Contribution of Nicotine to Acute Endothelial Dysfunction in Long-Term Smokers

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OBJECTIVES

The aim of this study was to determine whether nicotine, a constituent of cigarette smoke, contributes to acute endothelial dysfunction after smoking one cigarette.

BACKGROUND

Animal studies suggest that nicotine might cause an impairment of endothelium-dependent vasodilatation via an increase in oxidative stress.

METHODS

Sixteen healthy smokers were entered into a randomized, observer-blinded crossover study comparing the effects of nicotine nasal spray (1-mg nicotine) and cigarette smoke (1-mg nicotine, 12 mg tar) on vascular reactivity in the brachial artery. Using high-resolution ultrasound, flow-mediated dilation (FMD) and endothelium-independent, nitroglycerin-induced dilation were assessed at baseline and 20 min after the administration of nicotine (spray or cigarette).

RESULTS

In response to similar increases in nicotine serum levels, FMD values declined from 10.2 ± 4.4% to 6.7 ± 4.0% after the spray (mean difference: −3.6 ± 2.0%, 95% confidence interval: −4.6; −2.5, p < 0.0001) and from 9.4 ± 3.8% to 4.3 ± 2.8% after the cigarette (−5.1 ± 2.6%, −6.5; −3.7, p < 0.0001). Nitroglycerin-induced dilation remained similar within both periods. Performing a period effect analysis of variance, a significant influence on FMD was found for the mode of administration (p = 0.017) and the baseline value (p = 0.021). The effect on FMD was more pronounced after the cigarette than after the spray (estimated average effect difference: 1.9% FMD). Oxidation parameters did not increase significantly after nicotine spray or tobacco exposure.

CONCLUSIONS

These results demonstrate that nicotine alone causes acute endothelial dysfunction, although to a lesser extent than smoking a cigarette of the same nicotine yield. However, the precise mechanisms by which nicotine leads to this altered vascular reactivity remain unclear. (J Am Coll Cardiol 2002;39:251–6) © 2002 by the American College of Cardiology

Cigarette smoking is an established risk factor for cardiovascular disease and the leading preventable cause of coronary artery disease (CAD) and death in most industrialized countries (1). However, it remains unclear whether the increased occurrence of atherosclerosis in smokers is caused by nicotine or by other components of tobacco smoke. Conflicting results have been obtained with regard to the causal role of nicotine for ischemic events. Several reports suggest that nicotine might cause angina pectoris as well as myocardial infarction (MI) (2). However, transdermal nicotine does not affect platelet activation and catecholamine release compared with placebo, as shown by Benowitz et al. (3). Moreover, in a randomized, double-blind, placebo-controlled study among high-risk outpatients with cardiac disease, transdermal nicotine did not cause a significant increase in cardiovascular events (4). Accordingly, smokeless tobacco use did not increase the risk of MI and did not affect common carotid intima thickness, which is associated with CAD (5–7).

Endothelial dysfunction, an early marker of atherosclerosis, has been observed in chronic smokers as well as after acute cigarette smoking (8–10). However, the precise mechanisms causing this altered vascular reactivity are not fully understood and may be complex. Furthermore, the mechanisms causing acute endothelial dysfunction may be different from those leading to chronic endothelial dysfunction (11). Animal studies examining the effects of nicotine on endothelial function produced controversial results. Chronic nicotine exposure did not effect peripheral vascular reactivity in the rat, neither at low nor at high concentrations (12). In contrast, endothelium-dependent dilation of arterioles in the hamster was modestly impaired by infusion of low concentrations of nicotine and markedly by high concentrations causing an increase in nicotine plasma levels (13). Moreover, an impairment of endothelium-dependent arteriolar dilation has been observed in the hamster after chronic exposure to nicotine and after acute infusion of nicotine (14,15). Reversibility of these effects by superfusion with superoxide dismutase suggested a causal role of oxidative stress (15). The concept that nicotine causes endothelial dysfunction via an increase in oxidative stress...
has been recently supported by a study demonstrating that exposure of endothelial cells to nicotine in vitro and in vivo increases superoxide anion production and endothelial adhesiveness for monocytes (16). It is further conceivable that cigarette smoke contains other substances such as carbon monoxide, inducing significant effects on vascular reactivity (17). Recently, it has been shown that acute local exposure to nicotine impairs endothelium-dependent vasodilation in human veins in vivo (18). The aim of this study was to determine whether nicotine alone causes acute endothelial dysfunction in the brachial artery of long-term smokers and whether nicotine replacement therapy is less harmful for the integrity of endothelial function than smoking a cigarette containing the same amount of nicotine.

