Modulation of Nitric-Oxide Synthase by Nicotine

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ABSTRACT

Effects of nicotine on arterial endothelium-dependent relaxations mediated by nitric oxide are controversial. Experiments were designed to test the hypothesis that nicotine can directly alter activity of endothelial nitric-oxide synthase (eNOS). NOS from aortic endothelial cells of untreated dogs and recombinant eNOS, neuronal NOS, and inducible NOS were used for these experiments. NOS activity was determined as conversion of \( \text{L-[^3H]} \text{arginine to L-[^3H]} \text{citrulline in the absence or presence of nicotine (10}^{-7} \text{–} 10^{-5} \text{M}) \) in vitro. In separate assays, concentrations of cofactors NADPH, FAD, and tetrahydrobioprotein were reduced by half to assess for possible interaction with nicotine. With enzyme from aortic endothelial cells, total and calcium-dependent accumulation of citrulline increased by 30% in the presence of \( 10^{-5} \text{ M nicotine. Nicotine dose dependently also increased citrulline accumulation by recombinant eNOS and neuronal NOS but not inducible NOS. Effects of nicotine on accumulation of citrulline by isolated eNOS and recombinant eNOS were further modulated by changes in the concentration of NADPH in the incubation solution. Our data demonstrate a significant effect of nicotine on eNOS-mediated citrulline accumulation. These results suggest that effects of nicotine on production of nitric oxide may depend on NADPH or oxygen radical interactions with NOS and thus may explain, in part, inconsistent findings of changes in production of endothelium-derived nitric oxide with nicotine administration.

Smoking is a leading cause of cardiovascular disease, accounting for 30% of cardiac deaths in the United States each year (Ockene and Miller, 1997). Although nicotine is the addictive component of cigarette smoke, it should be recognized that smoking is not equivalent to nicotine. In fact, nicotine is but one of several thousand components of cigarette smoke. Given nicotine’s widespread use in tobacco products and in over-the-counter nicotine patches and gum, and the prescription products nasal spray and nicotine inhaler, it is important to determine effects of nicotine on the cardiovascular system independent of tobacco smoking.

Effects of nicotine on the cardiovascular system are multifactorial, reflecting activity of nicotinic receptors centrally and on peripheral autonomic ganglia (McPhail et al., 1998). In addition, effects of nicotine on endothelial cells and expression of endothelium-dependent relaxations are controversial (Li and Duckles, 1993; Li et al., 1994; Mayhan and Patel, 1997; Mayhan and Sharpe, 1999; Clouse et al., 2000b; Miller et al., 2000). These conflicting results reflect in part effects of nicotine on several endothelium-derived factors contributing to vascular tone, including changes in production of prostaglandins, nitric oxide, endothelin, bradykinins, and leukotrienes (Toda, 1975; Bull et al., 1988; Toda and Okamura, 1992; Suzuki et al., 1994).

Nitric oxide is a major endothelium-derived relaxing factor of the arterial circulation; thus, any perturbation in the synthesis of nitric oxide could potentially have significant effects on blood pressure, flow, and vascular resistance (Knowles, 1996). Although direct infusion of nicotine may reduce nitric oxide-mediated relaxations of mesenteric arteries, inhaled nicotine may maintain circulating nitric oxide in humans (Mayhan and Patel, 1997; Miller et al., 1998; Mayhan and Sharpe, 1999). Nicotine could alter production of nitric oxide in several ways. Nicotine could change production of nitric oxide directly through nicotinic-receptor activation of nitrooxidergic nerves (Toda and Okamura, 1992), endothelial cells (Macklin et al., 1998), or bypass receptor activation by directly interacting with biochemical pathways in endothelial cells. Alternatively, nicotine could alter activity of nitric-oxide synthase (NOS) indirectly through production of oxygen-derived free radicals (Mayhan and Sharpe, 1999). Therefore, because nicotine can cross cell membranes, it is possible that nicotine could directly affect production of nitric oxide through interaction with the enzyme NOS. Therefore, experiments were designed to test the hypothesis that nicotine directly affects the enzymatic conversion of L-arginine to L-citrulline by nitric-oxide synthase.

