NADPH Oxidase and the Cardiovascular Toxicity Associated with Smoking

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Smoking is one of the most serious but preventable causes of cardiovascular disease (CVD). Key aspects of pathological process associated with smoking include endothelial dysfunction, a prothrombotic state, inflammation, altered lipid metabolism, and hypoxia. Multiple molecular events are involved in smoking-induced CVD. However, the dysregulations of reactive oxygen species (ROS) generation and metabolism mainly contribute to the development of diverse CVDs, and NADPH oxidase (NOX) has been established as a source of ROS responsible for the pathogenesis of CVD. NOX activation and resultant ROS production by cigarette smoke (CS) treatment have been widely observed in isolated blood vessels and cultured vascular cells, including endothelial and smooth muscle cells. NOX-mediated oxidative stress has also been demonstrated in animal studies. Of the various NOX isoforms, NOX2 has been reported to mediate ROS generation by CS, but other isoforms were not tested thoroughly. Of the many CS constituents, nicotine, methyl vinyl ketone, and α,β-unsaturated aldehydes, such as, acrolein and crotonaldehyde, appear to be primarily responsible for NOX-mediated cytotoxicity, but additional validation will be needed. Human epidemiological studies have reported relationships between polymorphisms in the CYBA gene encoding p22phox, a catalytic subunit of NOX and susceptibility to smoking-related CVDs. In particular, G allele carriers of A640G and -930[A/G] polymorphisms were found to be vulnerable to smoking-induced cardiovascular toxicity, but results for C242T studies are conflicting. On the whole, evidence implicates the etiological role of NOX in smoking-induced CVD, but the clinical relevance of NOX activation by smoking and its contribution to CVD require further validation in human studies. A detailed understanding of the role of NOX would be helpful to assess the risk of smoking to human health, to define high-risk subgroups, and to develop strategies to prevent or treat smoking-induced CVD.

Key words: Smoking, Cigarette, NADPH oxidase, Reactive oxygen species, Oxidative stress, Cardiovascular disease

INTRODUCTION

Smoking is a human habit of immense global scale. It is a risk factor of life-threatening diseases and thus is the leading cause of preventable death. Smoking harms nearly every organ of the body, adversely affects the health statuses of smokers and causes a variety of diseases. Of these, the most well recognized health risks are cancers, respiratory diseases, and cardiovascular disease (CVD). CVDs are

the leading cause of death in most countries and account for 30~40 percent of all deaths. Smoking is one of the most well-established risk factors of CVD and accounts for one tenth of cardiovascular mortality worldwide. Smoking causes stroke and coronary heart disease commanding a majority of CVD, and has been estimated to increase their developmental risks by 2 to 4 fold (1-3). According to a pooled analysis of 21 cohorts in Asia, smoking contributed to the deaths of 2 million adults over the age of 45 in 2004, which corresponded to a 1.4-fold increase in mortality. Furthermore, 31% of smoking-related deaths were attributable to CVDs (4).

Many studies conducted over decades allow an understanding of the mechanisms underlying smoking-related CVD. Key aspects of the pathological process include endothelial dysfunction, the prothrombotic state, inflammation, altered lipid metabolism, and hypoxia (3). Although the molecular bases of these events have not been fully elucidated, a num-

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ber of mechanisms have been suggested and multiple molecular events are known to be involved in the etiologic link between smoking and CVD. One of the major contributors is the oxidative stress caused by reactive oxygen species (ROS). Indeed, smoking stimulates ROS production, which is related with various cardiovascular events, such as a decrease in nitric oxide (NO) bioavailability, induction of the prothrombotic state, the initiation and progression of inflammatory responses, and the oxidation of lipoproteins. Smoke itself contains high levels of ROS (5), but due to their short half lives, they do not reflect the magnitude of systemic events caused by smoking (6). Smoking stimulates ROS production in biological systems and the NOX family of ROS-generating NADPH oxidases has been suggested to produce this ROS. In this review, we summarize current information regarding the generation of smoking-induced ROS by NADPH oxidase and discuss perspectives regarding future study briefly.

There are many types of tobacco products including cigarettes, electronic cigarettes, cigars, hooka, and smokeless tobaccos such as chewing tobacco and snuff. Nevertheless, cigarettes are the most common tobacco products in Korea and in most other countries. Hence, most experimental studies have been conducted using cigarette smoke (CS). In this review, we also focus on cigarette smoking, and if not specified, smoking and smoke indicate cigarette smoking and CS.

