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Identification of stable cytotoxic factors in the gas phase extract of cigarette smoke and pharmacological characterization of their cytotoxicity

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ABSTRACT

Smoking is a major risk factor for atherosclerotic vascular diseases, but the mechanism for its genesis is unknown. We have recently shown that the gas phase of cigarette smoke (nicotine- and tar-free cigarette smoke extract; CSE) likely to reach the systemic circulation contains stable substances which cause cytotoxicity like plasma membrane damage and cell death in cultured cells, and also that the plasma membrane damage is caused through sequential activation of protein kinase C (PKC) and NADPH oxidase (NOX) and the resulting generation of reactive oxygen species (PKC/NOX-dependent mechanism), whereas cell death is caused through PKC/NOX-dependent and -independent mechanisms. To identify these stable substances, the CSE was prepared by passing the main-stream smoke of 10 cigarettes through a Cambridge glass fiber filter, trapping of the smoke in a vessel cooled at −80 °C, and subsequent dissolution in 10 ml of water. The CSE was fractionated into nine fractions using reversed-phase HPLC, and each fraction was screened for cytotoxicity in cultured cells, using propidium iodide uptake assay for cell membrane damage and MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] reduction assay for cell viability. The cytotoxicity was positive in two of the nine fractions (Fr2 and Fr5). After extraction of the active fractions into dichloromethane, GC/MS analysis identified 2-cyclopenten-1-one (CPO) in Fr5 but none in Fr2. After derivatization of the active fractions with O-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride, GC/MS analysis identified acrolein, acetone and propionaldehyde in Fr2, and methyl vinyl ketone (MVK) in Fr5. After 4-h incubation, authentic acrolein and MVK induced concentration-dependent cytotoxicity with EC_{50} values of 75.9 ± 8.2 and 47.0 ± 8.0 μM (mean ± SEM; $n = 3$), respectively, whereas acetone, propionaldehyde and CPO were without effect. However, after 24-h incubation, CPO induced concentration-dependent cytotoxicity with an EC_{50} value of 264.0 ± 16.9 μM ($n = 3$). The concentrations of acrolein, MVK and CPO in the CSE were 3368 ± 334, 2429 ± 123 and 392.9 ± 31.8 μM ($n = 4$), respectively, which were higher than the cytotoxic concentrations. The cytotoxicity of acrolein and MVK consisted of plasma membrane damage and decreased cell viability: the plasma membrane damage was totally prevented by treatment with an inhibitor of PKC or NOX, whereas the decreased cell viability was only partially prevented by these inhibitors. The cytotoxicity of CPO consisted only of decreased cell viability, which was totally resistant to these inhibitors. These results show that acrolein and MVK are responsible for the acute cytotoxicity of the CSE through PKC/NOX-dependent and -independent mechanisms, whereas CPO is responsible for the delayed cytotoxicity of the CSE through a PKC/NOX-independent mechanism.

Abbreviations: BIS I, 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide; CSE, cigarette smoke extract; CPO, 2-cyclopenten-1-one; DMEM, Dulbecco's modified Eagle's medium; DPI, diphenyleneiodonium chloride; ESI, electrospray ionization; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; MVK, methyl vinyl ketone; NOX, NADPH oxidase; PFBOA, O-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride; PI, propidium iodide; PKC, protein kinase C; ROS, reactive oxygen species.

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1. Introduction

Smoking has been implicated as a major risk factor for cardiovascular diseases such as stroke and coronary artery disease (Ambrose and Barua, 2004; Steenland et al., 1996). The vasculature develops proinflammatory and proatherogenic phenotype in response to smoking. It is generally believed that increased production of reactive oxygen species (ROS) plays a central role in vascular inflammation and atherogenesis (Griendling et al., 2000; Sorescu et al., 2002), but less is known about the oxidative stress that is induced in the vasculature by cigarette smoke.

