Differential Effects between Cigarette Total Particulate Matter and Cigarette Smoke Extract on Blood and Blood Vessel

Jung-Min Park1, Kyung-Hwa Chang1, Kwang-Hoon Park1, Seong-Jin Choi2, Kyuhong Lee2, Jin-Yong Lee2, Masahiko Satoh3, Seong-Yu Song4 and Moo-Yeo Lee1

1College of Pharmacy, Dongguk University, Goyang, Korea
2Inhalation Toxicology Research Center, Korea Institute of Toxicology, Jeongeup, Korea
3Laboratory of Pharmaceutical Health Sciences, School of Pharmacy, Aichi Gakuin University, Nagoya, Japan
4Department of Public Health Science, Graduate School, Korea University, Seoul, Korea

(Received August 4, 2016; Revised August 16, 2016; Accepted August 18, 2016)

The generation and collection of cigarette smoke (CS) is a prerequisite for any toxicology study on smoking, especially an in vitro CS exposure study. In this study, the effects on blood and vascular function were tested with two widely used CS preparations to compare the biological effects of CS with respect to the CS preparation used. CS was prepared in the form of total particulate matter (TPM), which is CS trapped in a Cambridge filter pad, and cigarette smoke extract (CSE), which is CS trapped in phosphate-buffered saline. TPM potentiated platelet reactivity to thrombin and thus increased aggregation at a concentration of 25–100 µg/mL, whereas 2.5–10% CSE decreased platelet aggregation by thrombin. Both TPM and CSE inhibited vascular contraction by phenylephrine at 50–100 µg/mL and 10%, respectively. TPM inhibited acetylcholine-induced vasorelaxation at 10–100 µg/mL, but CSE exhibited a minimal effect on relaxation at the concentration that affects vasoconstriction. Neither TPM nor CSE induced hemolysis of erythrocytes or influenced plasma coagulation, as assessed by prothrombin time (PT) and activated partial thromboplastin time (aPTT). Taken together, CS affects platelet activity and deteriorates vasomotor functions in vitro. However, the effect on blood and blood vessels may vary depending on the CS preparation. Therefore, the results of experiments conducted with CS preparations should be interpreted with caution.

Key words: Smoking, Cigarette, Total particulate matter (TPM), Cigarette smoke extract (CSE), Cardiovascular toxicity

INTRODUCTION

Smoking is a human habit of global scale and the leading cause of preventable death. Smoking is harmful to nearly every organ of the body and causes a variety of deadly diseases. Of these, the most well-recognized health risks are cancers, respiratory disorders and cardiovascular diseases (CVD) (1,2). Owing to the contribution of smoking, CVD are the leading cause of death in most countries and account for 30–40 percent of all deaths (3). Smoking-caused CVD include coronary heart disease (CHD), stroke, atherosclerosis, aortic aneurysm, peripheral vascular disease and subclinical CVD, such as increased carotid intima–media thickness, intermittent claudication and lacunar infarcts (4). Numerous studies investigated the pathological processes related with smoking-caused CVD, and revealed that the key aspects of them include endothelial dysfunction, prothrombotic state, inflammation, altered lipid metabolism, and hypoxia (5). Indeed, the major consequences of smoking include enhanced platelet reactivity, altered vascular function, increased coagulability of blood plasma, and oxidative stress to the circulatory system (1). The molecular bases of these events have not been fully clarified, but a variety of mechanisms have been suggested and multiple molecular events must be involved in the etiologic link between smoking and CVD.

