Time and dose effect of transdermal nicotine on endothelial function

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Miller, Virginia M., W. Darrin Clouse, Britt H. Tonnessen, Umar S. Boston, Sandra R. Severson, Sue Bonde, Kevin S. Rud, and Richard D. Hurt. Time and dose effect of transdermal nicotine on endothelial function. Am J Physiol Heart Circ Physiol 279: H1913–H1921, 2000.—Nicotine patches are available as an over-the-counter medication for aid in smoking cessation. This study was designed to determine how nicotine patch therapy over time and dose ranges used in smoking cessation programs in humans affects endothelium-dependent relaxations. Dogs were treated with nicotine patches (11, 22, or 44 mg/day) for 2 and 5 wk. Circulating nicotine and oxidized products of nitric oxide (NOx) were measured. Coronary arteries were prepared for measurement of isometric force and aortic endothelial cells were prepared for measurement of mRNA or NO synthase (NOS) activity. Circulating nicotine increased with increasing concentrations of nicotine patches. After 5 wk of treatment with 22 mg/day patches, circulating NOx was reduced but NOS activity was increased. NOS mRNA was similar among groups. Only after 5 wk of treatment with 22 mg/day patches were endothelium-dependent relaxations reduced to α1-adrenergic agonists, ADP, and the calcium ionophore A-23187. These results suggest a time and biphasic dose effect of nicotine treatment on endothelium-dependent responses that may be related to bioavailability of NO. This complex relationship of duration and dose of nicotine treatment may explain, in part, discrepancies in effects of nicotine on endothelium-dependent responses.

SMOKING IS THE LEADING CAUSE of cardiovascular disease, accounting for 30% of cardiac deaths in the United States (12). Nicotine is the addictive component of cigarette smoke (3). However, it should be recognized that harmful cardiovascular effects of cigarette smoke, which contains thousands of known carcinogens, is not equivalent to nicotine consumption (11, 26). Indeed, 2-yr exposure of rats to inhaled nicotine did not result in significant hypertension nor gross vascular or cardiac pathology (48).

Transdermal nicotine is now available over the counter as a therapeutic modality for smoking cessation, making its use less well supervised than when it was a prescription medicine. It is important to understand more fully the effects of transdermal nicotine on vascular function at tissue and cellular levels. Circulating nicotine could affect vascular function through activation of central and/or peripheral nicotinic receptors associated with sympathetic and parasympathetic fibers to the heart and blood vessels (27). In addition, nicotine could affect vascular function directly through modulation of vascular endothelium (5, 42, 44, 47). Effects of direct infusion or subcutaneous absorption of nicotine on functions of arterial endothelium are controversial, with no changes, increases, and decreases in endothelium-dependent relaxations described (21–23, 25, 26). In human smokers, nicotine nasal spray for smoking cessation may sustain increases in circulating nitric oxide (NO) (29). Differences in responses to nicotine may depend on the anatomic origin of the blood vessels studied and the route of administration, dose, and duration of nicotine exposure. Effects of transdermal nicotine treatment on functions of arterial endothelium are not known; therefore, experiments were designed to determine dose effects of transdermal nicotine on the function of coronary arterial endothelium and aortic endothelial NO synthase (NOS). Transdermal nicotine was tested at doses within and double those used for conventional smoking-cessation programs (7–22 mg) in humans (10). On the basis of other animal studies, it was hypothesized that transdermal nicotine would dose-dependently decrease endothelium-dependent responses in coronary arteries.

METHODS

Animals. 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 No. 86-23, Revised 1996). Dogs were either untreated (controls) or treated with transdermal nicotine patches (Lederle Laboratories, Pearl River, NY) of 11, 22, or 44 mg/day (two 22-mg patches) doses. Patches were applied to shaved necks and were changed daily. None of the animals exhibited signs of nicotine toxicity.

