HOKKAIDO UNIVERSITY

Title	Carbonyl Compounds in the Gas Phase of Cigarette Mainstream Smoke and Their Pharmacological Properties
Author(s)	Horinouchi, Takahiro; Higashi, Tsunehito; Mazaki, Yuichi et al.
Citation	Biological and pharmaceutical bulletin, 39(6), 909-914 https://doi.org/10.1248/bpb.b16-00025
Issue Date	2016-06
Doc URL	https://hdl.handle.net/2115/62431
Type	journal article
File Information	39_b16-00025.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Current Topics

Recent Advances in Research on Bioactive Ingredients in Cigarette Smoke

Carbonyl Compounds in the Gas Phase of Cigarette Mainstream Smoke and Their Pharmacological Properties

Takahiro Horinouchi,* Tsunehito Higashi, Yuichi Mazaki, and Soichi Miwa

Department of Cellular Pharmacology, Hokkaido University Graduate School of Medicine; North 15, West 7, Kita-ku, Sapporo 060–8638, Japan.

Received January 8, 2016

Cigarette mainstream smoke is composed of gas and tar phases and contains >4000 chemical constituents, including nicotine and tar. The substances in the gas phase but not in the tar phase can pass through the airway epithelial barrier, enter the systemic circulation *via* the pulmonary circulation, and increase systemic oxidative damage, leading to the development of cigarette smoking-related diseases such as atherosclerosis. Recently, we identified some stable carbonyl compounds, including acrolein (ACR) and methyl vinyl ketone (MVK), as major cytotoxic factors in nicotine- and tar-free cigarette smoke extract (CSE) of the gas phase. CSE, ACR, and MVK induce protein kinase C (PKC)-dependent activation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) and subsequent generation of reactive oxygen species (ROS) *via* NOX, causing plasma membrane damage and cell apoptosis. CSE, ACR, and MVK also trigger carbonylation of PKC, which is an irreversible oxidative modification. Cell damage and PKC carbonylation in response to treatment with CSE, ACR, or MVK are abolished by thiol-containing antioxidants such as *N*-acetyl-L-cysteine and reduced glutathione. Thus pharmacological modulation of PKC and NOX activities and the trapping of ROS are potential strategies for the prevention of diseases related to cigarette smoking.

Key words cigarette smoke extract (CSE); acrolein (ACR); methyl vinyl ketone (MVK); protein kinase C (PKC); reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX); protein carbonylation

1. INTRODUCTION

Cigarette smoking is a potential risk factor for cardiovascular diseases and cancers.[1,2] Cigarette mainstream smoke is a dynamic aerosol that contains >4000 chemical constituents, including nicotine and particulate matter (tar).[3] The constituents in the cigarette smoke aerosol are differentially distributed in the tar (particulate) phase and the gas phase. The components of these phases are simultaneously delivered to the active smoker, but their impact on smoker's health differs. The tar phase is composed predominantly of electrically charged semi-liquid particles ranging from 0.1 to 1 μm in diameter (average=0.2 μm).[2] The mean diameter of the particles rapidly increases due to their coagulation. Therefore these inhaled particles do not pass through the alveolar walls and exert local rather than systemic toxicity in limited organs such as the lung, tongue, and pharynx. On the other hand, long-lived components of the gas phase can pass into the bloodstream through the pulmonary circulation, leading to acute and late systemic actions.[1,2] It is important to identify constituents of the gas phase and to determine their toxic properties so as to understand systemic toxicity related to cigarette smoking.

The tar and gas phases of cigarette mainstream smoke can be separated by a filter, typically a standard glass-fiber Cambridge filter, which traps the particles with a size >0.1 μm from cigarette smoke.[4] Thus the tar phase and gas phase are defined as material that is trapped on the filter and that passes through the filter, respectively.[4–6] To characterize the pharmacological properties of the gas phase of cigarette smoke, several studies have employed nicotine- and tar-free cigarette smoke extract (CSE), which is prepared by bubbling cigarette mainstream smoke in aqueous solution such as phosphate-buffered saline after passage through the Cambridge filter.[5–9] The method for preparing the nicotine- and tar-free CSE will be discussed in a separate review by Higashi *et al.* in this issue.[10]

In this review, we will briefly address the components of the tar and gas phases of cigarette mainstream smoke and their impact on smokers' health. Then, we will highlight recent insights into the pharmacological properties of gas phase constituents, with main focus on stable carbonyl compounds identified in the nicotine- and tar-free CSE.[6,7]

