Reactive Oxygen Species Are Involved in Smoking-Induced Dysfunction of Nitric Oxide Biosynthesis and Upregulation of Endothelial Nitric Oxide Synthase

An In Vitro Demonstration in Human Coronary Artery Endothelial Cells

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Background—Our group has previously shown that human umbilical vein endothelial cells exposed to smokers’ serum decreased nitric oxide (NO) production and endothelial nitric oxide synthase (eNOS) activity in the presence of increased eNOS expression. In the present study, we examined whether these observations extended to human coronary artery endothelial cells (HCAECs). In addition, the role of reactive oxygen species in the observed alterations was examined.

Methods and Results—HCAECs were incubated with serum from 10 nonsmokers and 15 smokers for 12 hours with or without the addition of either polyethylene glycol-superoxide dismutase (PEG-SOD, 300 U/mL), PEG-SOD+PEG-catalase (1000 U/mL), chelerythrine (3 μmol/L), or tetrahydrobiopterin (20 μmol/L). At the end of incubation, NO, eNOS protein, and eNOS activity were measured from the same culture. HCAECs incubated with smokers’ serum alone showed significantly lower NO production (P<0.05) and eNOS activity (P<0.005) but higher eNOS expression (P<0.005) compared with nonsmokers. In smokers, addition of PEG-SOD, PEG-SOD+PEG-catalase, or tetrahydrobiopterin significantly (P<0.05) improved NO levels and eNOS activity. Interestingly, in the same smokers, a significant decrease in eNOS expression was only seen with the addition of PEG-SOD+PEG-catalase (P<0.05) and treatment with PEG-SOD alone insignificantly increased eNOS expression.

Conclusions—The present study indicates that in vitro, HCAECs show similar changes in NO biosynthesis as human umbilical vein endothelial cells when exposed to smokers’ serum and also confirms that oxidative stress plays a central role in smoking-mediated dysfunction of NO biosynthesis in endothelial cells. Furthermore, these data support other studies suggesting a role for hydrogen peroxide in the upregulation of eNOS. (Circulation. 2003;107:2342-2347.)

Key Words: smoking • nitric oxide • nitric oxide synthase • oxidative stress

Dysfunctional nitric oxide (NO) biosynthesis, as indicated by reduced endothelial-dependent vasodilatation, precedes the morphological changes in the vessel wall associated with various atherosclerotic risk factors, including smoking.¹ ³ In a recent investigation, our group demonstrated that smoking was associated with reduced endothelial-dependent vasodilatation and that the serum from these same smokers decreased NO availability and endothelial nitric oxide synthase (eNOS) activity but upregulated eNOS protein in human umbilical vein endothelial cells (HUVECs).³ Although HUVECs are a well-recognized model for investigating endothelial function, they are of venous origin. Because atherosclerotic changes are uncommon in veins, a study in arterial endothelial cells was warranted for a more appropriate pathophysiological interpretation of our previous observations.

Excessive generation of reactive oxygen species (ROS) such as superoxide (O₂⁻), hydrogen peroxide (H₂O₂), and their products has been implicated as a final common pathway for the development of endothelial dysfunction by various cardiovascular risk factors, and ROS may be responsible for the observed changes in NO biosynthesis, including the upregulation of eNOS.⁴–⁶ Specifically, recent studies have proposed H₂O₂ as a potential inducer of eNOS upregulation.⁶ The protein kinase C (PKC) pathway has also been implicated in increasing eNOS expression,⁷ as well as increasing O₂⁻ and H₂O₂ production, especially in the setting of diabetes.⁸ ⁹ However, a fundamental pathophysiological difference between smoking and other risk factors is that, in cigarette smoking, a substantial amount of free radicals are introduced exogenously and cigarette smoke contains >4000 known and >100 000 unknown constituents.¹⁰ Therefore, it

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is unknown whether these same mechanisms are also involved in the smoking-mediated dysfunction of NO biosynthesis.

The aims of the present study were to extend our previous observations in an arterial cell line (human coronary artery endothelial cells, HCAECs) and, with the use of cell-permeable-polyethylene glycol-superoxide dismutase (PEG-SOD), PEG-SOD+PEG-catalase, chelerythrine (a PKC inhibitor), and tetrahydrobipterin (BH₄), to investigate the potential role of free radicals in the observed smoking-related alterations of the NO biosynthetic pathway.