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**Blood samples.** To validate systemic absorption of nicotine in treated dogs, nicotine concentration ([nicotine]) and cotinine concentration ([cotinine]), the major metabolite of nicotine, were measured in venous blood before initiation of treatment and again at 6 h (peak) and 24 h (trough) after patch placement. These time points were selected based on previous studies of pharmacokinetics of transdermal nicotine (34). Nicotine was measured in serum by mass spectrometry, previous studies of pharmacokinetics of transdermal nicotine treatment and again at 6 h (peak) and 24 h (trough) after treatment in treated dogs, nicotine concentration ([nicotine]) plus 14.7 nM [3H]cotinine, 54 mM L-valine, 1.2 mM MgCl₂, 1 mM NaDHP, 10 μM BH₄, 2 μM FAD, 50 U/ml calmodulin, and 50 mM Tris-HCl buffer. Each incubation buffer was subdivided into three tubes, adding either 0.83 mM CaCl₂, 1 mM ethylene glycol-EGTA, and 2 mM l-NMMA to respectively assess total, calcium-independent, and non-specific activity. Exogenous nicotine was not added to any of the incubation buffers. The reaction was started by adding sample homogenates and placing in a shaker bath for 1 h at 37°C. The reaction was terminated by adding 1.5 ml of ice-cold HEPES buffer (pH 5.5). Columns (Poly-Prep chromatography columns, Bio-Rad) were prepared by adding Dowex suspension, which retains the charged species of L-[3H]citrulline but allows l-[3H]citrulline to pass through. The elute was circumvented into Anti-Flux solution (Perkin-Elmer, Meriden, CT) in scintillation vials. l-[3H]citrulline activity was measured using a Beckman 6800 liquid scintillation counter. NOS activity was expressed as picomoles of l-[3H]citrulline produced per milligram of protein per hour. Calcium-dependent activity was measured as total activity minus calcium-independent activity, correcting for nonspecific activity.

**RT-PCR.** RT-PCR was performed on total RNA extracted from aortic endothelial cells using RNA STAT-60 reagent (Tel-Test B, Friendswood, TX). The supernatant was removed, and the RNA pellet was washed with 1 ml of 75% ethanol, air dried, and reconstituted in diethylpyrocarbonate (DEPC)-treated water. [RNA] was determined by measuring absorbance at 260 nm in a spectrophotometer (Beckman DU 640, Fullerton, CA). DNase I digestion (amplification grade, Life Technologies, Rockville, MD) of the RNA preparation was performed to eliminate genomic DNA. DNase treatment of 2 μg of total RNA was carried out with 2 μl DNase buffer (200 mM Tris-HCl at pH 8.4, 500 mM KCl, and 20 mM MgCl₂) plus 2 μl of amplification grade DNase I (Life Technologies) for 15 min at room temperature. DNase I was then inactivated by heating to 65°C after addition of 2 μl of 25 mM EDTA.

Total RNA was transcribed to cDNA following the manufacturer’s instructions and the oligo(dT) priming method. Reverse transcription was performed using SuperScript Pre-amplification System (Life Technologies) after addition of 1 μl of oligo(dT)₁₂₋₁₈ primers to hybridize to 3’-poly(A) tails on mRNA (70°C for 10 min), followed by 7 μl of reaction mixture (2 μl 10× PCR buffer, 2 μl 25 mM MgCl₂, 1 μl dNTP mix, and 2 μl 0.1 M dithiothreitol) (42°C for 5 min) and 1 μl SuperScript II RT (42°C for 50 min). The reaction was terminated by heating to 70°C for 15 min followed by incubation with RNase H for 20 min at 37°C. Target cDNA was next amplified by PCR: 35 cycles of denaturation (94°C for 45 s), annealing (60°C for 45 s), and polymerization (72°C for 60 s). The primers used, 5’-TCAACAGTACTACAGCTCC and 3’-GT-GTTGCGAGTGTAGTGA, detected endothelial cell NOS (ecNOS). A 251-bp product was visualized on 2% agarose gel electrophoresis. Glyceraldehyde-3-phosphate dehydrogenase primers were added to verify the efficiency of cDNA synthesis. Control reactions performed in the absence of RT were negative for genomic DNA.