NADPH OXIDASE AS A SOURCE OF ROS IN THE CARDIOVASCULAR SYSTEM

There are a variety of ROS sources in living organisms such as the mitochondrial electron transport chain, cyclooxygenase, lipoxygenase, xanthine oxidase, cytochrome P450 oxidases, monoamine oxidase, and NADPH oxidase (7). NADPH oxidase (NOX) is a membrane-bound enzyme complex that catalyzes the electron transfer from NADPH to molecular oxygen as shown in the following reaction. ROS generation is the primary and sole function of NOX, unlike other ROS-producing enzymes which generate ROS as only a part of their functions.

$$NADPH + 2O_2 \rightarrow NADP^- + H^+ + 2O_2^{\cdot-}$$
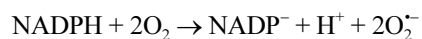
NOX was originally found in phagocytic leukocytes such as neutrophils and macrophages. It participates in respiratory burst during immune responses by producing superoxide. NOX in phagocytes is composed of membrane bound catalytic subunits such as gp91phox and p22phox, and regulatory cytosolic subunits such as p47phox, p67phox, p40phox, and Rac. NOX is latent under normal circumstances, but upon activation, its regulatory cytosolic subunits translocate to the membrane and associate with membrane bound catalytic subunits. The assembled enzyme complex then produces superoxide via the one electron reduction of the

oxygen molecule by gp91phox using NADPH as the electron donor. During the last two decades, NOX activity has been reported in non-phagocytic cells and additional isoforms of gp91phox have been discovered. Seven NOX isoforms have been identified in human and named NOX1, -2, -3, -4, -5, and dual oxidase (DUOX) 1 and 2. The originally identified phagocytic NOX has now been named NOX2 unreasonably. NOX exhibits tissue-specific expression patterns and distinct subcellular localizations that are isoform dependent. Furthermore, each isoform works with different regulatory subunits and has a specific regulatory mechanism, and thus, the isoforms probably have quite different pathophysiological functions. NOX isoforms are found throughout the cardiovascular system, including endothelial cells, vascular smooth muscle cells, adventitial fibroblasts, cardiac myocytes, and blood cells such as phagocytic leukocytes and platelets. NOX1, -2, -4 and -5 are important in the cardiovascular system. All four are expressed in endothelium, NOX1, -4, and -5 in vascular smooth muscle cells (VSMC), and NOX2 and -4 in adventitial fibroblasts (8). NOX2 and -4 have been reported in cardiomyocytes (9). Detailed reviews of the biochemistry and pathophysiology of NOX has been recently published (7,10).

As its etiological roles have been demonstrated, NOX is viewed as a potential therapeutic target. Furthermore, the harmful effects of various toxicants are known to be mediated by NOX, and increasing numbers of xenobiotics and environmental factors are reported to exert their toxic effects by stimulating NOX-dependent ROS generation. These include chemical factors like heavy metals, organic solvents, diesel exhaust particles, and physical factors such as ionizing and ultraviolet radiation. CS has also been demonstrated to stimulate NOX-mediated ROS production in the cardiovascular system and been implicated in the development of CVD (6,11). Epidemiological studies have investigated the relationship between polymorphisms of genes encoding NOX subunits and the cardiovascular toxicity of smoking, but unfortunately, the conclusions of these studies are disputed.

IN VITRO STUDIES ON THE INVOLVEMENT OF NOX IN THE CARDIOVASCULAR TOXICITY OF CS

NOX-dependent ROS generation by CS. Although ROS formation by CS was reported decades ago, NOX-mediation was firstly demonstrated by Jaimes *et al.* (11), who reported the contribution made by NOX to cigarette smoke extract (CSE)-induced ROS formation in pulmonary arterial endothelial cells. Their findings were subsequently reproduced in the cardiovascular system (Table 1). Orosz *et al.* (2007) exposed carotid arteries and aortas isolated from rats to commercially available total particulate matter (TPM) of CS at concentrations of 0.04 to 40 mg/ml for 6 hrs (6). TPM treatment resulted in ROS production measured by lucigenin and dichlorofluorescein (DCF), and this ROS genera-

Table 1. Experimental evidence of NOX activation by CS exposure in blood vessels and vascular cells