**METHODS**

**Study design.** This is an observer-blinded, randomized crossover study examining the effects of nicotine nasal spray and cigarette smoke on vascular reactivity in random order. Subjects received two sprays of a nicotine nasal spray (Nicotrol NS, Pharmacia and Upjohn, Vienna, Austria) or smoked one cigarette (Camel Filters, R. J. Reynolds Tobacco Co., Winston-Salem, North Carolina). Each 10-ml spray bottle contains 100-mg nicotine (10 mg/ml) in an inactive vehicle. After priming the delivery system for Nicotrol NS, each actuation of the unit delivers a metered dose spray containing approximately 0.5 mg of nicotine. The smoking machine determined the nicotine yield for one cigarette as 1-mg nicotine. An additional randomized, observer-blinded crossover study compared the effects of nicotine nasal spray and nicotine nasal spray placebo on flow-mediated dilation (FMD) in the brachial artery. Studies were always conducted at the same time of day (between 3 PM and 5 PM) after fasting for ≥6 h. Subjects stopped smoking ≥2 h before the study and did not change smoking habits from day 1 to day 2. Blood samples were drawn 5 min before and 10 min after the administration of nicotine nasal spray or the ending of smoking a cigarette. Flow-mediated dilation and nitroglycerin-induced dilation (NMD) were evaluated at baseline and 20 min after the administration of a nicotine nasal spray or ending of smoking a cigarette on two subsequent days.

**Assessment of FMD and blood flow.** Endothelium-dependent FMD after reactive hyperemia and endothelium-independent NMD were examined in the brachial artery according to the method described by Celermajer et al. (19). Using high-resolution ultrasound (7.5-MHz linear array transducer), measurements of the right brachial artery were taken at rest after lying quietly for at least 10 min after cuff deflation, after completing suprasystolic compression (250 mm Hg for 4.5 min) of the right upper arm and after sublingual application of 0.8-mg nitroglycerin. Scans of the brachial artery were taken proximal to the bifurcation of the radial and the ulnar artery by the same ultrasound operator. Diameter measurements were taken from one media-adventitia interface to the other for at least three times at baseline and every 20 s after reactive hyperemia and after administration of nitroglycerin. The maximum FMD and NMD diameters were calculated as the average of the three consecutive maximum diameter measurements after hyperemia and nitroglycerin, respectively. The FMD and NMD were then calculated as the percent change in diameter compared with baseline. The impairment of FMD after the application of nicotine (deltaFMD) was estimated as the percent change in FMD values compared with baseline. To verify that suprasystolic compression of the brachial artery caused adequate increases in blood flow, flow velocity was measured at rest and within 15 s after cuff deflation. Blood flow was calculated by multiplying the velocity time integral by the heart rate and the vessel cross-sectional area (3.14 × D^2/4). Reactive hyperemia was then calculated as percent change in flow during hyperemia compared with baseline.

**Abbreviations and Acronyms**

- ANOVA = analysis of variance
- CAD = coronary artery disease
- CI = confidence interval
- DIENE = conjugated dienes
- FMD = flow-mediated dilation
- HPLC = high-pressure liquid chromatography
- LPO = lipid hydroperoxides
- MDA = plasma malondialdehyde
- MI = myocardial infarction
- NMD = nitroglycerin-induced dilation
- NO = nitric oxide
- TBARS = thiobarbituric acid-reactive substances

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**Study population.** Sixteen young healthy smokers were enrolled into the study. No participant had a history of hypercholesterolemia, hypertension or diabetes mellitus. None of the participants took antioxidative drugs before or during this study. The investigations conformed to the principles outlined in the Declaration of Helsinki, and the local human subjects committee approved the study protocol. Written, informed consent was obtained from all participants.

**Determination of nicotine in serum by high-pressure liquid chromatography (HPLC)/mass spectrometry.** (S)-(−)-nicotine 98% was purchased from Aldrich (Vienna, Austria); nicotine-methyl-d3 as internal standard was obtained from Cambridge Isotope Laboratories, Inc. (Andover, Massachusetts). Acetonitrile LiChrosolv, ammonium acetate p. a. and n-butyl acetate HPLC grade was purchased from Merck (Darmstadt, Germany), and methanol Chromasolv was obtained from Riedel de Haen (Selze, Germany).