Methods

Subjects and Study Design

Men with a history of active smoking (n=15) were matched for age and sex with individuals who had never smoked (n=10). All subjects were free of other cardiovascular risk factors (ie, hypertension, diabetes, low HDL cholesterol, hyperlipidemia, or a family history of premature vascular disease) and were not taking any medication.

Subjects were requested to abstain from smoking and caffeinated food or drink overnight (6 to 8 hours). The next morning, venous blood was collected, and the serum was isolated and stored at −70°C until use. Analysis for the lipid profile and glucose was done in the hospital’s clinical laboratory, and the serum cotinine level was determined by a commercially available kit (STC Technologies). The study protocol was approved by Saint Vincent Catholic Medical Centers of New York Ethics Committee, and written informed consent was obtained from each subject.

Endothelial Cell Culture and Treatment

HCAECs were purchased from Clonetics and were cultured using a slight modification of a method described previously. In brief, 40,000 cells were plated in 12-well, flat bottom, tissue culture plates with complete endothelial growth media (EGM; Clonetics) and grown to ~85% confluence at 37°C in 5% CO₂. The confluent cells were then incubated with an equal volume of serum and EGM (ie, 400 μL of serum and 400 μL of EGM; total volume, 800 μL) for 12 hours (37°C; 5% CO₂), with or without the addition of PEG-SOD (300 U/mL), PEG-SOD+PEG-catalase (1000 U/mL), chelerythrine (3 μmol/L), or BH₄ (20 μmol/L). All treatments (PEG-SOD, PEG-catalase, BH₄, and chelerythrine) were made fresh before the experiments to their desired concentrations by dissolution in EGM. In the respective wells, HCAECs were pretreated for 30 minutes with these substances before the serum was added.

All experiments were done within the second passage in our laboratory after obtaining cells from the vendor (third passage). Each set of experiments in smokers and nonsmokers (with or without treatment) were done simultaneously using the same batch of HCAECs.

Determination of NO Levels

After a 12-hour incubation, NO levels in the cell-culture supernatants were determined by the chemiluminescence method using a NO analyzer (Sievers, Model 280), as previously described. The NO level in each sample was expressed as μmol/L per mg of total protein after adjusting for the background NO level in each sample and the total protein concentration in each well.

Determination of eNOS Protein Concentration

The eNOS protein concentration of the HCAECs in culture was determined in each experiment by using a commercial ELISA kit (R&D Systems). The cells that were used to detect 12-hour NO production were washed twice with phosphate buffer, and cells were lysed with lysis buffer from the kit. The assay was performed using 100 μL of each cell lysate by following the manufacturer’s instructions. All samples were tested in duplicate and expressed as pg/mg total protein after adjusting for the total protein concentration in each well.

Determination of Total Protein Concentration

The total protein in each well was determined from the same cell lysates generated for the eNOS protein using the micro-Lowry method, with a slight modification, and by using a commercial kit (Sigma). In brief, 100 μL of each standard and samples were added to a 96-well plate (in duplicate). Ten microliters of desoxycholate (1.5 mg/mL) solution was added to each well, and the plate was incubated on a horizontal shaker at room temperature for 10 minutes; this was followed by the addition of 10 μL of trichloroacetic acid (72%) solution. The plate was centrifuged at 210g for 5 minutes, and the supernatant was discarded without dislodging the pellet. Then, to each well, 100 μL of Lowry reagent was added, mixed, and incubated at room temperature for 20 minutes. At the end of the incubation period, 50 μL of Folin and Ciocalteu’s phenol red reagent was added to each well and incubated again at room temperature for 30 minutes. The absorbance was read at 620 nm. The protein concentrations of the unknown samples were extrapolated from the calibration curve constructed with the known standards.