Target	Exposed substance	Effect on blood vessels or vascular cells	Tool for NOX inhibition	Restoration of effect by NOX inhibition	Ref.
Rat aorta and carotid artery	CSC	Increase in $O_2^{\cdot-}$ production	DPI	restored	
Rat carotid artery	CSC	Increased expression of iNOS, TNFα and IL-1β Increase of ICAM-1 mRNA	DPI, apocynin	restored	(6)
Rat CAEC	CSC	Increase in NF-κB activation and adhesion to monocytes	DPI, apocynin	restored	
Mouse AEC and BMEPC	CSE	Increase in $O_2^{\cdot-}$ production Decrease of NO production and VEGF-induced migration	NOX2 KO	restored	(12)
Bovine PAEC	CSE	Increase in $O_2^{\cdot-}$ production	DPI, apocynin, gp91ds-tat	restored	(11)
Human PAEC, bovine PAEC and bovine PAEH	CSE	Increase of $O_2^{\cdot-}$ production and $ONOO^-$ formation	DPI	restored	
HUVEC	CSE + LDL	Increase in $O_2^{\cdot-}$ production and decrease in NO bioavailability	apocynin, VAS2870, NOX4 KD	restored	(13)
Mouse CEC	CSE + IL-1β	Increase in H_2O_2 production, PGE_2 synthesis and mPGES-1 expression	DPI, apocynin, NOX2 KD	restored	(16)
HUVEC, TrHBMMEC	CSE + IL-1β and TNF-α	Increase of H_2O_2 generation and COX-2 expression Phosphorylation of Akt and p38	DPI, apocynin, NOX2 KD	restored	(15)

AEC, aortic epithelial cells; BMEPC, bone marrow epithelial progenitor cells; CAEC, coronary arterial endothelial cells; CEC, cardiac endothelial cells; COX, cyclooxygenase; CS, cigarette smoke; CSC, cigarette smoke condensate; CSE, cigarette smoke extract; DPI, diphenyleneiodonium; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule 1; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; KD, knockdown; KO, knock-out; LDL, low density lipid; mPGES-1, microsomal-prostaglandin E synthase-1; mRNA, messenger RNA; NF-κB, nuclear factor-κB; NO, nitric oxide; NOX, NADPH oxidase; PAEC, pulmonary arterial endothelial cells; PAEH, pulmonary arterial epithelial cells homogenates; PGE_2, prostaglandin E_2; p38, p38 mitogen-activated protein kinase.; TNFα, tumor necrosis factor-α; TrHBMMEC, transformed human bone marrow microvascular endothelial cell line; VEGF, vascular endothelial growth factor.

tion was prevented by pretreatment with the pharmacologic NOX inhibitor diphenyleneiodonium (DPI), implying that ROS originated from NOX. NOX-mediated oxidative stress was confirmed in other sets of experiments using different vessel types from diverse animal species, including mice aortas (12) and pulmonary arteries from mice, cattle, and human (11).

NOX-dependent ROS generation has been mostly studied *in vitro*. In human umbilical vein endothelial cells (HUVEC), CSE produced superoxide, which was abolished by pretreatment with the NOX inhibitors apocynin or VAS2970 or gene silencing using p47phox-specific siRNA (13). Most chemical inhibitors cannot differentiate NOX isoforms, but the effectiveness of siRNA for p47phox indicates that NOX1 and/or NOX2 are mainly responsible for CSE-induced ROS generation, because p47phox is used only by these isoforms. Similarly, endothelial progenitor cells (EPC) isolated from NOX2-knockout mice were resistant to superoxide production by CSE, indicating a major role of NOX2. In addition, ROS production by CS has been confirmed in vascular smooth muscle cells (6,14).

In other experiments, additional stimuli were required to activate NOX, and CS was only capable of producing ROS when combined with other vasoactive compounds. In mouse cardiac endothelial cells, tobacco smoke (TS) exhibited only a minimal effect on NOX, but when combined with interleukin-1β (IL-1β), it strongly induced the translocation of p47phox to cellular membranes, and thereby, stimulated NOX and induced ROS production. Similarly, CSE needed IL-1β or tumor necrosis factor α (TNF-α) to activate NOX in human bone marrow microvascular endothelial cells or HUVEC. In addition to cytokines, low-density lipoprotein (LDL) exhibited synergistic activity with CS (13). In HUVEC, CS or LDL alone induced NOX-dependent ROS production minimally, but in combination strongly potentiated ROS generation (13). Accordingly, CS appears to interact with other vasoactive substances to activate NOX, which suggests ROS generation by CS may be dependent on microenvironments in the cardiovascular system.