2. CHEMICAL SUBSTANCES IDENTIFIED IN TAR AND GAS PHASES OF CIGARETTE MAINSTREAM SMOKE AND THEIR IMPACT ON SMOKERS' HEALTH

Table 1 summarizes representative substances identified in the tar and gas phases of cigarette smoke. The tar phase contains well-known constituents such as nicotine, tar, tobacco-specific *N*-nitrosamines (TSNA), and polynuclear aromatic hydrocarbons (PAH). Nicotine is the major addictive substance of cigarettes, and a precursor for TSNA including 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone and *N'*-nitrosonornicotine, which are important carcinogens related to cigarette smoking.[1] Tar contains stable free radicals that can cause oxidative damage to DNA, proteins, and lipids, leading to tissue injury.[4] PAH as well as TSNA possess carcinogenic activity.[1]

* To whom correspondence should be addressed. e-mail: horinouc@med.hokudai.ac.jp

© 2016 The Pharmaceutical Society of Japan

Table 1. Representative Constituents Identified in Gas Phase and Tar Phase of Cigarette Mainstream Smoke

Phase	Constituents	References
Gas phase	Major components	
	Carbon monoxide	1)
	Reactive oxygen species	4)
	Carbonyls	
	Acetone	1, 6)
	Acrolein	1, 6)
	Acetaldehyde	1)
	Butylaldehyde	1)
	Crotonaldehyde	1, 7)
	Formaldehyde	1)
	Propionaldehyde	1, 6)
	Methyl ethyl ketone	1)
	Methyl vinyl ketone	6, 7, 41)
	2-Cyclopenten-1-one	6)
	Others	
	Hydrogen cyanide	1)
	Ammonia	1)
	Nitric oxide	1)
Tar phase	Major components	
	Nicotine	1)
	Tar	1)
	Catechol	1)
	Tobacco-specific *N*-nitrosamines	
	4-(*N*-Nitrosomethylamino)-1-(3-pyridyl)-1-butanone	1)
	N′-Nitrosonornicotine	1)
	N-Nitrosoanatabine	1)
	N-Nitrosoanabasine	1)
	Polynuclear aromatic hydrocarbons	
	Benz[*a*]anthracene	1)
	Benzo[*a*]pyrene	1)
	Metals	
	Arsenic	1)
	Nickel	1)

The gas phase contains highly reactive oxygen species (ROS) and various carbonyl compounds that play a significant role in cigarette smoke toxicology. Although ROS such as superoxide ($O_2^{\cdot-}$) and hydroxyl radicals ($^{\cdot}OH$) in the gas phase are partially scavenged by antioxidants present in the epithelial lining fluid covering airway epithelial cells, the remaining components react with their plasma cell membranes, causing direct tissue injury.[11] ROS in the gas phase seem responsible for local rather than systemic toxicity of cigarette smoke, since ROS are too reactive and short-lived to reach the systemic circulation through the pulmonary circulation.[12–14]

In contrast to ROS, carbonyl compounds, which are relatively stable, extremely hydrophilic, and chemically reactive, pass through the airway epithelial barrier, enter the systemic circulation, and increase systemic oxidative stress and oxidative damage, contributing to the development of chronic diseases such as atherosclerosis, diabetes, and chronic obstructive pulmonary disease (COPD).[15–17] In addition, carbonyl compounds can induce carbonylation of proteins, which is an irreversible oxidative damage often associated with loss of protein function and accumulation of damaged or unfolded proteins.[18] Cigarette smoke may induce endogenous ROS production *via* continuous activation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) in inflammatory cells including macrophages, neutrophils, and epithelial cells, leading to chronic inflammatory conditions such as COPD.[19] Therefore it appears reasonable to assume that stable carbonyl compounds in the gas phase of cigarette smoke are involved in the systemic toxicity of cigarette smoking.

Our recent study with liquid chromatography/mass spectrometry and gas chromatography/mass spectrometry has revealed that acrolein (ACR), methyl vinyl ketone (MVK), and 2-cyclopenten-1-one, all of which are stable carbonyl compounds, are major cytotoxic factors in nicotine- and tar-free CSE.[6] With respect to the impact of carbonyl compounds present in the gas phase on human health, we focus on the cytotoxic effects of CSE, ACR, and MVK, and their mechanisms.

