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Full Paper

Comparison of cytotoxicity of cigarette smoke extract derived from heat-not-burn and combustion cigarettes in human vascular endothelial cells

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ABSTRACT

The present study compared the properties of mainstream smoke generated from heat-not-burn (HNB) cigarettes and a combustion cigarette (hi-lite™ brand). Three types of cigarette heating devices were used to generate cigarette smoke at different heating temperatures [Ploom S™ (200 °C), glo™ (240 °C), and IQOS™ (300–350 °C)]. Mainstream smoke was generated using the following puffing regimen: volume, 55 mL; duration, 3 s; and interval, 30 s. The rank order of particulate phase (nicotine and tar) amounts trapped on a Cambridge filter was Ploom S < glo < IQOS < hi-lite. Heated cigarette-derived smoke extract (hCSE) from the devices except for Ploom S, and burned CSE (bCSE) decreased mitochondrial metabolic activity (glo < IQOS < hi-lite) in human vascular endothelial cells. Furthermore, the cytotoxicity was reduced by removing the particulate phase from the mainstream smoke. Endothelial nitric oxide synthase activity was reduced by nicotine- and tar-free CSE of IQOS and hi-lite (IQOS < hi-lite), but not Ploom S and glo. These inhibitory effects were diminished by removing the carbonyl compounds from the mainstream smoke. These results indicated that the cytotoxicity of hCSE was lower than that of bCSE in vascular endothelial cells.

1. Introduction

Conventional cigarette smoking is a known risk factor for cardiovascular disease, stroke, and chronic obstructive pulmonary disease.[1,2] Cigarette smoke contains over 4000 chemicals and is divided into two phases: the particulate phase, which mainly consists of nicotine, tar, and water and a gas phase.[3,4] Both phases contain bioactive substances which are generated during the process of cigarette combustion.[5] The tar in the particulate phase contains stable free radicals that can cause oxidative damage to DNA, proteins, and lipids, leading to tissue injury.[6] The gas phase contains relatively stable carbonyl compounds, such as ACR, that enter the systemic circulation, and increase systemic oxidative stress.[4,7,8] Recently, we demonstrated that n/t-free bCSE and ACR cause oxidative stress in human vascular endothelial cells, leading to endothelial dysfunction characterized by a reduction in eNOS activity and NO production.[9]

The strategy of tobacco harm reduction has been established to reduce smoking-related health risks.[10] Strategies for reducing the harmful effects of smoking combustion cigarette include the use of nicotine-containing HNB cigarettes. Accumulating evidence indicates that during smoking, lower amounts of bioactive substances are generated by HNB cigarette products than by combustion cigarettes.[11,12] Several studies reported that although ACR was detected in mainstream smoke from HNB cigarette products, the amount generated decreased by > 90% compared with that of combustion cigarettes.[12–14] These facts indicate that the use of HNB cigarette products reduces endothelial dysfunction caused by ACR generated during smoking. However, an in vitro study with rats exposed to mainstream cigarette smoke via nose cone has revealed that the smoke from a single HNB cigarette impairs endothelial function comparably to smoke from a combustion cigarette.[15] Thus, the usefulness of HNB cigarette in tobacco harm reduction remains controversial.

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Abbreviations

ACR	acrolein
ARP	aldehyde reactive probe
bCSE	burned cigarette-derived smoke extract
BH4	tetrahydrobiopterin
CaSR	Ca^{2+}-sensing receptor
CORESTA	Cooperation Centre for Scientific Research Relative to Tobacco
CX-572	Carboxen-572
DAF-2	4,5-diaminofluorescein
DAF-2T	triazolofluorescein
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
eNOS	endothelial nitric oxide synthase
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
hCSE	heated cigarette-derived smoke extract
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HNB	heat-not-burn
HRP	horseradish peroxidase
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NO	nitric oxide
NOX	nicotinamide adenine dinucleotide phosphate oxidase
n/t-free	nicotine- and tar-free
PVDF	polyvinylidene fluoride
SA-HRP	horseradish peroxidase-conjugated streptavidin
SDS	sodium dodecyl sulfate

HNB cigarette products currently available in Japan can be classified into two categories based on the heating temperature: high- and low-temperature heating devices (200–350 °C and 30–40 °C, respective). High-temperature heating devices include the Ploom S™ brand (heating temperature, 200 °C; Japan Tobacco Inc.), glo™ (240 °C; British American Tobacco Plc.), and IQOS™ (300–350 °C, Philip Morris International Inc.). Ploom S and glo produce an aerosol by heating the cigarette from outside, whereas IQOS heats the cigarette inside with a heater blade. The health risk associated with smoking HNB cigarettes possibly varies among devices used, because the heating temperature during vaporization affects the concentration of carbonyl compounds in cigarette aerosols.[16,17]

The purpose of this study was to characterize the properties of mainstream smoke generated from heated and burned cigarettes. To prepare hCSE and bCSE, mainstream smoke from heated and burned cigarettes was generated by manual smoking using the CORESTA approach.[18] To clarify the cytotoxicity of hCSE and bCSE on human vascular endothelial cells, mitochondrial metabolic activity, eNOS activity, NO production, and protein carbonylation were evaluated.

2. Materials and Methods

2.1. Materials

Cigarette heating devices [Ploom S (Japan Tobacco Inc., Tokyo, Japan), glo series 2 (British American Tobacco Japan Ltd., Tokyo, Japan), and IQOS 3 multi (Philip Morris Japan Ltd., Tokyo, Japan)], HNB cigarettes (Mevius regular for Ploom S, Kent bright for glo, and Marlboro regular for IQOS), and a combustion cigarette (hi-lite containing 17 mg of tar and 1.4 mg of nicotine per cigarette, Japan Tobacco Inc.) were used in this study. A Cambridge filter was obtained from Borgwaldt KC GmbH. (Hamburg, Germany). CX-572 Rezorian™ Cartridge, DMEM, HEPES, and anti-FLAG M2 antibody were purchased from Sigma–Aldrich Co. LLC. (St. Louis, MO, USA). FBS and Dynabeads™ protein G were procured from Thermo Fisher Scientific Inc. (Waltham, MA, USA). The ARP was obtained from Dojindo Laboratories (Kumamoto, Japan). EDTA-free protease inhibitor cocktail, EDTA-free phosphatase inhibitor cocktail, and penicillin-streptomycin mixed solution were purchased from Nacalai Tesque Inc. (Kyoto, Japan). DAF-2 was purchased from Goryo Chemical, Inc. (Sapporo, Japan). The CellTiter 96™ Aqueous One solution cell proliferation assay kit for the MTS reduction assay was procured from Promega Corporation (Madison, WI, USA). Anti-phosphorylated (phospho)-eNOS (Ser^{1177}) and anti-total eNOS antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Anti-GAPDH antibody was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). HRP-conjugated anti-rabbit IgG and anti-mouse IgG secondary antibodies and SA-HRP were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). HRP-conjugated anti-FLAG peptide (DDDDK) antibody (FLAG-HRP) was procured from Medical and Biological Laboratories Co., Ltd. (Aichi, Japan). All other reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2. Preparation of hCSE and bCSE

To treat human vascular endothelial cells with high concentrations of CSE, the components of mainstream cigarette smoke were extracted with Krebs–HEPES solution [140 mM sodium chloride (NaCl), 3 mM potassium chloride (KCl), 2 mM calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), 1 mM magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), 11 mM D-(+)-glucose, and 10 mM HEPES; pH 7.4], which is assay buffer. The mainstream smoke of HNB cigarettes and hi-lite was generated at seven puffs per one cigarette using the puffing regimen parameters of the CORESTA approach (55 mL, 3 s, and 1 puff/30 s for puff volume, duration, and interval, respectively).[18] Smoke of 42 puffs was bubbled into 5 mL ice-cold Krebs–HEPES solution (Fig. 1); that is, the smoke of six cigarettes (7 puffs from one cigarette per trial) was generated to prepare the CSE solution of 42 puffs. The concentration of each CSE solution prepared with the smoke of 42 puffs was considered to be 100% individually. In some experiments, the particulate phase (nicotine, tar, and water) and carbonyl compounds in the mainstream smoke were removed by passing the stream through a Cambridge filter and a CX-572 cartridge, respectively. To estimate the amount of particulate phase generated, the resulting Cambridge filter was dried for 6 h in an oven at approximately 25 °C and <20% relative humidity.

