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Heavy and Light Cigarette Smokers Have Similar Dysfunction of Endothelial Vasoregulatory Activity

An In Vivo and In Vitro Correlation

Rajat S. Barua, MD,*† John A. Ambrose, MD, FACC,* Lesley-Jane Eales-Reynolds, PHD,† Mary C. DeVoe, RN,* John G. Zervas, RCDS,* Dhanonjoy C. Saha, PHD*

New York, New York and Guildford, United Kingdom

OBJECTIVES The goal of this study was to investigate the dose-dependent effects of active cigarette smoking on endothelial nitric oxide (NO) and endothelin-1 (ET-1) biosynthesis.

BACKGROUND Limited studies have suggested that active cigarette smoking may be associated with a dose-dependent reduction of endothelium-dependent vasodilation (EDV). The underlying biochemical changes that cause this dose-specific effect, such as changes in the endothelial NO biosynthetic pathway and ET-1 production, have not been examined.

METHODS Flow- and nitroglycerin-mediated reactivity of the brachial artery were measured in eight nonsmokers, seven light smokers (≤1 pack/week) and eight heavy smokers (≥1 pack/day), and their sera were added to confluent (~85%) monolayers of human umbilical endothelial cells (HUVECs) for 12 h. Basal and substance P-stimulated NO and basal ET-1 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.

RESULTS Serum cotinine level and pack-years of smoking were significantly lower in light smokers compared with heavy smokers ($p < 0.006$ and $p < 0.004$, respectively). There were no significant differences between heavy smokers and light smokers in EDV ($p = 0.52$), basal- ($p = 0.70$) and stimulated-NO production ($p = 0.95$), eNOS protein ($p = 0.40$) and eNOS activity ($p = 0.63$). Compared with nonsmokers, all the parameters were significantly altered in both of the smokers' groups. No differences were found in nitroglycerin-mediated vasodilation and in vitro ET-1 production among the three groups.

CONCLUSIONS These results indicate light smoking may have similar detrimental effects on EDV and NO biosynthetic pathway as does heavy smoking. These data may have important implications concerning the amount of active cigarette exposure that imparts cardiovascular risk. (J Am Coll Cardiol 2002;39:1758–63) © 2002 by the American College of Cardiology Foundation

Cigarette smoking is a recognized risk factor for cardiovascular disease and is known to promote the development of atherosclerosis and thrombosis (1,2). Endothelial dysfunction and early artherogenesis, increased procoagulation and platelet activity as well as hypertension and vasospasm have all been documented either clinically or in various experimental models (1–3). While this association between smoking and cardiovascular risk has clearly been demonstrated, an unanswered question related to smoking is whether or not there is a linear dose effect in active smokers. Early epidemiologic studies suggested a direct dose effect with a greater risk for heavy smokers in comparison with lighter smokers (4). However, several recent large epidemiologic studies, although showing a trend for more cardiovascular events in heavier smokers, have failed to find a significant dose-dependent correlation between the risk of cardiovascular disease and the number of cigarettes smoked or the pack-years of smoke exposure (5,6). Furthermore, smoking as few as one to four cigarettes per day was associated with a doubling of risk for coronary artery disease in the Nurses' Health Study (7).

It has been shown that cigarette smoking is associated with a reduction of endothelium-dependent vasodilation (EDV) (8–16). However, data regarding the dose-dependent effects of smoking on EDV are limited and inconclusive (8,13–15). Celermajer et al. (8,13) suggested that both active and passive smoking were associated with a dose-dependent decrease in EDV, but in other studies this dose-dependent effect was not evident (14,15). We have previously described a physiologic in vitro model to study the effects of smoking on the NO biosynthetic pathway and validated this model with in vivo measurements of EDV of the brachial artery (16). In the present study, the effects of light and heavy cigarette smoking exposure on endothelial cell (EC)-derived vasoregulatory factors (nitric oxide [NO], endothelin-1 [ET-1]) as well as EDV of the brachial artery were assessed to determine whether or not there is a dose-dependent effect of active smoking on endothelial vasoregulatory function.

From *Saint Vincent Catholic Medical Centers, New York, New York; and †the School of Biomedical and Life Sciences, University of Surrey, Guildford, United Kingdom.

