Adverse Effects of Cigarette Smoke on NO Bioavailability
Role of Arginine Metabolism and Oxidative Stress

Wei-Zhong Zhang, Kylie Venardos, Jaye Chin-Dusting, David M. Kaye

Abstract—Endothelial dysfunction is a hallmark of cardiovascular disease, and the L-arginine:NO pathway plays a critical role in determining endothelial function. Recent studies suggest that smoking, a well-recognized risk factor for vascular disease, may interfere with L-arginine and NO metabolism; however, this remains poorly characterized. Accordingly, we performed a series of complementary in vivo and in vitro studies to elucidate the mechanism by which cigarette smoke adversely affects endothelial function. In current smokers, plasma levels of asymmetrical dimethyl-arginine (ADMA) were 80% higher ($P=0.01$) than nonsmokers, whereas citrulline (17%; $P<0.05$) and N-hydroxy-L-arginine (34%; $P<0.05$) were significantly lower. Exposure to 10% cigarette smoke extract (CSE) significantly affected endothelial arginine metabolism with reductions in the intracellular content of citrulline (81%), N-hydroxy-L-arginine (57%), and arginine (23%), while increasing ADMA (129%). CSE significantly inhibited (38%) arginine uptake in conjunction with a 34% reduction in expression of the arginase transporter, CAT1. In conjunction with these studies, CSE significantly reduced the activity of eNOS and NO production by endothelial cells, while stimulating the production of reactive oxygen species. In conclusion, cigarette smoke adversely affects the endothelial L-arginine NO synthase pathway, resulting in reduced NO production and elevated oxidative stress. In conjunction, exposure to cigarette smoke increases ADMA concentration, the latter being a risk factor for cardiovascular disease. (Hypertension. 2006;48:278-285.)

Key Words: smoking ■ endothelium ■ metabolism

Atherosclerotic coronary and cerebrovascular disease are leading causes of death and disability in the Western world, and cigarette smoking has been clearly identified as a risk factor for coronary artery disease (reviewed by Ambrose and Baruwa8). In this context, measures of endothelial function have been associated with cardiovascular outcome,2 and smoking has been widely associated with reduced endothelial function.3 The endothelium plays a central role in the modulation of vascular tone, the inhibition of platelet aggregation and vascular smooth muscle proliferation, and a key participation in angiogenesis under appropriate conditions. NO is well recognized as playing a pivotal part in these endothelial properties, being produced by the endothelial isoform of NO from its preferred substrate L-arginine. In this context, provision of sufficient L-arginine is critical for the sustained production of NO supply,4 mediated in endothelial cells (ECs) principally by the type 1 cationic amino acid transporter (CAT1). Previous studies indicate that deleterious actions of cigarette smoke on endothelial function could be mitigated by supplemental L-arginine;5 however, to date, the precise basis for this interaction remains unclear. One explanation is that cigarette smoke exerts an inhibitory effect on components of the L-arginine:NO pathway to influence NO production. In addition, potential effects of cigarette smoke on the availability of intracellular L-arginine may also influence the survival of ECs in the setting of oxidative stress and progressive atherosclerosis.6

Cigarette smoke contains a large number of toxic and potentially toxic elements that have the capacity to affect EC function, smooth muscle proliferation, and activation of inflammatory cells in addition to the direct modification of lipids and proteins,1,7,8 in part because of a substantial amount of free radicals.1 As such, cigarette smoke has been reported to induce many cellular effects, including DNA damage and apoptosis, activate protein kinase C, and inducing of interleukin-8 expression.9,10 In the present study, we aimed to evaluate the cellular and molecular mechanism(s) by which cigarette smoke alters endothelial function. Specifically, we investigated the effects of cigarette smoke extract (CSE) on the major pathways of arginine and NO metabolism in endothelial cells using a series of complementary approaches. In particular, we evaluated the effects of CSE on arginine transport and the intracellular concentrations of arginine and related amino acids, in conjunction with an investigation of the actions of CSE on NO synthase (NOS) and NO production, as well as the production of reactive oxygen species (ROS). In conjunction, we evaluated the full plasma concentration profile.

