. In line with this finding, previously we demonstrated that TNF-α -induced endothelial NF-κB activation can also be prevented by catalase and NAD(P)H inhibitors. 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.

## 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. 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**

**Other 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**