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Oxidative stress and accelerated vascular aging: implications for cigarette smoking

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1. ABSTRACT

Cigarette smoking is the major cause of preventable morbidity and mortality in the United States and constitutes a major risk factor for atherosclerotic vascular disease, including coronary artery disease and stroke. Increasing evidence supports the hypothesis that oxidative stress and inflammation provide the pathophysiological link between cigarette smoking and CAD. Previous studies have shown that cigarette smoke activates leukocytes to release reactive oxygen and nitrogen species (ROS/RNS) and secrete pro-inflammatory cytokines, increases the adherence of monocytes to the endothelium and elicits airway inflammation. Here we present an overview of the direct effects of water-soluble cigarette smoke constituents on endothelial function, vascular ROS production and inflammatory gene expression. The potential pathogenetic role of peroxynitrite formation, and downstream mechanisms including poly (ADP-ribose) polymerase (PARP) activation in cardiovascular complications in smokers are also discussed.

Keywords

Endothelium; Cigarette Smoking; Smokers; Tobacco; Senescence; Inflammation; Gene Expression; Redox Status; Peroxynitrite; PARP-1; poly polymerase; ADP-ribose; PARP

2. INTRODUCTION

In the United States, an estimated 45.8 million adults (~22.5%) are current smokers and more than 400 000 people die from tobacco smoke–related illnesses each year. There is overwhelming evidence that tobacco smoking is an important factor contributing to premature vascular aging. The cardiovascular morbidity and mortality induced by cigarette smoking exceeds that attributable to lung cancer: 190 000 cardiovascular disease deaths per year are related to cigarette smoke, out of which 37 000 to 40 000 deaths are attributable to second hand smoke exposure (1). Although the precise molecular basis of smoking-induced vascular injury remains unclear, increasing evidence supports the hypothesis that oxidative-

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nitrosative stress and inflammation provide the pathophysiological link between cigarette smoking and coronary artery disease (CAD) (2-24).

Cigarette smoke contains reactive oxidants, which can get into the bloodstream and cause macromolecular damage in the endothelial cells. Cigarette smoking also elicits a marked activation of leukocytes and platelets, which can also contribute to the oxidative vascular damage in smokers. Circulating cigarette smoke constituents were also shown to induce and activate ROS producing enzyme systems within the vascular wall. The mechanisms by which endothelial oxidative stress leads to vascular inflammation and development of atherosclerosis have been the subject of intense studies in this field. This review focuses on the emerging evidence that reactive oxygen species (ROS) and activation of inflammatory pathways play a central role in accelerated cardiovascular aging in smokers, and discusses some of the signaling pathways involved in cigarette smoking-induced vascular inflammation.

3. CIGARETTE SMOKE-INDUCED VASCULAR OXIDATIVE-NITROSATIVE STRESS

The causes of cigarette smoking-induced vascular oxidative stress are likely multifaceted. Cigarette smoke can be divided into 2 phases: particulate matter and gas-phase smoke, which contain high concentrations of ROS, NO, peroxynitrite, and/or free radicals of organic compounds (25-39). In addition to these short-lived, highly reactive substances, inhaled particles encountered in cigarette smoke, especially in the presence of ROS (4) may evoke an inflammatory response in the lung activating immunocytes to produce ROS and promoting the production of proinflammatory cytokines. It has also been suggested that aqueous cigarette tar extracts contain pro-oxidant substances that have the potential to increase cellular production of ROS (11, 25, 26, 40-43). These include a semiquinones, hydroquinones, and quinones (25, 26), a series of α,ß-unsaturated aldehydes, such as acrolein and crotonaldehyde, α,ß-unsaturated ketones, and a number of saturated aldehydes (40-42). These water soluble components of cigarette smoke are likely to reach the systemic circulation and can directly promote vascular oxidative stress in systemic vascular beds. This hypothesis was supported by clinical and animal studies showing that cigarette smoke produces generalized endothelial dysfunction in virtually every vascular bed (2, 21, 44-47), which is usually an indicator of an increased oxidative stress.

Several lines of evidence suggest that cigarette smoke constituents can directly activate vascular ROS production. Clinical and animal studies have demonstrated that cigarette smoke (CS) produces generalized endothelial dysfunction by reducing NO bioavailability (2, 21, 44-48). It is generally accepted that tonic release of NO from the endothelium exerts vasculoprotective and cardioprotective effects, such as maintenance of normal coronary blood flow, inhibition of platelet aggregation and inflammatory cell adhesion to endothelial cells and disruption of pro-inflammatory cytokine-induced signaling pathways. Importantly, coronary vasodilatory capacity was shown to be impaired in smokers lowering the ischemic threshold and even short-term smoking increases the coronary vasomotor tone and markedly reduces the myocardial flow reserve (46, 47). Substantial evidence is accumulating suggesting that decreased NO bioavailability and increased generation of reactive oxygen species (ROS), including that of superoxide ($O_2^{\cdot -}$; Figure 1), plays a critical role in cigarette smoke-related endothelial dysfunction (2, 7, 49, 50). Also, CSE is known to induce endothelial apoptosis by activating caspase-3 (51). In endothelial cells eNOS pre-activation by L-arginine substantially reduces CS-induced endothelial apoptosis; whereas eNOS inhibition accentuated CS-induced endothelial apoptosis (51). Thus, ROS-mediated reduction of endogenous NO production seems to be an important mechanism in smoking-induced endothelial damage (51). Because SOD enzymes catalyze the removal of $O_2^{\cdot -}$ with a

rate constant of 2×10^9 $mol^{-1} \cdot L \cdot s^{-1}$, 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 (48).

