Note

Cigarette Smoke Extract and Its Cytotoxic Factor Acrolein Inhibit Nitric Oxide Production in Human Vascular Endothelial Cells

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Acrolein (ACR), a highly reactive α,β -unsaturated aldehyde, is a major cytotoxic factor in nicotine- and tar-free cigarette smoke extract (CSE). There are conflicting results regarding endothelial functions despite the fact that both CSE and ACR cause cellular damage. Several lines of evidence indicate that CSE impairs endothelium-derived nitric oxide (NO)-dependent vasodilation by reducing the activity and protein expression of endothelial NO synthase (eNOS), whereas ACR elicits endothelium-dependent vasorelaxation by increasing the production of NO and expression of eNOS. To clarify whether CSE and its cytotoxic factor ACR cause endothelial dysfunction, this study examined the effects of CSE and ACR on human vascular endothelial EA.hy926 cells. CSE and ACR reduced the phosphorylation of eNOS at serine (Ser)¹¹⁷⁷ and total expression of eNOS. The CSE- and ACR-induced decrease in the phosphorylation and expression of eNOS was counteracted by glutathione (reduced form), an antioxidant. Basal NO production was inhibited by CSE, ACR, *N*^G-nitro-L-arginine methyl ester (a competitive eNOS inhibitor), and nominally Ca^{2+} -free solution supplemented with BAPTA-AM (a membrane permeable Ca^{2+} chelator). These results indicate that CSE and ACR increase oxidative stress, and reduce NO production by reducing the activity and total protein level of eNOS.

Key words acrolein; cigarette smoke extract; endothelial nitric oxide synthase; nitric oxide; endothelial dysfunction; oxidative stress

INTRODUCTION

Cigarette smoking is a major risk factor for cardiovascular diseases, such as arteriosclerosis.^{1,2)} Cigarette mainstream smoke contains more than 4000 chemical constituents, including nicotine and particulate matter (tar).³⁾ Recently, we identified stable carbonyl compounds, including acrolein (ACR) and methyl vinyl ketone (MVK), as major cytotoxic factors in nicotine- and tar-free cigarette smoke extract (CSE).⁴⁾ Several lines of evidence suggest that CSE, ACR, and MVK cause plasma membrane damage and cell apoptosis *via* Ca^{2+} - and protein kinase C-dependent activation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) and the subsequent NOX-mediated generation of reactive oxygen species.^{3–6)}

Endothelial dysfunction is characterized by (i) a reduction in endothelial nitric oxide (NO) bioavailability, which is due to the direct inactivation of NO, (ii) a decrease in the activity or expression of endothelial NO synthase (eNOS), and (iii) uncoupling of eNOS, leading to the impairment of endothelium-derived NO-dependent vasodilation.^{7–9)} Studies have indicated that CSE induces the impairment of endothelium-dependent vasodilation, which can be attributed to a reduction in the activity and protein expression of eNOS.^{8–10)} Whereas, ACR elicits endothelium-derived NO-dependent vasorelaxation by increasing eNOS expression and NO production.^{11,12)} These results suggest that the cigarette smoking-related endothelial dysfunction is due to cytotoxic factors other than ACR. However, the action of ACR on endothelial cells remains controversial, because ACR causes endothelial cell injury and death, both of which contribute to endothelial dysfunction.^{13,14)}

To clarify whether CSE and ACR are involved in endothelial dysfunction, this study examined their effects on human vascular endothelial cells.

MATERIALS AND METHODS

Materials HI-LITETM brand (Japan Tobacco Inc., Tokyo, Japan) cigarettes containing 17 mg of tar and 1.4 mg of nicotine per cigarette was used in the study. ACR and glutathione (reduced form, GSH) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM), *N*^G-nitro-L-arginine methyl ester (L-NAME), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were procured from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, U.S.A.). 1,2-Bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM) was bought from Dojindo Laboratories (Kumamoto, Japan). 4,5-Diaminofluorescein (DAF-2) was obtained from Goryo Chemical, Inc. (Sapporo, Japan). Anti-phospho-eNOS (serine (Ser)¹¹⁷⁷) antibody, anti-total eNOS antibody, anti-phospho-Akt (Ser⁴⁷³) antibody, and anti-total Akt antibody were procured from Cell Signaling Technology Inc. (Beverly, MA, U.S.A.). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, U.S.A.). Horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G (IgG) secondary antibody and HRP-conjugated anti-mouse IgG secondary antibody were procured from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, U.S.A.). The other reagents used were of the highest grade in