ABBREVIATIONS: NOS, nitric-oxide synthase; eNOS, endothelial NOS; BH4, tetrahydrobioprotein; nNOS, neuronal NOS; iNOS, inducible NOS.
Materials and Methods

Isolation of eNOS from Aortic Endothelial Cells. Adult male mongrel dogs (20–30 kg) were maintained in accordance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the National Institutes of Health (NIH Publication 86-23, revised 1996).

A 5-cm segment of descending thoracic aorta was removed from anesthetized (30 mg/kg pentobarbital i.v.) male mongrel dogs and placed in cold, modified Krebs-Ringer-bicarbonate solution of the following millimolar composition: 118.3 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 K₂HPO₄, 25.0 NaHCO₃, 0.026 calcium disodium edetate, 11.1 glucose. Endothelial cells were then immediately scraped from each aorta’s surface and placed in 1 ml of homogenate buffer (50 mM Tris-HCl, 320 mM sucrose, 0.1 mM EDTA, two tablets Complete protease inhibitor, pH 7.8) and snap frozen in liquid nitrogen. Samples were stored at −70°C.

To prepare NOS-containing extract, 100 μg/ml phenylmethylsulfonyl fluoride was added to the aortic cells that were then homogenized for 10 s using a tissue homogenizer (Tekmar, Cincinnati, OH). Homogenates were centrifuged at 2000g for 10 min at 4°C to remove cellular debris. To collect the crude NOS extract, the supernatant was run through a desalting column (Bio-Rad, Hercules, CA). NOS extract from two to four individual animals was pooled to provide adequate protein concentration for each assay. A small aliquot of pooled samples was set aside to measure protein concentration using the bicinchonic acid protein assay reagent kit (Pierce, Rockford, IL) and adequate protein concentration for each assay. A small aliquot from that of Myatt et al. (1993) and previously published by our group (Wang et al., 1997; Jeppsson et al., 1998). The standard incubation buffer consisted of 5 μM L-arginine plus 14.7 mM [³H]-l-arginine, 54 mM L-valine, 1.2 mM MgCl₂, 1.0 mM NADPH, 10 μM BH₄, 2.0 μM FAD, 50 U/ml calmodulin, and 50 mM Tris buffer. When nicotine was added to an assay, 20 μM of freshly diluted (S)-nicotine (Sigma Chemical, St. Louis, MO) replaced a volume of Tris in the incubation buffer to give final nicotine concentrations of 10⁻⁷ to 10⁻³ M. In some experiments, standard molar concentration of NADPH, FAD, or BH₄ were reduced in the incubation buffer. Each incubation buffer was subdivided into three tubes, adding either 0.83 mM CaCl₂, 1.0 mM EGTA, or 2.0 mM L⁻²-hydroxyethylpiperazine-N'⁻²-ethanesulfonic acid, 8 mM EDTA, pH 5.5. Columns (Poly-Prep chromatography columns; Bio-Rad) were prepared by adding Dowex suspension, which retains the highly charged L-³H]-arginine, but allows L⁻¹³C]citrulline to pass through. The elute was collected into Opti-Fluor solution (Packard, Meriden, CT) in scintillation vials. L-³H]Citrulline activity was measured using a Beckman 6800 liquid scintillation counter. Knowing the specific activity ratio (150,960 dpm/pmol), NOS activity was expressed as picomoles of L⁻¹³C]citrulline produced per milligram of protein per hour. Calcium-dependent activity was measured as total minus calcium-independent activity, correcting for nonspecific activity.