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Abbreviations and Acronyms

ANOVA = analysis of variance
EC = endothelial cells
EDV = endothelium-dependent vasodilation
EGM = endothelial growth media
eNOS = endothelial nitric oxide synthase
ET-1 = endothelin-1
HUVEC = human umbilical endothelial cells
mRNA = messenger ribonucleic acid
NO = nitric oxide
PLSD = protected least significant difference
SP = substance P

METHODS

Subjects and study design. Male volunteers with a history of active smoking were matched for age and gender with individuals who had never smoked. Active smokers were subclassified into two groups: light smokers (with smoking habit of ≤1 pack/week) and heavy smokers (with smoking habit of ≥1 pack/day). All subjects were free of other cardiovascular risk factors (i.e., hypertension, diabetes, low high-density lipoprotein, hyperlipidemia or a family history of premature vascular disease) and were not taking any medication.

Subjects were requested to abstain from smoking and foods or caffeinated drinks for 6 to 8 h (overnight). On arrival in the morning, subjects were allowed to rest for 20 min. Baseline blood pressure and heart rate were recorded, and blood was collected from the anticubital vein. This was followed by an in vivo assessment of endothelial function by utilizing ultrasonography to measure the brachial artery diameter at rest, during reactive hyperemia (leading to flow-mediated endothelium-dependent dilation) and after administration of 400 μg of sublingual nitroglycerin (an endothelium-independent dilator) as previously described (8,16). All ultrasonographic scans were obtained utilizing the same equipment (Acuson L10 6-11 MHz transducer and Acuson Aspen System, Mountain View, California).

Blood for in vitro and biochemical studies (lipid profile and serum cotinine) was collected in vacutainer tubes and centrifuged at 1,776 *g* (3,000 rpm) for 15 min (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 ELISA kit (STC Technologies, Bethlehem, Pennsylvania).

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.

Determination of EDV and endothelium-independent vasodilation. All brachial artery scans were read by a single, experienced, ultrasonographer (J. G. Z.) who was blinded to the identity of the participants and stage of the experiment. The validated method of analysis has been published previously (8,16). In brief, a pneumatic cuff was inflated around the right forearm to ~300 mm Hg for 4.5 min followed by cuff deflation. Flow-mediated EDV was calculated by dividing the maximum vessel diameter at 50 to 60 s after cuff deflation with average baseline diameter.

Endothelium-independent dilation was calculated as the maximum vessel diameter at 3 to 4 min after nitroglycerin administration divided by average baseline diameter. The arterial diameter was measured using ultrasonic calipers for four cardiac cycles for each condition and was averaged. Diameter measurements were taken at end diastole, coincident with the R wave on a continuously recorded electrocardiographic trace. Results were expressed as percentage changes from the baseline.

EC culture and treatment. Primary human umbilical endothelial cells (HUVEC) from a single donor were purchased from Clonetics (BioWhittaker, Walkersville, Maryland). Human umbilical endothelial cells were cultured using the method described previously (16). In brief, 20,000 cells/well were plated in 24-well, flat bottom, tissue culture plates (Primaria, Baxter Scientific Products, Springfield, New Jersey) with complete endothelial growth media (EGM) (Clonetics) and grown to ~85% confluence at 37°C in 5% CO_2. The supernatant was removed from confluent cells and incubated with an equal volume of serum and EGM for 12 h (37°C; 5% CO_2). All in vitro experiments were carried out, during the second passage of the HUVECs, by the same investigator (R. S. B.) who was blinded to the identity of the participant and the clinical data.

Determination of NO production, endothelial NO synthase (eNOS) expression and eNOS activity. As described above, HUVECs were treated for 12 h with equal volumes of serum and EGM (total volume: 400 μl) in 24-well tissue culture plates. After 12 h, basal NO production in the supernatant was determined. To measure stimulated NO production in culture, the supernatants were removed after 12 h and the cells washed twice with Dulbecco's phosphate buffered saline (Gibco BRL, Grands Island, New York). Fresh EGM was added to each well followed by stimulation with 10^{-6} M substance P (SP) (Sigma, St. Louis, Missouri) for 30 min. Cell culture supernatants were collected and stored at −70°C until analyzed. Nitric oxide concentration in each sample was determined by the chemiluminesence method as described previously using a NO analyzer (Sievers, Model # 280, Boulder, Colorado) and expressed as nM after adjusting for background NO levels (16).

The eNOS protein concentration of the HUVECs in culture was determined using a commercial ELISA kit (R&D Systems, Minneapolis, Minnesota) and expressed as pg/ml as published previously (16).