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Fig. 1. Time Course of the 5% Cigarette Smoke Extract (CSE)- and 50 μ M Acrolein (ACR)-Induced Decrease in the Basal Phosphorylation Level of Endothelial Nitric Oxide (NO) Synthase (eNOS) at Ser¹¹⁷⁷ in Human Umbilical Vein Endothelial EA.hy926 Cells

(A, D) The upper panels are representative immunoblots with the anti-phospho-eNOS (Ser¹¹⁷⁷) antibody to determine the level of phosphorylated eNOS (indicated as α -p-eNOS). The middle panels are representative immunoblots with the anti-total-eNOS antibody to determine the level of total (phosphorylated and nonphosphorylated) eNOS (indicated as α -t-eNOS). The lower panels are representative immunoblots with the anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody to determine the level of GAPDH (indicated as α -GAPDH) as the internal control. (B, E) The histograms present the changes in the relative ratio of the phosphorylated eNOS levels to the total eNOS expression levels (p-eNOS/t-eNOS) in the absence or presence of CSE and ACR. The phospho/total eNOS ratio was normalized by considering the ratio in control cells as 100%. The data are presented as mean $\pm$ standard error of the mean (S.E.M.) of the results obtained from four independent experiments (* p < 0.05 and ** p < 0.01 compared with the vehicle-treated cells). (C, F) The histograms present the changes in the relative ratio of the total eNOS levels to the total GAPDH expression levels (t-eNOS/GAPDH). The total eNOS/GAPDH ratio was normalized by considering the ratio in control cells as 100%. The data are presented as mean $\pm$ S.E.M. of the results obtained from four independent experiments (* p < 0.05 and ** p < 0.01 compared with the vehicle-treated cells).

terms of purity.

Preparation of CSE CSE was prepared as described previously.⁵⁾ Briefly, the mainstream smoke of four cigarettes (one cigarette per trial) was continuously sucked through a standard glass-fiber Cambridge filter at a constant flow rate (1.050 L min⁻¹) using an aspiration pump to remove tar and nicotine. The remaining gas phase of the cigarette smoke was bubbled into 15 mL of phosphate-buffered saline (PBS). The concentration of this CSE solution was considered 100%.

Cell Culture Human umbilical vein endothelial EA.hy926 cells were obtained from Dr. Yuko Suzuki (Hamamatsu University School of Medicine, Japan) with permission from Dr. Cora-Jean S. Edgell (University of North Carolina at Chapel Hill, U.S.A.) and cultured in DMEM supplemented with 10% FBS (v/v), penicillin (100 units mL⁻¹), and streptomycin

(100 μ g mL⁻¹) at 37°C under humidified air with 5% CO₂.

Measurement of Activity and Expression of eNOS and Akt The activity and expression of eNOS and Akt were determined by Western blotting. Cells grown in six-well plates were serum-starved for 24 h before the experiment. The cells were treated with vehicle (PBS), CSE, or ACR in Krebs-HEPES solution (140 mM NaCl, 3 mM KCl, 2 mM CaCl₂·2H₂O, 1 mM MgCl₂·6H₂O, 11 mM D-(+)-glucose, and 10 mM HEPES; pH of the solution was adjusted to 7.4 with NaOH). To examine the effects of 300 μ M GSH and its vehicle (1% PBS) on the CSE- and ACR-induced changes in the phosphorylation and expression of eNOS and Akt, the cells were treated with GSH or vehicle for 0.5 h prior to adding CSE and ACR. The primary antibodies bound to proteins (phospho-eNOS (Ser¹¹⁷⁷), total eNOS, phospho-Akt (Ser⁴⁷³), total Akt,