The HPLC system consisted of Jasco Pu 980 pump, Jasco LG-98002 Ternary Gradient Unit, CMA/260 degasser and a Merck–Hitachi As–4000 intelligent auto sampler equipped with 20-μl sample loop and six-port valve.
The mass spectrometer used was a VG Quattro (Micromass, Altrincham, United Kingdom) operating in positive APCI (atmospheric pressure chemical ionization) mode and using electrospray inlet. The corona discharge needle voltage was set to 3.5 kV; source temperature was 100°C, and mobile phase was acetonitrile/methanol/10 mM ammonium acetate (53/32/15, v/v/v), pH 7.8. The chromatographic column used for the separation was Merck Purospher Star RP-18 endcapped, 30 × 2 mm, and the chromatographic separation was performed at ambient temperature.

A sample preparation consisted of 500 μl serum spiked with 100 μl of internal standard (400 pg/μl) prepared in methanol and extracted with 1 ml of n-butyl acetate for 10 min. The mixture was centrifuged for 5 min, and the organic phase was transferred into the clean 1.5-ml Eppendorf vial. The organic solvent was evaporated in Speedvac concentrator at ambient temperature to near dryness. The residue was dissolved in 100 μl of mobile phase, and 20 μl of it was injected into the chromatographic system.

The increase of nicotine (delta nicotine) after the application of nicotine was calculated as the percent change in nicotine compared with baseline. Determination of oxidation parameters. The measurements of thiobarbituric acid reactive substances (TBARS) are based on the reaction of malondialdehyde (MDA), a secondary breakdown product of lipid hydroperoxides (LPO), with thiobarbituric acid. The assay was performed as described previously (20). Lipid hydroperoxides were determined by a method described by Tateishi et al. (21) using a commercial kit from Kamya company (Seattle, Washington). Peroxidation of polyunsaturated fatty acids leads to the formation of a conjugated diene (DIENE) system, with a characteristic ultraviolet absorption maximum at 234 nm. The measurement of DIENE was performed as described previously (22). Plasma malondialdehyde concentrations were measured after derivatization by thiobarbituric acid and separation on HPLC (MDA-HPLC), as reported previously (23,24).

Statistical analyses. Results are expressed as mean ± SD. Paired t tests (baseline vs. post-treatment values) were performed to examine whether the administration of nicotine has an effect on FMD. The 95% confidence interval (CI) for the mean difference (post − baseline values) was calculated for both periods. Analysis of variance (ANOVA) with the factors mode of administration (2 levels: cigarette, coded with 1 and spray coded with 0), day of administration (2 levels: first and second day), sequence (2 levels: spray/cigarette and cigarette/spray), gender and the interaction between sequence and gender, the random factor patient within sequence and gender (16 levels) and the covariable baseline FMD value was performed for the post-treatment difference to examine the different effect of the nicotine nasal spray and the cigarette smoke on FMD. The same ANOVA model was used to examine the different effects of nicotine nasal spray and nicotine nasal spray placebo on FMD. The ANOVA model was calculated using proc glm with the test option within the SAS computer package (Release 6.12, SAS Institute, Cary, North Carolina). Paired t test was performed to examine whether nicotine serum levels differed between the spray period and the cigarette period. Correlation analysis was performed to examine the association of changes in nicotine serum levels with the impairment of FMD. Analysis of variance with the results were considered significant at p < 0.05.

RESULTS

Clinical and biochemical parameters. Sixteen chronic smokers (13.4 ± 6.5 cigarettes per day, 4.3 ± 2.4 pack years), eight men and eight women, were enrolled into this study. Subjects were characterized as follows: age: mean ± SD: 24.9 ± 1.9 years, body mass index: 21.5 ± 2.6, total serum cholesterol: 4.69 ± 0.98 mmol/l, low-density lipoprotein cholesterol: 2.55 ± 0.80 mmol/l, high-density lipoprotein cholesterol: 1.52 ± 0.44 mmol/l, triglycerides: 1.35 ± 0.76 mmol/l. Levels of nicotine were similar in both study groups at baseline (spray vs. cigarette: 13.78 ± 8.09 ng/ml vs. 13.25 ± 4.57 ng/ml, p = 0.805) and after application of nicotine (spray vs. cigarette: 19.91 ± 16.63 ng/ml vs. 18.98 ± 6.93 ng/ml, p = 0.764). Both nasal spray and cigarette smoke resulted in significant increases of nicotine serum levels (spray: p = 0.027; cigarette: p = 0.003). With regard to TBARS, DIENE, LPO and MDA-HPLC, no significant increases were observed either after nicotine nasal spray or after cigarette smoking.