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of L-arginine and related amino acids in smokers in comparison with that in normal control subjects.

**Methods**

**Clinical Samples**

Blood samples were drawn from 12 otherwise healthy male current smokers (defined as ≥20 cigarettes per day) and 10 male nonsmokers for the determination of plasma levels of arginine, citrulline, N-hydroxy-L-arginine (NOHA), and asymmetrical dimethylarginine (ADMA). No subject received any medication, none were obese (body mass index >30), and none had a history of renal impairment. Samples were obtained in the overnight fasted state. Subjects were not provided with specific dietary instruction before the fasting period. Smokers were advised to refrain from smoking during the fasting period, before blood sampling. Plasma cotinine levels were measured as an index of cigarette smoke exposure. Determination of plasma arginine and related metabolites was determined using high-performance liquid chromatography (HPLC) as described below. The study was approved by the Alfred Hospital Ethics Review Committee under guidelines of the National Health and Medical Research Council of Australia. All of the subjects gave written informed consent.

**Cell Culture and Reagents**

Where indicated, human ECs (EA.hy926) or bovine aortic endothelial cells (BAEC, passage 3 to 6) were plated in 6-well, flat bottom, tissue culture plates with DMEM containing 10% FBS and grown to ~90% confluence. All of the chemicals and reagents were purchased from Sigma unless otherwise specified. We performed experiments using 2 types of endothelial cells to avoid the potential for results bias based on 1 type of cell. Similar to BAECs, EA.hy926 cells have also been shown to produce NO and to robustly transport L-arginine.

**Preparation for CSEs**

Five cigarettes (Winfield, ≤16 mg of tar per cigarette; Winfield Tobacco Co) without filters were combusted using a syringe-driven apparatus. The smoke from each cigarette (burned over 5 minutes) was then bubbled through 30 mL of DMEM, which provided a stock concentration of CSE for the concentration-response study; with or without the following treatment reagent(s) in PBS (pH 7.4); with or without the following treatment reagent(s) in PBS (pH 7.4); with or without the following treatment reagent(s) in PBS (pH 7.4); with or without the following treatment reagent(s) in PBS (pH 7.4); with or without the following treatment reagent(s) in PBS (pH 7.4).

**Determination of SE Toxicity and Cell Viability**

The concentration-dependent effect of CSE on endothelial cell viability was initially established. Cells were exposed to CSE that had been prepared as above in culture medium, over a series of CSE concentrations from 0% to 20% for 10 minutes at 37°C in triplicate. For bisindolylmaleimide I ([BIM-I] Molecular Probes). ROS production was measured during and after treatment with 10 nM BIM-I; for 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) treatment, 4 mmol/L DTNB was included in the medium; and for N-acetyl-L-cysteine (NAC) treatment, 10% CSE plus 0.8 mmol/L NAC were included in the medium. At the conclusion of the incubation period, each culture well was washed twice with ice-cold PBS and then lysed in 0.1% Triton X for subsequent liquid scintillation spectroscopy, as well as for protein content analysis as below.

**NOS Activity Assay and Western Analysis**

NOS activity was determined in vitro by determining the generation of citrulline and NOHA by purified endothelial NOS (eNOS) and inducible NOS ([iNOS] Sigma). The reaction mixture consisted of eNOS or iNOS as appropriate, 10 μmol/L BH4, 1 mmol/L dithiothreitol, 10 μg/mL calmodulin, 4 μmol/L flavin adenine dinucleotide, 4 μmol/L flavin mononucleotide, 2 μmol/L L-arginine, 1 mmol/L CaCl2, and 50 mmol/L Tris-HCL (pH 7.4), with CSE at a concentration of 0%, 2%, 4%, 10%, or 20% for concentration-response study; with 10% CSE plus 1 mmol/L NAC for the NAC treatment; and with 2 mmol/L DTNB for the DTNB treatment were included, respectively, in reactions. The reaction proceeded for 20 minutes at 37°C, stopped by adding EDTA with a final concentration of 20 mmol/L. The supernatant was dried under N2 gas and reconstituted with HPLC mobile phase (acetone:isopropanol:water:ACN). The formation of citrulline and NOHA was analyzed as described previously by us. One unit of enzyme produces 1 mmol total of citrulline and NOHA, and the activity was normalized with total cellular proteins, expressed as units per milligram of protein.