Previous studies identified NAD (P)H oxidase (s) and xanthine oxidase as two potential intracellular sources of $O_2^{\cdot-}$ in various cell types that may be induced by components of cigarette smoke (2, 6, 49). We have recently shown that both *in vivo* chronic cigarette smoke exposure and *in vitro* treatment with aqueous cigarette smoke extract (CSE) elicit significant endothelial dysfunction in rat carotid arteries, which could be reversed by inhibition of the NAD (P)H oxidase (48). This finding accords with the increased NAD (P)H oxidase-dependent $O_2^{\cdot-}$ generation in these vessels upon CSE exposure (48). 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 (48). Further evidence for the central role for NAD (P)H oxidase activation in cigarette smoke-induced vascular oxidative stress came from the observations that CSE increased the expression of $gp91^{phox}$ subunit of NAD (P)H oxidase in rat arteries (48). Also, in human pulmonary artery endothelial cells gp91 docking sequence-tat peptide (similar to apocynin) was reported to inhibit CSE-induced $O_2^{\cdot-}$ generation (49). In this context it is important to note that NAD (P)H oxidase activation has been linked to the development of CAD in various pathophysiological conditions, including aging. We have previously shown that both endothelial cells and vascular smooth muscle cells exhibit an up-regulated $O_2^{\cdot-}$ generation in vessels of cigarette smoke-exposed animals (48). Similarly, CSE challenge elicited oxidative stress in both cell types (48), mimicking the effects of *in vivo* exposure to cigarette smoke. Recent studies support the idea that CSE *in vitro* may induces NAD (P)H oxidase (s) in other cell types as well (2, 6, 49). Cigarette smoke contains more than 4000 known components and at present it is unclear, which components activate NAD (P)H oxidase. Numerous experimental and epidemiological studies have demonstrated that polycyclic aromatic hydrocarbons (PAHs), which are major constituents of cigarette tobacco tar, are able to induce various cellular enzyme systems involved in ROS metabolism. Although nicotine may impair endothelium-mediated vasodilation in microvessels (52), in our recent studies it could not mimic the effect of serum from cigarette smoke-exposed rats or CSE on endothelial ROS production (48). Also, although nicotine at high concentrations may cause pro-inflammatory gene expression in cultured coronary endothelial cells (such as up-regulation of angiotensin-I converting enzyme, tissue-type plasminogen activator, plasminogen activator inhibitor-1 (53)), it does not mimic the effect of smoking on the endothelial expression of ICAM-1, matrix metalloproteinases and many other inflammatory mediators (53). A recent 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 (49). 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. It is now well established that NAD (P)H oxidase (s) are a major source of ROS in vascular cells and an increased NAD (P)H oxidase activity is responsible for enhanced endothelial $O_2^{\cdot-}$ production in aging and in pathophysiological conditions associated with accelerated vascular aging (54) such as, hyperhomocysteinemia (55) and hypertension (56, 57). Activity of arterial NAD (P)H oxidase is regulated by PKC-dependent signaling pathways (56, 57) and one could hypothesize that PKC activation by CS components (12, 58, 59) may contribute to the increased NAD (P)H oxidase activity observed in CS-exposed animals. Because NAD (P)H oxidase seems to represent a common pathway eliciting endothelial oxidative stress, further studies are needed to test the hypothesis that smoking will aggravate vascular injury in pathophysiological conditions associated with a high basal NAD (P)H oxidase activity (e.g. hypertension).

Mitochondria are also important sources of ROS in the cardiovascular system. There is growing evidence that cigarette smoke constituents impair mitochondrial function and elicit mitochondrial oxidative stress (Figure 2) in various cell types (60-65), including cardiovascular tissues (66). A recent study demonstrated that acrolein, a major toxicant in cigarette smoke, causes oxidative mitochondrial damage (61). *In vitro* treatment with CSE caused loss of cellular ATP and rapid depolarization of mitochondrial membrane potential, followed by apoptotic cell death (62). In smokers a higher level of oxidative mtDNA damage has been observed (66-68). These data support the hypothesis that cigarette smoke-induced mitochondrial damage and dysfunction may contribute an increased risk for cardiovascular disease in smokers. It should be noted that in addition of the NAD (P)H oxidase and mitochondria, other cellular sources (such as cytochrome P_{450}, which may be induced by cigarette smoke constituents (69)) can also produce significant amounts of ROS, however, the role of these enzymes in CSE-induced vascular oxidative stress is not well understood. In parenchymal tissues a striking cigarette smoke-induced depletion of GSH content was shown (70-75). In addition, acrolein also readily inactivates thioredoxin and thioredoxin reductase (76), which effects have the potential to modulate redox signaling mechanisms.

