Basic Science Reports

Nicotine Does Not Influence Arterial Lipid Deposits in Rabbits Exposed to Second-Hand Smoke

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Background—Second-hand smoke (SHS) accelerates atherogenesis and impairs vascular function. The role of nicotine in this process has not been defined.

Methods and Results—To examine the potential effects of nicotine on atherogenesis and vascular function, 48 rabbits receiving a 0.5% cholesterol diet were randomized to control (cholesterol diet only), SHS from nicotine-standard research cigarettes (SHS-ST), and SHS from nicotine-free research cigarettes (SHS-NF). The SHS rabbits were exposed to 48 nicotine-standard (12 animals) or nicotine-free (12 animals) cigarettes/d, 5 d/wk for 10 weeks. Air carbon monoxide and particulates and plasma carboxyhemoglobin were significantly higher in the 2 SHS groups than the control group (P<0.001). The SHS-ST group had significant increases in plasma nicotine and cotinine compared with the other groups (P<0.001). There was no difference in serum lipids. Lipid lesions were increased in both SHS groups (54±5% [SEM] aorta and 66±4% pulmonary artery, 53±7% and 69±4%, and 39±4% and 43±3% in the SHS-ST, SHS-NF, and control groups, respectively: P=0.049 aorta and P<0.001 pulmonary artery).

Conclusions—SHS exposure increased arterial lipid lesions, but nicotine did not contribute significantly to this effect. This effect is presumably due to other combustion products in the smoke. (Circulation. 2001;104:810-814.)

Key Words: smoking ■ atherosclerosis ■ vasodilation

Epidemiological studies show that second-hand smoke (SHS) increases the risk of fatal and nonfatal deaths from heart disease by \( \approx 30\% \).\(^1\) SHS is associated with a deterioration in the elastic properties of the aorta\(^2\) and coronary endothelial dysfunction.\(^3\) We previously found that SHS from 96 Marlboro cigarettes per day increased the development of atherosclerosis in rabbits.\(^4\) Passive smoking is associated with progression of carotid intima-media thickness measured by ultrasound in humans.\(^5\) SHS may be responsible for adverse effects on the heart and vessels by increasing vasoconstriction, reducing vasorelaxation, increasing myocardial oxygen demand or oxygen free radical generation, enhancing thrombosis, or depressing nitric oxide (NO) synthesis. Nicotine, the psychoactive ingredient of tobacco, is responsible for the addictive effects of smoking. The present study compares the adverse effects of SHS from research cigarettes containing nicotine and nicotine-free research cigarettes on vascular function and atherogenesis.

Methods

Study Design

Forty-eight male New Zealand White rabbits (average weight 2.1±0.06 [SEM] kg) were housed individually with free access to water and fed a 0.5% cholesterol diet including 3% soybean oil for the whole study period. The rabbits were assigned randomly to 3 groups: control (cholesterol diet only, \( n=24 \)), SHS exposure to nicotine-standard research cigarettes (SHS-ST, \( n=12 \)), and SHS exposure to nicotine-free research cigarettes (SHS-NF, \( n=12 \)). Body weight and food intake were measured and recorded. All rabbits were euthanized at the end of the study with an injection of 130 mg/kg pentobarbital via the marginal ear vein.

The study protocol was approved by the Committee for Animal Research of the University of California San Francisco in accordance with Public Health Service Policy and Animal Welfare Regulations.

Monitoring SHS Exposure

A total of 48 research cigarettes (either nicotine standard or nicotine free, Ultratech Corp) were smoked per day by a smoking machine (RM1/G, Heinr. Borgwald GmbH) in an exposure chamber as previously described.\(^6\) Four filter cigarettes were used every 30 minutes for 6 h/d, 5 d/wk, for 10 weeks. Three small fans were used to circulate the smoke in each well-mixed SHS exposure chamber (1.92×1.92×0.97 m, model H5500, BioClean, Duo Flo, Laboratory Products Inc), which approximates the interior volume of a midsize car (3.7 m\(^3\)). A model L15 carbon monoxide (CO) Personal Exposure System (Langan Products, Inc) with a resolution of 0.5 ppm CO (range, 0 to 128 ppm) was used to measure average air CO, and a Miniram PDM-3 Optical Scattering Particle Monitor (MIE, Inc, resolution 10 \( \mu \text{g/m}^3 \)) was used for measurement of average total particulates. Concentrations of plasma nicotine and cotinine were determined by gas chromatography with nitrogen-phosphorus detection modified for simultaneous extraction of nicotine and cotinine.\(^7\)
Blood carboxyhemoglobin (COHb) was measured with a Radiometer ABL TMS20 Blood Gas System. The blood samples were collected at the end of a day of SHS exposure.

