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

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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-

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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 polytetrafluorethylene 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 $MgCl_2$, 0.8 mM Na_2HPO_4, 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 $MgCl_2$, 0.8 mM Na_2HPO_4, 10 mM HEPES, 5.6 mM glucose, 1 mM $CaCl_2$, 0.3% bovine serum albumin; pH 7.4), the platelet concentration was adjusted to 2×10^8 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 the buffy 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 5×10^8 cell/mL for hemolysis study.

Erythrocyte suspension was treated 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. TPM or CSE was treated to plasma for 60 min. PT or aPTT was measured with a Coagulator2 coagulation analyzer (Behnk Elektronik, Norderstedt, Germany) using thromboplastin-D or $CaCl_2$ 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 KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25.0 mM $NaHCO_3$, and 11.1 mM glucose, pH 7.4). The tissue bath was maintained at 37°C and continuously aerated with 95% O_2/5% CO_2. 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^{-6} 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^{-9}-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% CO_2, 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 1×10^5 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.

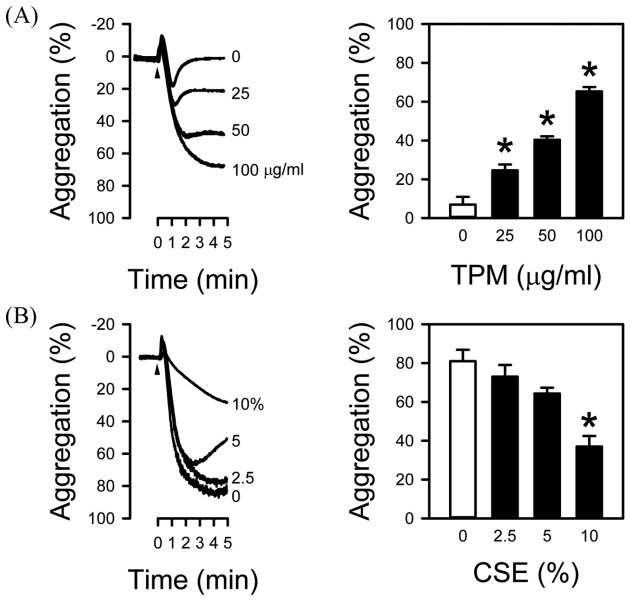

Fig. 1. Effect of CS on platelet aggregation. Platelets were treated with the indicated concentrations of TPM (A) or CSE (B) for 5 min. Aggregation was induced by the addition of thrombin (arrowheads). Representative tracings (left panels) and the aggregation percentages relative to the control (right panels) are presented. Values are means ± standard errors (n = 3). *$P <$ 0.05 *vs.* control.

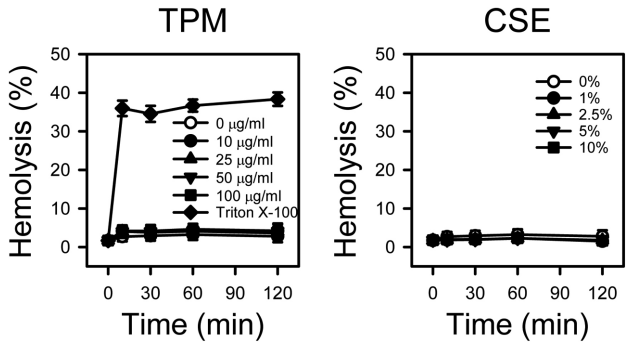

Fig. 2. Hemolysis test of erythrocytes exposed to CS. Erythrocyte suspension was incubated with the indicated concentrations of TPM or CSE, and the hemolysis was assessed at 10, 30, 60, and 120 min after treatment. 0.1% Triton X-100 was treated to erythrocytes as a positive control. Values are means ± standard errors (n = 3).

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.

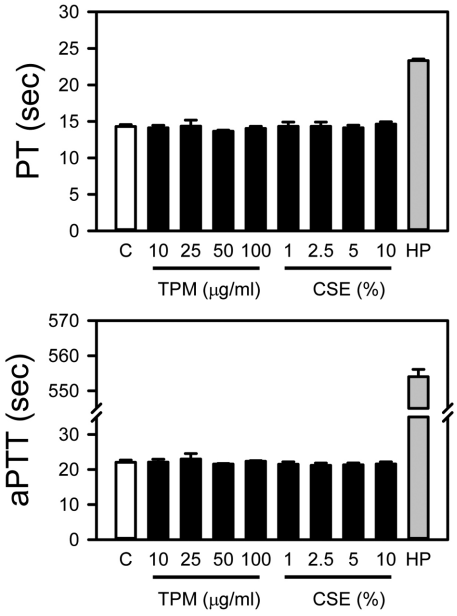

Fig. 3. Effect of CS on plasma coagulation. Blood plasma was treated with TPM or CSE. PT and aPTT were measured after 60 min. Plasma incubated with 3 IU/mL heparin for 5 min was used as a positive control. Values are means ± standard errors (n = 3).