3. DIFFERENT SENSITIVITY OF VARIOUS CELL LINES TO NICOTINE- AND TAR-FREE CSE

Since the substances in the gas phase of cigarette mainstream smoke can act on numerous cells *via* the systemic circulation, it is important to know the cytotoxic effects of nicotine- and tar-free CSE on several types of cells. The cytotoxic effects vary depending on the cell types used in a cell viability assay.[5] The rank order of potency for CSE-induced reduction of cell viability is Chinese hamster ovary cells>U937 human monocytes=A7r5 rat aorta smooth muscle cells=human lung small cell carcinoma SBC-3 cells>C6 rat glioma cells=human embryonic kidney HEK293T cells. In contrast, human cervical carcinoma HeLa cells, RAW264.7 mouse macrophages, EA.hy926 immortalized human umbilical vein endothelial cells, and human lung adenocarcinoma A549 cells are resistant to CSE. The different sensitivity of these cell lines to CSE may reflect a difference in the resistance to oxidative stress and oxidative damage. For quantitative and qualitative investigations of the cytotoxic effects of CSE and its components, C6 rat glioma cells are more convenient because of the superior accuracy and reproducibility of the results.[5,8]

4. INVOLVEMENT OF PROTEIN KINASE C-DEPENDENT ACTIVATION OF NADPH OXIDASE IN THE CYTOTOXIC EFFECTS OF CSE, ACR, AND MVK

Our recent studies with pharmacological and molecular biological approaches have provided evidence that nicotine- and tar-free CSE, ACR, and MVK stimulate *de novo* generation of ROS *via* protein kinase C (PKC)-dependent activation of NOX in the target cells, leading to ROS-dependent plasma membrane damage and cell apoptosis.[5,6,8,9]

PKC is a family of serine/threonine kinases that regulate a multitude of cellular processes, including proliferation, differentiation, apoptosis, inflammation, and migration.[20,21] The PKC family comprises ten distinct isozymes that can be divided into three subfamilies such as conventional PKC isozymes (α, βI, βII, and γ), novel PKC isozymes (δ, ε, η, and θ), and atypical PKC isozymes (ζ and ι/λ), based on their structural properties and co-factor requirements for activation (see review by Igumenova).[21] The hallmark of PKC activation is its translocation to cellular endomembranes, including the plasma membrane. Nicotine- and tar-free CSE induces translocation of PKCα from cytosol to the plasma membrane as well

as plasma membrane damage and cell apoptosis, all of which are inhibited by chelating intracellular Ca^{2+} but not extracellular Ca^{2+}.[9] Both ACR and MVK also cause plasma membrane damage,[6] cell apoptosis,[6] and translocation of PKCα to the plasma membrane (unpublished data). As expected, the cytotoxic effects of CSE, ACR, and MVK are prevented by a PKC inhibitor, bisindolylmaleimide I.[6,8,9] These results indicate that Ca^{2+}-dependent activation of PKCα is required for the cell damage induced by CSE, ACR, and MVK. There is accumulating evidence that other PKC isozymes also play important roles in the cellular responses to cigarette smoke. CSE and ACR induce activation and translocation of PKCζ into the nucleus, leading to lung inflammation.[22] Activation and translocation of PKCη to the plasma membrane are essential for CSE-induced apoptosis *via* upregulation of caspase 3 and 8 in human lung fibroblasts (MRC-5 cells).[23] In addition, CSE-induced apoptosis of MRC-5 cells is inhibited by PKCα but promoted by PKCζ.[24] PKCε activation in response to co-exposure to cigarette smoke and alcohol decreases airway epithelial cell cilia beating.[25]

Nicotine- and tar-free CSE, ACR, and MVK cause PKC-dependent plasma membrane damage and cell apoptosis, which are inhibited by an NOX inhibitor (diphenyleneiodonium) and thiol-containing antioxidants (*N*-acetyl-L-cysteine [NAC] and reduced glutathione [GSH]), indicating the involvement of NOX.[6,8] NOX generates $O_2^{\cdot-}$, which is immediately dismutated by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2). H_2O_2 is then converted to $^{\cdot}OH$ through a metal-catalyzed reaction known as the Fenton reaction.[26] Our pharmacological study has shown that $^{\cdot}OH$ is involved in CSE-induced plasma membrane damage, while $O_2^{\cdot-}$ and/or H_2O_2 are responsible for CSE-induced cell apoptosis.[8]