Biochemical Analyses
Total serum cholesterol and triglyceride levels were measured by quantitative enzymatic assays (Sigma Diagnostics). HDL cholesterol (HDL-C) was determined by quantitative measurement with HDL-C reagent after precipitation of other lipoprotein classes (PTA/MgCl₂, Sigma Diagnostics). Serum lipid measurements were done with a spectrophotometer. As a measurement of cumulative exposure of arterial walls to cholesterol by week, the area under the cholesterol-time curve was calculated in cholesterol-weeks (mg/dL×weeks).

Four hearts from each group were collected to measure NOₓ (combination of total NOₓ, nitrite, and nitrate). NOₓ was then immediately for analysis. The nitrite and nitrate were reduced by use of vanadium (III) and hydrochloric acid at 90°C. NOₓ was then purged from the solution to measure the peak of NOₓ by chemiluminescence (NOA 280, Sievers Instruments Inc; detection limit 1 nmol/L per mL nitrate).

Vascular Reactivity In Vitro
Vascular tension was measured in intact aortic rings suspended in organ chambers. After injection with pentobarbital, aortic segments 4 to 5 mm long were immediately dissected and placed into a bath containing warm (37°C) Krebs bicarbonate solution bubbled with a gas mixture of 95% O₂ and 5% CO₂. All rings were gradually stretched over a period of 60 minutes to a preload of 4 g. Acetylcholine and calcium ionophore A23187 were used to measure vascular endothelium-dependent relaxation, and nitroglycerin was used for endothelium-independent relaxation. The dose that induced a half-maximal contraction (EC₅₀) was determined by increasing the concentration of phenylephrine in half-log increments from 10⁻⁸ to 10⁻⁴ mol/L. The vasorelaxation response was expressed as percent relaxation of the contraction induced by EC₅₀ phenylephrine.

Morphological Studies
After removal of the aortic ring, the whole aorta was removed from its origin (2 cm distal to the aorta valve) to the bifurcation of the internal iliac arteries and the pulmonary artery from its beginning at the pulmonary valve to just above the bifurcation. The vessels, including the rings used for the study of vascular reactivity, were opened by a linear vertical incision, fixed in a formalin solution, stained with Sudan IV lipophilic dye, and photographed. The intimal lipid lesions in the aorta were measured quantitatively by planimetry to estimate the percentage of Sudan IV–stained regions in photographs.

Statistical Analysis
All values are expressed as mean±SEM. Comparisons of the differences among the groups were done with 1-way ANOVA with pairwise multiple comparisons by the Student-Newman-Keuls test (α=0.05 family error rate). Correlation coefficients were used to relate continuous variables. A value of P<0.05 was considered statistically significant.

Results
One rabbit in the nicotine-free cigarette group died and was excluded from the study. All other rabbits showed a modest weight gain during the study period. There were no significant differences in body weight gain (average 1.54±0.04 kg, P=0.198) or food consumption (184±4 g/d, P=0.799) among the groups.

Monitoring SHS Exposure
Average CO air, particulates (Table 1), and plasma COHb (Table 2) in the 2 SHS groups were significantly higher than the nonexposed control group. There were significant elevations of air CO, particulates, and plasma COHb in the SHS-NF group compared with the SHS-ST group, perhaps

### Table 1. SHS Monitoring

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>SHS-NF (n=7)</th>
<th>SHS-ST (n=7)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air CO, ppm</td>
<td>1.87±0.10</td>
<td>53.36±5.97</td>
<td>44.91±1.81</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Air particulates, mg/m²</td>
<td>0.68±0.20</td>
<td>35.21±2.39</td>
<td>24.08±3.79</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Subgroups are by Student-Newman-Keuls test. Values are mean±SEM.