After appropriate treatment, ECs were exposed to lysis buffer containing protease inhibitor mixture for 30 minutes at room temperature. Samples were then sonicated for 10 minutes and centrifuged for 10 minutes at 1000 rpm at 4°C. Soluble extracts were collected and stored at −80°C until analysis. Samples (20 μg) were subjected to SDS-PAGE analysis using a 4% to 15% linear gradient gel (Bio-Rad). After transfer to nitrocellulose membranes, immunoblotting was performed for eNOS (1:1000, Becton Dickinson) and iNOS (1:1000, Becton Dickinson). Appropriate secondary antibodies were then used (both at 1:1000), and signal detection was performed by chemiluminescence. Equal sample loading was confirmed by Western analysis for β-tubulin (data not shown).

**NO Production and ROS Production in ECs**

ECs were plated in black 96-well plates with clear bottoms (Viewplates) and were serum deprived 24 hours before treatment. When cells were 95% to 100% confluent, they were treated with either 10% smoke extract in DMEM or various concentrations of hydrogen peroxide (10 μmol/L to 1 mmol/L final) in DMEM for 60 minutes. Control wells had DMEM only. Black seals were added to the bottom of the plates before the addition of any fluorescent probes. NO production was measured during and after treatment with 5 μmol/L 4-aminomethylmalino-2,7'-dichlorofluorescein diacetate ([DAF-FM] Molecular Probes). ROS production was measured during and after treatment with 10 μmol/L 2,7'-dichlorodihydrofluorescein diacetate (Molecular Probes). Oxidation of DAF-FM by NO and oxidation of 2,7'-dichlorodihydrofluorescein diacetate by ROS produces the fluorescent product 2',7'-dichlorofluorescein, which was measured on a fluorescence microplate reader at 495 nm excitation and 538 nm emission. For NO and ROS production during CSE and H2O2 treatment, fluorescent probes were made up in DMEM and were present throughout the treatment incubation time. Fluorescence was measured after 60 minutes of treatment. For measurement of NO and ROS production after treatments, probes were made up in warm PBS. Cells were washed with warm PBS, incubated with the relevant probe for 20 minutes at 37°C, and fluorescence measured. Data are presented as raw fluorescent units and percentage of control raw fluorescent units.

**Dimethylarginine Dimethylaminohydrolase Activity Assay**

For detection of endothelial dimethylarginine dimethylaminohydrolase (DDAH) activity, the endothelial extract was made with the above cultured ECs by washing twice with cold PBS and quickly freezing at ~−70°C for 1 hour, followed by homogenization. Cellular debris was removed by centrifugation at 10 000g for 10 minutes. The DDAH reaction mixture consisted of the cell extract, 5 mmol/L ADMA, with or without the following treatment reagent(s) in PBS (pH 7.4): 0.8 mmol/L NAC plus 10% CSE for the NAC treatment and 4 mmol/L DTNB for the DTNB treatment were included, respectively, in reactions. The reaction was incubated at 37°C for 1 hour and stopped.
by adding cold ethanol (3-fold volume to the reactant). The aliquot was recovered after being spun at 4°C for 10 minutes and dried under N2 gas. The residues were reconstituted with HPLC mobile phase (acetonitrile:methanol:dH2O, 4:3:3, vol/vol/vol) for citrulline quantification with the HPLC method as described previously.15 One unit of enzyme produces 1 nmol of citrulline, and the activity was normalized with total cellular proteins, expressed arbitrarily as units per milligram of protein.