The cDNA was next quantified using the PCR-MIMIC technique (Clontech, Palo Alto, CA). A MIMIC (nonhomologous internal standard) DNA was constructed by performing two rounds of PCR amplification. In the first reaction two...
Table 1. Circulating concentrations of nicotine and cotinine in dogs treated with transdermal nicotine patches for 2 and 5 wk

<table>
<thead>
<tr>
<th>Sample Time</th>
<th>Nicotine, ng/ml</th>
<th>Cotinine, ng/ml</th>
</tr>
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<tbody>
<tr>
<td>Peak, 6 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotine</td>
<td>8.9 ± 2.8</td>
<td>15.7 ± 8.1</td>
</tr>
<tr>
<td>Cotinine</td>
<td>(n = 9)</td>
<td>(n = 7)</td>
</tr>
<tr>
<td>Trough, 24 h</td>
<td>1.3 ± 0.5†</td>
<td>9.5 ± 6.9</td>
</tr>
<tr>
<td>Nicotine</td>
<td>(n = 9)</td>
<td>(n = 7)</td>
</tr>
<tr>
<td>Cotinine</td>
<td>(n = 9)</td>
<td>(n = 7)</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. *Statistical significance from 11 and 22 mg/day treatment groups, P < 0.05; †Statistical significance from values at 6 h within treatment groups, P < 0.05.

RESULTS

Blood analysis. Circulating [nicotine] and [cotinine] were not detectable in blood from untreated dogs. With nicotine patching, both [nicotine] and [cotinine] increased 6 h after patching and significantly declined within 24 h only in the 11 mg/day treatment group (Table 1). At 6 h, circulating [nicotine] increased with increasing [nicotine] in the patch (Table 1). Steady-state levels were achieved by 3 wk, an effect that persisted out to 5 wk. [Cotinine] was similar with 11 and 22 mg/day patching and decreased by ~50% during the 24-h period. With 44 mg/day treatment, [cotinine] was significantly elevated compared with other treatment groups at 6 h after patching and decreased by 50% by 24 h.

Circulating [NOx] did not increase significantly over baseline pretreatment levels (10.9 ± 0.8 nM, n = 24) in any group after 2 wk of treatment with nicotine (Table 2). However, after 5 wk of treatment, [NOx] was significantly decreased in dogs treated with 22 mg/day nicotine patches compared with either that measured in untreated dogs or dogs treated with 44 mg/day patches (Table 2).

Organ-chamber experiments. Maximal contractions to KCl (60 mM) were not affected by dose or duration of nicotine patch treatment (Table 3). L-NMMA caused <2 g change in baseline tension in rings with endothelium from any group.

Table 2. Circulating concentrations of NOx in dogs treated with transdermal nicotine patches for 2 and 5 wk

<table>
<thead>
<tr>
<th>Duration</th>
<th>Treatment Dose, mg/day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11</td>
</tr>
<tr>
<td>2 wk</td>
<td>n = 9</td>
</tr>
<tr>
<td>6 h</td>
<td>10.3 ± 3.5</td>
</tr>
<tr>
<td>24 h</td>
<td>8.9 ± 0.9</td>
</tr>
<tr>
<td>5 wk</td>
<td>n = 9</td>
</tr>
<tr>
<td>6 h</td>
<td>10.0 ± 1.1</td>
</tr>
<tr>
<td>24 h</td>
<td>9.5 ± 0.5</td>
</tr>
</tbody>
</table>

Data are shown as means ± SE (in nanomol/ml plasma); n, number of samples from different dogs. *Statistical significant difference from baseline values (10.9 ± 0.8 nM, n = 24) by ANOVA, P < 0.05.
Endothelium-dependent relaxations to the α2-adrenergic agonist were concentration dependent and were not altered significantly by dose of nicotine patching at 2 wk of treatment (Fig. 1). After 5 wk of treatment, variability among relaxations to UK-14304 was significantly increased, and maximal relaxations in rings from dogs treated with 22 mg/day patches were less than those from untreated dogs and from dogs treated with 44 mg/day patches (Fig. 1). Incubation of arteries with L-NMMA significantly inhibited relaxations in all groups to the same extent (Fig. 1).