2.3. Cell culture

Human 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, USA) and cultured in DMEM supplemented with 10% FBS (*v*/*v*), penicillin (100 units mL^{-1}), and streptomycin (100 μg mL^{-1}) at 37 °C in a humidified atmosphere with 5% CO_2. EA.hy926 cells stably expressing C-terminally FLAG-tagged human CaSR (CaSR-FLAG) were generated as described previously.[19] The cell culture medium was removed and replaced by Krebs–HEPES

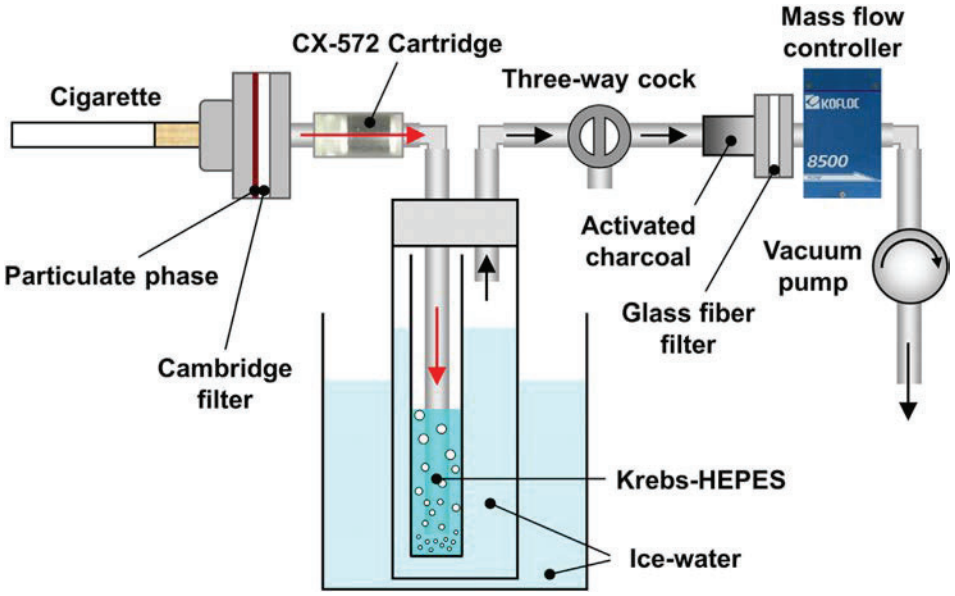

Fig. 1. Experimental setup for preparation of CSE. Mainstream smoke of HNB cigarettes and a combustion cigarette was generated using puffing regimen parameters of CORESTA approach (55 mL puff volume, 3 s puff duration, and 1 puff/30 s). Smoke was sucked for 3 s/puff at a constant flow rate of 1.1 L min^{-1} using a vacuum pump. Flow rate was regulated using mass flow controller (MODEL 8500 series, KOFLOC Corp., Kyoto, Japan). Three-way cock was manually switched on for 3 s/puff. Smoke was bubbled into 5 mL ice-cold Krebs–HEPES solution. Cambridge filter and CX-572 cartridge were used when necessary.

solution with or without the indicated concentrations of CSE immediately before the start of the following experiments.

2.4. Evaluation of effects of hCSE and bCSE on mitochondrial metabolic activity

Mitochondrial metabolic activity was assessed by measuring MTS reduction activity using the CellTiter 96 Aqueous One solution cell proliferation assay kit. Briefly, EA.hy926 cells were seeded into 24-well plates at a density of approximately 8×10^4 cells per well and cultured in DMEM supplemented with 10% FBS for 2 days. The cells grown to 100% confluence were washed with warmed PBS [137 mM NaCl, 2.68 mM KCl, 8.1 mM sodium hydrogen phosphate (Na_2HPO_4), and 1.47 mM potassium dihydrogen phosphate (KH_2PO_4); pH 7.4], and then treated with Krebs–HEPES solution with or without the indicated concentrations of CSE for 4 h. For example, when the cells are treated with 80% CSE, the cell culture medium is replaced by a mixture with 800 μL of 100% CSE solution (which is prepared with Krebs–HEPES solution, as described above) and 200 μL of fresh Krebs–HEPES solution. To evaluate the short-term effects of hCSE/bCSE, MTS reagent was added to each well after treatment with hCSE/bCSE for 4 h and the cells were incubated for an additional 2 h. When estimating the long-term effects of hCSE/bCSE, the cells were treated with 80% CSE for 4 h, and then the CSE solution was replaced by fresh DMEM supplemented with 10% FBS. After culture for 24 h, the MTS reduction activity of the cells was assessed as above. The supernatant was transferred to 96-well plates, and subsequently, the amount of reduced MTS in the supernatant was measured as the absorbance at 492 nm using a microplate reader (Multiskan FC microplate reader, Thermo Fisher Scientific Inc.).

2.5. Evaluation of effects of hCSE and bCSE on activity and expression of eNOS

EA.hy926 cells were seeded into six-well plates at a density of approximately 4×10^5 cells per well and cultured in DMEM supplemented with 10% FBS for 24 h. The cells were serum-starved in DMEM for 24 h prior to the experiment. After serum starvation, the cells grown to 100% confluence were washed with warmed PBS and then treated with Krebs–HEPES solution with or without the indicated concentrations of CSE for 4 h. After treatment, cells were washed with ice-cold PBS buffer and lysed in radioimmuno precipitation assay buffer [150 mM NaCl, 1.5 mM $MgCl_2$, 50 mM Tris–HCl (pH 6.8), 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS] supplemented with EDTA-free protease inhibitor cocktail and EDTA-free phosphatase inhibitor cocktail. Whole cell lysates were mixed with SDS sample buffer [62.5 mM Tris–HCl (pH 6.8), 10% glycerol, 5% 2-mercaptoethanol, 2.5% SDS, and 0.1% bromophenol blue] and analyzed using western blotting as described previously.[20] The primary antibodies [phospho-eNOS (Ser^{1177}), total eNOS, and GAPDH] bound to proteins on PVDF membranes (Immobilon™-P, pore size 0.45 μm, Millipore Corp., Bedford, MA, U.S.A.) were detected using HRP-conjugated secondary antibodies and Pierce western blotting substrate (Thermo Fisher Scientific Inc.).[9]