NOX-mediated ROS production by CS leads to a variety of cell-type dependent responses. CS seems to stimulate proinflammatory or proliferative signals in vascular cells.

Table 2. NOX activation by CS constituents in cardiovascular cells

Target	Exposed substance	Effect on cells	NOX inhibition	Restoration of effect by NOX inhibition	Ref.
Bovine PAEC	Acrolein	Increase in $O_2^{\cdot-}$ production	DPI	Restored	(11)
Rabbit cVSMC	Nicotine	Increase of $O_2^{\cdot-}$ production and PDE-5 expression	Apocynin	Restored	(14)
Mouse cardiomyocyte	Crotonaldehyde	Increase in H_2O_2 production	Apocynin	Restored	(18)

CS, cigarette smoke; cVSMC, cavernosal vascular smooth muscle cells; DPI, diphenyleneiodonium; NOX, NADPH oxidase; PAEC, pulmonary arterial endothelial cells; PDE, phosphodiesterase.

CSE induced proinflammatory cytokine production in rat carotid arteries and this was prevented by NOX inhibitors, suggesting the involvement of NOX in CS-induced cytokine production and inflammation (6). CSE also facilitated adhesion between rat coronary arterial endothelial cells and monocytes (6), and induced COX-2 expression and the activations of mitogen-activated protein kinases (MAPK) in HUVEC and in human bone marrow microvascular endothelial cells, respectively (13). Moreover, nicotine stimulated phosphodiesterase-5 in cavernosal vascular smooth muscle cells derived from rabbit penises (14,15). All these effects were abolished by NOX inhibitors or by suppressing NOX expression. The productions and regulations of prostanoids are mediated by CS. CS altered the balance between PGI_2 and PGE_2 in cardiac endothelial cells by reducing PGI_2 generation and by increasing the synthesis and activity of microsomal prostaglandin E synthase-1, which are under regulation of NOX. In addition, NOX mediated the endothelial dysfunction and vascular remodeling caused by CS in mouse heart (16), and in a study on aortic endothelial cells from NOX2-knockout mice, it was found that NOX2 enhanced ROS production and the cellular migration elicited by CSE (12).

CS constituents activating NOX. CS is a mixture of thousands of identified and unidentified constituents generated from tobacco combustions, which raises the question, which constituents of CS are responsible for activating NOX?. However, only a limited number of studies have tried to identify responsible compounds systemically. Instead, most studies conducted on the subject have examined stable compounds known to be abundant in CS, such as nicotine, acrolein, crotonaldehyde, α,β-unsaturated ketones, saturated aldehydes, and quinones, which potentially could produce ROS (6).

Noya *et al.* (2013) assumed that the gas phase of CS is capable of reaching the systemic circulation and demonstrated that it is cytotoxic in vascular system via the sequential activations of protein kinase C and NOX (17). In addition, they tried to indentify cytotoxic constituents by cytotoxicity assay-guided CSE fractionation, and concluded that stable substances, such as methyl vinyl ketone (MVK)

and acrolein, were responsible for NOX-mediated cytotoxicity. Other *in vitro* studies attributed the cardiovascular toxicity of CS to stable, water-soluble components of CSE, and suggested acrolein and crotonaldehyde act as NOX activators (Table 2) (6,11,14,18).

Acrolein is one of the most abundant and crotonaldehyde is another α,β-unsaturated aldehyde in CS, and both stimulated ROS production by activating NOX in mouse cardiomyocytes (18) and in endothelial cells from bovine pulmonary arteries (11). Some controversy exists regarding the role played by nicotine in NOX activation. In cavernous VSMCs, nicotine stimulated superoxide production was inhibited by an NOX inhibitor (14), but in rat carotid and coronary arteries nicotine had no effect on CSE-induced peroxide hydrogen production (6). Although several CS constituents have been suggested to be responsible for NOX activation, they may not be the only constituent capable of activating NOX. Systematic, logical approaches will undoubtedly reveal the constituents of CS responsible for activating NOX.