### Table 2. Biochemical Measurements and Vasorelaxation

<table>
<thead>
<tr>
<th></th>
<th>Control (n=24)</th>
<th>SHS-NF (n=11)</th>
<th>SHS-ST (n=12)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma COHb, %</td>
<td>1.4±0.2</td>
<td>3.8±0.3</td>
<td>2.7±0.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Plasma nicotine, nmol/L</td>
<td>4.0±0.5</td>
<td>14.1±1.6</td>
<td>339.0±74.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Plasma cotinine, nmol/L</td>
<td>6.7±2.9</td>
<td>23.2±2.2</td>
<td>269.3±24.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triglyceride, mmol/L</td>
<td>7.8±0.4</td>
<td>8.9±0.5</td>
<td>9.2±0.8</td>
<td>0.160</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>79.6±4.4</td>
<td>94.4±7.6</td>
<td>95.5±8.6</td>
<td>0.109</td>
</tr>
<tr>
<td>Chol-wk, mmol/L-wk</td>
<td>666±29</td>
<td>701±52</td>
<td>769±54</td>
<td>0.206</td>
</tr>
<tr>
<td>HDL-C, mmol/L</td>
<td>1.3±0.1</td>
<td>1.5±0.3</td>
<td>0.9±0.1</td>
<td>0.119</td>
</tr>
<tr>
<td>Myocardial NOₓ, μmol/L</td>
<td>18.7±4.9</td>
<td>7.9±0.9</td>
<td>6.6±1.1</td>
<td>0.032</td>
</tr>
<tr>
<td>Ach-max, %†</td>
<td>−66±15</td>
<td>−49±9</td>
<td>−49±6</td>
<td>0.559</td>
</tr>
<tr>
<td>Ach-10⁻⁷, %†</td>
<td>−25±6</td>
<td>−15±5</td>
<td>−12±4</td>
<td>0.194</td>
</tr>
</tbody>
</table>

Chol-wk indicates cholesterol-weeks. Values are mean±SEM. Subgroups by Student-Newman-Keuls test are underlined.

* n=4 in each group.
† Acetylcholine-induced maximal relaxation and acetylcholine 10⁻⁷-induced vasorelaxation.
related to a longer burning time for the nicotine-free cigarettes.

The plasma concentrations of nicotine and cotinine in the SHS-ST group were much higher than in the SHS-NF and control groups (Table 2).

**Biochemical Analyses**

There were no significant differences in total serum cholesterol, triglyceride, HDL-C, or cholesterol-weeks among the 3 groups. Acetylcholine induced less maximal endothelial vasorelaxation among the 3 groups. Acetylcholine induced less maximal endothelial-dependent vasorelaxation among the 3 groups. Acetylcholine induced less maximal endothelial-dependent vasorelaxation among the 3 groups. Acetylcholine induced less maximal endothelial-dependent vasorelaxation among the 3 groups.

**Vascular Reactivity In Vitro**

There were no significant differences in phenylephrine-induced maximal vasoconstriction and calcium ionophore A23187–induced (endothelium-dependent) and nitroglycerin-induced (endothelium-independent) vasorelaxation among the 3 groups. Acetylcholine induced less maximal endothelial-dependent relaxation in both SHS groups than in control (−49±6% in SHS-ST and −49±9% in SHS-NF versus −66±15% in control, t=0.559, Table 2) or at the concentration of 10−7 mol/L acetylcholine (−12±4% and −15±5% versus −25±6%, t=0.194, Table 2), but these differences were not statistically significant (Figure 1).

**Morphological Studies**

The lipid lesions in the aorta and pulmonary artery were 39±4% and 43±3% (control), 54±5% and 66±4% (SHS-ST), and 53±7% and 69±4% (SHS-NF) (t=0.049 for aorta and t<0.001 for pulmonary artery, Figure 2). The rabbits in both SHS groups had significantly more lipid deposits, but nicotine did not contribute further to lipid lesions in either the aorta or the pulmonary artery.

There was a positive correlation between aortic lipid lesions and phenylephrine-induced maximal vasoconstriction (r=0.318, P=0.029). Aortic lesions were also negatively correlated to acetylcholine-induced maximal relaxation (r=−0.382, P=0.010), relaxation response at 10−7 mol/L acetylcholine (r=−0.409, P=0.005), and calcium ionophore A23187–induced maximal relaxation (r=−0.563, P<0.001).

**Discussion**

The major finding of this study is that SHS exposure significantly increased arterial lipid lesions, but nicotine did not make them worse. SHS breaks down the serum antioxidant defense, leading to lipid peroxidation, LDL modification, and accumulation of LDL cholesterol.8 In humans, SHS is significantly related to increased carotid artery intima-media thickness by B-mode ultrasound.5 Our data are consistent with these findings.

In contrast to our previous work,4 which showed that SHS (from 96 Marlboro cigarettes/d) significantly adversely affects vascular endothelial function, we did not find significant changes in vascular reactivity in the present study. This difference may be due to the lower dose (48 research cigarettes/d) in this study or may indicate that SHS from Marlboro cigarettes is more toxic than SHS from research cigarettes.