In addition to the increased levels of $O_2^{\cdot -}$ and H_2O_2 (which have been implicated in pro-atherogenic vascular phenotypic alterations (77-79), including induction of pro-inflammatory gene expression (80-86)), a large body of experimental evidence accumulated over the past 15 years indicates that peroxynitrite ($ONOO^-$) generation from NO and $O_2^{\cdot -}$ represents a major threat to the functional integrity of the vascular endothelium (55, 87-89). Cigarette smoke contains peroxynitrite that can penetrate into the blood stream and also contains agents that lead to increased ONOO- production within the cells (90-94). Importantly, CSE-induced increased $ONOO^-$ generation has been recently demonstrated in cultured endothelial cells (49). The damaging potential of peroxynitrite is explained by its peculiar chemistry involving direct oxidation as well as radical-mediated nitration reactions. These properties allow peroxynitrite to significantly alter the function of a considerable number of proteins (e.g. GSH peroxidase, myeloperoxidase), to degrade membrane structure by peroxidizing lipids, to turn off crucial metabolic functions within mitochondria, and to inflict serious damage to nucleic acids, activating a major pathway of cell injury and inflammation orchestrated by the nuclear enzyme PARP (87). Once severe enough to overwhelm repair mechanism, these various cytotoxic effects commit cells to death, either through the necrotic or apoptotic pathway. Peroxynitrite can also activate/modulate cell signaling pathways. The ability of peroxynitrite to nitrate tyrosine residues can interfere with signaling processes depending on tyrosine phosphorylation (87). Peroxynitrite was also shown to modulate MAP kinase pathways as well (87). There are also studies extant directly implicating peroxynitrite in activation of NF-κB -dependent signaling pathways (95). As a result, previous studies have shown that $ONOO^-$ can mediate a wide range of pro-inflammatory effects (95-102). We and others have shown that in coronary arteries expression of TNFα, which orchestrates pro-atherogenic vascular phenotypic changes (103), is frequently up-regulated in conditions associated with increased $O_2^{\cdot -}$ and $ONOO^-$ production, such as hyperhomocysteinemia (55), aging (104) and hypertension. Thus, future studies should elucidate the possible link between cigarette smoking, peroxynitrite generation and up-regulation of pro-inflammatory cytokine expression.

4. CIGARETTE SMOKE-INDUCED ENDOTHELIAL DNA DAMAGE

Cigarette smoking was reported to increase oxidative DNA modification in humans (105) and laboratory animals. These studies suggested that a link exists between oxidative DNA modification, accelerated aging and cancerogenesis. Earlier studies focused on cigarette smoking-induced DNA damage in the lung, however, it soon became obvious that systemic

exposure to circulating cigarette smoke constituents results in an increased presence of elevated levels of DNA adducts in tissues not directly exposed to tobacco smoke. The literature is mainly focused on 8oxodG lesions caused by circulating cigarette smoke constituents (e.g. measurements of increased urinary excretion of 8-hydroxy-2'-deoxyguanosine in smokers (105)). It has been shown that cigarette smoke extracts increases similar DNA damage in cultured lung fibroblasts and endothelial cells (106) (Figure 3). Because endothelial cells *in vivo* represent the first line of defense against circulating toxic agents, it was expected that significant oxidative DNA damage can occur in the vasculature *in vivo*. Indeed, apart from 8oxodG, previous studies demonstrated the presence of cigarette smoking-related polycyclic aromatic hydrocarbon (PAH)-DNA adducts in human internal mammary artery specimens from smokers (107). The levels of these adducts are about 100 times less frequent than 8oxodG. However, the half-life of PAH-DNA adducts (at least in the lung) is much longer, about 1-2 years (108). Taken together, these findings provide strong evidence that cigarette smoke constituents can induce oxidative DNA damage in the cardiovascular system. Whereas most studies focus on oxidative DNA modifications, it has become clear that DNA repair processes have equal importance for accelerated aging. Repair of oxidative DNA damage is extensive and differences in DNA repair are proposed to be important for cancerogenesis and premature aging as well. There are a large number of enzyme systems that recognize oxidative DNA modifications and start a multistep process of repair. Previous research has suggested that there a positive correlation between maximum longevity and the rate or fidelity of DNA repair. Our studies comparing relative rate of DNA repair in long and short lived species of primates (human vs. marmoset; Podlutsky and Austad unpublished data 2007), rodents (white-footed mouse vs. house mouse (109)) and bat species indicate that long-lived species have superior DNA repair compared to related short-lived species. In humans many of the enzyme systems involved in DNA repair exhibit single nucleotide polymorphisms (SNPs), which are associated with an increased risk for cancer development. The relationship between cigarette smoke-induced oxidative DNA damage in the lung and parenchymal tissues and carcinogenesis are widely appreciated and there is good reason to believe that DNA damage also contribute to cardiovascular pathophysiological alterations. An important hypothesis put forward by Ames also suggest a direct link between oxidative DNA modification and the aging process (110-112). Thus, future studies should elucidate whether inter-individual differences in DNA repair capacity may determine susceptibility to CAD and accelerated cardiovascular aging in smokers.