NOX is not only the major source of ROS ($O_2^{\cdot-}$, H_2O_2, and $^{\cdot}OH$) but also a potential substrate for PKC.[27] Seven members of the NOX family have been identified in mammals: NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, and DUOX2.[28] The NOX family comprises seven catalytic subunits (NOX1-5, DUOX1, and DUOX2), regulatory subunits (p22^{phox}, p40^{phox}, p47^{phox}, p67^{phox}, NOX organizer 1, and NOX activator 1), and binding proteins (small guanosine 5′-triphosphatases (GTPases) [RAC1 and RAC2] and polymerase δ-interacting protein 2); the composition of NOX isoform varies depending on its core catalytic subunit. As for the regulation of enzyme activity by PKC, NOX2 is the best-characterized member of this family.[27,28]

NOX2 is composed of a six membrane-spanning catalytic NOX2 subunit known as gp91^{phox}, a two membrane-spanning p22^{phox} subunit, and cytosolic proteins p40^{phox}, p47^{phox}, p67^{phox}, RAC1, and RAC2. Under resting condition, the catalytic subunit and p22^{phox} form an inactive complex referred to as cytochrome b_{558}, while other subunits (p40^{phox}, p47^{phox}, and p67^{phox}) form a trimer in the cytosol. In human neutrophils, phosphorylation of the catalytic subunit by PKC enhances the interaction of NOX2 with the cytosolic components (p47^{phox}, p67^{phox}, and RAC2) and its diaphorase activity.[29] In addition, phosphorylation of p47^{phox} by PKC facilitates translocation of p47^{phox} complexed with p40^{phox} and p67^{phox} to the plasma membrane, and subsequent association between the membrane-associated cytochrome b_{558} and the cytosolic trimer, resulting in formation of an active enzyme.[29] PKC-mediated phosphorylation of p40^{phox} on serine 315 or threonine 154 is increased during NOX2 activation in human neutrophils, where the phosphorylation state of p40^{phox} strongly correlates with the level of superoxide production.[30] Whereas p40^{phox} activates NOX by increasing the affinity of p47^{phox} for cytochrome b_{558},[31] phosphorylation of p40^{phox} on threonine 154 alone by PKC inhibits NOX activation by PKC-phosphorylated p47^{phox},[30] indicating inhibitory and stimulatory effects of p40^{phox} on NOX activity. In primary human monocytes, phosphorylation of p67^{phox} by PKCδ increases $O_2^{\cdot-}$ production by NOX2.[32] Detailed molecular aspects and pathophysiology of NOX have been extensively described elsewhere.[27,28]

As already described, gas phase components of cigarette smoke induce PKC-dependent NOX activation followed by ROS generation.[5,6,8,9] A recent study has demonstrated that a particulate (tar) phase of cigarette smoke extract (PPCSE) also activates PKC/NOX/ROS signaling.[33] In mouse brain endothelial cells, PPCSE activates PKCδ *via* phosphatidylcholine phospholipase C (PC-PLC) but not phosphatidylinositide PLC. PPCSE-induced activation of PKCδ stimulates NOX-mediated ROS generation, which in turn activates a platelet-derived growth factor receptor (PDGFR)/phosphatidylinositol 3-kinase (PI3K)/Akt pathway. The PPCSE-induced activation of PC-PLC/PKCδ/NOX/ROS-dependent PDGFR/PI3K/Akt upregulates the inducible form of heme oxygenase that exacerbates early brain injury produced by intracerebral hemorrhagic stroke often related to cigarette smoking.[33] Thus PKC and NOX are potential targets for prevention of smoking-related toxicity.

5. PROTEIN CARBONYLATION AS A POSSIBLE MECHANISM UNDERLYING PKC ACTIVATION BY CSE, ACR, AND MVK

Although nicotine- and tar-free CSE, ACR, and MVK are suggested to activate PKC, the activation mechanism remains unclear. A possible mechanism underlying the activation of PKC by CSE, ACR, and MVK is carbonylation of proteins. The gas phase of cigarette smoke contains a large number of α,β-unsaturated aldehydes, including ACR and MVK,[1,6] and such reactive aldehydes can react with the side chains of cysteine, lysine, and histidine residues of proteins *via* Michael-addition reactions where their electrophilic C=C double bond is added to the sulfhydryl group of cysteine, the ε-amino group of lysine, and the imidazole group of histidine, resulting in carbonylation of proteins.[18] In addition, ROS generated by NOX can introduce carbonyl groups into proteins *via* a variety of oxidative pathways, contributing to protein carbonylation. An excellent review on the reaction pathways involving protein carbonylation is available.[18]

