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Corrigendum

Corrigendum to "Chronic administration of nicotine-free cigarette smoke extract impaired endothelium-dependent vascular relaxation in rats via increased vascular oxidative stress" [J Pharmacol Sci 118 (2012) 206–214]

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Full Paper

Chronic Administration of Nicotine-Free Cigarette Smoke Extract Impaired Endothelium-Dependent Vascular Relaxation in Rats via Increased Vascular Oxidative Stress

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Abstract. Cigarette smoking has been implicated in the initiation and progression of cardiovascular disorders and atherosclerosis. Here, we examined the effects of nicotine-free cigarette smoke extract (CSE) on the regulation of cardiovascular function. Rats were subcutaneously administered PBS or nicotine-free CSE at 0.05 to 1.5 mL/day per rat for 4 weeks. Blood pressure, cardiac function, and vascular responsiveness were measured at 4 weeks after administration. Furthermore, acute effects of nicotine-free CSE were also studied in the aorta isolated from normal rats. Blood pressure and left ventricular systolic pressure (LVSP) were significantly increased in the nicotine-free CSE–administered rats, but heart rate, dP/dt_{max}, and dP/dt_{min} were not affected. Endothelium-dependent relaxation by acetylcholine (ACh) in the nicotine-free CSE–treated rats was significantly attenuated compared to PBS-treated rats, but endothelium-independent relaxation by sodium nitroprusside (SNP) did not differ. Pretreatment with superoxide dismutase restored the attenuated ACh-induced relaxation. Contractions by phenylephrine, angiotensin II, and KCl did not differ between two groups. In vitro acute nicotine-free CSE treatment did not alter the response to ACh or SNP. These results suggest that chronic nicotine-free CSE administration impairs endothelial function by increased production of superoxide derived from the vascular wall components other than smooth muscles and induces slight hypertension accompanied with LVSP elevation.

Keywords: chronic administration, nicotine-free cigarette smoke extract, endothelial function, superoxide

Introduction

Epidemiological studies indicate that cigarette smoking is one of the independent risk factors for hypertension, atherosclerosis, and ischemic heart diseases (1 – 4). Endothelial dysfunction is thought to be an early sign of atherosclerosis (5) and that is observed in rodent models of cardiovascular diseases (6 – 8). Impairment of vascular endothelial function has been reported in chronic smokers (9 – 11). Nitric oxide (NO) is an intercellular signal–mediating molecule in the cardiovascular, immune, and nervous systems (12). In the cardiovascular system, NO plays a crucial role in the regulation of vascular tone (13, 14) and in protecting against the development of atherosclerosis (15). NO bioavailability is decreased in cigarette smokers (16, 17), but the underlying mechanism was not clarified. Physiological and pharmacological effects of nicotine, a major component of cigarette smoke, are well established, and it is reported that treatment with nicotine impairs endothelial function by decreasing NO bioavailability in the experimental animals (18, 19). Cigarette smoke contains numerous vasoconstricting and vasorelaxing components other than nicotine (20, 21), and cigarette smoke extract (CSE) exposure affects vasomotor responses of isolated arteries (22, 23). Recently nicotine-free CSE has attracted attention in the pathogenesis of vascular disorders caused by smoking (24 – 26). However, the effect of cigarette smoke components other than nicotine on regulation of cardiovascular function is not sufficiently examined. Therefore, in the present study, acute and chronic effects of nicotine-free CSE on the vascular responsiveness were

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examined in the isolated rat aorta. Furthermore, its chronic effects on the body weight, blood pressure, and cardiac function were also examined in the rat.

Materials and Methods

Materials

Drugs used were acetylcholine (ACh; Daiichi-Sankyo, Tokyo); superoxide dismutase (SOD), L-phenylephrine, indomethacin (Sigma, St. Louis, MO, USA); sodium nitroprusside (SNP; Nacalai Tesque, Kyoto); calcium ionophore A23187 (Boehringer Ingelheim, Ingelheim, Germany); papaverine hydrochloride (Nichi-iko, Toyama); N^G-nitro-L-arginine (L-NA), angiotensin II (Peptide Institute, Minoh); and L-arginine (Kanto Chemical, Tokyo).

Animals

Six-week-old male Sprague-Dawley rats (Charles River Japan, Inc., Yokohama) were fed standard chow and water ad libitum and were housed according to institutional guidelines at the Shiga University of Medical Science. These studies were approved by the Animal Care and Use Committee at Shiga University of Medical Science. Rats were subcutaneously administered 0.05 to 1.5 mL of nicotine-free CSE or phosphate-buffered saline (PBS, pH 7.4) once a day for 4 weeks. Rats administered PBS were used as the control animals.