**Evaluation of CAT1 mRNA Expression With RT-PCR**

The total cellular RNA was extracted from the above cultured and with or without CSE-treated ECs with a modified method of Chomczynski and Sacchi.16 RT-PCR amplification of the RNA was used to construct cDNA fragments of CAT1 determined by TaqMan reverse transcription (PE Biosystems) in triplicate for each sample. CAT1 primers and probe sequences were designed using primer express 1.5 with the following primer sequences, 5'-CACTCTCTTCCTTTCCAACCAT-3' and 5'-CTTTCCTCTTTGACCTGAADDA-3'. The Cq was calculated for both cultured ECs treated in the absence and presence of CSE.

**Preparation Cell Lysate for HPLC and Protein Analysis**

We added 0.990 mL of cold ethanol and 10 μL of homoaarginine (1 mmol/L) into each of the above treated wells and bathed at 4°C overnight. All of the residues in each well were harvested, and 20 μL were used to determine the protein content as described below. The remaining was then centrifuged for 10 minutes (10 000g) at 4°C, and the supernatant was freeze-dried under liquid nitrogen for HPLC analysis.

**Determination of Intracellular Contents of Arginine and Related Metabolites With HPLC**

The HPLC method for simultaneous determination of arginine and 7 metabolites was used as described previously by us.15 In brief, the above dried sample was reconstituted with 100 μL of methanol and spun at 10 000×g at 4°C for 15 minutes. Ten microliters of the supernatant were mixed with 70 μL of borate buffer (pH 9.5) and 20 μL of 7.5 mmol/L of orthophthalaldehyde solution, which included 11.5 mmol/L of 3-mercaptopropionic acid, and 20 μL of that mixture was injected into HPLC. A peak ratio of the analyte to homoaarginine (used as an internal standard) was used together with a constructed standard curve for quantification. All of the analytes were normalized with total cellular proteins expressed as nanomoles per milligram of protein.

**Protein Assay**

Protein concentrations were measured by Bio-Rad protein assay kit (Bio-Rad) using BSA as a standard.

**Statistical Analysis**

Data are presented as mean±SD. All of the experiments were performed in triplicate, unless otherwise indicated. Between-group comparisons were performed using an unpaired Student t test. A 2-sided P<0.05 was considered statistically significant.

**Results**

**Effect of Smoking on Plasma Arginine and Related Metabolites**

Consistent with their history of cigarette smoking, the plasma level of cotinine in the smoking group was 1277±182 nM, whereas it was not detectable in any of the nonsmokers. As demonstrated in Table 1, the plasma levels of ADMA were significantly elevated in smokers compared with nonsmokers, whereas plasma levels of citrulline and NOHA were lower in smokers. Plasma arginine tended to be lower in smokers, although the difference was not statistically significant.

**Effects of CSE on Intracellular Arginine, Related Metabolites, and Arginine Transport**

It has been reported previously that CSE has a strong direct cytotoxic effect on the alveolar L2 cell.17 Accordingly, we first investigated the cytotoxicity of CSE to ECs. At a CSE concentration >50% in the medium, no viable ECs were found after 24-hour exposure. In contrast, at a CSE concentration of 25%, more than half of the EC population was adhesive to the culture dishes and viable (positive with the trypan blue exclusion test). Treated with 10% CSE, ECs showed no significant loss of adhesion or morphological change, and they showed a >99% viability. Accordingly, 10% CSE was used for cell culture studies unless otherwise indicated.

To study the effect of CSE exposure on arginine metabolism, with particular emphasis on the NO pathway, we analyzed arginine metabolic profiles in human endothelium cells in the absence or presence of CSE. The profiles consisted of intracellular concentrations of citrulline, NOHA, arginine, Nε-monomethyl-arginine (NMMA), ADMA, symmetric Nε,Nε-dimethyl-arginine, ornithine, and agmatine. We found that 24-hour exposure to CSE elicited a significant reduction in the intracellular concentrations of arginine, citrulline, NOHA, and SDMA indicates symmetric dimethylarginine. Data from 8 separate experiments.