Burning cigarettes produces cigarette smoke (CS) through combustion, pyrolysis and other chemical reactions. CS includes thousands of chemicals, which have largely unde-
fined properties and biological activities. Generation and collection of the cigarette smoke (CS) is a prerequisite to the toxicology study, especially in vitro CS exposure study. The concentration and constituents of CS and CS preparations vary depending on burning conditions and collection methods, respectively. Accordingly, over the decades, many efforts have been made to develop and set standard procedures for the reproducible generation of CS samples and the representative collection of constituents of CS. As a result, widely accepted methods are currently available, although there are still debates about the most appropriate and best approaches. Among them, International Organization for Standardization (ISO) 3308 “Routine analytical cigarette-smoking machine”, which is based on the Federal Trade Commission (FTC) protocol, is regarded as a general standard for mainstream CS generation (6). CS trapping methods are available to generate total particulate matter (TPM), tar- or nicotine-free dry particulate matter (Tar or NFDPM), cigarette smoke condensate (CSC), gas/vapor phase (GVP) and whole smoke (WS), depending on the method used for collection (7). Each CS preparation contains different constituents with various concentrations and thus exhibit distinct toxicity to biological system.

In this study, the in vitro effect on blood and blood vessels was tested with two CS preparations. CS was generated following standard procedure and was prepared as forms of total particulate matter (TPM), a CS collected on filter pad and cigarette smoke extract (CSE), a CS trapped in aqueous solution. They are CS preparations used most widely in in vitro toxicological studies (8). TPM and CSE were examined for their cytotoxicity toward erythrocytes, reactive oxygen species (ROS) generating capability in endothelial cells, and the effects on platelet aggregation, plasma coagulation and vasomotor function. All experiments were performed with both CSE and TPM in parallel and the results were analyzed and compared.

MATERIALS AND METHODS

Reagents. Thrombin, phenylephrine, acetylcholine and heparin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hematologic reagents for prothrombin time (PT) and activated partial thromboplastin time (aPTT) measurements were obtained from Fisher Diagnostics (Middletown, VA, USA). 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1) was from Seoul Clinical Genomics (Seoul, Korea). All other chemicals used were of the highest purity available and purchased from standard suppliers.

Preparation of total particulate matter (TPM) and cigarette smoke extract (CSE). TPM and CSE were prepared as described in our previous studies (9). University of Kentucky 3R4F reference cigarettes were smoked on a 30-port smoking machine (CH Technologies, Westwood, NJ, USA) at 35-mL puff volume, 2-sec puff duration and 60 sec between puffs, in conformity with ISO standard 3308. TPM in mainstream smoke from 30 cigarettes was collected on the 44 mm Cambridge filter pad (GE Healthcare, Little Chalfont, UK). TPM trapped on the filter, which was 149.5 mg, was eluted with 7.5 mL dimethyl sulfoxide for 30 min with shaking to make up 20 mg/mL. The resulting TPM was filtered through a 0.45-µm polytetrafluoroethylene filter (Merck Millipore, Darmstadt, Germany). CSE was generated by passing mainstream smoke from 30 cigarettes through an impinger containing 30 mL phosphate-buffered saline (PBS) for 5 min. CSE and TPM were dispensed and kept at −80°C until use. As a standard constituent, nicotine was analyzed with a 6410B triple quadrupole liquid chromatography-mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). Nicotine concentrations were 1,452 µg/mL and 19.5 µg/mL for TPM and CSE, respectively.

Animals. All animal experiments were conducted in accordance with protocols approved by the Ethics Committee of Animal Service Center at Dongguk University. Male Sprague-Dawley rats (5–6 weeks of age) were purchased from Daehan Biolink (Eumseong, Korea) and acclimated for 1 week before experiments. The laboratory animal facility was maintained at a constant temperature and humidity with a 12-hr light/dark cycle. Food and water were provided ad libitum.

Preparation of washed platelets (WP) and platelet aggregation studies. WP were prepared, and aggregation experiments were performed as previously described (10). Briefly, rat blood was collected from the abdominal aorta of rats anesthetized with ether using acid-citrate-dextrose (ACD; 85 mM trisodium citrate, 66.6 mM citric acid, 111 mM glucose) as an anticoagulant (ACD : blood = 1 : 6). After centrifugation at 250 g for 15 min, platelet-rich plasma (PRP) was obtained from the supernatant. Platelets were spun down by further centrifugation of PRP at 500 g for 10 min and washed once with washing buffer (138 mM NaCl, 2.8 mM KCl, 0.8 mM MgCl₂, 0.8 mM Na₂HPO₄, 10 mM HEPES, 0.55 mM glucose, 22 mM trisodium citrate, 0.35% bovine serum albumin; pH 6.5). After resuspending the platelet pellets in suspension buffer (138 mM NaCl, 2.8 mM KCl, 0.8 mM MgCl₂, 0.8 mM Na₂HPO₄, 10 mM HEPES, 5.6 mM glucose, 1 mM CaCl₂, 0.3% bovine serum albumin; pH 7.4), the platelet concentration was adjusted to 2 × 10⁸ platelets/mL.