2.6. Evaluation of hCSE/bCSE-induced carbonylation of CaSR

EA.hy926 cells stably expressing CaSR-FLAG were seeded into 10-cm dishes at a density of approximately 8×10^5 cells per dish and cultured in DMEM supplemented with 10% FBS for 3 days. The cells grown to 100% confluence were washed with warmed PBS buffer, and then treated with Krebs–HEPES solution with or without 80% hCSE/bCSE for 1 h at 37 °C. Then, the cells were washed with ice-cold PBS and lysed in NP-40 buffer [150 mM NaCl, 50 mM Tris–HCl (pH 7.4), and 1% NP-40] supplemented with an EDTA-free protease inhibitor cocktail. CaSR-FLAG in the lysate was collected by immunoprecipitation with Dynabeads™ protein G, as described previously.[20] The Dynabead-binding CaSR-FLAG was suspended in washing buffer (PBS supplemented with 0.1% NP-40), and then incubated with 5 mM ARP for 2 h at 37 °C. The resulting Dynabeads were washed three times with a washing buffer. The CaSR-FLAG proteins bound to the Dynabeads were eluted in elution buffer (50 mM glycine, pH 2.8) for 30 min at 37 °C, followed by

incubation with SDS sample buffer for 30 min at 37 °C. Proteins in the immunoprecipitated samples were analyzed using western blotting. The carbonylated CaSR-FLAG and immunoprecipitated total CaSR-FLAG on the PVDF membranes were detected using SA-HRP and FLAG-HRP, respectively.

2.7. Evaluation of effects of hCSE and bCSE on NO production

NO production in EA.hy926 cells was assessed using the fluorescent NO indicator, DAF-2, which reacts with NO to produce a fluorescent triazole adduct triazolofluorescein DAF-2T.[19,21] EA.hy926 cells were seeded into 24-well plates at a density of approximately 8×10^4 cells per well and cultured in DMEM supplemented with 10% FBS for 2 days. The cells grown to 100% confluence were washed with warmed PBS buffer and then treated with Krebs–HEPES solution with or without for 3 h. After treatment, 1 μM DAF-2 was added to each well and the cells were incubated for an additional 30 min. 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, USA) at excitation and emission wavelengths of 488 nm and 520 nm, respectively.

2.8. Data analysis

All results are presented as means ± standard error of the mean (SEM). The significance of the difference between mean values was analyzed using GraphPad Prism software (version 7.00, GraphPad Software, San Diego, CA, USA) using the Student's paired *t*-test or two-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. A P-value < 0.05 was considered significantly different.

3. Results

3.1. Amount of particulate phase trapped on Cambridge filters

Mainstream smoke was generated from heated cigarettes (Ploom S, glo, and IQOS) and a burned cigarette (hi-lite) using a manual smoking apparatus (Fig. 1) for the preparation of hCSE/bCSE and determination of the particulate phase. Fig. 2A shows the comparison of the color of the Cambridge filters before and after passage of the smoke from 91 puffs. The change in the color of the Cambridge filter after exposure to the smoke from Ploom S was slight compared to before exposure. In contrast, tan to brown particulate matter was deposited on the Cambridge filters after passage of the smoke from glo, IQOS, and hi-lite. The increases in the weight of the filters after drying at 25 °C for 6 h were 6 ± 1 mg, 16 ± 1 mg, 56 ± 3 mg, and 228 ± 2 mg for Ploom S, glo, IQOS, and hi-lite, respectively (Fig. 2B).

3.2. Effects of crude and n/t-free hCSE/bCSE on mitochondrial metabolic activity

MTS assay is based on the conversion of a tetrazolium salt into a colored formazan (which is the reduced form of MTS) by mitochondrial activity of living cells and can be used for in vitro evaluation of cell proliferation as well as cytotoxicity.[22] In this study, the decrease in MTS reduction activity by hCSE/bCSE is considered to be due to cytotoxicity rather than the suppression of cell proliferation, since the cells grown in 100% confluent are treated with hCSE/bCSE for 4 h. In addition, short-term and long-term effects of hCSE/bCSE were examined (see Materials and Methods).

As to acute damage, the MTS reducing activity was largely unaffected by crude hCSE and n/t-free hCSE produced by Ploom S at the indicated concentrations (Fig. 3A). In contrast, crude hCSE/bCSE from glo (Fig. 3B), IQOS (Fig. 3C), and hi-lite (Fig. 3D) caused a concentration-dependent decrease in MTS reducing activity in the following rank order of potency: glo hCSE < IQOS hCSE < hi-lite bCSE. The inhibitory effects were attenuated by removing the particulate phase from the mainstream smoke (Fig. 3B–D). The decrease in MTS reducing activity was not due to acute cell death, since no lactate dehydrogenase leakage, a well-known indicator of cell membrane integrity and cell viability,[23] was observe following treatment with crude hCSE/bCSE and n/t-free hCSE/bCSE (supplementary Fig. S1).

In terms of delayed damage, both crude hCSE/bCSE and n/t-free hCSE/bCSE induced a decrease in the MTS reducing activity and the efficacy of n/t-free hCSE/bCSE of each cigarette was less than that of corresponding crude hCSE/bCSE (supplementary Fig. S2).

3.3. Effects of crude and n/t-free hCSE/bCSE on eNOS activity and expression

To clarify whether crude hCSE/bCSE and n/t-free hCSE/bCSE from Ploom S (Fig. 4A–C), glo (Fig. 4D–F), IQOS (Fig. 4G–I) and hi-lite (Fig. 4J–L) caused endothelial dysfunction, the phosphorylation level of eNOS at Ser^{1177}, which is a stimulatory site for eNOS activity,[9,24] and the total eNOS protein level were analyzed using western blotting. Crude hCSE and n/t-free hCSE from Ploom S at concentrations up to 80% had little effect on the protein phosphorylation (Fig. 4B) and expression (Fig. 4C) of eNOS. Crude hCSE (80%) from glo decreased the eNOS phosphorylation level by 75%, and its inhibitory effect was almost abolished by removing the particulate phase (Fig. 4E). In contrast, the total eNOS protein expression was unchanged after treatment with crude hCSE and n/t-free hCSE from glo (Fig. 4F). Crude hCSE and n/t-free hCSE from IQOS at concentrations >40% significantly reduced the eNOS phosphorylation level, and the n/t-free hCSE was significantly less than the crude hCSE was (Fig. 4H). The n/t-free hCSE (80%) from IQOS slightly, but significantly reduced total eNOS protein levels (Fig. 4I). Crude bCSE and n/t-free bCSE induced a concentration-dependent decrease in protein phosphorylation (Fig. 4K) and expression levels of eNOS (Fig. 4L). The rank order of potency of the crude hCSE/bCSE in reducing eNOS activity was as follows: glo hCSE < IQOS hCSE < hi-lite bCSE.

3.4. Effects of n/t-free hCSE/bCSE on NO production

To clarify the effect of hCSE/bCSE on NO production, NO released from EA.hy926 cells was determined by measuring the fluorescence intensity of DAF-2T generated by the reaction of NO with DAF-2.[21] The effect of crude CSE could not be analyzed due to highly background of fluorescence, especially hi-lite bCSE. The fluorescence intensity of DAF-2T due to CaSR-mediated NO production[19] was significantly diminished by treatment with n/t-free hCSE (80%) from IQOS and n/t-free bCSE (80%) from hi-lite, whereas n/t-free hCSE (80%) from Ploom S and glo had no effect (Fig. 5).