Preparation of CSE

Nicotine-free CSE was prepared according to the previous papers (27, 28). Briefly, the mainstream of the cigarette (Frontier Light; Japan Tobacco Inc., Tokyo) was bubbled into PBS (1 mL per three cigarettes) to extract water-soluble components in the gas phase of cigarette smoke. Nicotine and tar were removed by passing the cigarette smoke through a Cambridge Filter before bubbling. It took approximately 5 min to consume one cigarette.

Measurement of blood pressure, heart rate, and cardiac function

Blood pressure, heart rate, and cardiac function were measured under anesthesia. Rats were anesthetized with intraperitoneal injection of sodium pentobarbital (50 mg/kg) and right common carotid arteries were isolated to insert a micromanometer-tipped catheter (SPR-320; Millar Instruments, Inc., Houston, TX, USA). The catheter was attached to a transducer control unit (TCB 500, Millar Instruments, Inc.) and connected to a data acquisition system (Power lab; AD instruments, Castle Hill, NSW, Australia). After recording of blood pressure and heart rate, the catheter was advanced into the left ventricle to record left ventricular systolic pressure (LVSP), left ventricle end-diastolic pressure (LVEDP), the maximum rate of left ventricular pressure rise (dP/dt_{max}), and the maximum rate of left ventricular pressure decline (dP/dt_{min}).

Isometric tension studies

Thoracic aortic was isolated and cut into strips with special care to preserve the endothelium as described previously (29). In some strips, endothelium was removed by gently rubbing the intimal surface with a cotton pellet. The strips were vertically fixed between hooks in the 10-mL organ bath containing the modified Ringer-Locke solution, which was aerated with a mixture of 95% O_2 and 5% CO_2 and maintained at 37°C ± 0.3°C. The hook anchoring the upper end of the strips was connected to the lever of a force-displacement transducer (Nihon Kohden Kogyo, Tokyo). The resting tension was adjusted to 2 g. The composition of the solution was 120 mM NaCl, 5.4 mM KCl, 2.2 mM $CaCl_2$,1.0 mM $MgCl_2$, 25.0 mM $NaHCO_3$, and 5.6 mM dextrose (pH 7.4). Indomethacin at a concentration of 10^{-5} M was added to prevent the synthesis of prostaglandins. All preparations were allowed to equilibrate for more than 60 min in the bathing medium, during which time the bath solution was replaced every 10 min with fresh medium. The strips for measurement of relaxation response were partially pre-contracted with phenylephrine, and then ACh (10^{-9} to 10^{-5} M) with or without SOD (200 U/mL), A23187 (3×10^{-9} to 10^{-6} M), or SNP (10^{-11} to 10^{-6} M) was cumulatively added to the bath to obtain the concentration–response curve. At the end of each experiment, papaverine (10^{-4} M) was added to attain the maximal relaxation. Relaxations induced by agonists relative to those caused by papaverine are presented. Contractile responses to KCl (30 mM), phenylephrine (10^{-9} to 10^{-6} M), or angiotensin II (10^{-9} to 10^{-6} M) were measured. Contractions induced by 30 mM KCl were taken as standards (100%) for the contractile responses to phenylephrine and angiotensin II. In a separate series of experiments, thoracic aorta were isolated from age-matched normal rats by the same procedure as described above, and strips were pre-incubated with 0.05% – 0.5% nicotine-free CSE for 30 min before measuring the relaxant responses to ACh and SNP. The 0.5% nicotine-free CSE would be equivalent or exceeds the nicotine-free CSE concentration in blood under subcutaneous administration of 1.5 mL nicotine-free CSE to the rat. According to the general principles of pharmacokinetics, we presumed that sustainably exposed concentration of nicotine-free CSE under subcutaneous administration is about 1/10 or less of the administered concentration. Therefore, the maximum exposure concentration of nicotine-free CSE for in vitro experi-

ments was determined as 0.5% based on the blood volume and body weight of rats.

Histologic study

Thoracic aorta was fixed with 10% formaldehyde (Wako Pure Chemical, Osaka) and embedded in paraffin. The sample was cut into 3.0-μm sections and stained with hematoxylin-eosin.

Statistical analyses

The results are expressed as the mean ± S.E.M. Statistical analyses were performed by Student's *t*-test for two groups and Tukey's method after one-way analysis of variance for more than three groups. *P*-values less than 0.05 were considered to be significant.