Platelet aggregation experiments were performed using a four-channel aggregometer (Chrono-log, Havertown, PA, USA). WP were pretreated with TPM or CSE for 5 min, and aggregation was induced by the addition of 0.14 or 0.05 U/mL thrombin to examine the anti-aggregatory or pro-aggregatory effect of CS, respectively. Aggregation was...
measured for 5 min after the application of thrombin.

**Preparation of erythrocytes and measurement of hemolysis.** The erythrocyte suspension was prepared, and hemolysis was measured, as previously described (11). Blood was withdrawn from the abdominal aorta of ether-anesthetized rats into a heparinized syringe. Plasma was obtained by centrifugation at 1,000 × g for 10 min, and theuffy coat was removed. Erythrocytes were washed twice with PBS composed of 6 mM sodium phosphate and 140 mM NaCl (pH 7.4). Erythrocytes were suspended in PBS to a final count of 5 × 10⁶ cell/mL for hemolysis study.

Erythrocyte suspension was prepared with TPM or CSE and was incubated in a shaking water bath at 37°C for the indicated times. An aliquot of suspension was taken out at specific time intervals and centrifuged for 1 min at 10,000 × g to obtain a supernatant. Hemolysis was assessed by measuring a light absorbance at 540 nm. Percent hemolysis was expressed as the ratio of the light absorbance at 540 nm for the reaction mixture compared to a standard sample hemolyzed with a hypotonic solution.

**Plasma coagulation test.** Coagulability of blood plasma was assessed by measuring PT and aPTT as described previously (12). Plasma was prepared from whole blood anticoagulated with 3.2% trisodium citrate by centrifugation at 1,500 × g for 10 min. PT or aPTT was measured with a Coagulator2 coagulation analyzer (Behnk Elektronik, Norderstedt, Germany) using thromboplastin-D or CaCl₂ and APTT-XL reagents (Fisher Diagnostics), respectively, according to the manufacturer’s instructions.

**Measurement of vascular tone using myograph.** Vascular contraction and relaxation were examined by myography as previously described (13). Briefly, thoracic aortas were carefully isolated from rats, cleaned of fat and adipose tissues, and cut into ring segments 2-3-mm long. The rings were then mounted in 5 mL tissue baths on a wire myograph (Multi Wire Myograph System-610 M; DMT, Aarhus, Denmark) filled with Krebs-Ringer solution (115.5 mM NaCl, 4.6 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 25.0 mM NaHCO₃, and 11.1 mM glucose, pH 7.4). The tissue bath was maintained at 37°C and continuously aerated with 95% O₂/5% CO₂. The rings were gradually stretched to an optimal resting tension of 2 g and then allowed to equilibrate for 30 min, after which they were stabilized by sequential exposure to 60 mM K⁺-PSS solution, 10⁻⁴ M phenylephrine and 10⁻⁶ M acetylcholine. The change in isometric tension was recorded and analyzed using a PowerLab 8/30 Data Acquisition System and LabChart pro software (ADInstruments, Colorado Springs, CO, USA). The contraction was expressed as a percentage of 60 mM KCl-induced contraction.

The aortic rings were treated with TPM or CSE, and the change in tension was observed for 60 min to determine whether TPM or CSE affects the basal tension, i.e., tonic tension. To examine the effects on vasoconstriction, aortic rings were treated with TPM or CSE for 60 min, after which contraction was induced by cumulative addition of 10⁻²-10⁻⁵ M phenylephrine to obtain concentration-contraction curves. The effects on vasorelaxation were tested by measuring the acetylcholine-induced relaxation of aortic rings that had been precontracted with 10⁻⁶ M phenylephrine.