**TABLE 2. Intracellular Concentrations (nmol/mg Protein) of Arginine and Related Metabolites**

<table>
<thead>
<tr>
<th>Group</th>
<th>Citrulline</th>
<th>NOHA</th>
<th>Arginine</th>
<th>NMMA</th>
<th>ADMA</th>
<th>SDMA</th>
<th>Ornithine</th>
<th>Agmatine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.654±0.392</td>
<td>0.316±0.200</td>
<td>39.086±7.428</td>
<td>0.026±0.015</td>
<td>0.044±0.016</td>
<td>0.071±0.044</td>
<td>1.070±0.943</td>
<td>0.096±0.054</td>
</tr>
<tr>
<td>CSE</td>
<td>0.124±0.047*</td>
<td>0.137±0.058*</td>
<td>30.803±5.713*</td>
<td>0.026±0.018</td>
<td>0.101±0.065*</td>
<td>0.102±0.067</td>
<td>0.562±0.188</td>
<td>0.046±0.025*</td>
</tr>
</tbody>
</table>

*P<0.05 vs control group.
agmatine and significant increase in that of ADMA (Table 2). No significant changes in the abundance of NMMA, symmetric NG,NG-dimethyl-L-arginine, or ornithine were apparent.

To determine the potential mechanism(s) by which the intracellular content of L-arginine was reduced by exposure to CSE, we next determined the effect of CSE exposure on 3H-L-arginine transport in ECs. Short-term (60 minutes) exposure to 10% CSE reduced 3H-L-arginine uptake by EAhy926 cells to 69% of that in control cells (P<0.05; Figure 1a). By comparison, exposure to 10 mmol/L L-lysine, which competes with arginine for transport, reduced 3H-L-arginine to 8% of baseline values. To exclude the possibility that these short-term actions of CSE on 3H-L-arginine were selective for EAhy926 cells, the effect of CSE was also examined in BAECs. In keeping with the studies in EAhy926 cells, 60 minutes of exposure to CSE also significantly reduced BAEC 3H-L-arginine uptake to 71% of control values (P<0.01). Exposure of ECs to 100 mmol/L H2O2 was entirely without effect on 3H-L-arginine uptake (data not shown).

To complement these studies, we also examined the influence of longer-term (24 hour) CSE exposure. In these studies, there was a 38% reduction in the accumulation of 3H-L-arginine (Figure 1b). In conjunction, as determined by RT-PCR analysis, there was a 34% reduction in CAT1 mRNA expression (P<0.01; Figure 1c).

In an attempt to investigate the possible role of ROS in the inhibitory action of CSE on arginine transport, we evaluated the effects of antioxidant and oxidizing agents of 3H-L-arginine transport. As shown in Figure 2, the inhibitory action of CSE on arginine transport was attenuated by combination with N-acetyl cysteine (NAC), a cell-permeable monothiol reducing agent. This observation was supported by the demonstration that DTNB, a cell-impermeable dithiolo oxidizing agent, significantly reduced (P<0.05) arginine uptake, consistent with a potential role for an active thiol residue in the CAT1 transporter.

Given the demonstration that the intracellular content of ADMA was increased in ECs exposed to CSE and that the plasma concentration of ADMA is increased in the plasma of smokers, we also examined the effect of CSE on DDAH activity. As demonstrated in Figure 3, CSE inhibited DDAH activity in a similar manner to that for that on the arginine transporter, and this was rescued by NAC and mimicked by DTNB.