5. CIGARETTE SMOKE-INDUCED VASCULAR INFLAMMATION

Several lines of evidence suggest that CS can induce inflammatory processes, in part, via induction of pro-inflammatory cytokines. CS exposure (especially the ultrafine particulate fraction) is known to activate circulating immunocytes in the lung, which then release pro-inflammatory cytokines. It appears that especially TNFα plays a crucial role in CS-induced pathophysiology (113). For example, mice lacking TNFα receptors (TNFR -/- mice) do not develop a pulmonary inflammatory infiltrate or matrix breakdown after CS exposure (114). Recent studies demonstrated that apart from being a target for circulating cytokines, vascular cells (endothelial and smooth muscle cells, fibroblasts) produce a wide range of cytokines (104, 115, 116), which orchestrate pro-atherogenic processes in an autocrine/paracrine manner. Recently we have shown that *in vivo* exposure of rats 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 (48). Importantly, these pro-inflammatory phenotypic alterations could also be mimicked by *in vitro* CSE challenge (Figure 4) (48). Recent studies suggest that exposure of cultured human endothelial cells to CSE or serum from smokers also results in pro-inflammatory gene expression (12, 21, 24, 117, 118). 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. The aforementioned findings clearly demonstrate that cigarette smoke components are able to elicit a pro-atherogenic microenvironment in the vascular wall in the absence of circulating factors and immunocytes. The findings that inhibition of NAD (P)H oxidase and administration of antioxidants can attenuate CSE-induced up-regulation of pro-inflammatory mediators, suggest a central role for NAD (P)H oxidase-derived ROS in CSE-induced vascular inflammation (48).

NF-κB is thought to induce the transcription of a large range of genes implicated in inflammation, including cytokines, chemokines and adhesion molecules (119-121). All of these factors are known to predispose arteries to atherosclerosis (122). NF-κB has been shown to be activated by increased levels of ROS in many cell types (123-129), providing an important link between oxidative stress and pro-inflammatory cytokine expression in blood vessels. In line with the evidence that cigarette smoke induces significant endothelial oxidative stress (Figure 1), CSE was shown to induce NF-κB activation in human coronary arterial endothelial cells (Figure 5A) (48). In addition, there are studies extant showing that CSE elicits NF-κB activation in human histiocytic lymphoma cells and other cell lines (130). A recent study also showed that NF-κB activity in peripheral blood mononuclear cells of smokers compared to non-smokers is significantly higher (131). CS was also shown to elicit rapid increases in whole-lung NF-κB activation (132) and gene expression of proinflammatory cytokines after cigarette smoke exposure (113), an effect that could be attenuated by pretreatment with the free radical scavenger SOD (132). The findings that apocynin and catalase were able to reduce CSE-induced activation of NF-κB (24) in endothelial cells (48) (Figure 5A) provide strong evidence that NAD (P)H oxidase-derived H_2O_2 promotes vascular inflammation via NF-κB activation. A central role for H_2O_2 in endothelial NFκB activation is suggested by the findings that administration of exogenous H_2O_2 can mimic the effect of CSE on NF-κB activity in endothelial cells (48, 133). NF-κB response elements are present in the promoter region of many inflammatory genes (such as iNOS and adhesion molecules) as well as many cytokines (e.g. the TNFα gene (134, 135) and the IL-6 gene (136)), which seem to be up-regulated by cigarette smoke. Interestingly, in a monocyte-macrophage cell line cigarette smoke-induced proinflammatory cytokine release seems to be regulated also by SIRT1 (a member of the sirtuin family of class III histone/protein deacetylases) by its interaction with NF-κB (137). In these cells CSE exposure resulted in time-dependent decreases in SIRT1 activity and levels, which was concomitant to increased NF-κB -dependent cytokine production (137). Importantly, resveratrol, an activator of SIRT1, inhibited CSE-mediated proinflammatory cytokine release in cultured monocytic cells (137). It is possible that SIRT1 also plays an important role in controlling vascular inflammatory processes (138), because in CSE-treated cultured coronary arterial endothelial cells resveratrol also attenuates the expression of inflammatory cytokines (Ungvari and Csiszar, unpublished data, 2006). SIRT1 also seems to be important for protecting endothelial cells from oxidative stress-induced apoptosis (139).

6. CIGARETTE SMOKE-INDUCED PARP ACTIVATION

In recent years, evidence has accumulated that another important mechanism mediating the deleterious effects of endothelial oxidative stress is the activation of poly (ADP-ribose) polymerase (PARP), an enzyme present in the nucleus of eukaryotic cells (140, 141). PARP activation has been demonstrated *in vitro* in various cells exposed to ROS (140, 141), as well as to $ONOO^-$. *In vivo*, endothelial and cardiac PARP activation has been shown to act as a common oxidant-induced effector in various pathophysiological conditions associated with accelerated cardiovascular aging (142-150). Importantly, CSE induces PARP activity in coronary arterial endothelial cells (Figure 6). CSE was also reported to induce PARP