Cigarette smoke is reported to contain over 4000 chemical constituents (Burns, 1991; Church and Pryor, 1985). Among these are ROS such as peroxynitrate and free radicals of organic compounds (Ambrose and Barua, 2004; Pryor and Stone, 1993a). These substances are highly reactive, but the area to be damaged by them seems to be limited because of their short half-lives, suggesting that they are not responsible for vascular inflammation and atherogenesis (Pryor and Stone, 1993b). In addition to ROS, cigarette smoke contains relatively stable substances that have the potential to stimulate cellular production of ROS (Pryor et al., 1998; Stedman, 1968). In this context, it has recently been reported that following exposure to cigarette smoke, cultured pulmonary artery endothelial cells generate superoxide anion ($O_2^{\bullet -}$), and it is suspected that a stable thiol-reactive compound, acrolein, is one of the candidate factors to trigger the generation of $O_2^{\bullet -}$, based on the similarity of the action of authentic acrolein to that of cigarette smoke (Jaimes et al., 2004; Orosz et al., 2007). Such stable substances could be carried throughout the systemic circulation and act in organs remote to the lungs to stimulate ROS generation (Cooper et al., 1992).

Cigarette smoke can be divided into two phases, the tar-phase and the gas-phase. The separation into two phases is usually performed by the use of a filter, typically a Cambridge glass-fiber filter that retains the particles larger than 0.1 μm (Pryor and Stone, 1993a). The fraction which is trapped on the filter is designated the tar-phase, while the fraction which goes through the filter is the gas-phase. Most of the previous works on toxic effects of cigarette smoke has been performed using whole smoke cigarette smoke extract (whole smoke CSE) (Hoshino et al., 2001, 2005; Jaimes et al., 2004; Orosz et al., 2007; Raij et al., 2001; Raveendran et al., 2005), which is prepared by bubbling cigarette smoke directly in aqueous solution and hence contains both the tar phase and gas phase. However, because most of the tar phase can be trapped by alveolar epithelium and because it could be substantially removed by a filter like the Cambridge glass-fiber filter, it is important to characterize the cytotoxic effects of the gas phase of the cigarette smoke, in order to know the actual toxic effects of cigarette smoking against human health. In fact, recent works have shown that cigarette smoke extract free of the tar phase and nicotine (nicotine- and tar-free CSE), which is prepared by bubbling cigarette smoke in aqueous solution after passage through a Cambridge glass-fiber filter, can oxidize LDL *in vitro* and promote atherosclerotic changes in aortas *in vivo* (Frei et al., 1991; Kunitomo et al., 2009; Yamaguchi et al., 2002). Furthermore, our recent works (Asano et al., 2012; Mai et al., 2012) have shown that (1) the nicotine- and tar-free CSE induces cytotoxicity like plasma membrane damage and cell apoptosis, (2) the plasma membrane damage is caused mainly through protein kinase C (PKC)-dependent activation of NADPH oxidase (NOX) and the resulting generation of ROS (designated PKC/NOX-dependent mechanism), (3) the cell apoptosis is caused partly through the PKC/NOX-dependent mechanism, with the remaining part being caused independently of PKC and NOX (PKC/NOX-independent mechanism). However, actual compounds in the nicotine- and tar-free CSE responsible for cytotoxicity are at present unknown. This is mainly because most of the previous works on toxic effects of cigarette smoke has been performed using whole smoke CSE containing nicotine and the tar-phase, and because chemical analysis of the CSE has been performed alone but not in combination with functional assay for cytotoxicity.

In the present study, we attempted to identify cytotoxic compounds in the nicotine- and tar-free CSE. For this purpose, we fractionated the CSE using HPLC, assayed each fraction for cytotoxic activity, and subjected the fractions with cytotoxic activity to analysis by LC–MS and GC–MS. We identified acrolein, methyl vinyl ketone (MVK) and 2-cyclopenten-1-one (CPO) as major cytotoxic compounds in the nicotine- and tar-free CSE, and demonstrated that among these, acrolein and MVK are mainly responsible for acute cytotoxic activities of the CSE, based on their concentrations in the CSE, the onset of cytotoxicity and pharmacology of their cytotoxicity such as the sensitivity to inhibitors of PKC and NOX which is similar to the nicotine- and tar-free CSE. We also demonstrated that CPO plays a role in the delayed cytotoxicity of the CSE, based on the onset of cytotoxicity and the pharmacology of its cytotoxicity such as the insensitivity to inhibitors of PKC and NOX.