**Effect of CSE on NOS Activity and NO Production**

Having demonstrated that the endothelial transport of arginine is reduced in the presence of CSE and that the intracel-
The intracellular content of NOHA (the NO intermediate) in ECs is reduced by CSE, we also evaluated the effect of CSE on NOS enzymatic activity and directly on NO production using the NO-sensitive fluorochrome, DAF. As shown in Figure 4a, CSE exerts a clear inhibitory effect on eNOS enzymatic activity in a concentration-dependent manner, whereas iNOS appeared relatively resistant to the effects of CSE. Consistent with this observation, CSE exposure for a period of 1 hour significantly inhibited the production of NO by ECs, whereas this effect was not reproduced by exposure to H2O2 (Figure 4b). The magnitude of reduction in NO generation after CSE exposure was similar to that resulting from exposure to H2O2 (Figure 4b). The magnitude of reduction in NO generation after CSE exposure was similar to that resulting from exposure to the NOS inhibitor L-NAME (88%±1%; P<0.01). Although CSE reduced NO production, it did not alter the expression of eNOS (Figure 4c), and iNOS remained undetectable (data not shown). Given the apparent lack of a direct effect of H2O2 on NO production, we considered whether the influence of CSE could be mediated via another reactive species, potentially contributing to the modification of key thiol groups of NOS. Figure 5 demonstrates the ability of NAC to attenuate the direct inhibitory effect of CSE on NOS enzymatic activity, whereas exposure to DTNB recapitulates the action of CSE.

Modulation of Oxidative Stress by Exposure to CSE in ECs
To evaluate whether the ROS-mediated effects of CSE exposure on arginine transport and NOS activity were mediated directly by the presence of free radicals within cigarette smoke itself or, alternatively, whether CSE exposure led to a state of increased oxidative stress in ECs, we determined ROS production after 60 minutes of exposure to CSE. Using the ROS-sensitive fluorochrome, we found that exposure to CSE significantly increased ROS production, whereas in contrast, exposure to H2O2 did not alter subsequent ROS production (Figure 6).

Discussion
Endothelial dysfunction is a key feature of many cardiovascular diseases and their attendant risk factor states including hypertension, atherosclerosis, heart failure, diabetes, smoking, and chronic renal failure. Importantly, recent studies incorporating physiological and biochemical measures of endothelial integrity have shown that a clear link between endothelial dysfunction and cardiovascular outcome exists. In regard to smoking, CSE has been shown to directly impair endothelial function production, although the mechanism remains uncertain. Understanding the mechanism(s) responsible for this effect is of major clinical importance, having the potential to identify new therapeutic targets within the endothelium.
In the present study, we aimed to more comprehensively evaluate the effect of CSE exposure on critical elements of the endothelial L-arginine–NO pathway, including arginine transport, NOS activity, and production, as well as key related metabolic pathways, particularly related to ADMA. Previous studies suggest that the effect of CSE may relate in part because of reduced NOS expression and enzymatic activity.\textsuperscript{13,22–24} Alternatively, Hutchison et al\textsuperscript{25} showed previously that arginine supplementation attenuated the endothelial dysfunction caused by exposure to cigarette smoke, suggesting that substrate availability for NOS may be a key mechanism for CSE-induced endothelial dysfunction.

Maintenance of normal arginine influx and metabolism is crucial for the continued production of NO by ECs. In this regard, the cationic amino acid transporter, CAT1, is one of the major transporters for endothelium cell. It is an integral membrane protein with 14 trans-membrane domains, with intracellular N and C termini.\textsuperscript{25} CAT1 protein is predicted to contain extracellular 15 cysteine residues.\textsuperscript{25,26} The constitutive CAT1 transporter localizes in the caveolae of ECs and can form a functional unit with eNOS at the caveolar membrane.\textsuperscript{27} In the present study, we found that CSE exposure exerted multiple actions, including the acute reduction in EC L-arginine transport and a more chronic reduction in arginine transport, accompanied by diminished CAT1 mRNA. Of note, it has been shown previously that arginine deprivation and cellular stress normally result in increased CAT1 mRNA expression,\textsuperscript{28} in contrast to the present study. In a similar manner, we have shown previously that CAT1 mRNA is significantly decreased in association with decreased arginine uptake in heart failure patients.\textsuperscript{29} The rapid actions of CSE suggest a posttranslational modification of CAT1, possibly involving 1 or more cysteine residues given the ability of N-acetyl cysteine to attenuate the effects of CSE and DTNB, a dithiol oxidizing agent, to mimic the actions of CSE. To our knowledge, however, this potential posttranslational mechanism for the regulation of CAT1 has not been reported previously.