IN VIVO STUDIES ON THE INVOLVEMENT OF NOX IN THE CARDIOVASCULAR TOXICITY OF CS

Animal studies. One of the difficulties associated with *in vivo* animal studies is that the exposure method used must accurately mimic human cigarette consumption. Several smoke exposure models have been developed and used for animal experiments. Perhaps the easiest exposure method involves the administration of aqueous CS to laboratory animals, and in one study, aqueous CS administered intravenously increased ROS and cyclooxygenase-2 levels in the aortas and carotid arteries of FVB mice, suggesting a proinflammatory effect (15).

The current standard method of CS exposure involves the use of an inhalation chamber in combination with a CS-puffing machine (19). In a study performed by Rafacho *et al.* (2011), rats inhaled the CS equivalent of 10 cigarettes in 30 min, twice daily for 60 days, and noted a significant NOX elevations in cardiac tissues (20). Orosz *et al.* (2007) examined ROS levels in and the vasomotor functions of the carotid arteries of rats exposed to 5 cigarettes daily for a

Table 3. Major consequences of NOX activation by CS

Target	Exposure method	Effect of smoking	NOX inhibition	Restoration of effect by NOX inhibition	Ref.
Rat carotid artery	CS inhalation	Increase in $O_2^{\cdot-}$ production Decrease in endothelium-dependent vasorelaxation	DPI	restored	(6)
Mouse hind limb ischemic muscles	CS inhalation	Increase in $O_2^{\cdot-}$ production, $ONOO^-$ formation and NOX expression Decreased eNOS expression Enhancement of VEGF-induced endothelial migration and tube formation Decrease in blood flow, capillary density	NOX2 KO	restored	(12)
Mouse plasma	CS inhalation	Decrease in plasma NO and antioxidants level	NOX2 KO	restored	
Human platelets from smokers	Cigarette smoking	Increase in AA-induced ROS production, NOX activity, 8-iso-PGF2α and platelet recruitment	NOX2ds-tat	restored	(23)

AA, arachidonic acid; CS, cigarette smoke; DPI, diphenyleneiodonium; eNOS, endothelial nitric oxide synthase; KO, knockout; NO, nitric oxide; NOX, NADPH oxidase; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor; 8-iso-PGF2α, 8-iso-prostaglandin F2α.

week by inhalation, and found that exposure by inhalation diminished endothelium-dependent vasodilation and elevated ROS levels in carotid arterial smooth muscle and endothelium. Moreover, this impaired vasorelaxation and increase in ROS levels were prevented by pretreatments with the NOX inhibitors apocynin and DPI (6). Summarizing, these results show CS inhalation deteriorates vascular function by enhancing the expression and activity of NOX.

Cardiovascular toxicity was tested in a pathologic animal model of surgically-induced unilateral hind limb ischemia. Mice with established ischemia were exposed to CS from one cigarette twice daily for two weeks, and CS was found to decrease total antioxidant capacity and NO levels in plasma, reduce eNOS expression, and increase NOX2 expression and ROS production in ischemic muscles, and thereby, aggravated ischemic damage of injured hind limbs. In concomitant experiments, the exacerbation of ischemic injury by CS was significantly abrogated in NOX2 knockout mice, suggesting a pathological role for NOX2 in CS-induced CVD (Table 3) (12).

Effect of maternal exposure to nicotine on offspring.
Smoking during pregnancy is associated with obstetric and fetal complications, such as stillbirth, spontaneous abortion, and sudden infant death syndrome, and it has been established that the nicotine component of CS is largely responsible for the chronic effects in offspring associated with maternal cigarette smoking. Although little is known about the direct effect of nicotine on NOX, maternal nicotine exposure throughout gestation induces NOX-mediated impairment of vascular function in offspring (21). The administration of nicotine to pregnant rats using an osmotic minipump enhanced angiotensin II-induced vasoconstriction and impaired endothelium-dependent NO-mediated vasodilatation in thoracic aortas isolated from 5-month-old male

offspring. Furthermore, these functional abnormalities could be restored by acute treatment of vessels with the NOX inhibitor apocynin or with the superoxide dismutase mimetic tempol. Indeed, antenatal exposure to nicotine augmented the expression of NOX2 in the aortas of offspring. It would appear that exposure to nicotine in CS during the antenatal stage increases the risk of CVD development in adult offspring. However, the mechanism involved is unclear.