Determination of eNOS Activity

The same cell lysates generated for the eNOS protein assay were used to determine the specific eNOS activity. This was done using a commercial kit (Calbiochem) and a method described previously. In brief, 100 μL of lysate was added to a reaction cocktail containing 25 μL of reaction buffer (50 mmol/L Tris-HCl, 6 μmol/L BH₄⁻, 2 μmol/L flavin adenine dinucleotide, and 2 μmol/L flavin adenine mononucleotide), 3 μL of [1H]-L-arginine (58 Ci/mmol, 1 μCi/μL; Amersham), 1 mmol/L NADPH, 75 μmol/L CaCl₂, and 0.1 μmol/L calmodulin. The incubation was performed at room temperature for 1 hour. The radioactivity was quantified using a liquid scintillation counter (Packard Instrument). Both eNOS concentration and eNOS activity assays were run simultaneously. The eNOS activity in each sample was adjusted to the specific amount of eNOS protein per milligram of total protein, and the specific activity of eNOS was expressed as picomolar-liters of citrulline per minute per picogram of eNOS protein per milligram of total protein.

Statistical Analysis

Data were normalized with linear transformation where appropriate and presented as the mean±SEM. The baseline values between nonsmokers and smokers were compared using the unpaired Student’s t test. The effect of various treatments between and within groups was compared either by ANOVA or repeated measures ANOVA where appropriate. For each ANOVA or repeated measures ANOVA, differences between individual treatments were compared with post hoc Fisher’s protected least significant difference (PLSD) or paired Student’s t test where appropriate. P<0.05 was considered statistically significant. All calculations were performed using the StatView statistical program (version 4.5, Abacus Concepts Inc).

Results

Clinical Characteristics of the Study Population

Baseline clinical characteristics of both smokers and nonsmokers are shown in the Table. There was no significant difference between the 2 groups with the exception of serum cotinine levels, which were significantly higher in smokers.

Effects of Smokers’ Serum and Various Treatments on NO Level

HCAECs exposed to smokers’ serum alone showed a significantly lower NO level in the cell culture supernatant compared with those exposed to serum from nonsmokers (Figure 1: 0.12±0.04 versus 0.31±0.10 μmol·L⁻¹·mg⁻¹ of total protein; P<0.05).
In nonsmokers, the addition of either PEG-SOD (0.35±0.20 μmol·L⁻¹·mg⁻¹ of total protein), PEG-SOD+PEG-catalase (0.24±0.10 μmol·L⁻¹·mg⁻¹ of total protein), chelerythrine (0.33±0.20 μmol·L⁻¹·mg⁻¹ of total protein), or BH₄ (0.39±0.30 μmol·L⁻¹·mg⁻¹ of total protein) did not have a significant effect on the NO level (Figure 1; repeated measures ANOVA, P=0.90).

In smokers, compared with baseline, a significant increase in NO levels was seen (Figure 1; repeated measures ANOVA, P<0.02) with PEG-SOD (0.37±0.09 μmol·L⁻¹·mg⁻¹ of total protein; paired students’ t test, P<0.02), PEG-SOD+PEG-catalase (0.36±0.10 μmol·L⁻¹·mg⁻¹ of total protein; paired students’ t test, P<0.02), and BH₄ (0.33±0.10 μmol·L⁻¹·mg⁻¹ of total protein; paired students’ t test, P<0.03) treatment. However, chelerythrine treatment alone did not have any significant effect (0.22±0.10 μmol·L⁻¹·mg⁻¹ of total protein; P=0.30).

**Clinical Characteristics of the Study Population**

<table>
<thead>
<tr>
<th></th>
<th>Nonsmokers (n=10)</th>
<th>Smokers (n=15)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>31±1</td>
<td>33±1.4</td>
<td>0.20</td>
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<tr>
<td>Total cholesterol, mg/dL</td>
<td>180±7</td>
<td>173±8</td>
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<td>LDL cholesterol, mg/dL</td>
<td>107±8.0</td>
<td>99±8.0</td>
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<tr>
<td>HDL cholesterol, mg/dL</td>
<td>49±2.5</td>
<td>54±3.0</td>
<td>0.50</td>
</tr>
<tr>
<td>LDL/HDL ratio</td>
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<td>1.9±0.2</td>
<td>0.33</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>116±22</td>
<td>110±12</td>
<td>0.80</td>
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<tr>
<td>Glucose, mg/dL</td>
<td>96±4.1</td>
<td>97±3.5</td>
<td>0.90</td>
</tr>
<tr>
<td>Cottinine, ng/mL</td>
<td>7±3</td>
<td>107±16</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

**Effects of Smokers’ Serum and Various Treatments on eNOS Protein Expression**

HCAECs treated with serum from smokers had a significantly higher eNOS protein expression compared with those treated with serum from nonsmokers (Figure 2; 3778±272 versus 2281±270 pg/mg of total protein; P=0.001).