How is the carbonylation of proteins associated with the activation of PKC? There are two possibilities: (i) carbonylation of regulator proteins of PKC activity; and (ii) carbonylation of PKC by itself. An example of the former is that acetaldehyde and malondialdehyde react with bovine serum albumin (BSA) in a synergistic manner, generating malondialdehyde–acetaldehyde–BSA adducts[34] that activate PKCα to enhance release of interleukin-8 in bovine bronchial epithelial cells[35] and secretion of urokinase-type plasminogen activator in hepatic stellate cells.[36] Thus, the formation of carbonyl compound-adducted proteins may play important roles in not only PKC activation by carbonyl compounds but also the initiation and

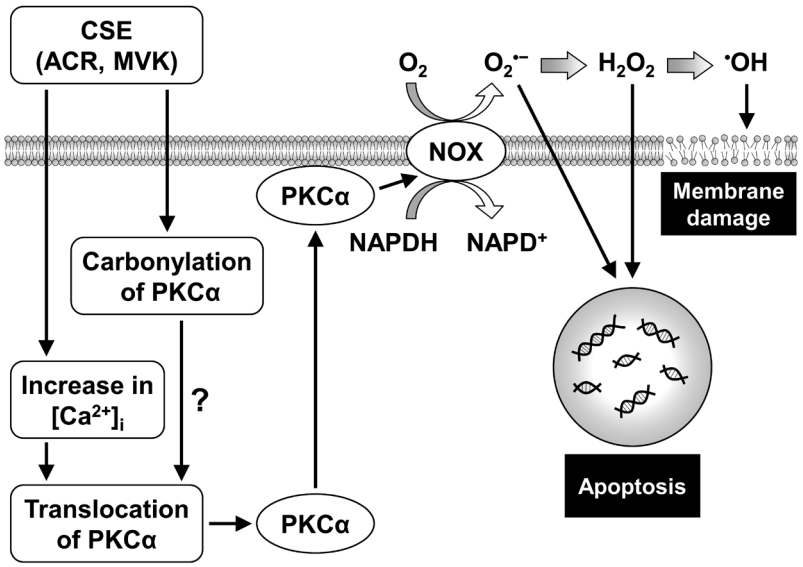


Fig. 1. Molecular Mechanism for Nicotine- and Tar-Free CSE-, ACR-, and MVK-Induced Cell Damage

The abbreviations are given in the text. ACR and MVK have been identified as stable cytotoxic factors in CSE.[6] CSE, ACR, and MVK induce an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), resulting in translocation of PKCα.[9] Carbonylation of PKCα triggered by CSE, ACR, and MVK may contribute to translocation of PKCα. PKC-dependent activation of NOX stimulates *de novo* generation of $O_2^{\cdot-}$. $O_2^{\cdot-}$ is converted to H_2O_2 by SOD, and the resulting H_2O_2 is converted to $^{\cdot}OH$ by the Fenton reaction. $^{\cdot}OH$ is responsible for plasma membrane damage, whereas $O_2^{\cdot-}$ and/or H_2O_2 are involved in cell apoptosis characterized by reduction of cell viability and DNA fragmentation.[8]

pathogenesis of diseases related to cigarette smoking. As an example of the latter, we have recently succeeded in detecting carbonylated PKCα in CSE-, ACR-, or MVK-treated C6 rat glioma cells (unpublished data) using a biotinylated reagent, called the aldehyde reactive probe, which specifically reacts with aldehyde/keto group in carbonyl-modified proteins.[37] The carbonylation of PKCα by CSE, ACR, and MVK is completely abolished by antioxidants such as NAC and GSH, indicating the involvement of oxidative mechanism.

In human PKC isozymes, 16–28 cysteine residues have been identified in the *N*-terminal regulatory domain, which contains one or two zinc-binding, cysteine-rich motifs with six cysteine residues for each motif: the C-terminal catalytic domain contains five to eight reactive cysteines.[38] Oxidative modifications of cysteine residues modulate PKC activity, implicating the regulation of PKC by redox signaling. Growing evidence indicates that PKC activity is regulated by reversible modifications, such as *S*-glutathiolation or *S*-cysteinylation, in an isozyme-specific manner: PKCα is inactivated by *S*-glutathiolation[39] whereas *S*-cysteinylation activates PKCδ and inactivates PKCε.[40] Although PKCα undergoes an irreversible modification (carbonylation) in addition to a reversible modification (*S*-thiolation), the impact of PKCα carbonylation on the regulation of enzyme activity and the target site(s) within the PKCα sequence remain unknown. Since conformational rearrangement of PKCα is required for its activation,[41] PKCα carbonylation associated with modification of protein structure may be a key step for PKCα activation.