**Effects of Nicotine on Lipids**

The effects of nicotine on lipoproteins have been studied in humans and in animals. One human study9 reported no evidence of an adverse effect of nicotine administered with chewing gum (2 mg, 8 times/d for 2 weeks) on lipids. A monkey study10 showed that long-term oral nicotine consumption (liquid diet with 6 mg · kg−1 · d−1 for 2 years) decreased HDL/total cholesterol ratio through enhancing lipolytic conversion of VLDL to LDL. The diminished removal of LDL would increase their deposition in the arterial wall.11 Nicotine was also found to increase lipolysis in heavy smokers.12 A rat study showed that nicotine at levels experienced by active smokers increased the synthesis and secretion of triglyceride-rich lipoprotein,13 exerting hyperlipidemic effects. We found no significant differences in lipids among the control, SHS, and SHS with nicotine-free cigarette groups. The fact that we did not observe an effect of nicotine on lipids in our rabbits suggests that the dose of nicotine in
this relatively short-term SHS exposure is below that which induces substantial effects on lipid metabolism.

**Effects of Nicotine on Vascular Endothelial Function**

Nicotine at doses observed in active smokers impairs vascular endothelial function. The mechanisms of endothelial cell injury are unclear. Increased vascular endothelial permeability, enhanced rate of cell loss or cell turnover, stimulation of sympathetic neurotransmission, and inhibition of NO synthase activity may be related to the impaired vascular function. The correlations between impaired endothelium-dependent vasorelaxation and arterial lipid lesions in this study most likely reflected functional or structural changes in response to the cigarette smoke. Decreased tissue NO levels were found in both SHS groups ($P=0.032$), regardless of nicotine exposure.

L-Arginine blocks the effect of SHS on vascular endothelial function in rabbits. L-Arginine also reverses nicotine-induced vasorelaxation to acetylcholine in rats. The reduction in endothelium-dependent vasorelaxation may be mediated, at least in part, through the degradation of released NO, by free radicals from the smoke. Nicotine has previously been reported to impair endothelium-dependent vasodilation in rats (25 or 50 μg/mL nicotine in drinking water for 15 days). Another study, however, failed to demonstrate significant adverse vascular effects of nicotine. Furthermore, a rat study found that components of cigarette smoke other than nicotine are responsible for endothelial dysfunction.

**Effects of Nicotine on Atherogenesis**

The rabbits in both SHS groups had significantly more lipid deposits in the aorta and pulmonary artery than the control group. Nicotine, however, did not increase lipid lesions in our study compared with the nicotine-free SHS group. Nicotine at doses observed in heavy smokers accelerated atherogenesis in rabbits. At levels observed in active smokers, nicotine enhanced the release of platelet-derived growth factor and transforming growth factor and may play a key role in the development and progression of atherosclerosis in active smokers. Nicotine can increase the rate of arterial endothelial cell turnover and endothelial permeability, then enhance entry of atherogenic lipoproteins into the arterial wall and accelerate atherogenesis. Several in vitro studies indicated that nicotine at levels observed in both active and passive smokers induced smooth muscle cell proliferation or changes in the vascular endothelium. The effects of nicotine and CO, however, are much smaller than the effects of whole smoke. In contrast, a 2-year rat study found that inhaled pure nicotine that yielded plasma nicotine concentrations of $\approx 100$ ng/mL (approximately twice the level we observed in our rabbits) did not induce atherosclerosis.

**Other Constituents of SHS**

There is evidence that other toxins in SHS contribute to adverse cardiovascular effects, particularly polycyclic aromatic hydrocarbons. Benzo(a)pyrene and 1,3-butadiene have been found to injure vascular endothelial function and initiate or accelerate the development of atherosclerosis. The heart tissue of smokers with cardiovascular disease contained elevated levels of aromatic DNA adducts. Chimney sweeps exposed to polycyclic aromatic hydrocarbons exhibited an excess mortality from ischemic heart disease. An association between exposure to tar and atherosclerosis was found by a Norwegian study in potroom workers. Butadiene, a gas in the vapor phase of SHS, accelerated atherosclerosis in cockerels. Thus, other constituents besides nicotine may play a more important role. For example, elevations of CO and particulates, which may be induced by longer burning time of the nicotine-free cigarettes, probably play some role in atherogenesis. We did not evaluate the influence of CO and particulates in this study, although other studies of CO have failed to demonstrate an atherogenic effect. Future studies need to address the effect of single elements of SHS on cardiovascular disease.

**Conclusions**

SHS exposure significantly increased arterial lipid lesions, but nicotine did not contribute to these adverse vascular effects of SHS. Other components of the SHS are presumably responsible. This result also suggests that the combustion products in air pollution may contribute to atherosclerosis.

**Acknowledgments**

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**References**


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