**Cell culture and ROS measurement.** Mouse brain endothelial cell line bEnd.3 was purchased from American Type Culture Collection (Manassas, VA, USA). bEnd.3 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were maintained in a humidified chamber at 37°C and 5% CO₂, and were subcultured when they reached 80-90% confluence.

ROS were quantified with the colorimetric probe WST-1. bEnd.3 cells were plated on multiwall plates at densities of 1 × 10⁵ cells/well of a 6-well plate and were grown overnight until 80-90% confluence were reached. Cells were treated with TPM or CSE in the presence of 500 µM WST-1, and absorbance at 450 nm was measured in 3-min intervals for 60 min using a SpectraMax M3 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

**Statistical analyses.** Means and standard errors (SE) of means were calculated for all experimental groups. Data were subjected to one-way analysis of variance followed by Dunn’s test to determine the significances of differences relative to controls. The statistical analysis was performed using SigmaPlot software ver. 13 (Systat Software, San Jose, CA, USA). P values of < 0.05 were considered statistically significant.

**RESULTS**

**Effect of TPM and CSE on platelet aggregation.** To test the effect of CS on platelet activity, WP were pretreated with TPM or CSE, and aggregatory response to thrombin was examined. Neither TPM nor CSE alone induced platelet aggregation up to 100 µg/mL or 10%, respectively (data not shown). However, 5-min pretreatment of TPM potentiated thrombin-induced aggregation, and such pro-aggregatory activity was concentration-dependent in a range of 25-100 µg/mL (Fig. 1A). In contrast, 2.5-10% CSE inhibited the platelet aggregation induced by thrombin in a concentration-dependent manner (Fig. 1B). Higher concentration than 100 µg/mL TPM or 10% CSE did not exhibited stronger effect (data not shown). The effect of CS on platelet function may vary depending on the CS preparation used.
Cytotoxicity test of erythrocytes exposed to TPM or CSE, and the coagulability of blood plasma treated with TPM or CSE.

Hemolysis was measured to examine the cytotoxicity of CS in erythrocytes. Erythrocyte suspension was treated with 10–100 µg/mL TPM or 1–10% CSE, and hemolysis was assessed at each time points. Neither TPM nor CSE caused hemolysis at any of the concentrations tested for up to 120 min (Fig. 2). 0.1% Triton X-100 was used as a positive control and was confirmed to induce hemolysis properly under this experimental condition.

The effect of CS on plasma coagulation was assessed by measuring the PT and aPTT, which represented the activities of the extrinsic pathway and the intrinsic pathway in coagulation, respectively. Blood plasma was treated with 10–100 µg/mL TPM or 1–10% CSE for 60 min, and PT and aPTT were assessed. However, PT and aPTT of TPM- or CSE-treated plasma were not different from those of control at all (Fig. 3). A positive control, 3 IU/mL heparin was confirmed to significantly prolong PT and aPTT. TPM or CSE alone did not induce coagulation directly (data not shown).

Effect of TPM and CSE on contraction and relaxation of blood vessels.

CS was tested for its effects on vascular tone and vasomotor function. Aortic rings were treated with CS, and the change in basal tone was measured. Treatment with TPM or CSE alone had no effect on tonic tension up to 100 µg/mL and 10%, respectively (data not shown). To test the effects on vasoconstriction, aortic rings were incubated with 10–100 µg/mL TPM or 1–10% CSE for 60 min and contraction was induced by the cumulative application of phenylephrine. TPM significantly reduced vasoconstriction at 50 and 100 µg/mL, although it was ineffective up to 25 µg/mL (Fig. 4A, left panel). CSE did not affect phenylephrine-induced contraction up to 5% but was capable of inhibiting vasoconstriction at 10%, the highest concentration tested (Fig. 4B, right panel).
To test the effect on vasorelaxation, aortic rings were treated with TPM or CSE for 60 min and precontracted with $10^{-6}$ M phenylephrine. Then, relaxation was induced by the cumulative addition of $10^{-9}$-$10^{-5}$ M acetylcholine. Pretreatment of TPM significantly reduced acetylcholine-mediated relaxation, which was concentration-dependent in a range of $10^{-9}$-$10^{-5}$ M acetylcholine. Values are means ± standard error (n = 4). *P < 0.05 vs. control.