Human studies conducted using the blood of smokers.
Although a number of *in vitro* and animal studies demonstrated NOX activation by CS, the clinical relevance remains to be confirmed. Loffredo *et al.* (2011) and Carnevale *et al.* (2011) found increases in the levels of soluble NOX2-derived peptide, a marker of NOX activation in sera and the translocation of p47phox from cytosol to membrane in platelets isolated from smokers, compared with nonsmokers (22,23). Carnevale *et al.* (2011) tested the function of NOX2 in platelets obtained from smokers and nonsmokers using the peptidic NOX2 inhibitor NOX2ds-tat. ROS production and platelet recruitment were greater and more extensive in smokers than in nonsmokers, and this difference was abolished by pretreating NOX2ds-tat (Table 3) (23).

POLYMORPHISMS OF THE NOX GENE AND SUSCEPTIBILITY TO THE CARDIOVASCULAR TOXICITY ASSOCIATED WITH SMOKING

Genetic polymorphisms influencing cardiovascular toxicity associated with smoking. Despite the causal relationship between smoking and CVD, not all smokers seem to be negatively influenced by CS (24). As is the case for most toxicants, individuals differ in terms of susceptibility to CS toxicity. Molecular epidemiologists have discov-

ered genetic factors influencing smoking-related CVD. For example, the methylation pattern of the F2RL3 gene, which encodes protease-activated receptor 4, is associated with a prognosis of stable coronary heart disease associated with CS (25). Furthermore, genetic polymorphisms are also related with susceptibility to CVD conferred by CS. Apolipoprotein E (ApoE) variants are an acknowledged example. The gene, *ApoE*, is polymorphic, with three major isoforms, ApoE2, ApoE3, and ApoE4. Among smokers, elevation of oxidized lipoprotein is prominent, and the risk of CVD was found to be higher for ApoE4 variant, ε4 allele carriers (25).

The cytochrome b-245, alpha polypeptide (CYBA) gene encoding p22phox has been studied recently. p22phox variants include C242T, A640G, C549T, $-930^{A/G}$, $-675^{A/T}$, $-852^{C/G}$, and $536^{C/T}$, and of these, C242T, A640G and, $-930^{A/G}$ are suspected to be associated with susceptibility to CVD, that is, hypertension, hypercholesterolemia, coronary artery disease (CAD), myocardial infarction, and cerebrovascular diseases (26). However, results are currently considered controversial.

A640G. A case-control study compared Polish Caucasian patients suffering from CAD with sex- and age-matched healthy volunteers (27). Among smokers, the risk of CAD was higher for G allele carriers [odds ratio (OR) 3.45, 95% confidence interval (CI) 1.63~7.38] than AA carriers (2.65, 1.04~6.81). Subgroup analysis revealed a synergism between smoking and the progression of hypercholesterolemia to CAD (8.02, 3.03~22.06 in G allele, and 4.05, 1.12~15.96 in AA). Intriguingly, the A640G genotype was not found to affect cardiovascular risk in the general population or among nonsmokers.

-930$^{A/G}$. Two research groups reported the resistance of the AA genotype of the $-930^{A/G}$ polymorphism to the cardiovascular toxicity of CS (28,29). Niemiec *et al.* (2014) investigated genotypes of the $-930^{A/G}$ polymorphism in a Polish Caucasian population. CS, as had already been shown, increased the risk of CAD (3.31, 2.26~4.83), but this relationship was observed in G allele carriers (3.38, 2.28~5.03) and not in AA carriers (1.23, 0.59~2.55) (28).

In a cross-sectional study conducted by Fan *et al.* (2009) carotid intima-media thicknesses (IMTs), a marker for subclinical atherosclerosis (30), were measured in healthy young Finns aged 24~39 (29). The mean IMT (mm) of smokers (mean ± standard deviation, 0.60 ± 0.09) was significantly higher than that of nonsmokers (0.58 ± 0.09) for the GG genotype, tended to be higher for the GA genotype [0.58 ± 0.09 in smokers and 0.57 ± 0.09 in nonsmokers ($p = 0.06$)], and was similar for the AA type (0.59 ± 0.09 in smokers and 0.59 ± 0.20 in nonsmokers). Furthermore, these findings agree with those of Niemiec *et al.* (2014), and suggest that G allele confers susceptibility to the cardiovascular toxicity of CS (28).