2. Materials and methods

2.1. Materials

The cigarettes used were the Hi-Lite (JT, Tokyo, Japan) containing 17 mg of tar and 1.4 mg of nicotine per cigarette. The reagents were purchased from the following sources: Cambridge filters from Heinr. Borgwaldt GmbH (Hamburg, Germany); CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] reduction assay) from Promega Corporation (Madison, WI, USA); acrolein, acetone, CPO, and 3-cyanopyridine from Wako Pure Chemical Industry (Osaka, Japan); MVK, O-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride (PFBOA), Hoechst 33342 and diphenyleneiodonium chloride (DPI) from Sigma–Aldrich (St. Louis, MO, USA); propidium iodide (PI) from Dojindo Laboratories (Kumamoto, Japan); 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide (BIS I) from Calbiochem (San Diego, CA, USA). The other reagents were of the analytical grade in purity.

2.2. Analysis by HPLC, LC/MS and GC/MS

The HPLC apparatus consisted of a LC-10A pump (Shimadzu, Kyoto, Japan), a reversed-phase column (Shim-Pack FC-ODS, 150 mm × 2 mm i.d., Shimadzu, Kyoto, Japan) and a photo-diode-array multiple-wavelength detector (SPD-M10A, Shimadzu, Kyoto, Japan). The mobile phase was water with a flow rate at 0.2 ml/min, and the absorbance was monitored at wavelengths of 210 nm and 254 nm. LC/MS analysis was performed using a LC/MS-2010A (Shimadzu, Kyoto, Japan) equipped with a reversed-phase column (Shim-Pack FC-ODS, 150 mm × 2 mm i.d., Shimadzu, Kyoto, Japan). Elution was isocratic with 0.1% (v/v) trifluoroacetic acid in water, and the absorbance was monitored at wavelengths of 210 nm and 254 nm. GC/MS was performed using a GC/MS-QP2010 (Shimadzu, Kyoto, Japan), equipped with a fused silica gel column DB-5, DB-WAX (30 m × 0.25 mm i.d. Agilent, CA, USA) at column temperature of 40–250 °C. High resolution mass spectrometry was performed on a LTQ-Orbitrap XL mass spectrometer (Thermo Scientific, CA, USA) with electrospray ionization (ESI) method, and the column temperature was set at 200 °C. For identification of a given compound based on retention time and mass spectrum by GC/MS, we referred to the NIST/EPA/NIH Mass Spectral Database (Shimadzu, Kyoto, Japan).

2.3. PFBOA-derivatization of carbonyl components

In some experiments, GC/MS analyses were conducted after derivatization of samples by PFBOA. PFBOA-derivatization was performed as described previously (Beranek et al., 2010). Briefly, PFBOA (10 mg/ml, 0.6 ml in water) was added to each fraction of HPLC (0.5 ml), and the mixture was allowed to stand at room temperature for 2 h. After addition of 600 mg of sodium chloride and 0.8 ml of 50% (v/v) sulfuric acid to the mixture to decompose excess PFBOA, the mixture was extracted with 1 ml of *n*-hexane. The organic phase was dehydrated with anhydrous sodium sulfate, and it was subjected to GC/MS analysis with 3-cyanopyridine as an internal standard.

2.4. Preparation of the nicotine- and tar-free cigarette smoke extract

The nicotine- and tar-free CSE was prepared as reported elsewhere (Asano et al., 2012; Yamaguchi et al., 2002), with a slight modification. Briefly, one cigarette per trial was fixed horizontally to be burned, and the main stream of the smoke was aspirated at a flow rate of 1.050 L/min, which was strictly regulated by the KOFLOC mass flow controller (MODEL 8300 series, Kojima Instruments Inc., Kyoto, Japan). The main stream of the smoke was passed through a Cambridge glass fiber filter, to remove the tar phase and nicotine from the cigarette smoke, and the remaining