ACh also caused concentration-dependent relaxations in all arteries with endothelium (Fig. 2). These relaxations were not altered statistically by nicotine treatment after 2 or 5 wk. Incubation of rings with l-NMMA to inhibit NO caused comparable rightward log shifts in all groups (data not shown, n = 5–7 in all groups).

ADP caused greater relaxation of rings with endothelium compared with rings without endothelium (Fig. 3). In rings with endothelium, relaxations to ADP were significantly shifted to the right of responses from rings from untreated dogs only after 5 wk of treatment with 22 mg/day patches (Fig. 3). After 5 wk of treatment, relaxations to ADP of rings with endothelium from dogs treated with 11 and 22 mg/day patches were shifted by l-NMMA to a greater extent than those from dogs treated with 44 mg/day patches (Fig. 4).

Relaxations of rings with endothelium to the calcium ionophore A-23187 from dogs treated with 22 and 44 mg/day nicotine patches for 5 wk were shifted to the right of responses from untreated dogs and dogs treated with 11 mg/day patches (Fig. 5). Incubation of the rings with l-NMMA caused comparable rightward shifts in all responses (data not shown, n = 6–8 per group). NO caused comparable concentration-dependent relaxations of rings without endothelium in all groups (Table 4).

**NOS activity.** Neither total nor calcium-dependent NOS activity differed among NOS derived from aortic endothelial cells of untreated dogs or dogs treated with transdermal nicotine for 2 wk. However, after 5 wk of treatment, NOS activity was significantly decreased in rings from dogs treated with 22 mg/day compared with those from untreated dogs and dogs treated with 44 mg/day patches. l-NMMA significantly inhibited relaxations to UK-14304 in arteries from all groups. *Statistically significant difference, P < 0.05.*

### Table 3. Contractions in coronary arteries with endothelium from untreated dogs and dogs treated with transdermal nicotine patches

<table>
<thead>
<tr>
<th>Dose, mg/day</th>
<th>2-Wk Treatment Duration, g Tension</th>
<th>5-Wk Treatment Duration, g Tension</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KCl (60 mM)</td>
<td>PGF&lt;sub&gt;2α&lt;/sub&gt; (2×10&lt;sup&gt;-6&lt;/sup&gt; M)</td>
</tr>
<tr>
<td>Untreated</td>
<td>9.4 ± 1.0</td>
<td>7.3 ± 1.4</td>
</tr>
<tr>
<td>11</td>
<td>9.1 ± 1.0</td>
<td>6.6 ± 0.7</td>
</tr>
<tr>
<td>22</td>
<td>8.1 ± 0.9</td>
<td>5.4 ± 0.6</td>
</tr>
<tr>
<td>44</td>
<td>10.0 ± 1.2</td>
<td>8.4 ± 0.8</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE; n = 6–12 dogs per condition.
treatment, total and calcium-dependent NOS activity was significantly greater ($P < 0.01$) in cells isolated from dogs treated with 22 mg/day patches compared with activity in cells from dogs treated with 11 and 44 mg/day patches (Fig. 6). Calcium-independent and nonspecific activities were not affected after either 2 or 5 wk treatment with nicotine patches.

**mRNA.** There were no statistically significant differences in mRNA for NOS in aortic endothelial cells from untreated dogs or dogs treated with transdermal nicotine for 5 wk (Fig. 7).

**DISCUSSION**

Results of this study indicate for the first time that effects of transdermal nicotine treatment on coronary arterial endothelial function are dependent on time and duration of nicotine patch treatment and the agonist used to stimulate the endothelial cells.

Nicotine dosing was within the range and double to that commonly used in smoking cessation programs (10). This resulted in serum [nicotine] within the range measured in humans undergoing nicotine patch therapy for standard smoking cessation therapy (range from 8–30 ng/ml with the 22 mg/day dosing) (1, 34). Plasma levels of nicotine in smokers range from 10.5–50 ng/ml nicotine (17, 34). Individual levels vary by weight, metabolism, and absorption. Cotinine, a major metabolite of nicotine, is measured frequently in humans as an indicator of nicotine exposure. Circulating levels of cotinine were elevated with nicotine treat-
ment but were lower in dogs than in humans treated with comparable doses of nicotine. Cotinine is psychobiologically inactive but its effects on the vasculature have not been defined. Therefore, it is not known at this time if cotinine, especially in animals treated with 44 mg/day patches, would antagonize effects of nicotine.