Results

Chronic treatment with nicotine-free CSE in vivo

Dose-related effects of nicotine-free CSE on vascular responses to ACh and SNP: After daily injection of nicotine-free CSE (0.05, 0.15, 0.5, and 1.5 mL/day) for 4 weeks, vascular responses to ACh and SNP were examined in the aorta isolated from rats. ACh-induced relaxation was significantly suppressed by nicotine-free CSE in rats given more than 0.15 mL/day, compared to control rats (0.5 mL/day, PBS) (Fig. 1A). On the other hand, SNP-induced relaxation did not differ among all 5 groups (Fig. 1B). For further analysis of the underlying mechanism, we examined the aorta isolated from the rats given 0.5 mL/day of nicotine-free CSE, in which ACh-induced relaxation was reduced at maximum.

Histology: Figure 2 shows hematoxylin-eosin staining of the aortas. No significant atherosclerotic changes were observed in aortas obtained from nicotine-free CSE–treated rats.

Effects on body weight, blood pressure, heart rate, and cardiac function: Daily administration of nicotine-free CSE (0.5 mL/day) for 4 weeks did not affect body weight of the rats compared to control rats (Table 1). The nicotine-free CSE treatment slightly but significantly increased systolic, diastolic, and mean blood pressure and LVSP compared to control rats. On the other hand, heart rate, LVEDP, dP/dt_{max}, and dP/dt_{min} were not different between control and nicotine-free CSE–treated rats.

Effects of nicotine-free CSE (0.5 mL/day) on vascular responses to ACh, A23187, SNP, KCl, phenylephrine, and angiotensin II: Chronic treatment of nicotine-free CSE (0.5 mL/day) reduced both ACh- and A23187-induced relaxation, which were abolished by endothelium denudation, but did not affect the SNP-induced relaxation (Fig. 3). Relaxation induced by ACh (75.0% ± 8.4%) was also reduced by treatment of L-NA (10^{-6} M: 19.5% ± 5.1%, 10^{-5} M: 0.0%), a NO synthase (NOS) inhibitor, and addition with 3×10^{-4} M L-arginine potentiated the relaxation (49.9% ± 6.7%) under treatment with L-NA (10^{-6} M). Pretreatment with SOD (200 U/mL) restored the attenuated ACh-induced relaxation in the nicotine-free CSE–treated group, and the degree of the relaxation was not significantly different from that of the control group (Fig. 4). Contractile responses to KCl, phenylephrine, or angiotensin II did not differ between control and nicotine-free CSE–treated groups (Fig. 5).

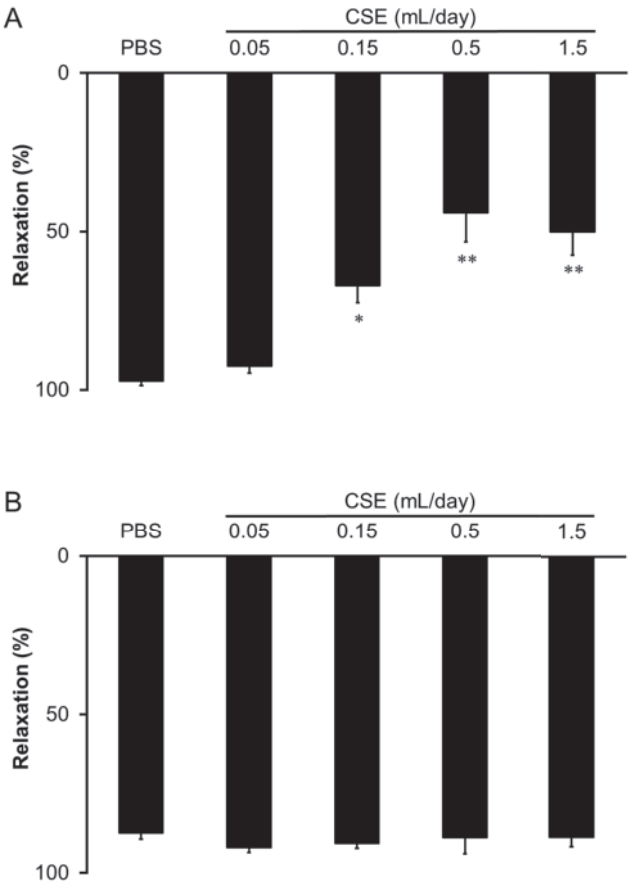

Fig. 1. Dose-related effects of chronic nicotine-free CSE treatment on relaxation by ACh (10^{-5} M, A) and SNP (10^{-8} M, B) in isolated rat aorta. 'PBS' and 'CSE' indicate responses of aorta isolated from rats injected with PBS (0.5 mL/day) or nicotine-free CSE (0.05 to 1.5 mL/day) daily for 4 weeks, respectively. Relaxations induced by 10^{-4} M papaverine were taken as 100%. Data represent the mean ± S.E.M. of 4 – 9 preparations. *$P < 0.05$, **$P < 0.01$ vs. PBS.