3.5. Measurement of n/t-free hCSE/bCSE-induced carbonylation of CaSR-FLAG

We sought to clarify whether n/t-free hCSE/bCSE from Ploom S, glo, IQOS, and hi-lite causes accumulation of carbonylated proteins, which are biomarkers of smoking-related oxidative stress.[25] The carbonylation level of the CaSR that promotes NO production[19] was measured using ARP, which binds to carbonylated proteins.[26,27] In CaSR-FLAG-expressing EA.hy926 cells not treated with hCSE/bCSE, CaSR-FLAG was slightly carbonylated (Fig. 6A). The carbonylation level remained unchanged following treatment with n/t-free hCSE (80%) from Ploom S for 1 h (Fig. 6B). In contrast, n/t-free hCSE/bCSE

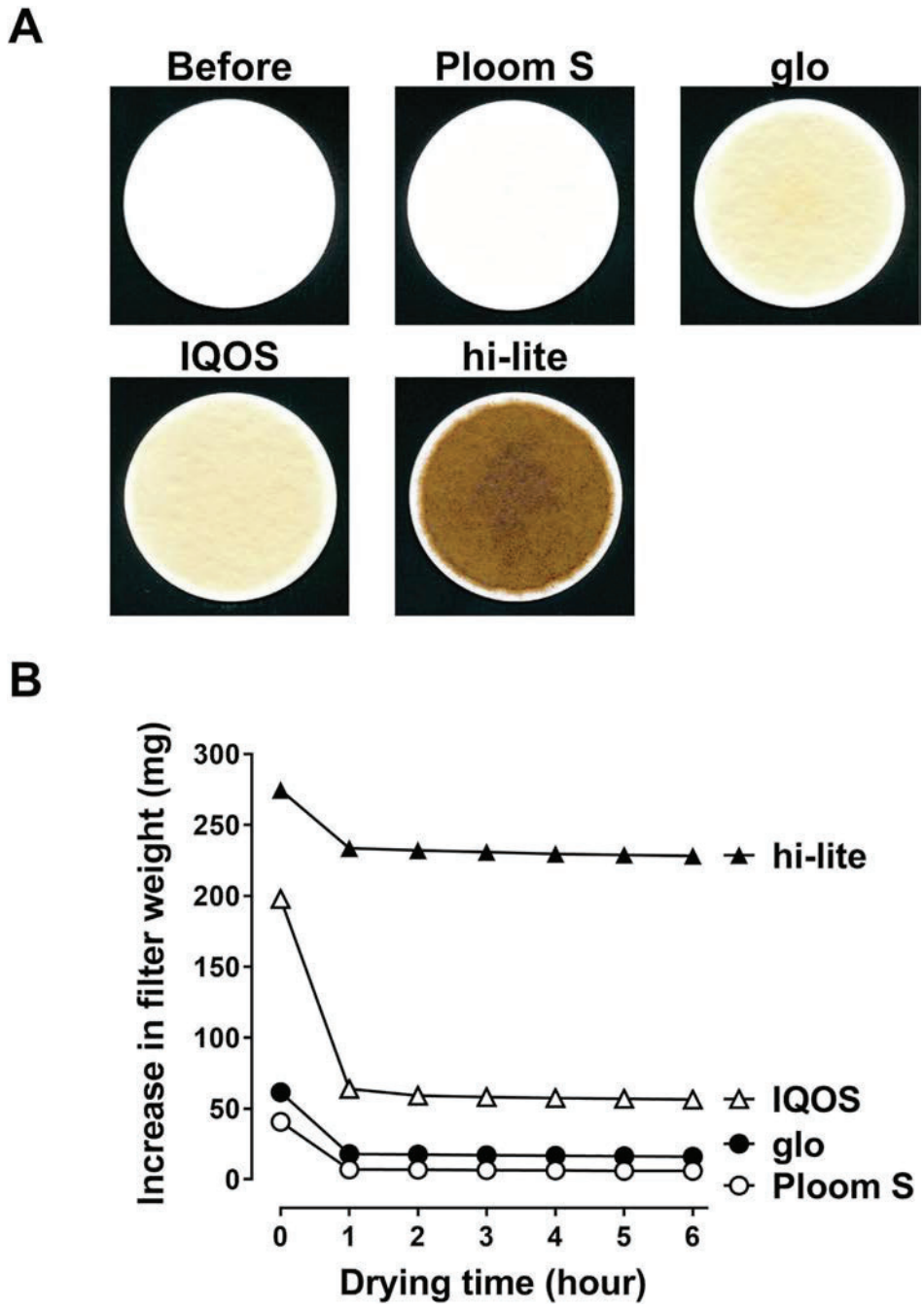

Fig. 2. Measurement of particulate phase trapped on Cambridge filters after collection of mainstream smoke of 91 puffs. (A) Representative images of Cambridge filters before and after collection of mainstream smoke (91 puffs/filter) of HNB cigarettes (Ploom S, glo, and IQOS) and a combustion cigarette (hi-lite). (B) Change in weight of particulate phase trapped on Cambridge filter during drying at approximately 25 °C and <20% relative humidity. Data are means ± standard error of the mean (SEM) of results of five independent experiments. When no error bar is shown, the error is smaller than the symbol.

(80%) from the cigarettes, except for Ploom S, facilitated the carbonylation of CaSR-FLAG with the following rank order of potency: glo hCSE < IQOS hCSE < hi-lite bCSE.

3.6. Effect of removal of carbonyl compounds from n/t-free mainstream smoke

A CX-572 cartridge was utilized to extract carbonyl compounds from mainstream cigarette smoke.[13,28] To prepare n/t- and carbonyl compound-free CSE, mainstream smoke from IQOS and hi-lite was passed through a Cambridge filter and a CX-572 cartridge. Carbonylation of CaSR-FLAG following treatment with n/t-free hCSE (80%) from IQOS and n/t-free bCSE (80%) from hi-lite for 1 h was inhibited by removing carbonyl compounds using the CX-572 cartridge (Fig. 7A and B). In addition, the elimination of carbonyl compounds restored the reduction in the activity and protein expression of eNOS induced by n/t-free hCSE and n/t-free bCSE (Fig. 7C–E).