The cell lysates generated for the eNOS protein assay were also used to determine the specific eNOS activity. This was done using a method described previously (16). 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/pg of eNOS protein.

Determination of ET-1 production. Nitric oxide and ET-1 were detected from the same cell-culture supernatant. Endothelin-1 concentrations were determined by using a commercial ELISA kit (Amersham, Arlington Heights, Illinois) and expressed as pg/ml after adjusting for background levels.

Statistical and power analyses. Results are presented as the mean ± SEM and all in vitro data are the average of duplicate measurements. One-way analysis of variance (ANOVA) was performed to compare values between nonsmokers, light smokers and heavy smokers. For each ANOVA, post-hoc Fisher PLSD (protected least significant difference) was performed to determine each individual group difference. The relationships between the degree of smoke exposure (as measured by the pack-year and serum cotinine levels) and EDV or NO were determined using linear regression analysis. A value of $p < 0.05$ was considered statistically significant.

As new in vitro methods have been described in this study, an accurate assumption for the difference between light and heavy smokers in vitro was difficult. For in vivo data, Celermajer et al. (8) have reported a mean absolute difference of 4% in EDV between very light (6.6%) and heavy (2.6%) smokers with a pooled SD of 2.9%. Although, the grouping criteria for smokers in their study were different from ours (pack-years of smoking as opposed to numbers of cigarette smoked), the pack-years of smoking in the corresponding groups were comparable between the two studies. Using their finding with the assumption that light smokers will have higher EDV compared with heavy smokers (i.e., one-sided), a sample size of seven to eight in each group had an 80% power at $\alpha = 0.05$. Power analyses were performed using the STPLAN statistical software (version 4.1, University of Texas M.D. Anderson Cancer Center, Dept. of Biomathematics, Houston, Texas), and all the other analyses were performed using the StatView statistical program (version 4.5, Abacus Concepts, California).

RESULTS

Clinical characteristics of the study population. There was no significant difference in age, systolic or diastolic blood pressure, heart rate, total cholesterol, low-density lipoprotein, high-density lipoprotein and triglyceride levels between nonsmokers, light smokers and heavy smokers. Serum cotinine levels, number of cigarette smoked per day and pack-years of smoking were significantly different between all the groups substantiating the smoking status of the study population (Table 1).

Brachial artery reactivity to flow and nitroglycerin. Baseline brachial artery diameters were not different between nonsmokers (4.2 ± 0.02 mm), light smokers (4.3 ± 0.03 mm) and heavy smokers (4.4 ± 0.01 mm) (ANOVA: $p = 0.6$). Flow-mediated EDV of the brachial artery was not significantly different between light smokers and heavy smokers (1.0 ± 1.0% and 0.1 ± 0.1%, $p = 0.52$), but both were significantly reduced compared with nonsmokers (6.0 ± 1.4%; ANOVA: $p < 0.001$ for the groups; Post-hoc Fisher PLSD: $p < 0.003$ for nonsmokers vs. light smokers, $p < 0.001$ for nonsmokers vs. heavy smokers). By contrast, the nitroglycerin-mediated (endothelium-independent) vasodilatory response of the brachial artery was not different between all the groups (19.1 ± 1.4% for nonsmokers, 22.4 ± 3.6% for light smokers and 15.7 ± 3.6% for heavy smokers, ANOVA: $p = 0.34$).

Table 1. Parameters of Smoke Exposure and Endothelial Function of the Study Population

	Nonsmokers (n = 8)	Light Smokers (n = 7)	Heavy Smokers (n = 8)
Cigarettes/day	—	1.8 ± 0.6	21 ± 1*
Pack-years	—	0.7 ± 0.2	13 ± 3*
Cotinine (ng/ml)	8 ± 3	140 ± 54†	286 ± 26‡§
Baseline diameter (mm)	4.2 ± 0.02	4.3 ± 0.03	4.4 ± 0.01
EDV (%)	6.0 ± 1.4	1.0 ± 1.0‖	0.1 ± 0.1¶
Nitroglycerin-mediated dilation (%)	19.1 ± 1.4	22.4 ± 3.6	15.7 ± 3.6

Values are expressed as mean ± SEM. *$p < 0.004$ for light smokers versus heavy smokers. Post-hoc Fisher protected least significant difference (PLSD): for serum cotinine levels: †$p < 0.02$ for nonsmokers versus light smokers; ‡$p < 0.0001$ for nonsmokers versus heavy smokers; §$p < 0.006$ for light smokers versus heavy smokers. Post-hoc Fisher PLSD: for endothelial-dependent vasodilation (EDV) (%): ‖$p < 0.003$ for nonsmokers versus light smokers; ¶$p < 0.001$ for nonsmokers versus heavy smokers.