Acute treatment with nicotine-free CSE in vitro

To examine the acute effects on vascular responsiveness, the isolated aorta was preincubated with nicotine-free CSE (0.05%, 0.15%, and 0.5%) for 30 min and then vascular responses to ACh and SNP were examined. Results are shown in Fig. 6. Nicotine-free CSE in a

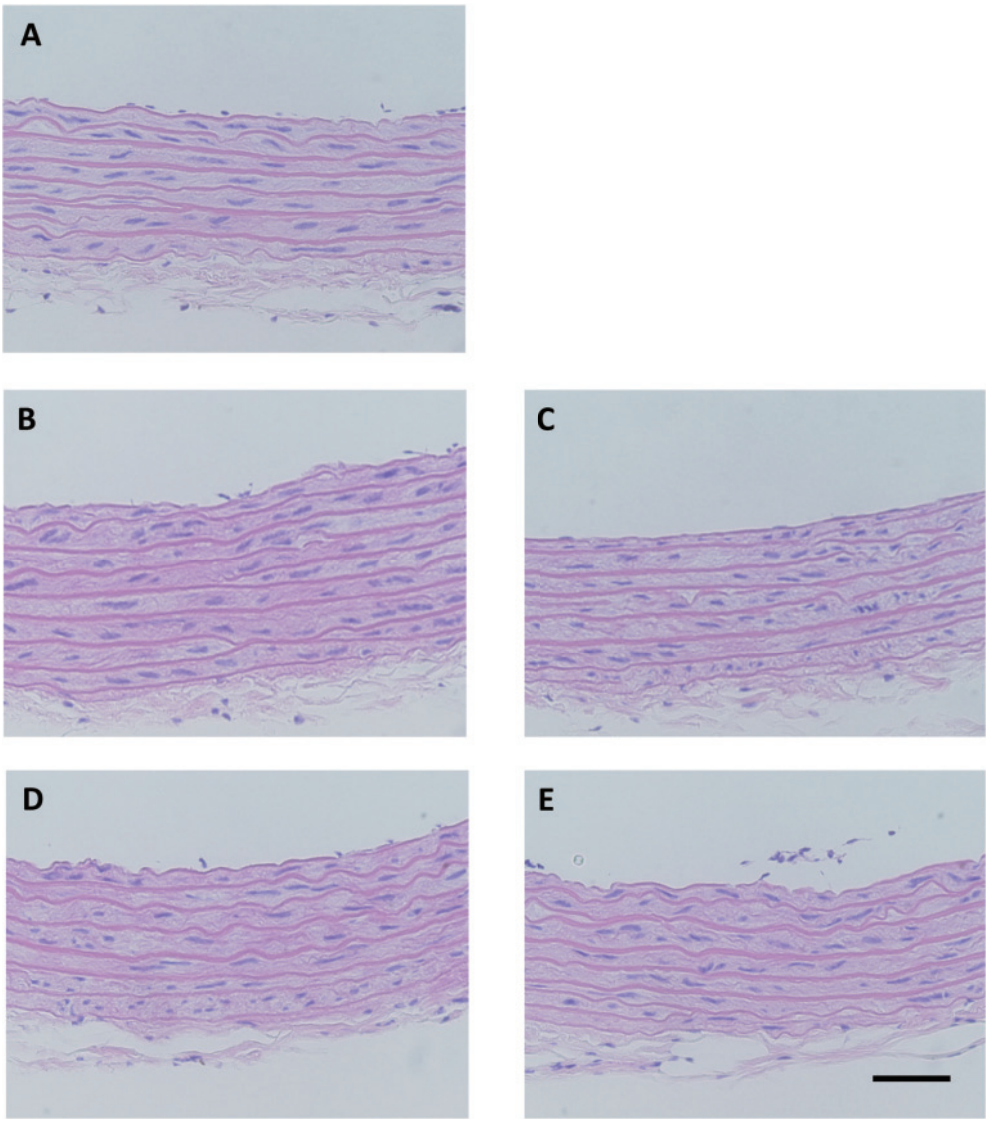

Fig. 2. Light microscopic observations of aorta isolated from rats injected with PBS (0.5 mL/day, A) or nicotine-free CSE (0.05, 0.15, 0.5, and 1.5 mL/day: B – E, respectively) daily for 4 weeks, respectively. The sample was cut into 3.0-μm sections and then stained with hematoxylin-eosin. Bar = 100 μm.

concentration up to 0.5% did not affect the relaxations caused by ACh and SNP.

