Dysfunctional Endothelial Nitric Oxide Biosynthesis in Healthy Smokers With Impaired Endothelium-Dependent Vasodilatation

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Background—The mechanisms involved in the dysfunction of both endothelium-dependent vasodilatation (EDV) and NO biosynthesis related to smoking are unclear. In this study, EDV was assessed in healthy smokers and nonsmokers in vivo and, using serum from the same individuals, was related to the NO biosynthetic pathway in vitro.

Methods and Results—Flow-mediated EDV of the brachial artery was measured in 23 male patients (8 nonsmokers and 15 smokers). Serum was collected, added to confluent (≈85%) monolayers of human umbilical vein endothelial cells (HUVECs), and incubated for 12 hours. Basal and substance P–stimulated NO production were measured. The HUVECs used for measuring basal NO production were lysed, and both endothelial NO synthase (eNOS) protein expression and eNOS activity were determined. EDV was lower in smokers compared with nonsmokers (P<0.001). HUVECs treated with serum from smokers compared with nonsmokers showed significantly lower basal (P<0.0001) and stimulated (P<0.02) NO production, higher eNOS expression (P<0.0001), but lower eNOS activity (P<0.004). There was a significant positive correlation between in vivo EDV and in vitro substance P–stimulated NO production (rho=0.57, P<0.01) and between basal NO production and eNOS activity (r=0.54, P<0.008) and a negative correlation between basal NO production and eNOS protein expression (r=−0.60, P<0.003).

Conclusions—This is the first study to combine an in vivo model with a near-physiological in vitro model to demonstrate an association between decreased NO production and reduced EDV. Cigarette smoking was associated with reduced EDV, NO generation, and eNOS activity in the presence of increased eNOS protein expression. (Circulation. 2001;104: 1905-1910.)

Key Words: smoking ■ nitric oxide ■ nitric oxide synthase ■ endothelium
free of other cardiovascular risk factors (ie, hypertension, diabetes, low HDL, 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 for 6 to 8 hours (overnight). On arrival in the early morning, subjects were allowed to rest for 20 minutes. Baseline blood pressure (BP) and heart rate (HR) were recorded, and blood was collected from the antecubital vein. This was followed by an in vivo assessment of endothelial function by using ultrasonography to measure the brachial artery diameter at rest, during reactive hyperemia (leading to flow-mediated endothelium-dependent dilatation), and after administration of 400 μg of sublingual nitroglycerin (an endothelium-independent dilator) as described elsewhere.2 All ultrasonographic scans were obtained by the same operator using the same equipment (Acuson L10 6- to 11-MHz transducer and Acuson Aspen System).

Blood for in vitro and biochemical studies (lipid profile and serum cotinine) was collected in Vacutainer tubes and centrifuged at 1776g (3000 rpm) for 15 minutes (4°C). The serum was collected and stored at −70°C until use. Analysis for the lipid profile was done in the hospital’s clinical laboratory, and serum cotinine concentrations were determined by a commercially available kit (STC Technologies).

The study protocol was approved by Saint Vincent Catholic Medical Centers of New York Ethics Committee. Written informed consent was obtained from each subject.

**Determination of Endothelium-Dependent and Endothelium-Independent Vasodilatation**

The brachial artery scans were read by the same experienced ultrasonographer, who was blinded to the identity of the participants and stage of the experiment. The validated analysis method used has been described elsewhere.2 In brief, a pneumatic cuff was inflated around the right forearm to ~300 mm Hg for 4.5 minutes followed by cuff deflation. Flow-mediated EDV was calculated by dividing the maximum vessel diameter at 50 to 60 seconds after cuff deflation by the average baseline diameter. Endothelium-independent dilatation was calculated as the maximum vessel diameter at 3 to 4 minutes after nitroglycerin administration divided by average baseline diameter. The arterial diameter was measured by using ultrasonic calipers for 4 cardiac cycles for each condition and then averaged. Results were expressed as percentage change from the baseline.