To test the effect on vasorelaxation, aortic rings were treated with TPM or CSE for 60 min and precontracted with $10^{-6}$ M phenylephrine. Then, relaxation was induced by the cumulative addition of acetylcholine. Pretreatment of TPM significantly reduced acetylcholine-mediated relaxation, which was concentration-dependent in a range of $10^{-9}$-$10^{-5}$ M acetylcholine. Values are means ± standard error (n = 4). *P < 0.05 vs. control.

**DISCUSSION**

Diverse methods for CS production and trapping have been developed for toxicological studies of smoking. For this study, mainstream CS was generated with 3R4F reference cigarettes and smoking machine in conformity with ISO standard 3308. TPM and CSE were prepared from mainstream CS by employing two different trapping methods. TPM and CSE have been widely used for routine *in vitro* toxicological assessment of CS. TPM of mainstream CS comprises approximately 4~9% of the total weight of CS and comprises water, nicotine and nicotine-free dry particulate matter. However, TPM excludes the gas and vapors in CS that pass through a Cambridge filter pad. Meanwhile, CSE is the PBS-soluble fraction of CS and thus contains water-soluble, gas- and vapor-phase constituents although it lacks particulate matter (6). Both TPM and CSE include polycyclic aromatic hydrocarbons, tobacco-specific nitrosamines, phenol and nicotine. Because the constituents of TPM and CSE differ, they may well exhibit distinct toxicological properties. The effects of TPM and CSE were therefore examined to clarify the influence of CS on blood and blood vessels.

In accordance with general speculation, substantial differences could be found in the effects on platelet aggregation and vascular relaxation between TPM and CSE. Smoking is generally known to render platelets vulnerable to pro-aggregatory stimuli in clinical and *in vivo* animal studies (14). However, CSE inhibited platelet aggregation induced by thrombin while TPM increased platelet activity (Fig. 1). Both TPM and CSE inhibited phenylephrine-induced vaso-
constriction, although the potency of this inhibition may not be equal (Fig. 5). However, CSE did not affect the relaxation at the concentrations which inhibit contraction, while TPM deteriorated vascular relaxation. Different from CSE, the effect of TPM on relaxation seems to be larger than that on contraction at low concentrations, suggesting that interfering relaxation may be more critical than damaging contraction.

Both TPM and CSE were capable of producing ROS in an endothelial culture system (Fig. 5). Seemingly, TPM appears to produce ROS more than CSE, but it is difficult to compare quantitative aspect directly. Intriguingly, TPM and CSE were able to produce ROS even in the absence of cells, suggesting that they generate ROS in extracellular aqueous solution. CS itself is well recognized to contain ROS and redox-active substances with ROS generating capability (15). Taken together, TPM and CSE seem to generate ROS directly or to stimulate cells to produce ROS indirectly. The chemical reactions by which CS produce ROS and the cellular sources responsible for CS-stimulated ROS production remain to be characterized. Neither TPM nor CSE caused hemolysis in this experimental condition (Fig. 2). Plasma coagulation as assessed by PT and aPTT was not affected by TPM or CSE (Fig. 3).

Different from in vivo animal experiments or clinical studies, CS preparation is of importance in in vitro study. Considering the results obtained in this study, the biological activity of CS may vary depending on the method by which CS is collected. Therefore, the experimental results using CS preparations should be interpreted with caution because they may not reflect in vivo CS exposure. Currently, it is not clear why TPM and CSE exhibit distinct effects on platelet aggregation and vasorelaxation. CS is known to contain more than 4,000 different kinds of chemicals (6). Future studies should help to clarify the constituents causing toxicity and the mechanisms underlying the effect on platelet and blood vessels.