Assays Using Recombinant eNOS, nNOS, and iNOS. NOS assays were repeated as described above using recombinant bovine eNOS (gift from Dr. William Sessa, Yale University, or purchased from Caymen Chemical Inc., Ann Arbor, MI), rat nNOS, and murine iNOS (Caymen Chemical Inc.) with the following modifications. Recombinant nNOS (14.8 and 1.4 μg of enzyme/tube), eNOS (0.9 μg of enzyme/tube), and iNOS (5.9 μg of enzyme/tube) were incubated in the absence and presence of nicotine (10⁻⁷, 10⁻⁵, and 10⁻³ M) with standard cofactor conditions for 3, 10, and 30 min. In a separate experiment, recombinant eNOS (0.9 μg of enzyme/tube) was incubated with varying concentrations of NADPH (0.5–1 mM) for 30 min. For experiments using recombinant enzymes, a single assay was performed in triplicate.

Statistical Analysis. All assays using nitric-oxide synthase isolated from canine aortic endothelial cells were performed in duplicate and results were averaged. Data are expressed as mean ± standard error; n refers to the number of separate assays. To provide adequate amounts of enzyme, each assay consisted of combined enzymes isolated from cells of two to four dogs. Assays using recombinant enzyme were performed in triplicate. Results for each assay were calculated as picomoles of L⁻¹³C]citrulline produced per milligram of protein per time. Unless stated otherwise, NOS activity was normalized to that obtained in the absence of nicotine (100%). For assays using NOS extracts from endothelial cells, one-way ANOVA was used to compare more than two means. Significance was determined by P < .05. If overall significance was detected, a post hoc Newman-Keuls test for paired comparisons was applied.

Results

Effects of Nicotine on NOS Isolated from Aortic Endothelial Cells

NOS isolated from canine aortic endothelial cells was incubated with nicotine for 60 min at 37°C. Total citrulline accumulation increased with increasing concentrations of nicotine, reaching statistical significance at 10⁻⁵ M nicotine (Fig. 1). Calcium-dependent citrulline accumulation in the presence of 10⁻⁵ M nicotine was 28.4% greater than activity in the absence of nicotine (control) (Table 1). Calcium-independent citrulline accumulation and nonspecific radioactivity were similar among treatment groups. Calcium-dependent citrulline accumulation significantly exceeded that of calcium-independent accumulation in all assays using isolated enzyme (P < .001, Table 1).

Effects of Nicotine on Recombinant NOS

Citrulline accumulation increased linearly with incubation time for recombinant eNOS and iNOS (Fig. 2). Nicotine dose dependently increased nNOS-mediated accumulation of citrulline after 10 min of incubation, reaching about 20 and 43% increase with 10⁻⁷ and 10⁻⁵ M nicotine, respectively, at this time point (Fig. 2). Citrulline accumulation with recombinant
eNOS increased between 10 and 16% after a 30-min incubation with all three concentrations of nicotine. Notably, no significant effect of nicotine was observed at earlier time points. Accumulation of citrulline with recombinant iNOS did not increase with nicotine and in contrast to accumulation of citrulline with nNOS and eNOS decreased by 13% with $10^{-3}$ M nicotine (Fig. 2).

**Possible Mechanism of Nicotine-NOS Interaction**

**Enzyme Isolated from Aortic Endothelial Cells.** To identify possible sites where nicotine might affect NOS, assays were performed using half the standard assay concentrations of NADPH, FAD, or BH$_4$. Decreasing the concentration of NADPH from 1.0 to 0.5 mM decreased total citrulline accumulation by 25% (Fig. 3). Reducing FAD (2.0–1.0 mM) or BH$_4$ (10.0–5.0 mM) did not reduce NOS citrulline accumulation significantly compared with that obtained using standard assay conditions (data not shown; $n = 4$ assays with each condition). Under conditions of reduced NADPH, but not FAD or BH$_4$, nicotine caused a statistically significant dose-dependent decrease in total and calcium-dependent citrulline accumulation (Fig. 4).

**Recombinant eNOS.** In the presence of $10^{-5}$ M nicotine, accumulation of citrulline by recombinant eNOS increased with concentrations of NADPH from 0.3 to 0.5 mM during a 30-min incubation (Fig. 5, top). After a 30-min incubation with NADPH (1 mM), citrulline accumulation by recombinant eNOS was increased by 50% with $10^{-5}$ M but remained at control levels with $10^{-3}$ M nicotine. However, in the presence of $10^{-3}$ M nicotine, accumulation of citrulline was 60% less than control (absence of nicotine) when NADPH was 0.3 mM (Fig. 5, bottom).