Effect of smoking on NO and ET-1 production. Human umbilical endothelial cells exposed to light smokers' and heavy smokers' serum in vitro showed a similar basal NO production (Fig. 1A; 1,418 ± 421 vs. 1,137 ± 267 nM, respectively, $p = 0.62$). However, compared with the nonsmokers' group, both were significantly lower (3,613 ± 459 nM; ANOVA: $p < 0.0004$ for the groups; post-hoc Fisher PLSD: $p < 0.001$ for nonsmokers vs. light smokers, $p < 0.0003$ for nonsmokers vs. heavy smokers).

Substance P-stimulated NO production was similar between the light smoker and heavy smoker group (Δ from baseline: 145 ± 87 vs. 121 ± 120 nM, respectively, $p = 0.95$), but, compared with the nonsmoker group, both were significantly lower (Δ from baseline: 1,057 ± 455 nM; ANOVA: $p < 0.05$ for the groups; post-hoc Fisher PLSD: $p < 0.04$ for nonsmokers vs. light smokers, $p < 0.03$ for nonsmokers vs. heavy smokers).

Basal ET-1 production by the HUVECs treated with nonsmokers', light smokers' and heavy smokers' serum were not different between the three groups (Fig. 1B; 283 ± 43 pg/ml, 326 ± 24 pg/ml and 282 ± 17 pg/ml, respectively, ANOVA: $p = 0.55$).

Effects of light and heavy smokers' serum on eNOS protein expression and eNOS activity in vitro. Human umbilical endothelial cells treated with light smokers' and