Discussion

Cigarette smoking is considered to cause vascular endothelial dysfunction followed by progression of atherosclerosis (1 – 3). Several reports of forearm blood flow responses indicate that endothelium-dependent dilation was impaired in smokers, but endothelium-independent responses to NO donor were similar to those in nonsmokers (10, 11, 30, 31). In animal experiments, chronic inhalation of nicotine-containing cigarette smoke is reported to impair ACh-induced relaxation and increase blood pressure in mice (32). Furthermore, both acute- and chronic-administration of nicotine impaired endothelium-dependent dilation in rodents (19, 33, 34). Therefore, nicotine in the cigarette smoke has been considered to cause endothelial dysfunction. On the other hand, cigarette smoke contains thousands of constituents including nicotine, tar, and a number of oxidants, which were included in both the particulate phase and gas phase of cigarette smoke (20). However, little is known about effects of cigarette smoke components other than nicotine on cardiovascular function. This study is the first to elucidate the effects of nicotine-free CSE on regulation of cardiovascular function in rats.

In smoking research, widely used models include

Table 1. Body weight, blood pressure, heart rate, and cardiac parameters in rats injected subcutaneously with PBS or CSE (0.5 mL/day) daily for four weeks

Parameters	PBS	CSE
Body weight (g)	415 ± 14	419 ± 10
Blood pressure (mmHg)		
Systolic	108 ± 2	128 ± 8*
Diastolic	83 ± 3	101 ± 5*
Mean	90 ± 3	109 ± 6*
Heart rate (beats/min)	324 ± 22	353 ± 10
LVSP (mmHg)	110 ± 2	129 ± 8*
LVEDP (mmHg)	0.13 ± 0.85	0.76 ± 0.76
dP/dt_{max} (mmHg/s)	6924 ± 401	7971 ± 609
dP/dt_{max} (mmHg/s)	−5235 ± 231	−6227 ± 513

Abbreviations: LVSP, left ventricular systolic pressure; LVEDP, left ventricle end-diastolic pressure; dP/dt_{max}, the maximum rate of left ventricular pressure rise; dP/dt_{min}, the maximum rate of left ventricular pressure decline; PBS, phosphate buffered saline; CSE, nicotine-free cigarette smoke extract. Each value represents the mean ± S.E.M. of 5 animals. *$P < 0.05$ vs. PBS.