**Discussion**

Results from these experiments demonstrate for the first time that nicotine may modulate citrulline accumulation from L-arginine by eNOS. This modulation in vitro is dose-related over a concentration range of $10^{-7}$ to $10^{-3}$ M nicotine. Concentrations of nicotine in blood of human smokers or abstinent smokers using nicotine products for smoking cessation or nonsmokers using nicotine treatments for ulcerative colitis range from $10^{-7}$ to $10^{-6}$ M (Bannon et al., 1989; Palmer et al., 1992; Hurt et al., 1993; Sandborn, 1999). Stimulatory effects of nicotine on NOS activity were observed consistently with calcium-dependent isoforms of the enzyme, i.e., eNOS and nNOS. This stimulatory effect of nicotine was associated with “protection” against enzyme inactivation during the in vitro assay, but was not apparent during the initial phase of enzyme activity. Furthermore, stimulatory effects with eNOS were observed with three different sources of enzyme: crude enzyme from canine aortic endothelial cells and recombinant enzyme from two different sources.

The mechanism by which nicotine affects NOS cannot be determined from these experiments. However, some inferences can be derived. Effects of nicotine on citrulline accumulation by eNOS were influenced by concentrations of NADPH in the incubation solution. The apparent discrepancy between the dose effect of NADPH and nicotine on citrulline accumulation for NOS from aortic endothelial cells and recombinant enzyme (Figs. 4 and 5a) may relate to the purity of the enzyme preparation. It is unclear as to whether interactions among nicotine, NADPH, and NOS are direct or result from interactions associated with the production of oxygen radicals (Miller et al., 1998; Vasquez-Vivar et al., 1998; Mayhan and Sharpe, 1999).

Differences in absolute concentrations of citrulline accumulation among preparations may reflect differences in the purity of the enzymes. It is unclear as to what other factors may be present in enzyme prepared from aortic endothelial cells that affect NOS activity. Protein yield was low when enzyme was extracted from canine endothelial cells and it was necessary to combine isolates from several dogs to yield sufficient protein for each assay. Enzyme activity under control conditions for extracted eNOS was within the range of that reported using the same extraction method (Myatt et al., 1993) where specific activity ranged from about 10 to 831 pmol/mg/45 min. And, as would be expected for eNOS isolated from nonarteriosclerotic or noninfected animals, iNOS or calcium-independent conversion was low.

What, then, might be the physiological implications of these observations? Modulation of citrulline accumulation and by implication nitric oxide by nicotine in these in vitro experiments ranged from 25 to 50% of control assay conditions. Although changes within this range may not seem dramatic for assays optimized for substrate and cofactor conditions, similar changes in vivo where such parameters are also modulated may be of consequence. Nicotine applied directly to endothelial cells alters production of a variety of vasoactive factors (Bull et al., 1988; Suzuki et al., 1994). However, effects of nicotine on endothelium-dependent responses in blood vessels is variable and may be related to mode of administration of nicotine (infusion compared with transdermal patches or osmotic minipump), dose, and duration of treatment (Li and Duckles, 1993; Li et al., 1994; Mayhan and Patel, 1997; Mayhan and Sharpe, 1999; Clouse et al., 2000b; Miller et al., 2000). Results of the present experiments support a biphasic effect of nicotine on NOS, which is dependent on concentrations of NADPH. These observations would be consistent with the in vivo literature and provide a possible mechanistic explanation for the in vivo results. In vivo, nicotine effects on cells independent of nico-