C242T. It is controversial how C242T genotype influences the cardiovascular toxicity associated with smoking (31-35). In a cross-sectional study, brachial flow-mediated dilation (FMD), an early characteristic of endothelial dysfunction, was measured in healthy young Finns (31). The mean FMD of T allele carriers was higher than in the total population. The mean FMD values of the CC, CT, and TT genotypes were 7.8 ± 4.4%, 8.2 ± 4.5% and 8.7 ± 4.5%, respectively ($p = 0.02$ for trend). The same pattern reappeared in a subgroup analysis of smokers ($p = 0.008$), but not in a similar analysis of nonsmokers ($p = 0.438$). Furthermore, this flat relationship between FMD and C242T polymorphism was significant only in ever-smokers ($p = 0.008$), not in non-smoking subjects ($p = 0.438$).

Another cross-sectional study performed in China also suggested resistance conferred by the T allele (32). In this study, the risk for metabolic syndrome was calculated for Chinese participants without CVD. The risk of having metabolic syndrome was found to be lower for T allele carriers than those with the CC genotype. Furthermore, this pattern was repeated in the smoker group (OR 0.194, 95% CI 0.058~0.651 in T allele; 1.21, 0.824~1.776 in CC), but not in the nonsmoker group. In smoker subgroup analysis based on dosages (pack-years), the same pattern as that observed in the total smoker group analysis was found only in smokers consuming no less than 25 pack-years (0.217, 0.026~0.474 in T allele; 1.345, 0.614~2.946 in CC), whereas no such correlation was observed in the < 25 pack-years group. Of the components of metabolic syndrome, plasma triglyceride levels were lower for T allele carriers than for the CC genotype in all subjects and in smokers (0.11 ± 0.12 mM/L for the T allele vs. 0.49 ± 0.04 mM/L for CC in smokers), while no significant difference was observed between genotypes in nonsmokers. These two cross-sectional studies, which both included a CVD-free population, demonstrated that the T allele of the C242T polymorphism has a protective effect in smokers.

A case-control study conducted on Chinese patients with coronary heart disease (CHD) also reported that the T allele confers resistance to CHD. However, unlike the two previously mentioned cross-sectional studies, this resistance was only observed among nonsmokers (33). Moreover, the risk of CHD was lower for T allele carriers in the total population and for nonsmokers [OR 0.25, 95% CI 0.12~0.53 for the T allele versus nonsmokers with the CC genotype]. However, no difference was observed between genotypes in smokers (2.04, 0.74~5.61 in T allele; 2.16, 1.58~2.95 in CC).

Unlike the studies mentioned above, others have reported conflicting results. In one study, the plasma level of malondialdehyde-modified LDL (MDA-LDL) was used as an indicator of lipid peroxidation in Japanese patients with CAD (34). MDA-LDL levels were higher in T allele carriers than in those with the CC genotype in the total popula-

tion and in nonsmokers (113.3 ± 46.7 U/I for the T allele, 91.5 ± 27.4 U/I for CC in nonsmokers, $p = 0.014$), but no genotype associated differences were observed in smokers (116.8 ± 40.9 for the T allele, 108.8 ± 42.0 for CC in smokers, $p = 0.901$). Niemiec *et al.* (2007) compared the odds of CVD in a case-control study conducted on Polish Caucasians, and smokers with hypercholesterolemia had a significantly higher risk of CAD when they had the T allele rather than the CC genotypes (OR 36.83, 95% CI 5.16~734.15 in T allele; 3.36, 1.20~9.76 in CC) (35).

The G alleles of the A640G and $-930^{A/G}$ polymorphisms indicate susceptible to CVD in smokers. However, in the case of C242T results are conflicting, presumably because of different ethnicities, distributions of genotypes, study designs, and unidentified factors (31-35). Currently, the relations between CYBA polymorphisms and susceptibilities to CVD by smoking remain to be confirmed.