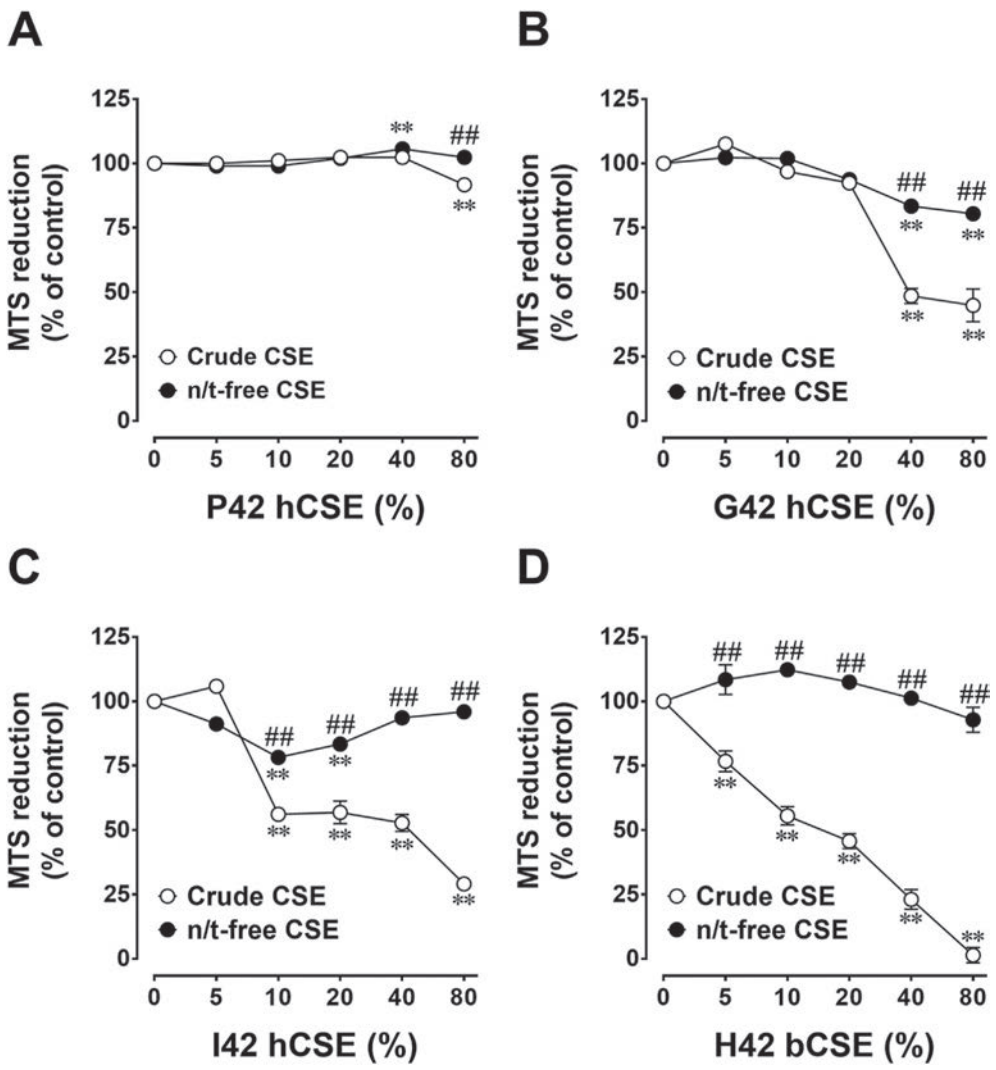

Fig. 3. Short-term effects of crude hCSE/bCSE and n/t-free hCSE/bCSE on MTS reducing activity in human endothelial EA.hy926 cells. Crude and n/t-free cigarette smoke of 42 puffs of Ploom S, glo, IQOS, and hi-lite was bubbled into 5 mL ice-cold Krebs–HEPES solution to prepare individual 100% CSE solution. EA.hy926 cells grown to 100% confluence were washed with warmed phosphate-buffered saline, and then treated with Krebs–HEPES solution with or without the indicated concentrations of CSE for 4 h. For example, when the cells are treated with 80% CSE, the cell culture medium is replaced by a mixture with 800 μL of 100% CSE solution (the solvent is Krebs–HEPES solution) and 200 μL of fresh Krebs–HEPES solution. After treatment for 4 h, MTS reagent was added to each well and the cells were incubated for an additional 2 h. MTS reducing activity of hCSE/bCSE-treated cells was represented as percentage of values of control cells. Data are means ± SEM of results of four to six independent experiments (*$P < 0.05$ and **$P < 0.01$, compared with control cells). When no error bar is shown, the error is smaller than the symbol.

4. Discussion

The use of HNB cigarette products is increasing in Japan.[29] The HNB cigarette products are composed of battery-operated devices and their cigarettes. In contrast to electronic cigarettes that vaporize a liquid containing a variety of chemical substances, including nicotine and glycerol,[16] the devices for HNB cigarettes heat the nicotine-containing cigarettes for inhalation of the resultant aerosol. The heating temperatures, which vary depending on the type of device, are lower than the combustion temperature of approximately 800 °C attained with smoking conventional combustion cigarettes. A comparative study of a HNB cigarette (maximum heating temperature of 350 °C) and a combustion cigarette has shown that the low operating temperature of HNB cigarettes produces a lower concentration of harmful constituents in the mainstream smoke than there is in the smoke of combustion cigarettes.[12] The present study demonstrated that increasing the heating temperature and combustion temperature increased the amount of particulate phase components generated [rank order: Ploom S (200 °C) < glo (240 °C) < IQOS (300–350 °C) < hi-lite (800 °C) (Fig. 2)]. This result is partially supported by evidence showing that the rank order of the content of nicotine generated from HNB cigarettes and combustion cigarettes is 510 μg/cigarette for glo <1200 μg/cigarette for IQOS <1900 μg/cigarette for combustion cigarette (reference cigarette).[13] The particulate phase contains well-known constituents such as nicotine, tar, and tobacco-specific N-nitrosamines (TSNA). Nicotine is the major addictive substance of cigarettes, and a precursor for TSNA which are carcinogens related to cigarette smoking.[4] Tar contains stable free radicals that increase oxidative stress.[6] Our results raise the possibility that the pharmacological and biological effects of HNB cigarette smoke are less than those of combustion cigarettes.

To test this notion, the short-term effects of hCSE and bCSE on mitochondrial metabolic activity were first evaluated using an MTS reduction assay. The short-term effect was defined as a change in MTS reducing activity immediately after treatment with hCSE/bCSE