Two weeks of treatment with nicotine (11–44 mg/day) patches did not alter endothelium-dependent relaxations in canine coronary arteries. These results are consistent with results from studies using a similar duration of nicotine treatment in rats (22, 23). However, results are in contrast with results found in hamster cheek-pouch arterioles, where nicotine treatment for 2 wk reduced endothelium-dependent relaxations (26). The difference in results could reflect differences in the endothelium of conduit compared with resistance arteries and final circulating [nicotine] or that the present studies were conducted in the presence of indomethacin. Indomethacin inhibits cyclooxygenase, a major source of reactive oxygen radical species, which could reduce the bioavailability of NO. Indeed, in hamster cheek-pouch arterioles, endothelium-dependent relaxations were restored in nicotine-

Table 4. Mean EC₅₀ relaxation to NO in coronary artery rings without endothelium from untreated dogs and dogs treated with transdermal nicotine

<table>
<thead>
<tr>
<th>Nicotine Treatment, mg/day</th>
<th>2-Wk Treatment, −log M</th>
<th>5-Wk Treatment, −log M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>6.8 ± 0.1</td>
<td>6.9 ± 0.1</td>
</tr>
<tr>
<td>11</td>
<td>6.3 ± 0.6</td>
<td>6.9 ± 0.2</td>
</tr>
<tr>
<td>22</td>
<td>6.4 ± 0.2</td>
<td>6.7 ± 0.3</td>
</tr>
<tr>
<td>44</td>
<td>6.5 ± 0.2</td>
<td>7.2 ± 0.2</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE; 5–6 dogs per group.
treated arteries when concentrations of oxygen-derived free radical were reduced by simultaneous administration of superoxide dismutase (26).

After 5 wk of treatment with transdermal nicotine, endothelium-dependent relaxations were reduced but the degree of inhibition was dependent on the agonist tested and the dose of nicotine treatment. For example, endothelium-dependent relaxations were most consistently inhibited in arteries from dogs treated with 22 mg/day nicotine. At this dose, circulating NOx was also reduced. Therefore, decreased endothelium-dependent relaxations are consistent with decreased release or bioavailability of NO from endothelial cells. At first these observations seem in contradiction with the augmentation of NOS activity in extracts of aortic endothelial cells from these dogs. However, increases in the amount and activity of the enzyme may reflect an upregulation due to decreased bioavailability of the end-product NO (expressed as decreased endothelium-dependent relaxations), which acts as a negative feedback regulator to reduce activity of the enzyme in the cell (15, 38). A limitation of this conclusion is that enzyme activity was not measured directly in coronary endothelial cells. However, despite this limitation, the results point to posttranscriptional regulation of NOS and NO with nicotine treatment for several reasons. First, mRNA for NOS was similar among groups. Second, if nicotine regulated NOS transcriptionally, then it might be expected for all endothelium-dependent responses to be uniformly reduced in dogs treated with 22 mg/day patches. This was not the case, because there was not a significant inhibition of relaxations to ACh. Posttranscriptional regulation of NOS may be through production of oxygen-derived free radicals (26), although direct scavenging of free radicals by superoxide dismutase was not tested in the coronary arteries from nicotine-treated dogs. Alternatively, unpublished observations from our laboratory suggest that nicotine may interact with NOS indirectly through interaction with oxygen radicals (47a). It is unlikely that arginine availability was limiting for NOS activity as inducible NOS was not increased significantly with nicotine treatment.