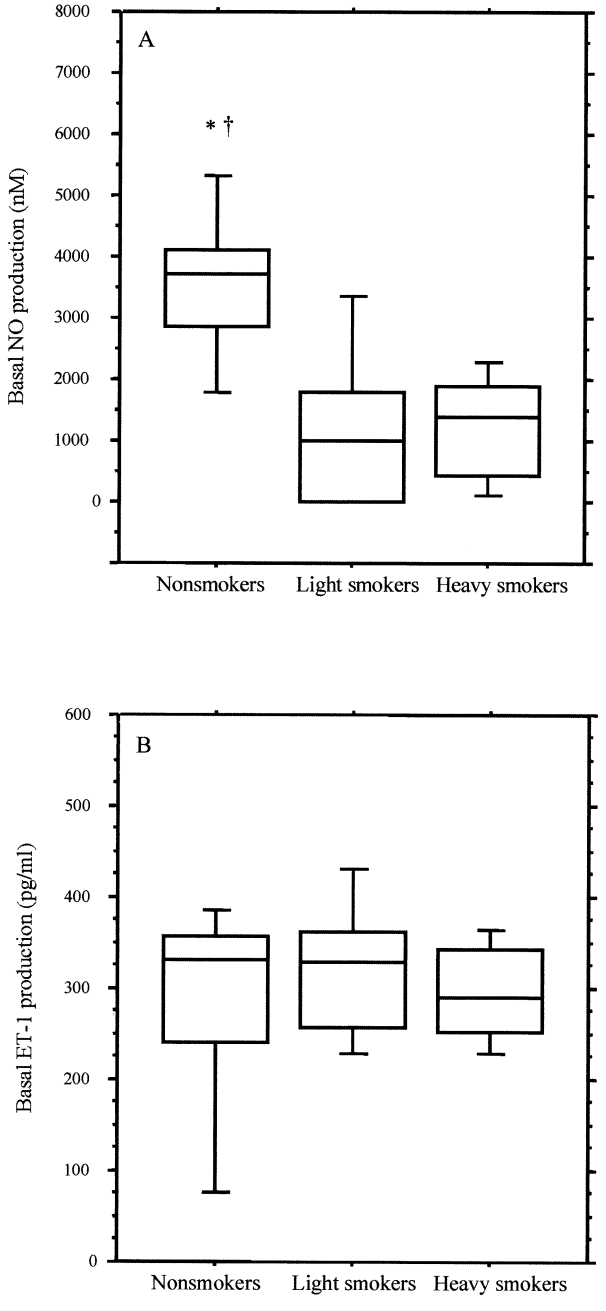

Figure 1. Effects of light and heavy smokers' serum on basal-nitric oxide (NO) and endothelin-1 (ET-1) production in vitro. Confluent (approximately 85%) human umbilical endothelial cells were incubated with an equal volume of medium and serum from nonsmokers (n = 8), light smokers (n = 7) or heavy smokers (n = 8) in 24-well plates. After 12-h incubation (37°C; 5% CO_2), the cell culture supernatant was collected. **(A)** Nitric oxide production in the cell culture supernatant was determined by a chemiluminescence method; **(B)** Endothelin-1 production in the cell culture supernatant was determined using an ELISA. **(A)** Basal NO production; one-way analysis of variance (ANOVA): group, $p < 0.001$. Post-hoc Fishers protected least significant difference (PLSD): *nonsmokers versus light smokers, $p < 0.001$; †nonsmokers versus heavy smokers, $p < 0.001$; light smokers versus heavy smokers, $p = 0.62$. **(B)** Basal ET-1 production; one-way ANOVA: group, $p = 0.55$. The **box** represent the interquartile range (between the 25th and 75th percentiles); the median is shown as a **horizontal bar within each box**. The **bars outside each box** show the range of 95% of all values.