6. CONCLUSION AND PERSPECTIVES

ACR and MVK, which are stable cytotoxic factors in nicotine- and tar-free CSE, activate the PKC/NOX/ROS signaling pathway, leading to plasma membrane damage and cell apoptosis (Fig. 1). In addition, such reactive carbonyl compounds can react with PKCα, causing its carbonylation—known as an irreversible oxidative modification. These findings indicate that pharmacological modulation of oxidative mechanisms by inhibitors for PKC and NOX, and by antioxidants such as NAC and GSH, may be an effective prophylactic strategy against diseases related to cigarette smoking.

Acknowledgments This study was supported in part by Grants-in-Aid for Challenging Exploratory Research [Grant 23659129] (to S.M.), for Scientific Research (B) [Grant 24390059] (to S.M.), for Scientific Research (C) [Grant 24590309] (to T. Horinouchi), and for Young Scientific Research (B) [Grant 26860166] (to T. Higashi) from Japan Society for the Promotion of Science (JSPS), and by a Grant from Smoking Research Foundation of Japan (to S.M.).

Conflict of Interest The authors declare no conflict of interest.

REFERENCES

1) IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Tabacco smoke and involuntary smoking. *IARC Monogr. Eval. Carcinog. Risks Hum.*, **83**, 1–1438 (2004).
2) Smith CJ, Fischer TH. Particulate and vapor phase constituents of cigarette mainstream smoke and risk of myocardial infarction. *Atherosclerosis*, **158**, 257–267 (2001).
3) Green CR, Rodgman A. The Tobacco Chemists' Research Conference: a half century forum for advances in analytical methodology of tobacco and its products. *Recent Adv. Tobacco Sci.*, **22**, 131–304 (1996).
4) Church DF, Pryor WA. Free-radical chemistry of cigarette smoke and its toxicological implications. *Environ. Health Perspect.*, **64**, 111–126 (1985).
5) Higashi T, Mai Y, Noya Y, Horinouchi T, Terada K, Hoshi A, Nepal P, Harada T, Horiguchi M, Hatate C, Kuge Y, Miwa S. A simple and rapid method for standard preparation of gas phase extract of cigarette smoke. *PLoS ONE*, **9**, e107856 (2014).