Fig. 2. Concentration-Response Relationship of the Cigarette Smoke Extract (CSE) (0.5 to 10%)- and Acrolein (ACR) (10 to 200 μ M)-Induced Decrease in the Basal Phosphorylation Level of Endothelial Nitric Oxide Synthase (eNOS) at Ser¹¹⁷⁷ in Human Umbilical Vein Endothelial EA.hy926 Cells

(A, D) The upper panels are representative immunoblots with the anti-phospho-eNOS (Ser¹¹⁷⁷) antibody to determine the level of phosphorylated eNOS (indicated as α -p-eNOS). The middle panels are representative immunoblots with the anti-total-eNOS antibody to determine the level of total (phosphorylated and nonphosphorylated) eNOS (indicated as α -t-eNOS). The lower panels are representative immunoblots with the anti-GAPDH antibody to determine the level of GAPDH (indicated as α -GAPDH) as the internal control. (B, E) The histograms present the changes in the relative ratio of the phosphorylated eNOS levels to the total eNOS expression levels (p-eNOS/t-eNOS) in the absence or presence of CSE and ACR. The phospho/total eNOS ratio was normalized by considering the ratio in control cells as 100%. The data are presented as mean $\pm$ S.E.M. of the results obtained from four independent experiments (* p < 0.05 and ** p < 0.01 compared with the vehicle-treated cells). (C, F) The histograms present the changes in the relative ratio of the total eNOS levels to the total GAPDH expression levels (t-eNOS/GAPDH). The total eNOS/GAPDH ratio was normalized by considering the ratio in control cells as 100%. The data are presented as mean $\pm$ S.E.M. of the results obtained from four independent experiments (* p < 0.05 and ** p < 0.01 compared with the vehicle-treated cells).

and GAPDH) on the cell membrane were detected with HRP-conjugated secondary antibodies and Pierce Western blotting Substrate (Thermo Fisher Scientific Inc.). The blots were exposed to Fuji medical X-ray film (FUJIFILM Corp., Tokyo, Japan). Optical density on the film was analyzed using National Institutes of Health Image J1.37 software.

Measurement of NO Production NO released from EA.hy926 cells was measured using DAF-2 (a fluorescent NO indicator) that reacts with NO to produce the fluorescent triazole adduct triazolofluorescein (DAF-2T).¹⁵ Cells cultured in 24-well plates were treated with the vehicle (5% PBS), 5% CSE, 50 μ M ACR, 300 μ M L-NAME in Krebs-HEPES solution, or 30 μ M BAPTA-AM in nominally Ca²⁺-free Krebs-

HEPES solution for 3 h, in the presence of 1 μ M DAF-2. The fluorescence intensity of the resultant DAF-2T was measured using a multi-mode spectroscopic reader (Spectra Max Paradigm; Molecular Devices, LLC., San Jose, CA, U.S.A.) at an excitation wavelength of 488 nm and emission wavelength of 515 nm.

Data Analysis All results are presented as mean $\pm$ standard error of the mean (S.E.M.) and n refers to the number of experiments. All statistical analyses were performed using GraphPad Prism software (version 7.00, GraphPad Software, San Diego, CA, U.S.A.). The data were analyzed using the one-way ANOVA, followed by Tukey's multiple comparison tests. The results were considered statistically significant at a

p value of <0.05.

RESULTS AND DISCUSSION

Effects of CSE and ACR on eNOS Activity and Expression Previous studies on the effects of cigarette smoking on endothelium by endothelium-derived NO-mediated vasorelaxation have reported conflicting results; that is, cigarette smoking leads to augmentation or attenuation of endothelial-derived NO-dependent vasorelaxation.^{16,17} In vascular endothelial cells, stimulation of G_q protein-coupled receptors, such as muscarinic M₃ receptor and Ca²⁺-sensing receptor (CaSR), induces an increase in intracellular Ca²⁺ concentration ([Ca²⁺]_i) to activate eNOS.^{16,18} eNOS is a phosphoprotein that is phosphorylated at multiple serine/threonine residues, including Ser¹¹⁷⁷.¹⁹ The phosphorylation of eNOS at Ser¹¹⁷⁷ leads to an increase in NO production *via* the eNOS activity.²⁰ Previously, we demonstrated that the phosphorylation of eNOS at Ser¹¹⁷⁷ and production of NO are facilitated by the stimulation of CaSR with extracellular Ca²⁺ in human umbilical vein endothelial EA.hy926 cells.¹⁸ To gain insights into the effect of cigarette smoke on endothelial function, here, we characterized the pharmacological properties of CSE and its cytotoxic factor ACR.