activity in alveolar epithelial cells (151). PARP-1 is known to participate in the regulation of gene transcription in many cell types (152). In particular, PARP-1 has been shown to play a key role in NF-κB-driven expression of pro-inflammatory cytokines (153). Recently, PAR-binding domains have been detected in NF-κB subunits (154) and a co-activator role of PARP-1 for NF-κB -dependent pro-inflammatory gene expression has been proposed (145, 155, 156). Consistent with the stimulatory effect of CSE on NF-κB activation, the potent PARP inhibitor PJ34 (157) provided significant anti-inflammatory effects in CSE-treated coronary arterial endothelial cells (Figure 4). Drugs inhibiting PARP-1 activity also reduce NF-κB-dependent transcription of TNFα in glial cells (153) and prevent pro-inflammatory gene expression in cultured endothelial cells (158). In addition, both NF-κB activity and NF-κB -driven transcription of pro-inflammatory cytokines is markedly impaired in PARP-1 knockout animals (159, 160). Despite the relevance of PARP-1 to transcriptional regulation of pro-inflammatory cytokines in cultured endothelial cells, the importance of PARP-1 activity in pro-inflammatory phenotypic alterations in coronary arteries induced by cigarette smoking remains to be established. In addition to the aforementioned pathways cigarette smoke exposure has been shown to activate a number of other redox-sensitive signaling pathways as well. For example, CSE was shown to activate MAP kinase pathways and stress activated protein kinase/c-Jun N-terminal protein kinases in endothelial cells (51).

7. CIGARETTE SMOKE-INDUCED ENDOTHELIAL ACTIVATION

Previous studies suggested that even moderate cigarette smoking leads to an activation of the circulating monocytes and their increased adhesion to the endothelium (161). Both *in vivo* exposure of rats to cigarette smoke and *in vitro* incubation of vessels with CSE enhance adhesion of activated monocytes to the endothelial surface (162-164) (Figure 5B). The role of water soluble components of cigarette smoke is supported by the findings that serum collected from smokers increases endothelial expression of adhesion molecules, including ICAM-1 (21, 24, 48, 164). Our recent findings support a role for NAD (P)H oxidase-derived H_2O_2 p in endothelial activation by cigarette smoke constituents (48). Also, increasing plasma vitamin C concentrations in smokers by oral supplementation decreased monocyte adhesion to values found in nonsmokers (165).

8. ACCELERATED VASCULAR AGING IN CIGARETTE SMOKERS

Epidemiological studies suggest that advanced age itself promotes the development of atherosclerosis and it seems that the cumulative effects of advanced age and cigarette smoking have deleterious consequences on cardiovascular morbidity and mortality in the elderly. Since Harmon proposed the original free radical theory of aging (166), considerable evidence has been published that cardiovascular aging in various tissues is associated with an increased oxidative-nitrosative stress and impaired bioavailability of vasoprotective NO (88, 89, 104, 109, 116, 167-175). Importantly, vascular NAD (P)H oxidase activity/ expression is up-regulated in aging (89, 169, 176), accompanied by a down-regulation of antioxidant mechanisms, such as ecSOD (167). On the basis of the aforementioned findings, we posit that aged arteries are more susceptible to cigarette smoke-induced oxidative stress. Our recent data that CSE elicits an exaggerated ROS production in aged vessels seems to support this premise (Csiszar and Ungvari, unpublished data 2006). Previously we have demonstrated that a pro-inflammatory shift develops in the vascular cytokine expression profile in aged coronary arteries (89, 104, 116). Importantly, expression of the pro-inflammatory cytokines TNFα, TNFβ (which acts on the same receptor as TNFα) and that of IL-6 increase in aged endothelial and/or smooth cells (89, 104, 177, 178) and recent studies suggest that NF-κB binding increases in aging (175, 179). Thus, we hypothesize that age-related oxidative/nitrosative stress induces chronic activation of NF-κB and/or PARP-1 (even in the absence of exogenous pro-oxidant factors). Because multiple intracellular

antioxidant and anti-inflammatory mechanisms (e.g. NO signaling) are likely to be impaired in aging (167, 180-182), we hypothesize that aged vessels are especially vulnerable to the pro-inflammatory effects of cigarette smoke. We propose that because the NF-κB/PARP system is already "primed" in aged vessels, cigarette smoke-induced oxidative stress will result in an enhanced vascular inflammatory response. More research on cigarette smoke-induced oxidative/nitrosative stress, pro-inflammatory vascular cytokine expression and endothelial activation in aged vessels is evidently needed. Future studies should determine whether advanced age renders endothelial and smooth muscle cells in coronary arteries more vulnerable toward the pro-oxidant, pro-inflammatory effects of cigarette smoke.

9. PERSPECTIVE

In conclusion, water soluble components of cigarette smoke increase NAD (P)H-oxidase derived ROS generation in endothelial and smooth muscle cell, which induce a pro-inflammatory vascular phenotype likely via mechanisms that involve NF-κB activation (Figure 7). It is likely that cigarette smoking-induced oxidative-nitrosative stress, PARP-1 activation and vascular inflammation will support atherosclerotic plaque formation in the coronary and carotid arteries increasing the morbidity of myocardial infarction and stroke. The available data support a role for novel anti-inflammatory treatments, including PARP-1 inhibitors, in pharmacological vasculoprotection in accelerated vascular aging (144, 149, 183).