6) Noya Y, Seki K, Asano H, Mai Y, Horinouchi T, Higashi T, Terada K, Hatate C, Hoshi A, Nepal P, Horiguchi M, Kuge Y, Miwa S. Identification of stable cytotoxic factors in the gas phase extract of cigarette smoke and pharmacological characterization of their cytotoxicity. *Toxicology*, **314**, 1–10 (2013).
7) Horiyama S, Takahashi Y, Hatai M, Honda C, Suwa K, Ichikawa A, Yoshikawa N, Nakamura K, Kunitomo M, Date S, Masujima T, Takayama M. Methyl vinyl ketone, a toxic ingredient in cigarette smoke extract, modifies glutathione in mouse melanoma cells. *Chem. Pharm. Bull.*, **62**, 772–778 (2014).
8) Asano H, Horinouchi T, Mai Y, Sawada O, Fujii S, Nishiya T, Minami M, Katayama T, Iwanaga T, Terada K, Miwa S. Nicotine- and tar-free cigarette smoke induces cell damage through reactive oxygen species newly generated by PKC-dependent activation of NADPH oxidase. *J. Pharmacol. Sci.*, **118**, 275–287 (2012).
9) Mai Y, Higashi T, Terada K, Hatate C, Nepal P, Horiguchi M, Harada T, Miwa S, Horinouchi T. Nicotine- and tar-free cigarette smoke extract induces cell injury *via* intracellular Ca^{2+}-dependent subtype-specific protein kinase C activation. *J. Pharmacol. Sci.*, **120**, 310–314 (2012).
10) Higashi T, Mai Y, Mazaki Y, Horinouchi T, Miwa S. A standardized method for preparation of gas phase extract of cigarette smoke. *Biol. Pharm. Bull.*, **39**, 898–902 (2016).
11) Hoshino Y, Mio T, Nagai S, Miki H, Ito I, Izumi T. Cytotoxic effects of cigarette smoke extract on an alveolar type II cell-derived cell line. *Am. J. Physiol. Lung Cell. Mol. Physiol.*, **281**, L509–L516 (2001).
12) Afri M, Gottlieb HE, Frimer AA. Superoxide organic chemistry within the liposomal bilayer, part II: a correlation between location and chemistry. *Free Radic. Biol. Med.*, **32**, 605–618 (2002).
13) Brzezinska AK, Lohr N, Chilian WM. Electrophysiological effects of $O_2^{\cdot-}$ on the plasma membrane in vascular endothelial cells. *Am. J. Physiol. Heart Circ. Physiol.*, **289**, H2379–H2386 (2005).
14) Mao GD, Poznansky MJ. Electron spin resonance study on the permeability of superoxide radicals in lipid bilayers and biological membranes. *FEBS Lett.*, **305**, 233–236 (1992).
15) Prokopczyk B, Hoffmann D, Bologna M, Cunningham AJ, Trushin N, Akerkar S, Boyiri T, Amin S, Desai D, Colosimo S, Pittman B, Leder G, Ramadani M, Henne-Bruns D, Beger HG, El-Bayoumy K. Identification of tobacco-derived compounds in human pancreatic juice. *Chem. Res. Toxicol.*, **15**, 677–685 (2002).
16) van der Toorn M, Rezayat D, Kauffman HF, Bakker SJ, Gans RO, Koëter GH, Choi AM, van Oosterhout AJ, Slebos DJ. Lipid-soluble components in cigarette smoke induce mitochondrial production of reactive oxygen species in lung epithelial cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.*, **297**, L109–L114 (2009).
17) van der Toorn M, Smit-de Vries MP, Slebos DJ, de Bruin HG, Abello N, van Oosterhout AJ, Bischoff R, Kauffman HF. Cigarette smoke irreversibly modifies glutathione in airway epithelial cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.*, **293**, L1156–L1162 (2007).
18) Dalle-Donne I, Aldini G, Carini M, Colombo R, Rossi R, Milzani A. Protein carbonylation, cellular dysfunction, and disease progression. *J. Cell. Mol. Med.*, **10**, 389–406 (2006).
19) Bernardo I, Bozinovski S, Vlahos R. Targeting oxidant-dependent mechanisms for the treatment of COPD and its comorbidities. *Pharmacol. Ther.*, **155**, 60–79 (2015).
20) Rosse C, Linch M, Kermorgant S, Cameron AJ, Boeckeler K, Parker PJ. PKC and the control of localized signal dynamics. *Nat. Rev. Mol. Cell Biol.*, **11**, 103–112 (2010).
21) Igumenova TI. Dynamics and membrane interactions of protein kinase C. *Biochemistry*, **54**, 4953–4968 (2015).
22) Yao H, Hwang JW, Moscat J, Diaz-Meco MT, Leitges M, Kishore N, Li X, Rahman I. Protein kinase Cζ mediates cigarette smoke/aldehyde- and lipopolysaccharide-induced lung inflammation and histone modifications. *J. Biol. Chem.*, **285**, 5405–5416 (2010).
23) Son ES, Kyung SY, Lee SP, Jeong SH, Shin JY, Ohba M, Yeo EJ, Park JW. Role of protein kinase C-η in cigarette smoke extract-induced apoptosis in MRC-5-cells. *Hum. Exp. Toxicol.*, **34**, 869–877 (2015).