**Endothelial Cell Culture and Treatment**

Primary HUVECs from a single donor were purchased from Clonetech (BioWhittaker). HUVECs were cultured with slight modification of a method described previously.16 Twenty thousand cells per well were plated in 24-well, flat-bottom tissue culture plates (Primaria, Baxter Scientific Products) with complete endothelial growth media (EGM) (500 mL endothelial cell basal media, 0.5 mL of 10 μg/mL human recombinant epidermal growth factor, 0.5 mL of 1 mg/mL hydrocortisone, 0.5 mL of 50 mg/mL gentamicin and 50 μg/mL amphotericin-B, 2 mL of 3 mg/mL bovine brain extract, and 25 mL of FBS) (Clonetics) and grown to 85% confluence at 37°C; 5% CO2. The supernatant was removed from confluent cells and incubated with equal volume of serum and EGM (total volume, 400 μL) in 24-well tissue culture plates. After 12 hours, basal NO production in the supernatant was determined. To measure stimulated NO production in culture, the supernatants were removed after 12 hours and the cells were washed twice with Dulbecco’s PBS (Gibco BRL-treated HUVECs). Fresh EGM was added to each well followed by stimulation with 10−5 mol/L substance P (SP) (Sigma) for 30 minutes.

In both experiments, cell culture supernatants were collected and stored at −70°C until analysis. All samples were tested in duplicate, and NO concentration was expressed as nmol/L after adjusting for background levels.

**Determination of Nitric Oxide Synthase Protein Concentration**

The eNOS protein concentration of the HUVECs in culture was determined by using a commercial ELISA kit (R&D systems). The cells that were used to detect basal (12-hour) NO production were washed twice with Dulbecco’s PBS, and cells were lysed with 400 μL of lysis buffer. The assay was performed using 100 μL of each cell lysate following the manufacturer’s instructions. All samples were tested in duplicate and expressed as pg/mL.

**Determination of Nitric Oxide Synthase Activity**

The same cell lysates generated for the eNOS protein assay were used to determine the specific eNOS activity. This was done using a slight modification of the method described by Bedt and Schmidt19 using a commercial kit (Calbiochem). In brief, 100 μL of lysis was added to a reaction cocktail containing 25 μL of reaction buffer (50 mmol/L Tris-HCl, 6 μmol/L BH4, 2 μmol/L FAD, and 2 μmol/L FMN) and a final concentration of 1 μL of [14H]-l-arginine (55 Ci/mmol, 1 μCi/μL; Amersham), 1 mmol/L NADPH, 75 μmol/L CaCl2, and 0.1 μmol/L calmodulin. The incubation was carried out at room temperature for 1 hour. The radioactivity was quantitated 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 detected by ELISA, and the specific activity of eNOS was expressed as pmol L-citrulline/min per pg of eNOS protein.

**Statistical Analysis**

Results are presented as mean±SEM, and all in vitro data are the average of duplicate measurements. The nonsmoker and smoker groups were compared using the unpaired Student’s t test. The relationship between basal NO production, eNOS protein expression, and eNOS activity was assessed using linear regression analysis. Correlation between in vivo flow-mediated EDV and in vitro SP-stimulated NO production was assessed by the Spearman’s rank correlation test. A value of P<0.05 was considered statistically significant. All calculations were performed using STATVIEW II (Abacus Concepts Inc) statistical program.

**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 two groups with the exception of serum cotinine levels.
Brachial Artery Reactivity to Flow and Nitroglycerin
Baseline brachial artery diameters were not different between smokers and nonsmokers (4.4±0.01 versus 4.2±0.03 mm, P=0.48). Flow-mediated EDV of the brachial artery was significantly reduced in smokers compared with nonsmokers (0.5±0.5% versus 6.1±1.5%, respectively, P<0.001). By contrast, the nitroglycerin-mediated (endothelium-independent) vasodilatory response of the brachial artery was not different between the two groups (19.2±1.4% versus 18.5±2.5%, respectively, P=0.87). The latter results indicate that subjects in the study group were apparently free of vascular smooth muscle disorder.

Effect of Smoking on Nitric Oxide Production
When the NO concentration in the serum from smokers was compared with that from nonsmokers, no significant difference was found (5473±858 versus 5691±737 nmol/L, respectively, P=0.58). Serum-treated, SP-stimulated HUVECs from the group of smokers showed markedly lower NO release in the cell culture supernatant than the nonsmokers group (Figure 1A; 1266±237 versus 3613±457 nmol/L, respectively, P<0.0001).

Serum-treated, SP-stimulated HUVECs from the group of smokers showed markedly lower NO release in the cell culture supernatant than the nonsmokers group (Figure 1B; increase from the baseline: 133±74 versus 1057±455 nmol/L, respectively, P<0.02).