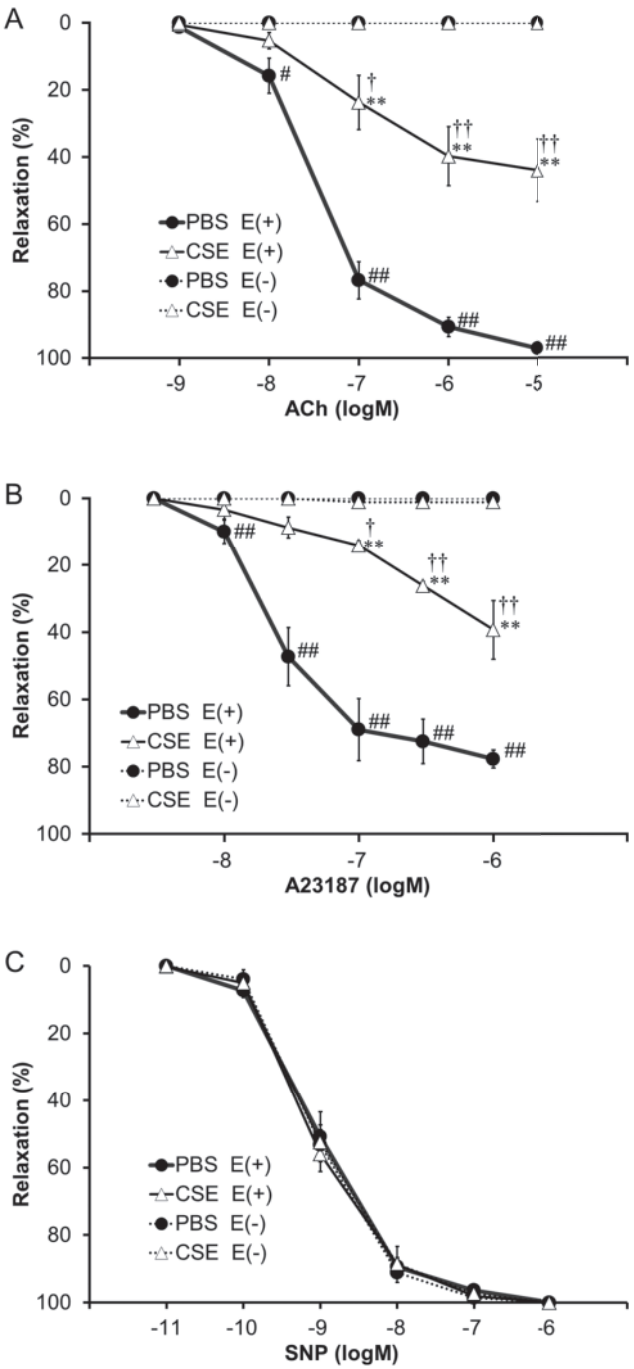

Fig. 3. Concentration–response curves for ACh (A), A23187 (B), and SNP (C) in aortic strips obtained from rats administered PBS or nicotine-free CSE (0.5 mL/day) daily for 4 weeks. E (+) and E (−) indicate the aortic strips with and without the endothelium, respectively. Relaxations induced by 10^{-4} M papaverine were taken as 100%. Data represent the mean ± S.E.M. of 4 – 7 preparations. **$P < 0.01$ vs. PBS E (+). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. the corresponding E (−) strips.

subjecting animals to inhalation of cigarette smoke and injection of CSE (25, 26, 32). Generally, administration of CSE solution to animals is an easier method to study the chronic effects of CSE compared to the exposure of animals to cigarette smoke using smoking apparatuses. Based on the results obtained from the present study, this procedure is convenient for examining the chronic effects of smoking on vascular function in vitro and ex vivo. In the present study, rats were given daily injections of different volumes of nicotine-free CSE (0.05, 0.15, 0.5, and 1.5 mL/day, s.c.) for 4 weeks, and then functional and morphological changes of aorta isolated from the rats were studied. Although endothelium-independent relaxation by SNP, a NO donor, was not affected by nicotine-free CSE at any volume, endothelium-dependent relaxations caused by ACh was significantly inhibited by nicotine-free CSE at a volume of more than 0.15 mL/day. The degree of inhibition did not change by nicotine-free CSE at more than 0.5 mL/day, so further experiments were performed in rats treated with 0.5 mL/day nicotine-free CSE for 4 weeks.

In the rats treated with 0.5 mL/day nicotine-free CSE for 4 weeks, ACh-induced relaxation was abolished by both endothelium denudation and pretreatment of L-NA, a NOS inhibitor. A similar result was obtained in the endothelium-dependent relaxation induced by A23187. On the other hand, endothelium-independent relaxation induced by SNP was not influenced by nicotine-free CSE treatment. Cigarette smoking is reported to decrease NO bioavailability by inhibiting NOS activity (17). These results indicate that chronic treatment of nicotine-free

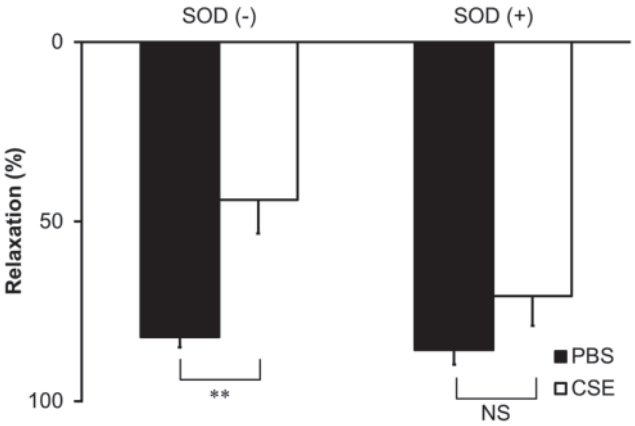

Fig. 4. Effect of SOD (200 U/mL) on the relaxation induced by ACh (10^{-5} M) in PBS- and nicotine-free CSE–treated rats. 'PBS' and 'CSE' indicate responses of aorta isolated from rats injected with PBS (0.5 mL/day) or nicotine-free CSE (0.5 mL/day) daily for 4 weeks, respectively. Relaxations induced by 10^{-4} M papaverine were taken as 100%. Data represent the mean ± S.E.M. of 6 – 8 preparations. $^{**}P < 0.01$ vs. PBS.

CSE affected the production of NO in the endothelium or the stability of released NO, and nicotine-free CSE did not impair the responsiveness to NO in the smooth muscle.

Increased superoxide generation is involved in the mechanism of endothelial dysfunction in the aorta from nicotine-free CSE–treated rats because the suppressed ACh-induced relaxation was restored by pretreatment with SOD. One may consider that vascular tissues isolated from rats treated chronically with nicotine-free CSE generated and spontaneously released a large amount of superoxide without stimulation, which attenuates the relaxation caused by NO released from the endothelium by ACh-induced stimulation. However, this may not be the case, because nicotine-free CSE treatment did not affect the relaxation induced by a NO donor, SNP. Therefore, NO inactivation by superoxide derived from vascular wall due to increase or activation of superoxide generating enzymes such as NADPH oxidase, xanthine oxidase, and so on is not likely to be involved in the endothelial dysfunction caused by chronic treatment with nicotine-free CSE. Furthermore, angiotensin II stimulates NADPH oxidase, which is known as one of the major sources of reactive oxygen species (ROS) generation in vascular wall (6, 35). However, contractions induced by angiotensin II did not differ between the control and nicotine-free CSE–treated rats.