As shown in Fig. 1, the phosphorylation of eNOS at Ser¹¹⁷⁷ decreased with time after treatment with 5% CSE (Figs. 1A, B) and 50 μM ACR (Figs. 1D, E). Total eNOS protein expression was unaffected by CSE treatment (Figs. 1A, C), but it was significantly reduced by ACR treatment for 2 and 4 h (Figs. 1D, F). Treatment with CSE (Figs. 2A, B) and ACR (Figs. 2D, E) for 4 h induced a concentration-dependent decrease in the phosphorylation of eNOS. Additionally, the total eNOS protein level declined after treatment with CSE (Figs. 2A, C) and ACR (Figs. 2D, 2F). These results suggest that CSE and ACR can decrease eNOS enzyme activity by inhibiting eNOS phosphorylation, and partially, by reducing total eNOS protein expression.

Effects of CSE and ACR on NO Production To confirm whether the CSE- and ACR-induced decrease in the activity and/or expression of eNOS led to a reduction in NO generation, we measured the fluorescence intensity of DAF-2T resulting from the reaction of DAF-2 with NO.¹⁵ As NO is continuously generated by CaSR-mediated increases in [Ca²⁺]_i and eNOS activity in the presence of extracellular Ca²⁺,¹⁸ the fluorescence intensity of DAF-2T, produced due to basal NO production, was decreased by treatment with 300 μM L-NAME (a competitive eNOS inhibitor)²¹ and nominally Ca²⁺-free solution supplemented with 30 μM BAPTA-AM (a membrane permeable Ca²⁺ chelator)⁵ (Fig. 3). In addition, the fluorescent intensity of DAF-2T was significantly reduced by 5% CSE and 50 μM ACR (Fig. 3). These results clearly indicate that CSE and ACR inhibit NO production. Our findings support the concept that cigarette smoking is deleterious to endothelial function. However, our results regarding the action of ACR contradict those of a previous study in which ACR stimulated NO production *via* an increase in [Ca²⁺]_i in human umbilical vein endothelial cells.¹¹ The reason for this discrepancy in the results is unknown, but it may be due to the difference in the type of endothelial cells used (primary cultured cells in the previous study¹¹) *versus* immortalized EA.hy926 cells in this study). Alternatively, CaSR-mediated responses observed in

Fig. 3. Effects of 2% PBS (vehicle), 5% CSE, 50 μM ACR, 300 μM L-NAME, and Nominally Ca²⁺-Free Solution Supplemented with 30 μM BAPTA-AM on NO Production in Human Umbilical Vein Endothelial EA.hy926 Cells

NO released from EA.hy926 cells was measured using DAF-2, which reacts with NO to produce its fluorescent triazole adduct DAF-2T. The fluorescence intensity of DAF-2T was calculated by subtracting the fluorescence intensity obtained in the absence of cells from that obtained in the presence of cells. The data are presented as mean ± S.E.M. of results obtained from seven independent experiments (***p* < 0.01 compared with the vehicle-treated cells).

EA.hy926 cells may have masked ACR-induced NO production if ACR has opposing effects on endothelial function—for example, stimulation and inhibition of NO generation.

Effects of GSH on the CSE- and ACR-Induced Changes in Phosphorylation and Expression of eNOS and Akt CSE and ACR can induce oxidative stress that contributes to endothelial dysfunction.¹³ It has been reported that antioxidants such as GSH and *N*-acetyl-L-cysteine reduce the cytotoxicity of CSE and ACR.^{5,13} Indeed, 300 μM GSH inhibited the 5% CSE- and 50 μM ACR-induced decreases in the phosphorylation and expression of eNOS (Figs. 4A–C). These results suggest that oxidative stress caused by CSE and ACR leads to a decline in the eNOS phosphorylation and expression.