Given the complex nature of CSE, we did not attempt to specifically identify the compound responsible for this action. Previous studies indicate that hydroxyl radicals are present in CSE in substantial quantities.\textsuperscript{30} This observation and the suggestion that the actions of DTNB are very similar to that of hydroxyl radicals\textsuperscript{31} raise the possibility that the rapid actions of CSE on arginine transport might be because of hydroxyl radicals. In a similar manner, it has been reported that \( \alpha \), \( \beta \)-unsaturated aldehydes, which are abundantly present in CSE, are highly reactive toward thiol, directly forming sulphydryl-ether linkages.\textsuperscript{32} Alternatively, protein kinase \( \mathrm{C} \)–mediated modification of arginine transport has been demonstrated previously.\textsuperscript{33} and CSE has been shown to activate protein kinase \( \mathrm{C} \) in non-ECs.\textsuperscript{34} However, we did not observe any diminution of the effects of CSE on arginine transport during coincubation with the protein kinase \( \mathrm{C} \) inhibitor BIM-I (data not shown).

As described above, we found that CSE was able to directly reduce eNOS enzymatic activity in a concentration-dependent fashion, in contrast to iNOS, which was not significantly affected. In concert, NO production was reduced by intact ECs, as assessed by DAF fluorescence measurements and by the intracellular concentration of the NO intermediate, N-hydroxy arginine. Moreover, this effect was blunted by NAC and was recapitulated by exposure to DTNB. Our findings in regard to the direct actions of CSE on NOS enzymatic activity are consistent with studies that predict that the catalytic domain of human eNOS contains 10 cysteine residues with 2 of them (Cys-94 and Cys-99) being responsible for coordinating an intermolecular interaction between 2 monomers.\textsuperscript{35} Although our observations are also consistent with previous reports,\textsuperscript{13,22–24} some longer-term studies\textsuperscript{13,22–24} suggest that a reduction in NOS activity parallels to some extent a decrease in eNOS content. In our study, we saw a more rapid onset of the inhibitory effect of CSE on NOS activity, using isolated purified eNOS enzyme. In our study, we did not observe a change in eNOS abundance during the 60-minute incubation, in which changes in NO production were already seen. In regard to the production of NO per se, however, it is not possible to determine the relative contribution of inhibition of NOS activity and the limitation of substrate availability. Of interest, we found that whereas exposure to CSE reduced the intracellular content of both NOHA and citrulline, consistent with reduced NOS activity and reduced arginine availability, the magnitude of reduction in citrulline was relatively more than NOHA. This may perhaps be explained by the citrulline–arginine cycle, reflecting the ability of many nonhepatic cells to convert citrulline to arginine.\textsuperscript{36} ECs may also express the neuronal isoform of NOS,\textsuperscript{37} although its role in endothelial NO production remains unclear and, accordingly, we did not test the effects of CSE on the neuronal isoform of NOS per se.

In the current study, we also found that after short-term exposure of ECs to CSE, production of ROS as measured by 2′,7′-dichlorofluorescein fluorescence was significantly elevated. Currently, key reactive species are considered to include hydrogen peroxide, the hydroxyl radical, superoxide, and peroxynitrite.\textsuperscript{38} Our data indicate that hydrogen peroxide did not recapitulate the effects of CSE in terms of the promotion of ROS production, suggesting that it was not the likely triggering component for elevated endothelial oxidative stress. In regard to the increased production of ROS after CSE exposure, we did not seek to specifically identify the cellular source. Well-recognized potential sources include NADPH oxidase, xanthine oxidase, uncoupled mitochondria, and uncoupled NOS.\textsuperscript{39} Given our finding that CSE reduced the intracellular content of arginine, it is possible that relative NOS uncoupling could have contributed to increased ROS generation.