The addition of either PEG-SOD (2779±439 pg/mg of total protein), PEG-SOD+PEG-catalase (2213±180 pg/mg of total protein), chelerythrine (2436±172 pg/mg of total protein), or BH₄ (2797±365 pg/mg of total protein) did not have any significant effect on eNOS expression in HCAECs treated with the serum from nonsmokers (Figure 2; repeated measures ANOVA, P=0.51).

In smokers, compared with baseline, a significant decrease in eNOS expression was seen only with PEG-SOD+PEG-catalase (3030±260 pg/mg of total protein; Figure 2; repeated measures ANOVA, P<0.004 for the group; paired students’ t test, P<0.02 versus baseline). The eNOS expression with PEG-SOD+PEG-catalase treatment was also significantly lower than with PEG-SOD alone (4030±288 pg/mg of total protein; paired students’ t test, P<0.005) and with chelerythrine (3832±188 pg/mg of total protein, paired students’ t test, P<0.005) treatment. When compared with baseline, treatment with PEG-SOD alone did not significantly increase eNOS expression (paired students’ t test, P=0.23). BH₄ treatment alone was partially effective in decreasing eNOS expression from baseline (3297±240 pg/mg of total protein; paired students’ t test, P=0.14).

**Figure 1.** Effects of smokers’ serum on NO production in vitro. Confluent (~85%) HCAECs were incubated for 12 hours with an equal volume of medium and serum from nonsmokers (n=10) or smokers (n=15), with or without treatment as indicated, in 12-well plates. NO production in the cell culture supernatant was determined by a chemiluminescence method. Results are presented as mean±SEM after adjusting for the background NO and the total protein in each well. Unpaired Student’s t test, *P<0.05 vs nonsmokers’ baseline; paired Student’s t test, †P<0.04 vs smokers’ baseline.
Effects of Smokers’ Serum and Various Treatments on eNOS Activity

The cell lysates used above were also used to determine eNOS activity. Specific eNOS activity for each sample was adjusted for the amount of eNOS protein per milligram of total protein, as detected by ELISA. The cell lysates from HCAECs treated with smokers’ serum alone showed significantly lower eNOS activity when compared with cells treated with nonsmokers’ serum alone (Figure 3; 0.26±0.03 versus 0.50±0.08 pmol L-citrulline · min⁻¹ · pg eNOS⁻¹ · mg of total protein⁻¹; \( P<0.004 \)).

In nonsmokers, the addition of PEG-SOD, PEG-catalase, chelerythrine, or BH₄ did not have any significant effect on eNOS activity (Figure 3; repeated measures ANOVA, \( P=0.85 \)).

In smokers, compared with baseline, a significant increase (Figure 3; repeated measures ANOVA for the group, \( P<0.007 \)) in the activity of each unit of eNOS was seen with PEG-SOD (0.37±0.09 pmol L-citrulline · min⁻¹ · pg eNOS⁻¹ · mg of total protein⁻¹; paired student’s \( t \) test, \( P=0.04 \)), PEG-SOD+PEG-catalase (0.41±0.06 pmol L-citrulline · min⁻¹ · pg eNOS⁻¹ · mg of total protein⁻¹; paired students’ \( t \) test, \( P<0.02 \)), and BH₄ (0.33±0.24 pmol L-citrulline · min⁻¹ · pg eNOS⁻¹ · mg of total protein⁻¹; paired students’ \( t \) test, \( P<0.05 \)) treatment. The addition of chelerythrine did not have any significant effect (0.29±0.03 pmol L-citrulline · min⁻¹ · pg eNOS⁻¹ · mg of total protein⁻¹; \( P=0.25 \)). Although with either PEG-SOD or BH₄, the activity of each unit of eNOS in smokers was increased from the baseline, it was still significantly lower compared with nonsmokers (ANOVA, \( P<0.05 \) for the group; post hoc Fisher’s PLSD, \( P<0.03 \) for nonsmokers’ baseline versus PEG-SOD, BH₄, or chelerythrine in smokers). Only with PEG-SOD+PEG-catalase did the activity of each unit of eNOS become similar to that of nonsmokers (post hoc Fisher’s PLSD, \( P=0.30 \) for nonsmokers’ baseline versus PEG-SOD+PEG-catalase treatment in smokers). Thus, in our model, scavenging of both \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) was essential to normalize the activity of each unit of eNOS protein in smokers.