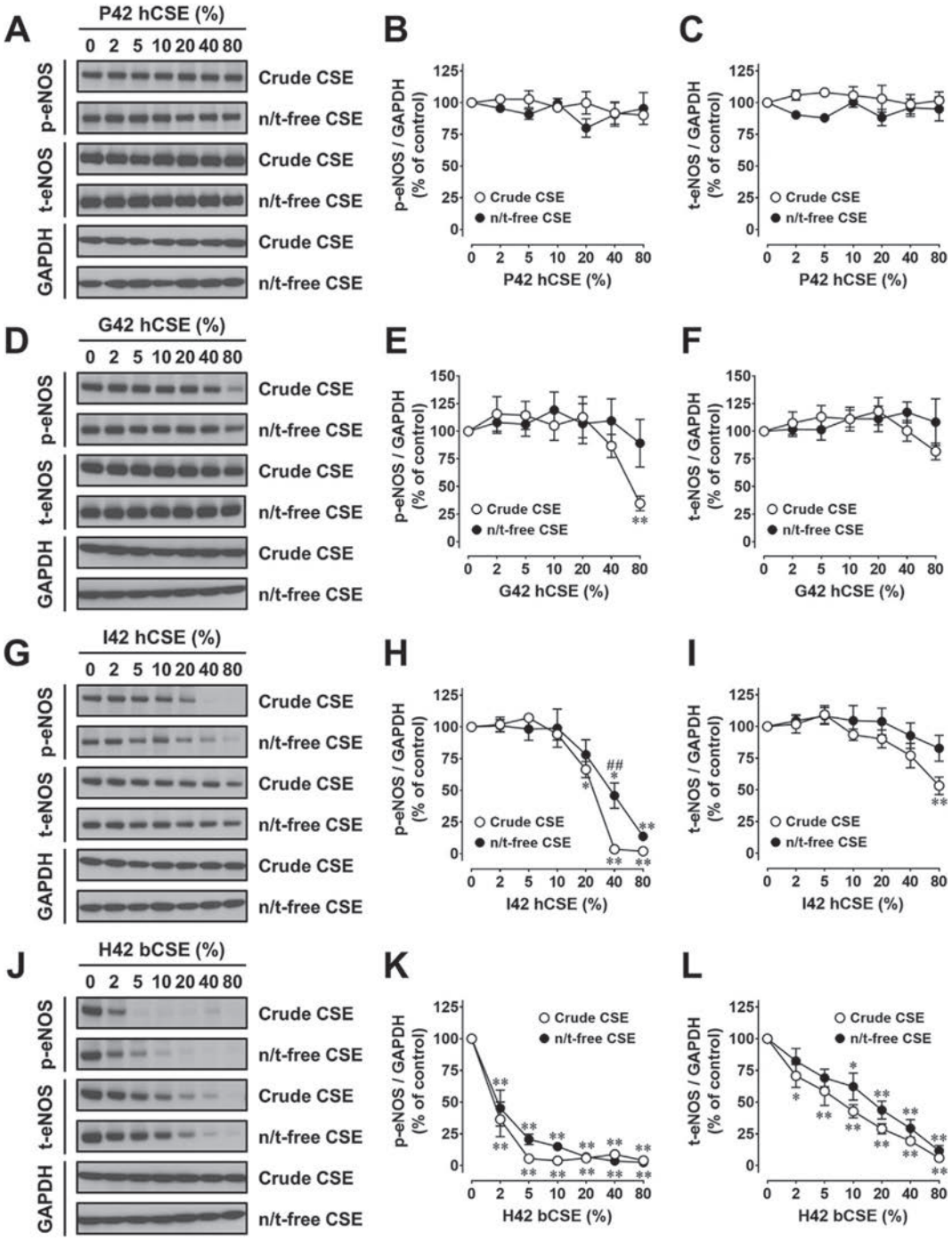

Fig. 4. Effects of crude and n/t-free hCSE/bCSE on eNOS phosphorylation at Ser^{1177} and total eNOS protein expression in human endothelial EA.hy926 cells. Crude and n/t-free cigarette smoke of 42 puffs of Ploom S, glo, IQOS, and hi-lite was bubbled into 5 mL ice-cold Krebs–HEPES solution to prepare individual 100% CSE solution (P42 hCSE for Ploom S, G42 hCSE for glo, I42 hCSE for IQOS, and H42 bCSE for hi-lite). Cells were treated with crude or n/t-free hCSE/bCSE at indicated concentrations for 4 h. (A, D, G, and J) Panels are representative immunoblots with anti-phosphorylated (phospho)-eNOS (Ser^{1177}), anti-total (phospho and nonphosphorylated)-eNOS (t-eNOS), and anti-GAPDH antibodies used to determine levels of phospho-eNOS, t-eNOS, and GAPDH, respectively. (B, E, H, and K) Ordinates indicate changes in relative ratio of expression levels of phospho-eNOS to total GAPDH (phospho-eNOS/GAPDH) in the absence or presence of CSE. The p-eNOS/GAPDH ratio was normalized to ratio of control cells (considered as 100%). Data are mean ± SEM of results of four independent experiments (**$P < 0.01$, compared with control cells; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, compared with corresponding crude CSE). When no error bar is shown, the error is smaller than the symbol. (C, F, I, and L) Ordinates indicate changes in relative ratio of t-eNOS levels to the total GAPDH expression levels (t-eNOS/GAPDH) in the absence or presence of CSE. The t-eNOS/GAPDH ratio was normalized to ratio of control cells (100%). Data are means ± SEM of results of four independent experiments (*$P < 0.05$ and **$P < 0.01$, compared with control cells). When no error bar is shown, the error is smaller than the symbol.

(see Material and Method). The crude hCSE and n/t-free hCSE from Ploom S (up to 80% of hCSE of 42 puffs) had no effect on MTS reducing activity (Fig. 3A), indicating that the concentration of harmful substances in the mainstream smoke from Ploom S was very low. The crude hCSE/bCSE from glo, IQOS, and hi-lite reduced the MTS reduction, at the following rank order of potency: glo hCSE < IQOS hCSE < hi-lite bCSE (Fig. 3B–D). The n/t-free hCSE/bCSE was less cytotoxic than the crude hCSE/bCSE, suggesting the

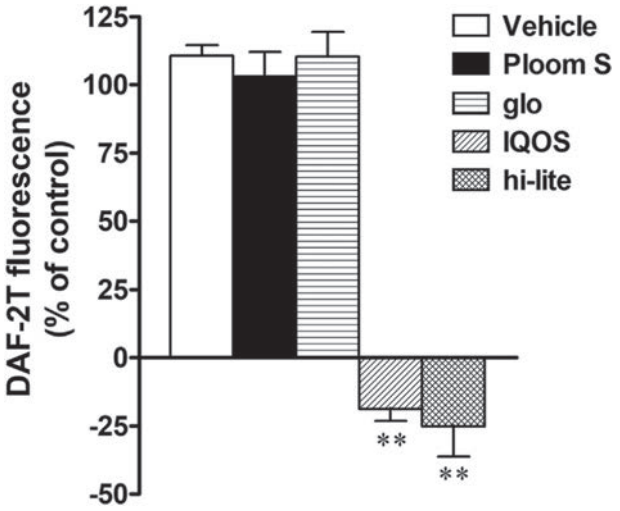

Fig. 5. Effects of n/t-free hCSE/bCSE on NO production in human endothelial EA.hy926 cells. Cells were treated with 80% hCSE/bCSE for 3 h, followed by incubation with DAF-2, which reacts with NO to produce fluorescent triazole adduct DAF-2T for 30 min. Fluorescence intensity of DAF-2T was calculated by subtracting that in the absence of cells from that in the presence of cells. Data are means ± SEM of eight independent experiments (**P < 0.01, compared with vehicle-treated cells).

particulate phase of the mainstream smoke was involved in the inhibition of mitochondrial metabolic activity. However, n/t-free hCSE of glo and IQOS caused a decrease in MTS reducing activity despite the removal of the particle phase from the mainstream smoke. This may be due to HNB cigarette-specific harmful carbonyl compounds, which are generated by the pyrolysis of carbohydrates contained in glycols used in HNB cigarettes. A more recent study showed that in human bronchial epithelial cells, the mainstream smoke of IQOS and combustion cigarette (3R4F reference cigarette) causes a reduction of cell viability with different potencies; that is, 45 puffs for IQOS and 2 puffs for 3R4F cigarette produce a 50% reduction of cell viability.[30] Taken together, these data suggest that an increase in the heating or combustion temperature increases the cytotoxicity of mainstream smoke.

Considering the above-mentioned results of the MTS reduction assay, cytotoxic substances seem to be less in the gas phase of mainstream smoke. Increasing evidence indicates that the gas phase contains harmful carbonyl compounds such as acetaldehyde and ACR,[3] which are generated by the thermal decomposition of carbohydrates contained in tobacco leaves and glycols used in HNB cigarettes.[31] In fact, the results regarding the long-term effects of crude and n/t-free hCSE/bCSE (80% of hCSE/bCSE of 42 puffs) have revealed that all hCSE/bCSE possess cytotoxic activity (supplementary Fig. S2). These findings suggest that cytotoxic factors in the gas phase cause delayed damage rather than acute injury in terms of mitochondrial function; that is, the function of cells exposed to CSE is no longer restored and it deteriorates further. However, no positive correlation was found between the heating or combustion temperature, which affects the generation of harmful carbonyl compounds by thermal decomposition, and the long-term effects of hCSE/bCSE. Therefore, the following experiments focused on the short-term effects of hCSE/bCSE. In addition, n/t-free hCSE/bCSE but not crude hCSE/bCSE was used to analyze NO production and carbonylation of CaSR, because the particulate phase of the mainstream smoke interfered with NO detection with the fluorescent NO indicator, DAF-2, and removal of carbonyl compounds from mainstream smoke with a CX-572 cartridge (data not shown).