FMD and blood flow responses. Flow-mediated dilation values declined from 10.2 ± 4.4% to 6.7 ± 4.0% after the spray (mean post/baseline difference: −3.6 ± 2.0%, 95% CI: −4.6; −2.5, p < 0.0001) and from 9.4 ± 3.8% to 4.3 ± 2.8% after the cigarette (mean post/baseline difference: −5.1 ± 2.6%, 95% CI: −6.5; −3.7, p < 0.0001) (Fig. 1). The changes in FMD were influenced by the mode of administration and the baseline value but not by the day of administration, the sequence, the gender or the sequence × gender interaction. On both days the mean FMD difference was less pronounced after the spray than after the cigarette (first day: −4.0 ± 1.7% vs. −5.2 ± 2.8%, second day: −3.1 ± 2.2% vs. −5.3 ± 2.8%). The estimated average effect difference was 1.9% FMD. Vessel size (baseline vs. after spray: 3.4 ± 0.7 mm vs. 3.4 ± 0.6 mm, p = 0.096; baseline vs. after cigarette: 3.4 ± 0.6 mm vs. 3.4 ± 0.5 mm, p = 0.669) and NMD (baseline vs. after spray: 15.3 ± 5.0% vs. 14.0 ± 5.5%, p = 0.124; baseline vs. after cigarette: 14.3 ± 4.5% vs. 13.8 ± 5.2%, p = 0.517) remained similar after the administration of 1-mg nicotine and were not affected by the mode of administration. Increases in blood flow during reactive hyperemia increased slightly but not significantly after the spray (430 ± 267% vs. 551 ± 384%, p = 0.058) and after the cigarette (402 ± 239% vs. 551 ± 384%, p = 0.216). The impairment of FMD was not related
to increases of nicotine serum levels after cigarette smoking and nicotine nasal spray, respectively.

Eight of the 16 healthy smokers returned to the laboratory and entered into a randomized, observer-blinded crossover study, comparing the effects of nicotine nasal spray (1-mg nicotine) and of nicotine nasal spray placebo on FMD in the brachial artery. Flow-mediated dilation values declined from 9.0 ± 2.7% to 4.6 ± 2.6% after the spray (mean post/baseline difference: -4.45 ± 2.46%, 95% CI: -6.50; -2.40, p < 0.0014) and from 10.2 ± 4.1% to 9.3 ± 2.8% after the nicotine nasal spray placebo (mean post/baseline difference: -0.88 ± 1.90%, 95% CI: -2.47; 0.71, p < 0.2320). Performing a period effect ANOVA, a significant influence on FMD was found only for the mode of administration (p = 0.023) but not for the baseline value, the day of administration, the sequence, the gender or the interaction between gender and sequence.

**DISCUSSION**

Our findings show that the administration of two sprays of nicotine nasal spray containing 1 mg of nicotine causes endothelial dysfunction in the brachial artery of chronic smokers, although to a lesser extent than smoking a cigarette with a machine-determined nicotine yield of 1-mg nicotine, suggesting that nicotine contributes to acute endothelial dysfunction after cigarette smoking.