In addition to possible direct interactions of nicotine with cellular processes, nicotine could alter endothelium-dependent relaxations through indirect mechanisms associated with activation of autonomic ganglia and release of neurotransmitters including ACh, noradrenaline, and NO (27, 45–47, 49). Such an indirect effect may explain why relaxations to α2-adrenergic stimulation with UK-14304 were reduced but dependent on the dose of nicotine treatment. Adrenergic receptors may be downregulated in response to increased release of adrenergic transmitter (7, 13). Although adrenergic receptors were not quantified in...
arteries from nicotine-treated dogs, stimulation of sympathetic neurons by nicotine may affect adrenergic receptor number and affinity. Nicotine treatment may also affect receptor-coupled regulatory proteins that stimulate NOS. α2-Adrenergic endothelium-dependent relaxations are associated with guanine nucleotide regulatory proteins which are inhibited by pertussis toxin (14, 28). These responses are sensitive to modulation by hypercholesterolemia and allotransplant rejection (36, 40). Endothelium-dependent relaxations initiated by ACh, which are insensitive to inhibition by pertussis toxin, were not significantly affected by nicotine treatment at any dose. This may reflect that ACh releases other endothelium-derived factors in addition to NO (6, 20, 32, 33).

There appears to be a biphasic dose relationship of nicotine treatment on selective endothelium-dependent responses. This conclusion is based on the observations that responses to UK-14304 and ADP were less sensitive in arteries from dogs treated with 22 mg/day patches but similar among arteries from untreated dogs and dogs treated with 11 and 44 mg/day nicotine. A similar biphasic dose-response relationship of nicotine treatment with endothelium-dependent responses was observed in saphenous veins from these same dogs (8) and in saphenous veins used as coronary artery bypass grafts (9). The mechanism of this biphasic effect of treatment dose is unclear. Nicotine may stimulate NOS directly (47a) or via specific receptor-coupled mechanisms for release of NO (8). Alternatively, because responses to the direct release of endothelium-derived factors by the calcium ionophore were inhibited similarly in coronary arteries from dogs treated with either 22 or 44 mg/day doses, other endothelium-derived factors not measured in this study could have been affected by the nicotine treatment (2, 5, 26, 41, 42). Whether this represents compensatory mechanisms similar to those associated with “nicotine tolerance” (2, 16) or antagonism of nicotine by cotinine remains to be determined. The observation of a complex dose relationship between nicotine and endothelium responses explains in part discrepancies in the literature regarding changes in vascular responses with nicotine.

Differences among studies in regard to effects of nicotine on endothelial function also may relate to the method by which nicotine was delivered. In the present study, circulating [nicotine] was cyclic with increases in concentrations within 6 h after application of a new patch and slow decline throughout the day. This pattern would correspond to that in humans using nicotine patches for smoking cessation therapy (1, 4, 17) and contrasted to other patterns obtained with either inhalation [which would result in rapid peaks (48)] or subcutaneous delivery by osmotic minipump [which would result in sustained levels (2, 16, 22, 23, 25, 26)].

In summary, results of this study provide direct evidence that treatment with transdermal nicotine alters endothelium-dependent responses in nondiseased coronary arteries in a time- and dose-dependent manner. Furthermore, whether or not an inhibition of endothelium-dependent relaxations was observed depended on the agonist used to elicit the response and the duration of treatment. How these changes in endothelial function observed in vitro affect coronary arterial function in vivo remains to be determined. However, smoking is a known risk factor for coronary artery disease in humans and adversely affects endothelial function (12, 18, 31, 39). Smoking cessation is known to reduce the risk for subsequent disease (12). Conventional nicotine patch therapy is safe for use in smokers with known coronary artery disease (19, 30, 50). In addition, inhalation of nicotine for up to 2 yr in rats did not result in gross arterial or coronary pathology (48). Therefore, it is likely that transdermal nicotine can continue to be used to aid smoking cessation with minimal detrimental effects (24, 37, 43). Results also caution against using general statements regarding effects of nicotine on endothelial function and its mechanism of action without attention to blood vessels studied, mode of delivery (tobacco or nontobacco; continuous or phasic; inhalation, intravenous, or cutaneous), dose, and duration of nicotine treatment.

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REFERENCES