Effects of Smokers’ Serum on eNOS Protein Expression and eNOS Activity In Vitro
To determine whether the observed changes in in vitro NO production by HUVECs were associated with a change in its eNOS protein, cells from the plates used to detect basal NO production were lysed and eNOS protein expression was determined. HUVECs treated with serum from smokers had significantly higher eNOS protein expression compared with the HUVECs treated with serum from nonsmokers (Figure 2A; 939.5±51.5 versus 440.8±58.1 pg/mL, respectively, P<0.0001).

To correlate basal NO production with enzymatic activity, the cell lysates above were used to determine eNOS activity. Specific eNOS activity for each sample was adjusted for the amount of eNOS protein as detected by ELISA. The cell lysates from HUVECs treated with smokers’ serum showed significantly lower eNOS activity compared with cells treated with nonsmokers’ serum (Figure 2B; 1.18±0.13 versus 1.92±0.19 pmol L-citrulline/min per pg eNOS, respectively, P<0.0004).

Relationship Between Nitric Oxide Production, eNOS Protein Expression, and eNOS Activity
On linear regression analysis, a significant positive correlation was found between in vitro basal NO production and eNOS activity (Figure 3A; r=0.54, P<0.008). On the other hand, in vitro eNOS protein expression showed a significant negative correlation with basal NO production (Figure 3B; r=−0.60, P<0.003). Additionally, eNOS protein negatively
correlated with eNOS activity (Figure 4; $r = -0.75$, $P < 0.0001$).

**Relationship Between Stimulated Nitric Oxide Production In Vitro and Endothelium-Dependent Vasodilatation In Vivo**

The relationship between in vivo EDV and in vitro SP-stimulated NO production was determined by the Spearman’s rank correlation test. A significant correlation was found between the in vivo and the in vitro model (Spearman’s rho = 0.57, $P < 0.01$).

**Discussion**

The association between cigarette smoking and vascular diseases is well documented, and there is a general consensus that it targets the vascular ECs. However, the exact mechanisms by which cigarette smoke alters EC function and induces cardiovascular pathology are not clear. NO, a heterodiatomic free radical, is primarily responsible for vasoregulatory and homeostatic functions of ECs. Several investiga-

![Figure 2](image1.png)

**Figure 2.** Effects of smokers' serum on eNOS protein expression and eNOS activity in vitro. Confluent (~85%) HUVECs were incubated with equal volume of medium and serum from smokers ($n=15$) and nonsmokers ($n=8$) in 24-well plates. After 12 hours, cell culture supernatant was collected and the HUVECs were lysed. A, eNOS protein concentration of the cell lysates was determined by ELISA. B, eNOS activity of the cell lysates was determined by detecting the conversion of [H] L-arginine to [H] L-citrulline. Results are presented as mean±SEM. Unpaired Student's t test: A, eNOS protein expression, *smoker versus nonsmoker, $P < 0.0001$; B, eNOS activity, *smoker versus nonsmoker, $P < 0.004$.

![Figure 3](image2.png)

**Figure 3.** Correlation between basal NO production and eNOS activity (A) and eNOS protein expression in vitro (B).

![Figure 4](image3.png)

**Figure 4.** Correlation between eNOS protein expression and eNOS activity in vitro.
tors have suggested that NO and its vasodilatory function are altered by cigarette smoking.\textsuperscript{1–6} However, conflicting data also exist. It has been reported that cigarette smoking is associated with impairment of EDV in both the brachial and coronary artery circulation.\textsuperscript{2–5} Using the NO antagonist (L-NMMA), several investigators have indirectly associated impairment of EDV in smokers with decreased NO.\textsuperscript{3–5} Conversely, other investigators have found no relationship between cigarette smoking and impaired EDV or decreased NO in various models.\textsuperscript{7,10,20–22} Similar disagreement also exists regarding the effect of cigarette smoke on eNOS mRNA, eNOS protein, and its activity. Both an increase and a decrease of eNOS mRNA and protein have been found in various experimental models.\textsuperscript{9,11–13} The majority of in vitro data has involved the use of CSE. However, this solution is probably not physiological, because in the circulation, cigarette smoke has to first bypass the lung. In addition, certain toxic components may be neutralized by the antioxidants present in the blood. Therefore, to simulate a physiological environment in the present study, serum was used as a vehicle of cigarette smoke exposure in vitro.