Discussion

The present study indicates that in our in vitro model, smokers’ serum caused a similar dysfunction in NO biosynthesis and upregulation of eNOS in HCAECs as was demonstrated previously in HUVECs. The improvement of NO level and eNOS activity with PEG-SOD, PEG-SOD+PEG-catalase, and BH₄ confirms that free radicals play a significant role in the smoking-related dysfunction of NO biosynthesis. The significant downregulation of eNOS protein seen with PEG-SOD+PEG-catalase suggests a potential role for \( \text{H}_2\text{O}_2 \) as a modulator of eNOS expression.

Cigarette smoke contains large quantities of free radicals. Although involvement of exogenous free radicals has been proposed in smoking-related dysfunctional NO biosynthesis, the effects of free radical scavengers in various smoking models have been inconsistent. In the present model, the increase in NO in the supernatant, as well as the increase in eNOS activity with cell-permeable PEG-SOD or PEG-SOD+PEG-catalase in smokers, indicates that ROS play a significant role in the smoking-mediated dysfunction of NO biosynthesis. The exact origin of free radicals in our model is unknown. The gas-phase radicals in cigarette smoke are only
stable for a short duration (seconds), but the tar-phase radicals are stable for hours to months. These tar-phase radicals, which can auto-oxidize to form \( \text{O}_2^- + \text{H}_2\text{O}_2 \) and \( \text{H}_2\text{O} \) in biological fluids, are a likely cause for the observed alterations in NO biosynthesis.

A reaction between \( \text{O}_2^- + \text{H}_2\text{O}_2 \) and NO gives rise to peroxynitrite (ONOO\(^-\)), which is known to avidly oxidize BH\(_4\), an essential cofactor for eNOS. A deficiency or oxidation of BH\(_4\) by ROS can uncouple the eNOS enzyme and cause the enzyme to produce \( \text{O}_2^- \) instead of NO, thereby increasing oxidative stress. In the present study, addition of exogenous BH\(_4\) in smokers also improved NO level and eNOS activity. Several investigators in both in vivo and in vitro models of smoking have previously demonstrated that addition of BH\(_4\) increases NO availability. All these data suggest that oxidation of BH\(_4\) could contribute to the dysfunctional NO biosynthesis in smokers. Thus, both exogenous and endogenous free radical generating mechanism(s) seem to participate in oxidative stress in smokers. Indeed, the effectiveness of cell-permeable PEG-SOD and PEG-catalase in our study and the lack of effect of non-cell-permeable SOD and catalase in other studies support a potential contribution of free radicals from endogenous source(s). Whether other endogenous free radical sources, such as the mitochondrial electron transport chain, xanthine oxidase, and NAD(P)H oxidase, are involved in smoking-mediated NO dysfunction is unknown and requires further investigation.

Several recent studies have shown that reduced NO biosynthesis in various cardiovascular pathological conditions may be associated with increased eNOS expression rather than a decrease in eNOS expression. Both \( \text{H}_2\text{O}_2 \) and PKC have been shown to be involved in the upregulation of eNOS in various models. In the present model, the significant downregulation of eNOS with PEG-SOD+PEG-catalase (causing conversion of \( \text{O}_2^- \) to \( \text{H}_2\text{O}_2 \) and then subsequent conversion to \( \text{H}_2\text{O} \) and \( \text{O}_2 \)), along with small increases in eNOS with PEG-SOD alone (causing conversion of \( \text{O}_2^- \) to \( \text{H}_2\text{O}_2 \)), suggest a role for \( \text{H}_2\text{O}_2 \) in the upregulation of eNOS. The exact source of \( \text{H}_2\text{O}_2 \) in our model is not known. It may have originated from components of cigarette smoke in the serum and/or from the conversion of \( \text{O}_2^- \) by endogenous SOD present in the extracellular or intracellular space. However, this study did not find that the chelerythrine (a PKC inhibitor) altered NO availability, eNOS activity, or eNOS expression. Therefore, PKC does not seem to play a significant role in smoking-mediated dysfunction of NO biosynthesis in the present model.