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Abbreviations

CAD	coronary artery disease
TNFα	tumor necrosis factor α
CS	cigarette smoke
CSE	cigarette smoke extract
PARP	poly (ADP-ribose) polymerase
NF-κB	nuclear factor κB
ROS	reactive oxygen species
PAH	polycyclic aromatic hydrocarbons

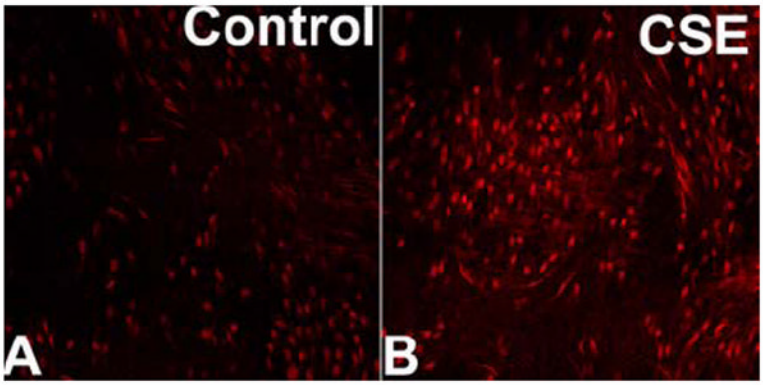

Figure 1.

Representative confocal images of nuclear ethidium bromide (EB) staining of *en face* preparations of rat aortas incubated with cigarette smoke extract (CSE; 4 μg/mL, for 6 h; Panel B) and untreated controls (Panel A). Vessels were incubated with the dye dihydroethidium, which produces a red nuclear fluorescence when oxidized to EB by $O_2^{\cdot -}$. On these 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 (original magnification: 20X).

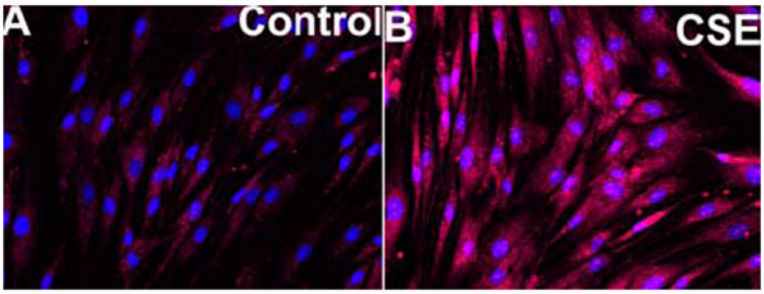

Figure 2.

Incubation with cigarette smoke extract (CSE) elicits mitochondrial oxidative stress. Representative fluorescent images show intensive MitoSox staining (red fluorescence) in CSE-treated cells (CSE; 4 μg/mL, for 6 h; Panel B), whereas MitoSox fluorescence is significantly weaker in mitochondria of untreated control cells (Panel A). Cells were incubated with the mitochondrion-targeted $O_2^{\cdot -}$ sensitive dye MitoSox (Invitrogen), which produces a red fluorescence in the mitochondria when oxidized by $O_2^{\cdot -}$. The DNA-binding dye Hoechst 33258 was used for nuclear counterstaining (original magnification: 20X).

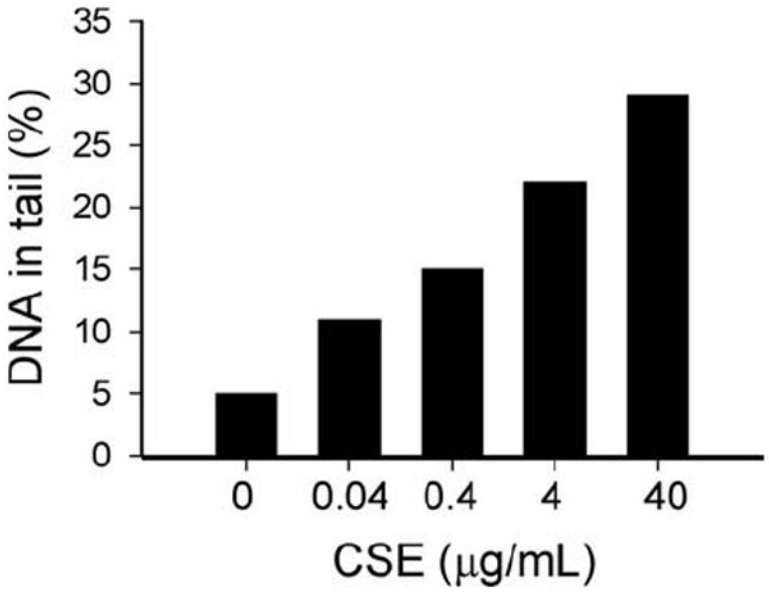

Figure 3.

Water-soluble cigarette smoke constituents elicit endothelial DNA damage. Coronary arterial endothelial cells were treated with cigarette smoke extract (CSE for 6 h). Then, cells were harvested and the extent of DNA damage was examined by single-cell electrophoresis ("comet assay") as we reported (109, 139). Damaged DNA migrates during electrophoresis from the nucleus towards the anode, forming a shape of a "comet" with a head (cell nucleus with intact DNA) and a tail (relaxed and broken DNA). Frequency distribution of tail DNA content in untreated control and CSE-exposed endothelial cells were obtained (median values for tail DNA content are shown in the graph).