Previous reports suggested that ROS contained in CSE itself impairs endothelial function (36, 37). However, in the present study, aortic strips were isolated 24 h after the final nicotine-free CSE administration to rats, and kept in an organ bath containing nutrient solution without nicotine-free CSE, and then strips were repeatedly washed by the solution. Furthermore, incubation of normal rat aorta with nicotine-free CSE for 30 min did not affect the

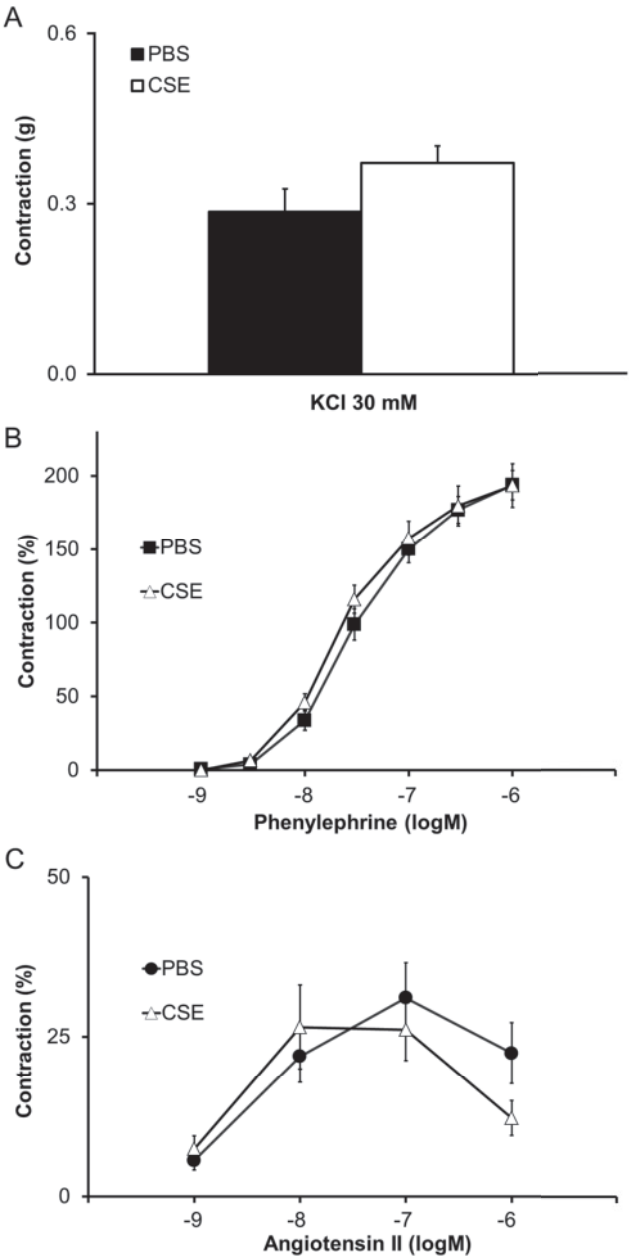

Fig. 5. Effects of chronic nicotine-free CSE (0.5 mL/day) treatment on the contractions caused by KCl (A), phenylephrine (B), and angiotensin II (C) in the isolated rat aorta. 'PBS' and 'CSE' indicate responses of aorta isolated from rats injected with PBS (0.5 mL/day) and nicotine-free CSE (0.5 mL/day) daily for 4 weeks, respectively. Contractions induced by 30 mM KCl were taken as 100% for phenylephrine- and angiotensin II–induced contractions. Data represent the mean ± S.E.M. of 6 – 8 preparations. No significant difference was found between PBS and nicotine-free CSE treatment.