Simultaneous observations of the in vivo (flow-mediated brachial artery reactivity) and in vitro (SP-stimulated NO production) model used in the study showed a significant reduction in NO production along with a reduction in EDV in the smokers’ group compared with the nonsmokers’ group. There was a significant correlation between both in vivo and in vitro systems. To our knowledge, the present study demonstrates, for the first time, a direct association between cigarette smoking and impaired EDV or decreased NO.\textsuperscript{13} The majority of in vitro data has involved the use of CSE. However, this solution is probably not physiological, because in the circulation, cigarette smoke has to first bypass the lung. In addition, certain toxic components may be neutralized by the antioxidants present in the blood. Therefore, to simulate a physiological environment in the present study, serum was used as a vehicle of cigarette smoke exposure in vitro.

The finding of reduced basal NO production by the HUVECs in the smokers’ group in the present model complements previous in vitro\textsuperscript{6} and in vivo\textsuperscript{3–5} findings that cigarette smoking affects basal NO production by ECs. However, no significant difference was found between serum NO of smokers and nonsmokers. Similarly, Rangemark and Wennmalm\textsuperscript{23} reported no difference in plasma nitrate (a metabolite of NO) between smokers and nonsmokers. These data support the notion that the bloodstream concentration of NO may be an unreliable measure of endothelium-derived NO. This parameter is affected by various factors, such as dietary intake, metabolism in the gut, denitrifying liver enzymes, inhalation of atmospheric gaseous nitrogen compounds, and renal function.\textsuperscript{24}

Analysis of eNOS protein expression in the present experimental model revealed a higher eNOS expression in HUVECs treated with serum from smokers. This concurs with a previous in vivo study in the rat.\textsuperscript{13} However, Su et al\textsuperscript{25} reported that CSE inhibited eNOS protein expression in pulmonary artery ECs. The differences noted between the two studies probably reflect differences in in vitro experimental models (nonphysiological versus near-physiological). A potential explanation for the observation seen in the present study is that a negative feedback mechanism may exist between NO levels and eNOS protein expression. Thus, decreased NO generation or availability may trigger increased eNOS expression. Several investigators\textsuperscript{25,26} have postulated a negative feedback mechanism between NO and eNOS activity, but data on eNOS protein are not available. In the present study, a strong negative correlation between basal NO production and eNOS protein expression in cultured HUVECs supports the existence of such mechanisms.

Analysis of eNOS activity from the same cell lysates showed decreased eNOS activity in HUVECs treated with serum from smokers. Basal NO production, eNOS protein, and eNOS activity were measured from the same culture. Thus, for each sample, a direct relationship of eNOS protein and its activity to NO production was determined. The eNOS activity positively correlated with basal NO production and negatively correlated with eNOS protein expression. Similarly, Wang et al\textsuperscript{12} found no correlation between eNOS protein and its activity in HUVECs isolated from pregnant smokers. Thus, in smokers there seems to exist a dissociation between eNOS protein expression and activity. Reduced eNOS activity in the presence of increased eNOS protein may be attributable to several factors, including the presence of inhibitors, lack of substrate and cofactors, production of nonfunctional enzyme, or an uncoupling effect on eNOS gene expression and enzyme activity.\textsuperscript{28,29}

**Study Limitations**

In the present study several potential limitations are recognized. First, the number of subjects enrolled in our study group was relatively small and included only male patients. Thus, the results may not be generalized. Second, flow-mediated EDV was correlated with in vitro SP-stimulated NO production. Although both stimulate NO production, the receptors that mediate the individual effects are different.\textsuperscript{27} It is possible that smoking differentially affects these receptors and their signaling process. Third, cigarette smoke–induced endothelial dysfunction is multifactorial and may be related to substrate deficiency, a reduction in eNOS synthase activity, a diminished release, or an enhanced degradation of NO.\textsuperscript{5} In the present study, the main focus was on NO production, eNOS protein expression, and eNOS activity. The influence of other factors in the present model cannot be excluded.

**Conclusion**

This study has taken a novel approach to confirm the association between decreased NO production and reduced EDV in cigarette smokers by combining in vitro and in vivo physiological models. In the in vitro model, cigarette smoking was associated with increased eNOS protein expression but reduced eNOS activity as well as decreased NO production. Additional investigation will be required to precisely elucidate the mechanisms responsible for the decreased eNOS activity seen in the presence of increased eNOS protein expression.

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**References**


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