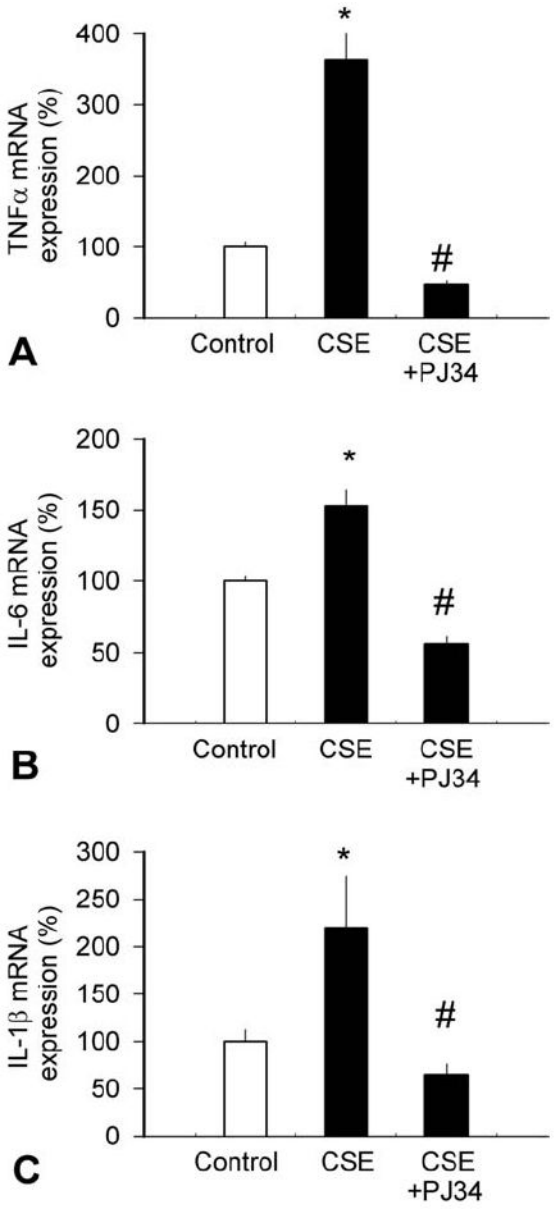

Figure 4.

Water-soluble cigarette smoke constituents up-regulate vascular inflammatory cytokine expression. Isolated rat carotid arteries were maintained in organoid culture in the presence and absence of cigarette smoke extract (CSE) and mRNA expression of TNFα (A), IL-6 (B) and IL-1β (C) were assessed by real-time QRT-PCR, as reported (48). β-actin was used for internal normalizations. Some vessels were pre-incubated with the potent PARP inhibitor PJ34 (142-144, 146). Data are mean ± S.E.M. (n=4-6 for each group). **P*<0.05 vs. untreated, # *P*<0.05 vs. CSE only.

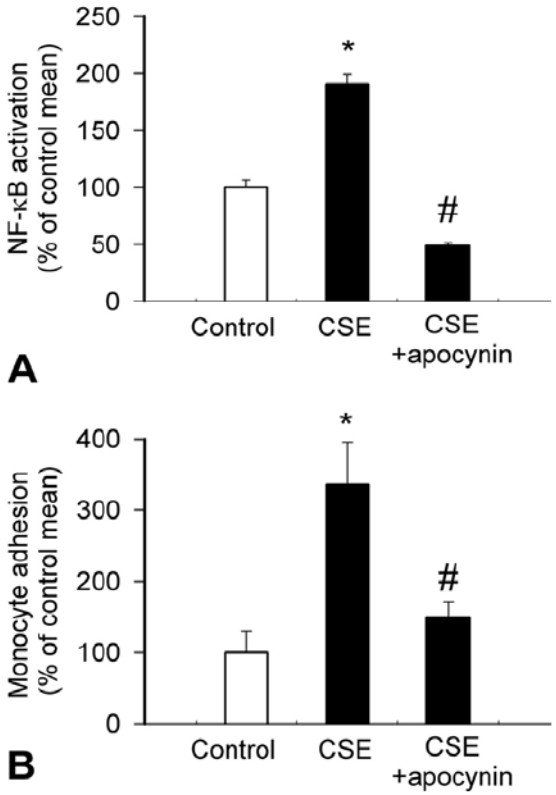

Figure 5.

Water-soluble cigarette smoke constituents elicit NF-κB activation and enhance monocyte adhesiveness via increasing NAD (P)H oxidase-derived ROS generation in primary coronary arterial endothelial cells (CAECs). Panel A: Reporter gene assay showing the effect of cigarette smoke extract (CSE) on NF-κB reporter activity in CAECs. The effects of pre-treatment with NAD (P)H oxidase inhibitor apocynin (3×10^{-4} mol/L) on CSE-induced NF-κB reporter activity in CAECs is also shown. 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. (Data are mean ± S.E.M. *p<0.05. vs. untreated; #P<0.05 vs. CSE only). 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 (3×10^{-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 CAECs with CSE significantly increased the adhesion of fluorescently labeled PMA-stimulated monocytes. Pre-treatment with the NAD (P)H oxidase inhibitor apocynin significantly attenuated CSE-induced monocyte adhesion. Data are mean ± S.E.M. *p<0.05. vs. control. #p<0.05 vs. CSE alone. Figure is redrawn based on data from reference (48).