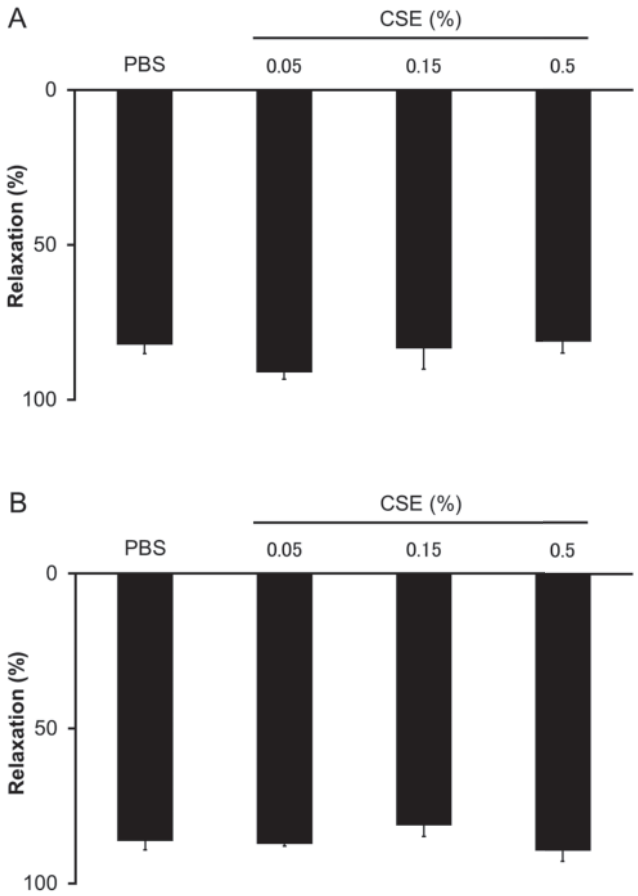

Fig. 6. Dose-related effects of acute nicotine-free CSE treatment on relaxations by ACh (A) and SNP (B) in the isolated rat aorta. The aortic strips were preincubated for 30 min with PBS or nicotine-free CSE (0.05%, 0.15%, and 0.5%) before the relaxant response to ACh (10^{-5} M) and SNP (10^{-8} M) was obtained. Relaxations induced by 10^{-4} M papaverine were taken as 100%. Data represent the mean ± S.E.M. of 4 – 9 preparations. No significant difference was found between PBS and nicotine-free CSE treatment.

ACh-induced relaxation. These results suggest that endothelial dysfunction observed in the present study is neither due to ROS nor due to endothelial degenerating agents such as saponin possibly present in the nicotine-free CSE. Therefore, we considered that chronic treatment with nicotine-free CSE tended to increase endogenous ROS generation in the vascular wall upon endothelial stimulation. In other words, ACh and A23187 stimulated the ROS generation from the vascular wall components other than smooth muscle cells. Both ACh and A23187 induces increase of intracellular Ca^{2+} concentrations in endothelial cells followed by eNOS activation via muscarinic ACh receptor stimulation and action as a calcium ionophore, respectively. Similar phenomenon that stimulation of endothelial cells by ACh and A23187 increases ROS generation was observed in the insulin-resistant rats in which eNOS is uncoupled by decreasing the ratio of BH_4/BH_2 in the vascular wall (38). Whether aortic eNOS was uncoupled or not in the nicotine-free CSE–treated rats needs further investigation.

Blood pressure was slightly but significantly increased in the nicotine-free CSE–treated rats. Impaired endothelium-dependent relaxation may be involved in the underlying mechanism. Although LVSP was significantly increased, LVEDP, dP/dt_{max}, and dP/dt_{min} were not deteriorated. Therefore, increase in LVSP seems to be a cardiac compensatory response to increased blood pressure, and nicotine-free CSE (0.5 mL/day, s.c.) treatment did not induce the cardiac dysfunction.

There are few reports that chronic nicotine-free CSE treatment affected the vascular disorders in the animal models. Eight-week, nicotine-free CSE administration increased aortic plaque lesion area in Watanabe heritable hyperlipidemic (WHHL) rabbits (25) and potentiated hyperplasia in balloon-injured rabbit carotid artery (26), which is related to a decrease of NO production. In the present study, our results indicated that nicotine-free CSE impaired endothelial function via insufficient NO bioavailability without atherosclerotic changes in healthy animals. Therefore, endothelial dysfunction by cigarette smoke components other than nicotine may be involved in the mechanism of initiation of atherosclerosis in chronic smoking. Yamaguchi et al. (39) suggested that stable peroxynitrite-generating species present in the nicotine-free CSE is a possible atherogenic factor. However, this may not be the case, since acute effects of nicotine-free CSE on endothelium-dependent relaxation were never observed under our experimental conditions. The substances in the nicotine-free CSE responsible for endothelial dysfunction remain to be identified.

In conclusion, the present study demonstrated that chronic nicotine-free CSE treatment impaired endothelial NO-dependent relaxation and elevated blood pressure in the rat. Scavenging of NO by superoxide generated and released from the vascular wall upon endothelial stimulation is partly involved in endothelial dysfunction induced by nicotine-free CSE. The precise mechanism needs to be clarified.

Acknowledgments

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