**Cigarette smoking and cardiovascular disease.** Chronic smokers are at increased risk of acute MI, unstable angina and sudden death (25). Moreover, cigarette smoking causes immediate constriction of epicardial coronary arteries and an increase in coronary artery resistance vessel tone despite an increase in myocardial oxygen demand (26), and the stimulation of coronary artery alpha-adrenergic receptors by circulating or locally released catecholamines may be responsible for these effects (27). On the other hand, the integrity of vascular endothelial cell function may play a key role in determining the vascular response to cigarette smoking (28,29). The mechanisms by which cigarette smoking leads to endothelial dysfunction are gradually being revealed. Oxidative stress may mediate this adverse effect since cigarette smoke contains large amounts of free radicals such as superoxide anion and hydroxyl radicals (30,31) that degrade nitric oxide (NO) released from the endothelium. Indeed, antioxidants such as vitamin E and C have been shown to attenuate the impairment of endothelium-dependent vasodilation after heavy cigarette smoking, due to an improvement of the antioxidant status (11,30). Recently, tetrahydrobiopterin supplementation has been shown to improve FMD in long-term smokers, supporting the concept that, in addition to the free radical burden of cigarette smoke, a dysfunctional NO synthase due to tetrahydrobiopterin depletion may contribute, at least in part, to endothelial dysfunction in smokers (32). Furthermore, inhaling a single cigarette decreased exhaled NO in current smokers, suggesting that it may inhibit the enzyme NO synthase whereas inhalation of NO itself and carbon monoxide, both constituents of tobacco smoke, had no effect on exhaled NO in nonsmoking controls (33).

**Nicotine and endothelial function.** The concept that nicotine causes endothelial dysfunction via an increase in
oxidative stress has been recently supported by animal studies demonstrating that chronic exposure to nicotine and acute infusion of nicotine cause an impairment of endothelium-dependent arteriolar dilation that can be restored by superperfusion with superoxide dismutase (15). Superoxide causes a loss of the vasodilatory action of NO and yields peroxynitrite at the same time. Subsequently, peroxynitrite causes endothelial cell damage via membrane lipid peroxidation (34) but also nitrates and inactivates PG12 synthase, leaving unmetabolized prostaglandin H2, which causes vasospasm via the Tx2A2/prostaglandin H2 receptor (35). Indeed, nicotine has been shown to decrease PG12 synthesis in the vascular endothelium, whereas it has no effect on thromboxane A2 production (36). However, the contribution of PG12 to endothelium-dependent relaxation may be less important than its platelet inhibitory effects. Indeed, at least in the porcine coronary circulation, PG12 contributes very little to the regulation of vascular tone (37). This study extends these findings by demonstrating that nicotine causes endothelial dysfunction in humans. In addition to nicotine, other constituents of cigarette smoke may contribute to acute endothelial dysfunction after cigarette smoking, since nicotine nasal spray caused less impairment of FMD than smoking a cigarette containing the same amount of nicotine. However, the estimated average intake of nicotine is 0.55 mg per nicotine nasal spray (1-mg nicotine) and 1.43 mg per cigarette (1-mg nicotine) (38).

Study limitations. A possible drawback of this study may be that the nicotine plasma kinetic after the cigarette differs from the kinetic after the spray and that this difference may be responsible for different effects on FMD. Indeed, Gourlay et al. (39) showed that both venous and arterial levels of nicotine after cigarette smoking are much higher and occur much quicker compared with nicotine nasal spray, although the nasal administration system has been developed to more closely approximate cigarette delivery for improved efficacy in clinical application and for more control in systemic testing of nicotine (40). However, in this study, using a protocol different from the one of Gourlay et al. (39), increases of venous nicotine serum levels were not significantly different between the study groups, and nicotine serum levels before the ultrasound examination were very similar in the spray period and in the cigarette period. Thus, mechanisms different from pharmacokinetic factors may have at least contributed to the more adverse effect of cigarette smoking on FMD compared with the nicotine nasal spray. Moreover, this study could not establish oxidative stress as a relevant mechanism for nicotine-induced endothelial dysfunction. Indeed, we cannot exclude that the number of patients enrolled in this study may have been insufficient to show significant increases in oxidation parameters. Thus, further studies enrolling a larger number of patients are required to determine whether nicotine causes endothelial dysfunction, at least in part, via an increase in oxidative stress. However, this study was not designed to examine the long-term effects of nicotine on endothelial function, and the results cannot necessarily be extrapolated to slow-release application of nicotine.

Summary and conclusions. The findings of this study demonstrate that nicotine causes acute endothelial dysfunction in long-term smokers and suggest that there may be other constituents of cigarette smoke that contribute to this adverse effect. However, the precise mechanisms responsible for this negative effect of nicotine on endothelial function remain unclear. We conclude that nicotine replacement therapy by nasal spray is less harmful for the endothelium than cigarette smoking but fails to preserve the integrity of endothelial function.

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