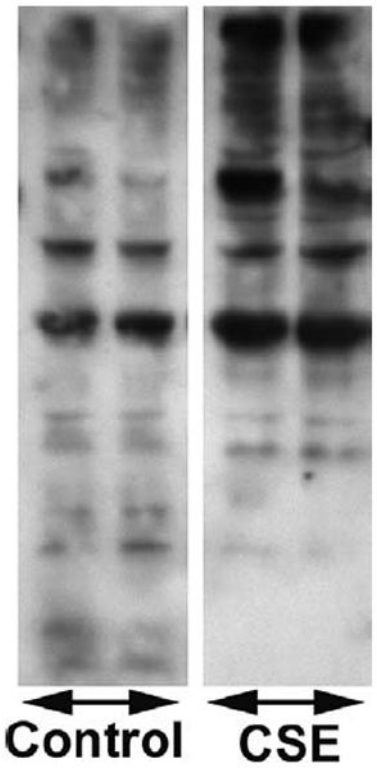

Figure 6.
Water-soluble cigarette smoke constituents elicit PARP activation in human coronary arterial endothelial cells. Endothelial cells were treated with cigarette smoke extract (CSE). Then, cells were harvested and the poly (ADP-ribose) polymer content was assessed by Western blotting.

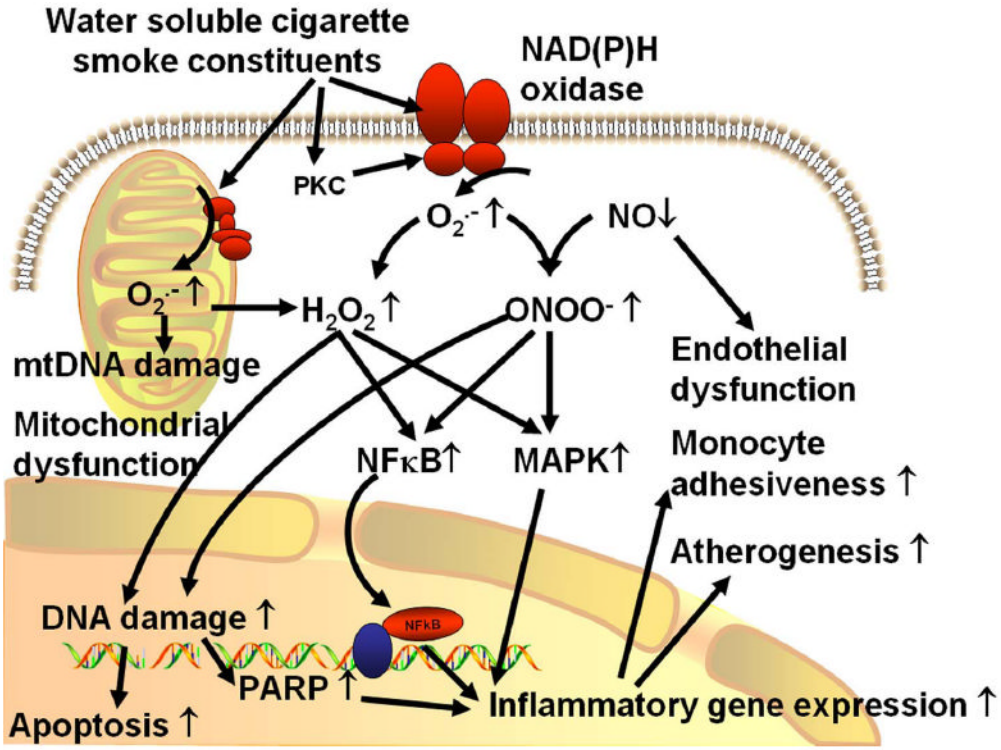

Figure 7.

Proposed scheme for the mechanisms by which water soluble components of cigarette smoke promote pro-inflammatory phenotypic alterations in the blood vessels. The model predicts that cigarette smoke induces an increased generation $O_2^{\cdot-}$ by NAD (P)H oxidase, which scavenges vasodilator NO resulting in an enhanced ONOO- formation and endothelial dysfunction. Cigarette smoke also impairs mitochondrial function eliciting mitochondrial oxidative stress. Increased cellular levels of NAD (P)H oxidase- and mitochondria-derived $O_2^{\cdot-}$, H_2O_2 and/or ONOO- activate redox-sensitive signaling pathways, including MAP kinases and the transcription factor NF-κB, up-regulating inflammatory gene expression. Oxidative-nitrosative stress (in particular, the enhanced ONOO- levels and increased production of hydroxyl radicals) also elicits nuclear DNA damage promoting endothelial apoptosis and activating the nuclear enzyme PARP-1, which importantly contributes to NF-κB -dependent regulation of gene transcription. The resulting pro-inflammatory vascular phenotype 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.