Our recent study demonstrated that n/t-free bCSE, which is prepared by continuous aspiration of smoke, and its cytotoxic factor

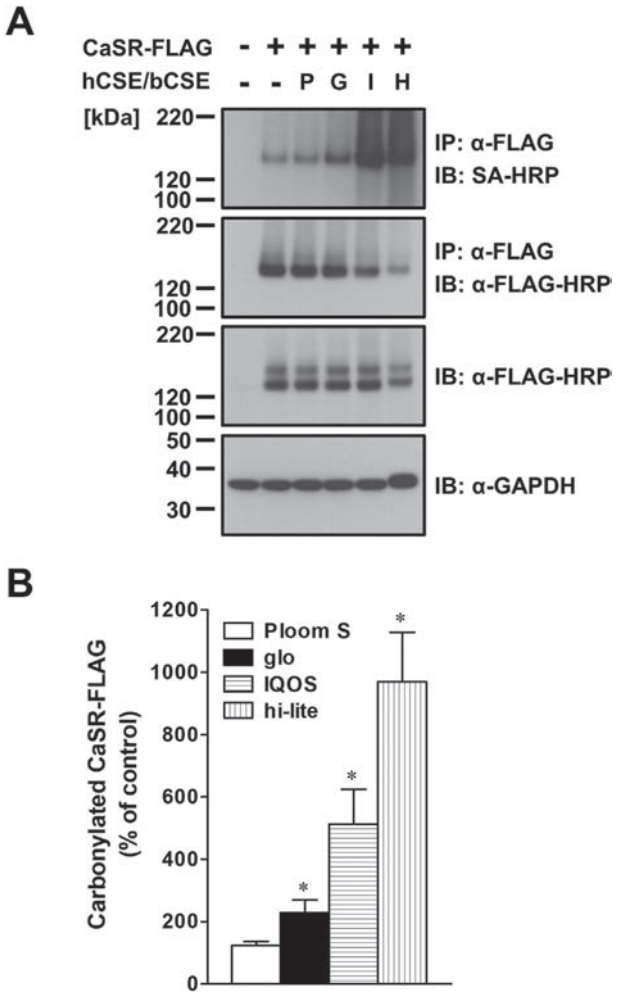

Fig. 6. Carbonylation of CaSR-FLAG proteins stably expressed in human endothelial EA.hy926 cells. Cells were treated with 80% hCSE/bCSE (P, Ploom S; G, glo; I, IQOS; and H, hi-lite) of 42 puffs for 1 h. Carbonylated CaSR-FLAG protein was labeled with ARP, which reacts with aldehyde and ketone groups to add biotin to the protein. Biotin-tagged carbonylated CaSR-FLAG was then detected using SA-HRP. (A) Upper panel is representative immunoblot to determine level of carbonylated CaSR-FLAG protein. Total CaSR-FLAG protein was immunoprecipitated (IP) using anti-FLAG antibody (IP: α-FLAG), and carbonylated CaSR-FLAG protein in immunoprecipitates was detected using SA-HRP (IB: SA-HRP). Second panel is representative immunoblot to determine level of total CaSR-FLAG protein in immunoprecipitates (IP: α-FLAG, IB: α-FLAG-HRP). Third panel is representative immunoblot to determine level of total CaSR-FLAG protein in whole cell lysates (IB: α-FLAG-HRP). Lower panel is representative immunoblot to determine level of total GAPDH in whole cell lysates (IB: α-GAPDH). (B) Histogram represents relative ratio of carbonylated CaSR-FLAG protein levels in hCSE/bCSE-treated cells to that in control cells. Data are presented as means ± SEM of results of four independent experiments (*P < 0.05, compared with control cells).

ACR, a highly reactive α,β-unsaturated aldehyde, decreased eNOS activity and NO production in human endothelial EA.hy926 cells.[9] In bovine pulmonary artery endothelial cells and human pulmonary artery endothelial cells, ACR in cigarette smoke has been reported to increase superoxide anion generation via activation of NOX, resulting in endothelial dysfunction attributable to a reduction in NO bioactivity.[32] The particulate phase (Fig. 2) and ACR[12,13] generated during smoking of HNB cigarettes was lower than that of conventional combustion cigarettes, which suggests that hCSE may have little or no effect on eNOS activity and NO production. Therefore, in the present study, we examined the effects of hCSE/bCSE on the phosphorylation of eNOS at Ser^{1177}, which positively regulates eNOS activity and NO generation.[19,33] The phosphorylation and protein