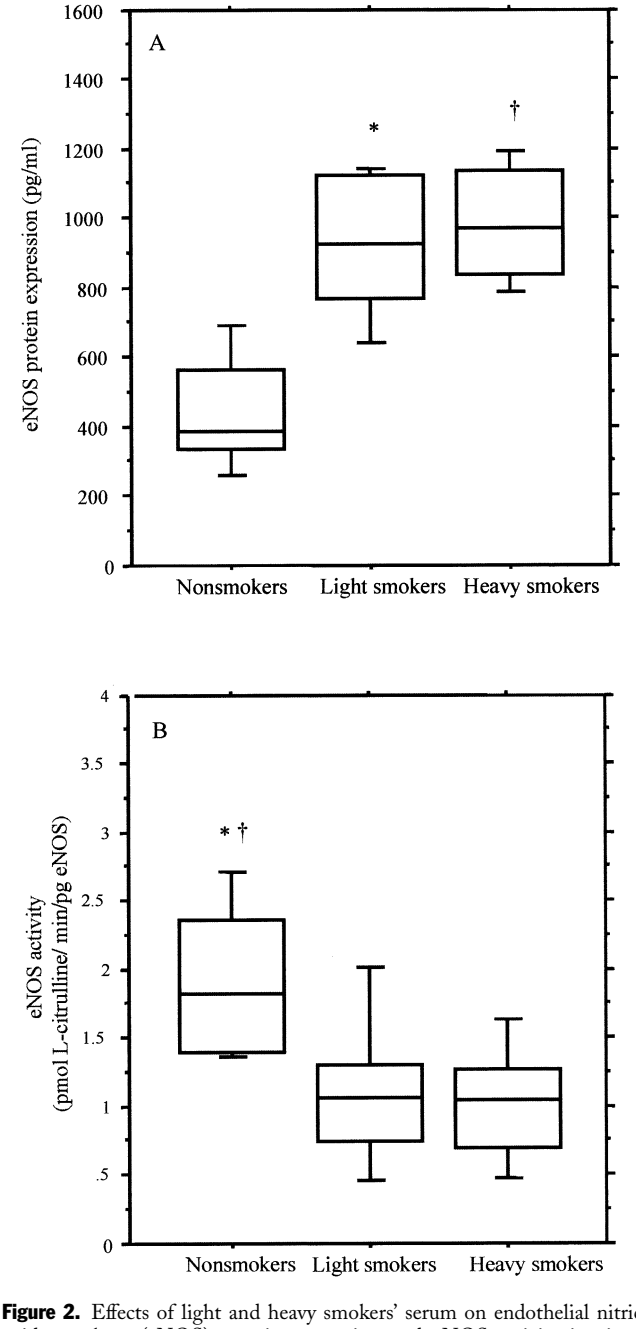

Figure 2. Effects of light and heavy smokers' serum on endothelial nitric oxide synthase (eNOS) protein expression and eNOS activity in vitro. Confluent (approximately 85%) human umbilical endothelial cells (HUVECs) were incubated with an equal volume of medium and serum from nonsmokers (n = 8), light smokers (n = 7) or heavy smokers (n = 8) in 24-well plates. After 12 h culture, supernatant was collected, and the HUVECs were lysed. **(A)** Endothelial nitric oxide synthase protein concentration of the cell lysates was determined by ELISA; **(B)** eNOS activity of the cell lysates was determined by detecting the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline. **(A)** Endothelial nitric oxide synthase protein expression; one-way analysis of variance (ANOVA): group, $p < 0.0001$. Post-hoc Fishers protected least significant difference (PLSD): *nonsmokers versus light smokers, $p < 0.0003$; †nonsmokers versus heavy smokers, $p < 0.0001$; light smokers versus heavy smokers, $p = 0.40$. **(B)** Endothelial nitric oxide synthase activity; one-way ANOVA: group, $p < 0.02$. Post-hoc Fisher PLSD: *nonsmokers versus light smokers, $p < 0.03$; †nonsmokers versus heavy smokers, $p < 0.004$; light smokers versus heavy smokers, $p = 0.63$. The **box** represents the interquartile range (between the 25th and 75th percentiles); the median is shown as a **horizontal bar within each box**. The **bars outside each box** show the range of 95% of all values.

heavy smokers' serum had similar eNOS protein expression (Fig. 2A; 888 ± 96 vs. 974 ± 50 pg/ml, respectively, $p = 0.40$). However, both smokers' groups showed significantly higher eNOS protein expression as compared with the nonsmokers (Fig. 2, A; 440 ± 58 pg/ml; ANOVA: $p < 0.0001$ for the groups; post-hoc Fisher PLSD: $p < 0.0003$ for nonsmokers vs. light smokers, $p < 0.0001$ for nonsmokers vs. heavy smokers).

The cell lysates utilized above were used to determine eNOS activity, and 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 either light smokers' or heavy smokers' serum showed similar eNOS activity (Fig. 2B; 1.25 ± 0.21 vs. 1.11 ± 0.16 pmol L-citrulline/min/pg eNOS, respectively, $p = 0.63$). However, both of these groups showed significantly lower eNOS activity as compared with the nonsmokers (Fig. 2, B; 1.92 ± 0.20 pmol L-citrulline/min/pg eNOS; ANOVA: $p < 0.02$ for the groups; post-hoc Fisher PLSD: $p < 0.03$ for nonsmokers vs. light smokers, $p < 0.006$ for nonsmokers vs. heavy smokers).

Relationship between degree of smoke exposure, EDV and NO production. Pack-years of smoke exposure represent long-term exposure, while serum cotinine represents short-term smoke exposure (two to four days). On linear regression analysis, no significant correlation was found between EDV and pack-years ($r = 0.14$, $p = 0.62$) or serum cotinine levels ($r = 0.20$, $p = 0.55$) in the smokers' groups. Similarly, no relationship could be found between in vitro NO production and serum cotinine levels ($r = 0.30$, $p = 0.33$) in the smokers' groups. These results suggest that dose-related detrimental effect of smoking on EDV and NO production in both short- and long-term may not be linear.

DISCUSSION

Impairment of EDV is one of the early steps that link cardiovascular risk factors and clinical events (17,18). Using a combined in vivo and in vitro model, we have previously demonstrated that smoking is associated with reduced EDV, which, in part, is a reflection of decreased production or bioavailability of endothelial NO (16). In this study, even a relatively small amount of active cigarette smoking appeared capable of overwhelming the biochemical and cellular process of endothelial NO biosynthesis in vivo and in vitro.

Degree of smoking, EDV and the NO biosynthetic pathway. Celermajer et al. (8) found a dose-dependent reduction of EDV in relation to pack-years of active smoke exposure. On the contrary, in our study both the active heavy and light smokers had a similar reduction in brachial artery EDV compared with nonsmokers. It should be noted that, in the present study, smokers were stratified into two groups by the number of cigarettes smoked as opposed to pack-years of smoking. Nevertheless, number of cigarettes smoked per day, pack-years of smoke exposure and serum cotinine levels were significantly lower in light smokers as compared with heavy smokers. Interestingly, in a later study, Celermajer et al. (13) reported that active and passive smoke exposure caused a similar degree of dysfunction in EDV of the brachial artery in the presence of significantly different salivary cotinine levels. Likewise, Sumida et al. (14), in addition to finding a similar reduction in vasodilatory function of the coronary arteries between active and passive smokers, also found a similar reduction in coronary artery vasodilatory function in individuals with heavy- and light-passive smoke exposure. More recently, Woo et al. (15) failed to a find a dose-dependent relation between the duration of passive smoke exposure and reduced EDV of the brachial artery. The above studies are consistent with our own in vivo findings and parallel our in vitro observations showing similar alterations in basal- and SP-stimulated NO production, eNOS protein expression and activity in both heavy and light smokers. These data suggest that active light smoking can induce equivalent detrimental biochemical changes leading to abnormal endothelial vasodilatory function as active heavy smoking.