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**TABLE 1**

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<th>Citrulline Accumulation</th>
<th>Nicotine Treatment</th>
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<tr>
<td></td>
<td>Untreated</td>
</tr>
<tr>
<td>Total</td>
<td>316.4 ± 113.7</td>
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<tr>
<td>Calcium dependent</td>
<td>291.7 ± 117.7</td>
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<td>Calcium independent</td>
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nicotine receptor activation are poorly understood and would depend on the concentration gradient for diffusion of nicotine into the cells. The concentration gradient would be influenced by dose, duration of treatment, and metabolism of nicotine. Effects of nicotine on NOS in vivo are the summation of nicotine’s effects on numerous other target sites in the central and peripheral nervous system. When administered acutely, nicotine acts as a vasoconstrictor in vivo. However, when administered chronically, vasodilatation may be observed (Bassenge et al., 1988). Potentially, nicotine could exert opposing effects on the same vessel. Alternatively, effects of nicotine in vivo either could be potentiated or masked by effects of metabolites, such as cotinine (Carty et al., 1996, 1997; Vainio et al., 1998; Rama Sastry et al., 1999). In spite of the complexity of these interactions, results of the present study showing in vitro effects of nicotine on NOS could explain, in part, the biphasic time and dose dependence of

**Fig. 2.** Calcium-dependent citrulline accumulation by recombinant nNOS (top), eNOS (middle), and calcium-independent accumulation for recombinant iNOS (bottom) in the absence (control) and presence of nicotine. Control, ○; 10⁻² M nicotine, ■; 10⁻³ M nicotine, ●; 10⁻⁴ M nicotine, ▲. Data are expressed as percentage of accumulation in the absence of nicotine (L-[³H]citrulline: nNOS = 88 nmol/mg of protein/30 min; eNOS = 18.1 nmol/mg of protein/30 min; iNOS = 34.3 nmol/mg of protein/30 min). Each data point represents the mean of triplicate assays from one experiment.

**Fig. 3.** Total citrulline accumulation by eNOS isolated from canine aortic endothelial cells incubated with standard and half concentrations of NADPH (0.5 mM) at 37°C for 1 h. Citrulline accumulation is shown as percentage of change from standard conditions (4.3 ± 0.8 nmol/mg of protein/60 min), set equal to 100%. Values are expressed as mean ± S.E. of four assays per condition. Halving NADPH reduced total citrulline accumulation by 25%. Each assay contained enzyme isolated and combined from four different dogs.

**Fig. 4.** Calcium-dependent citrulline accumulation by eNOS isolated from canine aortic endothelial cells in the absence (control) and presence of nicotine with half standard concentrations of NADPH in the incubation media. Values are expressed as percentage of calcium-dependent citrulline accumulation in the presence of reduced cofactor concentration (3.1 ± 0.4 nmol/mg of protein/60 min). Values are mean ± S.E., n = 4 assays for each column. Each assay contained enzyme isolated and combined from four different dogs. Asterisk denotes statistically significant difference from assays in the absence of nicotine (analysis of variance, P < .001 versus no nicotine, P < .05 versus 10⁻⁶ M).
action of nicotine with nNOS and iNOS may be possible given the structural and functional homology among the NOS enzymes. Effects of nicotine on iNOS were not as great as with nNOS or eNOS and very modest inhibition, rather than activation, was observed. This difference was not related to the kinetics of action because shorter incubation periods did not demonstrate activation of iNOS. Rather, differences in activation may relate to the binding characteristics of the NOS isoforms for cofactors or sensitivity to inhibition by oxygen free radicals.

Nicotine is available over-the-counter for use in smoking cessation. The use of transdermal nicotine has been proven safe in clinical smoking cessation trials for humans with coronary arterial disease (Joseph et al., 1996). In typical doses achieved by smokers, nicotine causes elevated alertness, mild elevation in blood pressure and heart rate, and gastrointestinal and urinary stimulation; these are all effects to which tolerance has been demonstrated. Results of this study establish a new mechanism by which nicotine may affect these functions through receptor-independent regulation of NOS. The observation that effects of nicotine on NOS may be related to cofactor interactions may explain inconsistent findings when nicotine is applied to endothelial cells in culture, or to different vascular beds in situ or in vivo. In addition, interactions of nicotine with nNOS and iNOS may provide insights into how this drug may alter neurological and immunological functions.

References


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