24) Park JW, Kim HP, Lee SJ, Wang X, Wang Y, Ifedigbo E, Watkins SC, Ohba M, Ryter SW, Vyas YM, Choi AM. Protein kinase Cα and ζ differentially regulate death-inducing signaling complex formation in cigarette smoke extract-induced apoptosis. *J. Immunol.*, **180**, 4668–4678 (2008).
25) Wyatt TA, Sisson JH, Allen-Gipson DS, McCaskill ML, Boten JA, DeVasure JM, Bailey KL, Poole JA. Co-exposure to cigarette smoke and alcohol decreases airway epithelial cell cilia beating in a protein kinase Cε-dependent manner. *Am. J. Pathol.*, **181**, 431–440 (2012).
26) Bergamini CM, Gambetti S, Dondi A, Cervellati C. Oxygen, reactive oxygen species and tissue damage. *Curr. Pharm. Des.*, **10**, 1611–1626 (2004).
27) Lassègue B, San Martín A, Griendling KK. Biochemistry, physiology, and pathophysiology of NADPH oxidases in the cardiovascular system. *Circ. Res.*, **110**, 1364–1390 (2012).
28) Drummond GR, Selemidis S, Griendling KK, Sobey CG. Combating oxidative stress in vascular disease: NADPH oxidases as therapeutic targets. *Nat. Rev. Drug Discov.*, **10**, 453–471 (2011).
29) Raad H, Paclet MH, Boussetta T, Kroviarski Y, Morel F, Quinn MT, Gougerot-Pocidalo MA, Dang PM, El-Benna J. Regulation of the phagocyte NADPH oxidase activity: phosphorylation of gp^{91}phox/NOX2 by protein kinase C enhances its diaphorase activity and binding to Rac2, $p67^{phox}$, and $p47^{phox}$. *FASEB J.*, **23**, 1011–1022 (2009).
30) Lopes LR, Dagher MC, Gutierrez A, Young B, Bouin AP, Fuchs A, Babior BM. Phosphorylated $p40^{PHOX}$ as a negative regulator of NADPH oxidase. *Biochemistry*, **43**, 3723–3730 (2004).
31) Cross AR. $p40^{phox}$ participates in the activation of NADPH oxidase by increasing the affinity of $p47^{phox}$ for flavocytochrome b_{558}. *Biochem. J.*, **349**, 113–117 (2000).
32) Zhao X, Xu B, Bhattacharjee A, Oldfield CM, Wientjes FB, Feldman GM, Cathcart MK. Protein kinase Cδ regulates $p67^{phox}$ phosphorylation in human monocytes. *J. Leukoc. Biol.*, **77**, 414–420 (2005).
33) Shih RH, Cheng SE, Hsiao LD, Kou YR, Yang CM. Cigarette smoke extract upregulates heme oxygenase-1 *via* PKC/NADPH oxidase/ROS/PDGFR/PI3K/Akt pathway in mouse brain endothelial cells. *J. Neuroinflammation*, **8**, 104 (2011).
34) Tuma DJ, Thiele GM, Xu D, Klassen LW, Sorrell MF. Acetaldehyde and malondialdehyde react together to generate distinct protein adducts in the liver during long-term ethanol administration. *Hepatology*, **23**, 872–880 (1996).
35) Wyatt TA, Kharbanda KK, Tuma DJ, Sisson JH. Malondialdehyde–acetaldehyde–adducted bovine serum albumin activates protein kinase C and stimulates interleukin-8 release in bovine bronchial epithelial cells. *Alcohol*, **25**, 159–166 (2001).
36) Kharbanda KK, Shubert KA, Wyatt TA, Sorrell MF, Tuma DJ. Effect of malondialdehyde–acetaldehyde–protein adducts on the protein kinase C-dependent secretion of urokinase-type plasminogen activator in hepatic stellate cells. *Biochem. Pharmacol.*, **63**, 553–562 (2002).
37) Chung WG, Miranda CL, Maier CS. Detection of carbonyl-modified proteins in interfibrillar rat mitochondria using *N*′-aminooxymethylcarbonylhydrazino-D-biotin as an aldehyde/keto-reactive probe in combination with Western blot analysis and tandem mass spectrometry. *Electrophoresis*, **29**, 1317–1324 (2008).
38) Gopalakrishna R, Jaken S. Protein kinase C signaling and oxidative stress. *Free Radic. Biol. Med.*, **28**, 1349–1361 (2000).
39) Ward NE, Stewart JR, Ioannides CG, O'Brian CA. Oxidant-induced *S*-glutathiolation inactivates protein kinase C-α (PKC-α): a potential mechanism of PKC isozyme regulation. *Biochemistry*, **39**, 10319–10329 (2000).

40) Chu F, Ward NE, O'Brian C. PKC isozyme *S*-cysteinylation by cystine stimulates the pro-apoptotic isozyme PKCδ and inactivates the oncogenic isozyme PKCε. *Carcinogenesis*, **24**, 317–325 (2003).
41) Sathishkumar K, Rangan V, Gao X, Uppu RM. Methyl vinyl ketone induces apoptosis in murine GT1-7 hypothalamic neurons through glutathione depletion and the generation of reactive oxygen species. *Free Radic. Res.*, **41**, 469–477 (2007).