ET-1 and smoking. Enothelin-1 is the most potent endogenous vasoconstrictor produced by both the ECs and vascular smooth-muscle cells (19). Available data on the effects of cigarette smoke or its components on ET-1 levels in the circulation or in various tissues are limited. Two early studies suggested that within the first 10 min of active smoking there is a rise in plasma or serum ET-1 level, which is followed by a decline over time (20,21). More recently, in a rat model, acute cigarette exposure was shown to increase the expression of ET-1 messenger ribonucleic acid (mRNA) in heart and lung tissues, but, with chronic cigarette exposure, the effect on the ET-1 mRNA in cardiovascular tissues became insignificant (22). Similarly, Barbera et al. (23) found both ET-1 expression in pulmonary arteries and endothelin content in lung tissue extracts were similar between smokers and nonsmokers. These data, taken together with our in vitro finding of no difference in basal ET-1 production among the three groups, suggest that the increase in ET-1 production in response to active smoking in the healthy individuals is probably a transient phenomenon and restricted only to the acute phase of smoking.

Implications of this study. Endothelial function encompasses the regulation of vasomotor tone, inflammation in the vessel wall and the balance of the thrombotic/thrombolytic factors at the lumen-wall interface (17,18). Nitric oxide, the potent vasodilator secreted by ECs, plays a pivotal role in regulation of all the components of endothelial function (17,18). Endothelin-1, the potent vasoconstrictor also secreted by ECs, opposes the effects of NO (18,19). Thus, a deficiency in NO or an increase in ET-1 can create an unfavorable atmosphere in the lumen-wall interface contributing to cardiovascular pathology. This study investigated the dose-related effect of active smoking on these two molecules. Our data on NO and EDV in active smokers

are consistent with the findings of other studies of active and passive smoke exposure and show that the dose-response curve for cardiovascular effects associated with tobacco smoke exposure may not be linear (5–7,14,24,25). Furthermore, data from the model utilized in the present study suggest that smoking appears to affect only the endothelial NO biosynthesis without significantly altering EC- specific ET-1 biosynthesis.

Study limitations. In the present study, several potential limitations are recognized. First, while similar in vivo and in vitro results in our study population substantiated our findings, the number of subjects enrolled in our study group was relatively small. Additional studies in a larger population may be required to exclude a smaller than hypothesized difference in biophysiology of EDV between light and heavy smokers. Second, in the present study only EC-specific ET-1 production was measured, but vascular-smooth muscle cells (19) also contribute to circulatory ET-1. Thus, our data should only be interpreted as an EC-specific response to smoking. Third, the cardiovascular effects of smoking leading to a clinical event depend on complex interactions involving alterations in the biology of the vessel wall, prothrombotic and proinflammatory molecules and cannot be explained solely by the results of this study.

Conclusions. This study indicates that both heavy and light active smoking (substantiated by pack years and serum cotinine) can cause a similar degree of endothelial vasoregulatory dysfunction in vivo and similar alterations in NO production, eNOS protein and its activity in vitro. Thus, even a small amount of active smoke exposure appears to have a significant effect on one of the early pathophysiologic indicators for atherosclerosis and supports other data that smoking even a small number of cigarettes has potentially deleterious effects contributing to cardiovascular risk. Furthermore, our study suggests that even a small amount of smoke exposure may initiate yet-to-be-defined cellular mechanisms that might overwhelm the biochemical process of NO biosynthesis. Additional investigations are required to elucidate such molecular and cellular mechanisms of endothelial dysfunction.

Reprint request and correspondence: Dr. John A. Ambrose, The Comprehensive Cardiovascular Center, Department of Medicine, Saint Vincent Catholic Medical Centers of New York, 170 West 12th Street, New York, New York 10011. E-mail: jambrose@saintvincentsnyc.org.

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