In conjunction with our observations on the effects of CSE on arginine transport and NO and ROS production, we also demonstrated that CSE increased the intracellular content of ADMA in ECs. ADMA has been recognized as a risk factor for vascular diseases\textsuperscript{40} and a maker of oxidative stress.\textsuperscript{40} In conjunction, elevated concentrations of ADMA are reported to diminish NOS activity,\textsuperscript{39} although this remains controversial and may correlate in some circumstances with the severity of endothelial dysfunction.\textsuperscript{41} Our results are, therefore, in concord with the hypothesis that oxidative stress can stimulate ADMA production\textsuperscript{42} via inhibition of DDAH ac-
tivity. Indeed, our study showed that the effects of CSE on DDHA activity could be attenuated by antioxidants. Moreover, this finding suggests that certain constituents of cigarette smoke, such as nicotine, would not explain the effects observed in our study. In regard to the potential mechanism for the action of CSE on DDHA activity, it has been shown that DDHA contains a reactive cysteine residue (Cys-249) at the active site and that this can be regulated by S-nitrosylation. Of note in the present study, although we found that whereas CSE caused a change in the cellular content of ADMA, NMMA levels were not changed. This may be because of its much lower content and/or because DDHA is more sensitive to ADMA than to NMMA with Michaelis constant values of 0.18 and 0.36 mmol/L, respectively.41

Consistent with findings on the intracellular levels of ADMA and the effects of CSE on DDHA activity, we found that the plasma levels of ADMA were substantially higher in smokers than those in nonsmokers, whereas plasma levels of citrulline, NOHA, and arginine were lower. The effect of smoking on plasma levels of ADMA has not been well characterized. In a large cross-sectional study, Eid et al41 suggested that ADMA levels were lower in smokers; however, this study was performed in an elderly population, in which there was a high frequency of other risk factors for cardiovascular disease, making any direct interpretation on the effect of smoking, per se, on ADMA levels impossible.

Interestingly although our study provides evidence for significant changes in the metabolism of arginine and NO, we did not observe a significant difference in resting blood pressure in smokers compared with controls. Endothelial dysfunction has been described in many cardiovascular conditions, including atherosclerosis and diabetes, for example, which alone do not necessarily cause hypertension, although it may facilitate the development of hypertension in conjunction with other environmental and genetic factors. Of note, even in the eNOS knockout mouse, blood pressure is only modestly elevated.46 This underscores the fact that vascular tone depends on the balance of vasodilators and vasoconstrictors, in conjunction with other key regulatory mechanisms, including the kidney and sympathetic nervous system.

Perspectives

Our study was designed to provide a comprehensive overview of the potential mechanisms that contribute to smoking-induced endothelial dysfunction and, potentially, into the pathogenetic process by which smoking causes atherosclerosis. In particular, we sought to demonstrate that alterations in L-arginine transport and metabolism, together with changes in NOS activity and ROS production, may occur concurrently, to ultimately alter NO bioavailability. Although our findings provide new biological insights into the basis for endothelial dysfunction, the fact remains that active cigarette smoking disturbs the integrity of the L-arginine: NO pathway at multiple key regulatory points. Therefore, our study provides further insight into the mechanisms responsible for the deleterious effects of cigarette smoking on endothelial function and also provides a mechanistic basis for a link between smoking and elevated ADMA levels in the context of cardiovascular disease.

Conclusions

The current study provides complementary observations performed in humans with detailed in vitro evidence that cigarette smoking markedly disturbs the integrity of the L-arginine: NO pathway at multiple key regulatory points. Therefore, our study provides further insight into the mechanisms responsible for the deleterious effects of cigarette smoking on endothelial function and also provides a mechanistic basis for a link between smoking and elevated ADMA levels in the context of cardiovascular disease.

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Disclosures

None.

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