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).

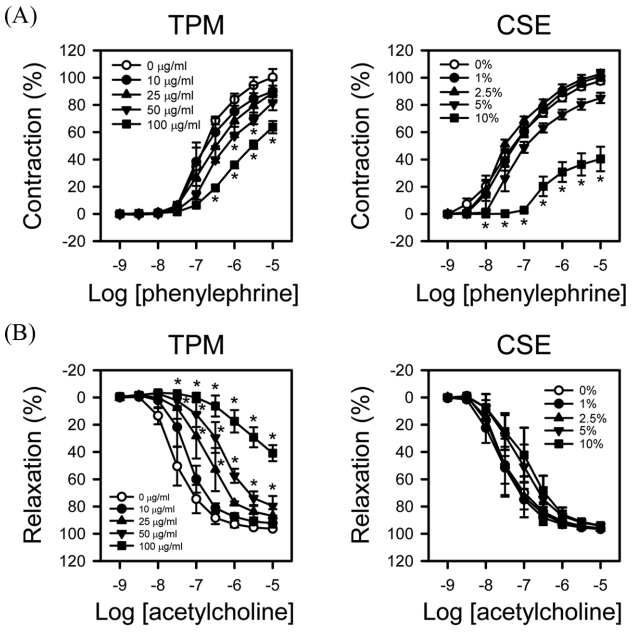

Fig. 4. Effect of CS on vasomotor function. Aortic rings were incubated with the indicated concentrations of TPM or CSE for 60 min. (A) Contraction was elicited by cumulative addition of 10^{-9}-10^{-5} M phenylephrine. (B) Aortic rings were precontracted with 10^{-6} M phenylephrine. After submaximal contraction was achieved, relaxation was induced by cumulative addition 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 10~100 μg/mL (Fig. 4B, left panel). In contrast, CSE did not exhibit any effect on relaxation at concentrations up to 10% (Fig. 4B, right panel).

ROS generation in bEnd.3 cells by TPM and CSE. CS was tested for its ROS-generating capability in bEnd.3, a brain endothelial cell line. ROS generation in bEnd.3 cells was measured with WST-1 for 60 min in the presence or absence of CS. Both TPM and CSE were capable of generating ROS in a range of 10~100 μg/mL and 1~10%, respectively (Fig. 5, black bars). On the whole, the amount of ROS formed by TPM was greater than that by CSE in these concentration ranges. Besides the ability to stimulate cells to generate ROS, CS itself contains ROS and stable substances with the potential to generate ROS (15). Although CS is proven to increase superoxide in bEnd.3 cells (Fig. 5, black bars), it is not clear whether all ROS detected originate from cells or not. Accordingly, ROS were measured in HBSS without bEnd.3 cells. Even in the absence of cells,

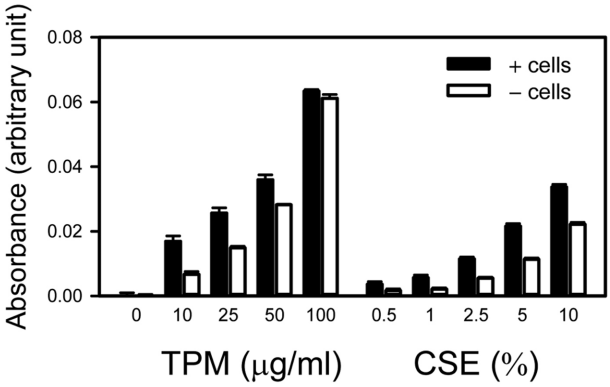

Fig. 5. CS-induced ROS generation in endothelial cells. CSE or TPM was treated to bEnd.3 cells (black bar) or HBSS without cells (white bars). Superoxide was measured with WST-1 for 60 min. Values are means ± standard errors (n = 4).

CSE and TPM increased ROS in a concentration-dependent manner (Fig. 5, white bars). The amounts of ROS formed by CSE and TPM corresponded to approximately two third and half of ROS measured in the presence of cells, respectively.

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.

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