ACKNOWLEDGMENTS

This research was supported by a grant (14182MFDS977) from Ministry of Food and Drug Safety in 2016.

REFERENCES

1. Ambrose, J.A. and Barua, R.S. (2004) The pathophysiology of cigarette smoking and cardiovascular disease: an update. J. Am. Coll. Cardiol., 43, 1731-1737.
2. Reilly, M.P. (2013) Tobacco-related cardiovascular diseases in the 21st century. Arterioscler. Thromb. Vasc. Biol., 33, 1458-1459.
3. Ezzati, M., Henley, S.J., Thun, M.J. and Lopez, A.D. (2005) Role of smoking in global and regional cardiovascular mortality. Circulation, 112, 489-497.
4. Centers for Disease Control and Prevention (US), National Center for Chronic Disease Prevention and Health Promotion (US) and Office on Smoking and Health (US) (2010) How tobacco smoke causes disease: The biology and behavioral basis for smoking-attributable disease: a report of the surgeon general, Centers for Disease Control and Prevention (US), Atlanta (GA).
5. National Center for Chronic Disease Prevention and Health Promotion (US) Office on Smoking and Health (2014) The health consequences of smoking-50 years of progress: a report of the Surgeon General, Centers for Disease Control and Prevention (US), Atlanta (GA).
6. Wright, C. (2015) Standardized methods for the regulation of cigarette-smoke constituents. Trends Anal. Chem., 66, 118-127.
7. Johnson, M.D., Schilz, J., Djordjevic, M.V., Rice, J.R. and Shields, P.G. (2009) Evaluation of in vitro assays for assessing the toxicity of cigarette smoke and smokeless tobacco. Cancer Epidemiol. Biomarkers Prev., 18, 3263-3304.
8. Kim, M., Han, C.H. and Lee, M.Y. (2014) NADPH oxidase and the cardiovascular toxicity associated with smoking. Toxicol. Res., 30, 149-157.
9. Kim, C.W., Go, R.E., Lee, H.M., Kwang, K.A., Lee, K., Kim, B., Lee, M.Y. and Choi, K.C. (2016) Cigarette smoke extracts induced the colon cancer migration via regulating epithelial mesenchymal transition and metastatic genes in human colon cancer cells. Environ. Toxicol. [Epub ahead of print].
10. Liu, Y., Park, J.M., Chang, K.H., Huh, H.J., Lee, K. and Lee, M.Y. (2016) AMP-activated protein kinase mediates the anti-platelet effects of the thiazolidinediones rosiglitazone and pioglitazone. Mol. Pharmaco., 89, 313-321.
11. Chung, S.M., Lee, J.Y., Lee, M.Y., Bae, O.N. and Chung, J.H. (2001) Adverse consequences of erythrocyte exposure to menadione: involvement of reactive oxygen species generation in plasma. J. Toxicol. Environ. Health A, 63, 617-629.
12. Kim, H., Oh, S.J., Kwak, H.C., Kim, J.K., Lim, C.H., Yang, J.S., Park, K., Kim, S.K. and Lee, M.Y. (2012) The impact of intratracheally instilled carbon black on the cardiovascular system of rats: elevation of blood homocysteine and hyperactivity of platelets. J. Toxicol. Environ. Health A, 75, 1471-1483.
13. Park, J.M., Lee, J.H., Na, C.S., Lee, D., Lee, J.Y., Satoh, M. and Lee, M.Y. (2016) Heartwood extract of Rhus verniciflua Stokes and its active constituent fisetin attenuate vasoconstriction through calcium-dependent mechanism in rat aorta. Biosci. Biotechnol. Biochem., 80, 493-500.
14. Barua, R.S. and Ambrose, J.A. (2013) Mechanisms of coronary thrombosis in cigarette smoke exposure. Arterioscler. Thromb. Vasc. Biol., 33, 1460-1467.
15. Pryor, W.A. and Stone, K. (1993) Oxidants in cigarette smoke. Radicals, hydrogen peroxide, peroxynitrate, and peroxynitrite. Ann. N. Y. Acad. Sci., 686, 12-27; discussion 27-28.