REMAINING ISSUES

Although a number of studies have suggested a role for NOX in the development of CVD by CS, evidence of a clinical relation is lacking. *In vitro* and animal studies have demonstrated NOX activation by CS, but few human studies have been performed. Because of the substantial amount of experimental evidence available, a well-designed clinical study could be undertaken. Major questions to be answered are; 1) Does smoking induce the activation or expression of NOX in smokers, and if so, 2) To what extent does NOX-originated ROS contribute to CVD by smoking. In addition, we need to know which NOX isoforms are responsible for the development of CVD by smoking. NOX isoforms exhibit distinct expression patterns and pathophysiological functions that are tissue and cell dependent. A number of *in vitro* studies and animal studies have shown that NOX2 is required for CS-mediated cardiovascular toxicity (6,12,14,22,23,36). Accordingly, it remains to be clarified whether NOX2 is the major isoform involved and whether other isoforms also contribute to CS toxicity. One of the technical problems in such studies is the lack of specificity of pharmacologic NOX inhibitors. In fact, most are non-specific NOX inhibitors and specific inhibitors of each isoform are not available. Therefore, genetic approaches may provide supplementary or alternative means of identifying the isoform responsible for CS toxicity. In addition, it needs to be determined whether individual susceptibility to cardiovascular toxicity associated with smoking can be ascribed to genetic polymorphisms of NOX. Actually, polymorphism studies could provide convincing, clinical evidence regarding the contribution of NOX to CVD by smoking, and studies involving human subjects or population studies might provide invaluable information.

CS contains more than 4,000 known chemicals constituents and hundreds of thousands of unidentified components

(5). Thus, it is difficult to identify the components responsible for activating NOX. As with many other studies on complex mixtures or extracts, the numbers of constituents and a lack of information regarding their bioavailabilities, distributions, and metabolic process make the identification of active principles difficult. Actual concentrations in blood and other tissues should also be considered to identify the actual activators of NOX. A conventional fractionation study has been attempted with some success, but its results need confirmation (16). Further systemic and logical approaches will provide opportunities for better understanding of CS constituents. One of the crucial points requiring careful consideration is that CS contains prooxidants and a substantial amount of ROS, and thus, there is a need to distinguish between preexisting ROS in CS and NOX-generated ROS. Oxidative stress can be induced by any ROS in CS or derived from NOX, and there may be other sources and other mechanisms of oxidative stress. NOX may not be only generator of ROS by CS (5).

In addition, the CS used in studies possibly contain different constituents, because CS were not prepared by the same methods. The different terms used, such as TS, CS, CSE, and CSC, lack specific definitions and CS are further confused by terms, such as whole smoke, TPM, or aqueous extract. Constituents probably also depend on cigarettes used for CS preparation, and the smoke generation and extraction methods used. Some research groups purchase CSC from pharmaceutical companies (6), whereas others self-produce CSE using a puffing machine and reference cigarettes (13,15,16) or unidentified cigarettes (11). Furthermore, the experimental conditions, such as exposure methods, concentrations, and durations of exposure, used also differ between studies. In practice, the above-mentioned differences prevent comparisons of results. Reference cigarettes are provided by the University of Kentucky Reference Cigarette Program (UKRCP), and standard methods for combustion and smoke extraction have been defined by International Organization for Standardization (ISO). However, not all the studies used or followed them and, in many cases, it is hard to refer them because of various purposes of studies. In addition to the qualitative aspects of testing materials, the exposure methods should be considered carefully. Smoke exposure methods are beyond the scope of this review, but it too is a critical issue influence the results of studies.

Generally, a detailed understanding of mechanisms aids the assessment of potential risk to human health, the definition of high-risk subgroups in human populations, and the development of preventative or therapeutic strategies. Although NOX activation by CS is well-established, comparatively little attention is paid to its mode of action. Mechanistic studies could enable more accurate assessments of cardiovascular risks and provide rationales for the development strategies to treat CVD caused by CS.

PERSPECTIVES

Over the last two decades, several studies have suggested that the NOX family of ROS generating enzymes play crucial functions in the development of CVD (10,37). NOX is now recognized as a major source for ROS causing CVD. NOX also mediates the toxicities of various toxicants via ROS generation. Cigarette smoke is a well-established cardiovascular toxicant, and presumably, diverse etiological mechanisms contribute to its toxicity. A growing body of evidence suggests NOX contributes to smoking-related CVD. However, the clinical relevance of NOX activation by smoking requires further validation, because human studies conducted to date do not sufficiently support its contribution to the development of CVD. If NOX does indeed play a critical role in the pathogenesis of CVD caused by smoking, we may be able to develop a strategy to prevent or treat CVD. Furthermore, the clinically effective NOX inhibitor currently under development may provide an invaluable tool for intervention.

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