Does ACR play a central role in the CSE-induced endothelial dysfunction? Several studies have indicated that ACR is one of the substances present in high level in CSE.^{3,4,13} We have reported that the concentration of ACR in the CSE, which was prepared by dissolving the gas phase of 10 cigarettes in 10 mL of water, was approximately 3.4 mM.⁴ It means that the estimated amount of ACR generated is 3.4 μmol per one cigarette. Therefore, the concentration of ACR in 5% CSE prepared in this study (see Materials and Methods) is estimated as 45 μM. As shown in Figs. 3 and 4B, 5% CSE was as effective as 50 μM ACR in terms of the reduction of eNOS phosphorylation and NO production. These results, taken together with the estimated concentration of ACR in 5% CSE, indicate that the inhibitory effect of CSE on the eNOS/NO axis is mainly due to ACR.

However, the phosphorylation of Ser⁴⁷³ of Akt, which is an upstream kinase of eNOS and phosphorylates Ser¹¹⁷⁷ of eNOS,²² were slightly reduced by 5% CSE but significantly elevated by 50 μM ACR (Figs. 4D, E). Interestingly, 5% CSE enhanced the Akt phosphorylation in the presence of 300 μM GSH (Figs. 4D, E). Fifty micromolar ACR induced a decrease in Akt expression, which was recovered by 300 μM GSH (Figs. 4D, F). These results indicate that ACR *per se* activates Akt, while oxidizing substance(s) other than ACR present in the CSE inhibits Akt activation. Further studies will be

Fig. 4. Effects of 1% PBS (Vehicle) and 300 μ M Glutathione (Reduced Form, GSH) on the 5% CSE- and 50 μ M ACR-Induced Changes in the Basal Phosphorylation Levels of Endothelial Nitric Oxide Synthase (eNOS) at Ser¹¹⁷⁷ (A–C) and Akt at Ser⁴⁷³ (D–F) in Human Umbilical Vein Endothelial EA.hy926 Cells

(A, D) The upper panels are representative immunoblots with the anti-phospho-eNOS (Ser¹¹⁷⁷) antibody and the anti-phospho-Akt (Ser⁴⁷³) antibody to determine the levels of phosphorylated eNOS (indicated as α -p-eNOS) and phosphorylated Akt (indicated as α -p-Akt), respectively. The middle panels are representative immunoblots with the anti-total-eNOS antibody and the anti-total-Akt antibody to determine the levels of total (phosphorylated and nonphosphorylated) eNOS (indicated as α -t-eNOS) and total Akt (indicated as α -t-Akt), respectively. The lower panels are representative immunoblots with the anti-GAPDH antibody to determine the level of GAPDH (indicated as α -GAPDH) as the internal control. (B, E) The histograms present the changes in the relative ratio of the phosphorylated eNOS or Akt levels to the total eNOS or Akt expression levels (p-eNOS/t-eNOS or p-Akt/t-Akt) in the absence or presence of CSE and ACR. The phospho/total eNOS or Akt ratio was normalized by considering the ratio in control cells as 100%. The data are presented as mean $\pm$ S.E.M. of the results obtained from four independent experiments (* p < 0.05 and ** p < 0.01 compared with the vehicle-treated cells; ## p < 0.01, between indicated columns). (C, F) The histograms present the changes in the relative ratio of the total eNOS or Akt levels to the total GAPDH expression levels (t-eNOS/GAPDH or t-Akt/GAPDH). The total eNOS or Akt/GAPDH ratio was normalized by considering the ratio in control cells as 100%. The data are presented as mean $\pm$ S.E.M. of the results obtained from four independent experiments (** p < 0.01 compared with the vehicle-treated cells; # p < 0.05 and ## p < 0.01, between indicated columns).

required to determine how CSE and ACR modulate the Akt/eNOS/NO pathway.

CONCLUSION

The present study provides evidence that CSE and ACR increase oxidative stress, and decrease NO production by reducing the activity and/or total protein level of eNOS. These findings suggest that (i) cigarette smoking leads to the attenuation of endothelial-derived NO-dependent vasorelaxation, and (ii) antioxidant compounds such as GSH may be effective in inhibiting cigarette smoking-related endothelial dysfunction.

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Conflict of Interest The authors declare no conflict of interest.

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