It is interesting to note that, in the present study, although the NO levels in smokers normalized to that of nonsmokers with PEG-SOD, PEG-SOD+PEG-catalase, or BH\(_4\) treatment, the activity of each unit of eNOS was still lower with either PEG-SOD or BH\(_4\) compared with nonsmokers. Only with PEG-SOD+PEG-catalase did the eNOS activity normalize to that of nonsmokers. Thus, the relatively higher eNOS protein expression with PEG-SOD or BH\(_4\) treatment in smokers compensated for its relatively lower activity, leading to normal NO levels. The cause of the relative lower activity with PEG-SOD or BH\(_4\) compared with PEG-SOD+PEG-
catalase in smokers was mostly likely attributable to the fact that PEG-SOD or BH4 did not scavenge H2O2. Although H2O2 seems to play a role in the upregulation of eNOS, it is a ROS and can give rise to hydroxyl radicals, which can affect the activity of eNOS protein. Therefore, it is not surprising that the scavenging of both O2·− and H2O2 in smokers had the most optimum response on eNOS activity in the present model.

A critical question remains as to why the increased expression of eNOS protein did not translate into an improvement of NO availability in smokers’ serum alone. In our study, the decreased eNOS activity of the HCAEC lysates treated with smokers’ serum alone, even after supplementation with various cofactors, indicates that upregulated eNOS was dysfunctional. This dysfunction of eNOS activity could be a direct and compound (O2⋅−, H2O2, and their derivatives) effect of ROS on the eNOS protein itself. It is conceivable that once structural changes in eNOS protein have occurred, addition of cofactors would be ineffective. However, our data suggest that H2O2 and its derivatives were the minor participants in this alteration. When PEG-SOD was added to smokers’ serum, all O2⋅− would have been converted to H2O2. This had only a mild detrimental effect on eNOS activity because NO availability was normalized, although at the expense of higher eNOS expression. Therefore, the most likely free radicals that caused a reduction of eNOS activity in HCAECs exposed to smokers’ serum alone were O2⋅− and its derivatives, such as ONOO−. Recently, Zou et al21 reported that the zinc-thiolate center of eNOS is 10 to 100 times more susceptible than BH4 to oxidative damage by ONOO−, and they also proposed that ONOO− releases zinc from eNOS protein and forms disulfide bonds between the monomers of eNOS, causing uncoupling of the enzyme. In addition, the duration (short versus long) and the amount of exposure to free radicals could also influence the catalytic activity of eNOS protein. Therefore, it is conceivable that persistent oxidative stress would decrease or uncouple the activity of upregulated eNOS. Additional studies would be required to identify the specific mechanism(s) for this dysfunction.

Study Limitations

First, in the present model, all subjects were male, and female hormones have been demonstrated to influence eNOS expression and its activity.22 Therefore, additional investigations will be required to establish whether the serum from female smokers would have similar effects on NO biosynthesis as in male smokers. Second, exogenous BH4 may act as an antioxidant directly,17,23 and the potential effect of BH4 cannot be excluded in the present model. Third, the focus of our study was the potential role of ROS in smoking-mediated NO dysfunction. Recently, asymmetric dimethylarginine, an endogenous inhibitor of L-arginine, was identified as an important cause of NO dysfunction.2 Its potential role in the present model cannot be excluded.

Conclusions

This study found a similar dysfunction of NO biosynthesis in HCAECs as was previously reported in HUVECs. Furthermore, the data from this study confirm that ROS are the most likely cause for the smoking-mediated dysfunction of the NO biosynthetic pathway and that H2O2 contributes to the upregulation of eNOS protein. However, this upregulation of eNOS may not translate into increased NO availability if the central mechanism, that is, oxidative stress due to ongoing formation of ROS, is not reduced.

References

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