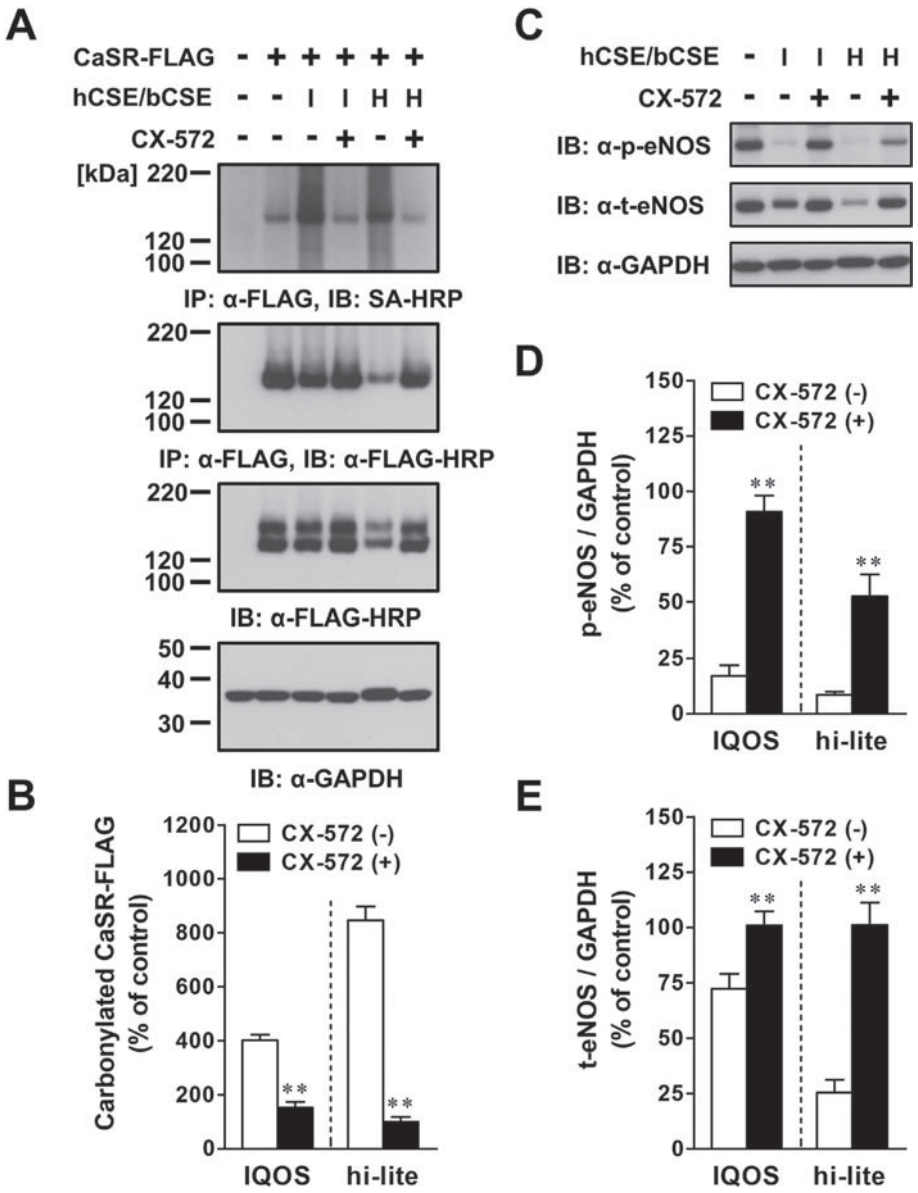

Fig. 7. CX-572 cartridge efficiently removed carbonyl compounds from mainstream smoke from IQOS and hi-lite. (A) Effect of removing carbonyl compounds on hCSE/bCSE-induced increase in CaSR-FLAG carbonylation level. Cells were treated with 80% hCSE/bCSE (I, IQOS and H, hi-lite) of 42 puffs for 1 h. (B) Histogram represents relative ratio of carbonylated CaSR-FLAG protein level in hCSE/bCSE-treated cells to that in control cells. Data are means ± SEM of results of four independent experiments [$^{**}P < 0.01$, compared with corresponding CX-572 (−)]. (C) Effect of removing carbonyl compounds on hCSE/bCSE-induced decrease in phosphorylated eNOS (phospho-eNOS) and total eNOS (t-eNOS) protein expression. Cells were treated with 80% hCSE/bCSE (I, IQOS and H, hi-lite) of 42 puffs for 4 h. (D) Ordinates indicate changes in relative ratio of phospho-eNOS levels to total GAPDH expression levels (phospho-eNOS/GAPDH) following hCSE/bCSE treatment. The p-eNOS/GAPDH ratio was normalized to control cells (considered as 100%). Data are means ± SEM of results of four independent experiments [$^{**}P < 0.01$, compared with corresponding CX-572 (−)]. (E) Ordinates indicate changes in relative ratio of total eNOS (t-eNOS) levels to total GAPDH expression levels (t-eNOS/GAPDH) following hCSE/bCSE treatment. The t-eNOS/GAPDH ratio was normalized to control cells (considered as 100%). Data are means ± SEM of results of four independent experiments [$^{**}P < 0.01$, compared with corresponding CX-572 (−)].

expression of eNOS were unaffected by crude hCSE and n/t-free hCSE from Ploom S (Fig. 4A–C). Indeed, NO production remained undisturbed after treatment with the n/t-free CSE from Ploom S (Fig. 5). These results suggest that the harmful effects of the mainstream smoke from Ploom S on the eNOS activity and NO generation were almost negligible. In contrast, crude hCSE/bCSE from glo, IQOS, and hi-lite reduced eNOS phosphorylation (Fig. 4) with different potencies (rank order: glo < IQOS < hi-lite), indicating that smoking HNB and combustion cigarettes increases the risk for endothelial dysfunction. The risk associated with the use of glo could be reduced by removing the particulate phase from the mainstream smoke because the n/t-free CSE from glo showed no inhibitory effects on eNOS phosphorylation (Fig. 4E) and NO production (Fig. 5). The reduction in eNOS phosphorylation following treatment with crude hCSE/bCSE from IQOS and hi-lite was partially inhibited by removing the particulate phase (Fig. 4). Furthermore, the n/t-free hCSE/bCSE from IQOS and hi-lite decreased NO production (Fig. 5). Indeed, arterial flow-mediated dilation, a measure of vascular endothelial function, has been reported to be impaired by IQOS smoke to the same extent as by combustion cigarette smoke.[15] These results

suggest that cytotoxic factors involved in the inhibition of eNOS activation and NO production are predominantly present in the gas phase of IQOS- and hi-lite-generated smoke. One of the candidates for the cytotoxic factors is ACR, which inhibits eNOS activation and NO production.[9] In fact, some studies have demonstrated that ACR is contained in the mainstream smoke of glo, IQOS, and hi-lite.[8,13]

The gas phase extract (n/t-free bCSE) from hi-lite includes many cytotoxic factors such as ACR[8] and increases oxidative stress.[9] ACR triggers protein carbonylation,[34,35] which is an irreversible oxidative modification.[3,25] The present study showed that n/t-free hCSE/bCSE from the cigarettes, except for that from Ploom S, facilitated carbonylation of CaSR with different potencies (rank order: glo < IQOS < hi-lite, Fig. 6). This observation implies that the carbonyl compounds in the smoke are predominantly formed at temperatures >240 °C. In fact, many varieties of carbonyl compounds, including ACR, have been detected in the mainstream smoke from glo and IQOS.[12,13] The ability of carbonyl compounds to cause protein carbonylation was confirmed by the finding that the CaSR carbonylation was inhibited by treatment with mainstream smoke using a CX-572 cartridge to scavenge carbonyl compounds and volatile organic compounds.[13] The present study with n/t- and carbonyl compound-free hCSE/bCSE demonstrated the involvement of carbonyl compound-induced oxidative stress in reducing the activity or expression of eNOS or both (Fig. 7). The oxidative stress-related endothelial dysfunction may be due to eNOS uncoupling that is associated with decreased cellular BH_4 resulting from the oxidation of BH_4.[36] It also remains to be determined whether the reduction in NO production was due to the carbonylation of CaSR involved in eNOS activation.

In summary, the present study provides evidence that an increase in the temperature during smoking leads to (i) an increase in the generation of the particulate phase and carbonyl compounds and (ii) enhancement of the cytotoxicity of mainstream smoke. Therefore, HNB cigarette products that operate at higher temperatures generate higher levels of harmful constituents.

5. Limitations

Tobacco companies claim that the use of HNB cigarette products reduce exposure to chemicals compared to combustion cigarettes. In the case of conventional cigarettes, the contents of tar and nicotine per cigarette are required by law to be displayed on the outer box of the cigarette, and hi-lite, a combustion cigarette, contains 17 mg of tar and 1.4 mg of nicotine per cigarette. Unlike conventional cigarettes, the method for measuring tar and nicotine levels in HNB cigarettes has not been established. Therefore, tobacco companies cannot provide the levels of tar and nicotine per HNB cigarette. However, it is reported that the tar and nicotine levels in IQOS are 7.47 mg and 0.5 mg per cigarette, respectively, while conventional 3R4F cigarette contains 7.98 mg of tar and 0.71 mg of nicotine per cigarette.[37] To verify the harmfulness and safety of HNB cigarette products, we have to know the relationship between the amount of chemical substances generated from HNB cigarettes and their pharmacokinetic profiles. In addition, long-term health effects of HNB cigarette smoking should be evaluated in the future. Most importantly, it cannot be interpreted that the reduction of harmful substances leads to the reduction of the health risk for users of HNB cigarette products.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jphs.2021.07.005.

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