Objectives: To determine whether secondhand smoke (SHS) induces pulmonary artery endothelial dysfunction, and whether dietary L-arginine supplementation is preventive.

Background: SHS causes coronary and peripheral arterial endothelial dysfunction.

Methods: The effects of L-arginine supplementation (2.25% solution) and SHS (10 weeks) on pulmonary vascular reactivity were examined in 32 rabbits fed a normal diet. Endothelium-dependent relaxation of precontracted pulmonary artery segments was studied using acetylcholine and calcium ionophore. Endothelium-independent relaxation was studied using nitroglycerin. Endothelial and serum L-arginine levels were measured by chromatography. In eight SHS-exposed and in eight control rats, pulmonary artery nitric oxide synthase (NOS) activity and arginase activity were studied using the titrated arginine to citrulline conversion assay.

Results: SHS reduced maximal acetylcholine-induced (p < 0.04) and calcium ionophore-induced (p < 0.02) relaxation. L-Arginine increased maximal acetylcholine-induced (p = 0.047) vasodilation. SHS and L-arginine did not influence nitroglycerin-induced relaxation. SHS reduced endothelial L-arginine (p = 0.04) but not serum L-arginine. L-Arginine supplementation increased endothelial (p = 0.007) and serum L-arginine (p < 0.0005). Endothelium-dependent relaxation induced by acetylcholine and calcium ionophore varied directly with endothelial (r = 0.67, r = 0.67) and serum L-arginine (r = 0.43, r = 0.45), respectively. SHS reduced constitutive NOS activity (p = 0.03).

Conclusions: SHS reduces pulmonary artery endothelium-dependent relaxation by decreasing NOS activity and possibly by decreasing endothelial arginine content. L-Arginine supplementation increases serum and endothelial L-arginine stores and prevents SHS-induced endothelial dysfunction. L-Arginine may offset the deleterious effect of SHS on pulmonary arteries by substrate loading of the nitric oxide pathway.

Key words: endothelium; L-arginine; nitric oxide; pulmonary artery; tobacco smoke

Endothelial cells continuously release endothelium-derived relaxing factor in a variety of vascular beds, including the pulmonary circulation.1–3 Endothelial production of nitric oxide is a major determinant of resting arterial tone in normoxic and hypoxic pulmonary arteries.1,3–5
Active tobacco smoking6 and secondhand smoke (SHS)7 cause endothelial dysfunction of systemic arteries in humans. Using a rodent model of controlled SHS exposure, we have demonstrated that SHS causes endothelial dysfunction of systemic arteries,8 and that long-term supplementation of L-arginine reduces aortic endothelial dysfunction in SHS-exposed normocholesterolemic rabbits.9

Long-term tobacco smoke exposure causes mechanical changes10 in small pulmonary arteries, and is the major cause of chronic obstructive lung disease. In vitro studies10 suggest that smoking may cause functional changes in the pulmonary vasculature as well. Endothelial nitric oxide-dependent relaxation may be impaired in the pulmonary arteries of patients with COPD.11 Mehta et al12 demonstrated that short-term administration of large IV doses of L-arginine, the substrate of nitric oxide synthase (NOS) and the precursor of nitric oxide, induces pulmonary vasodilation in patients with pulmonary hypertension.

The endothelial effects of SHS exposure and the potential benefits of long-term L-arginine supplementation have not been examined in the pulmonary circulation. The goal of this study was to characterize the effect of SHS exposure on pulmonary artery endothelial function and to determine whether long-term dietary L-arginine supplementation protects against endothelial dysfunction.

**Materials and Methods**

**Protocol**

The study protocol was approved by the Committee for Animal Research of the University of California at San Francisco, and was performed in accordance with the recommendations of the American Association for Accreditation of Laboratory Animal Care. Rabbits ingested a standard rabbit chow diet throughout the study. Thirty-two rabbits were randomized in a two-by-two design (with SHS and long-term L-arginine as factors) into four groups: normal rabbits, SHS exposure, L-arginine exposure, and SHS/L-arginine exposure. Sixteen rats were randomized to receive or not to receive SHS exposure, for NOS and arginase studies.

Rabbits were housed in individual cages. Rabbits randomized to SHS exposure (SHS and SHS/L-arginine groups) were placed in SHS-exposure chambers and were exposed to sidestream tobacco smoke, as previously described.13 Rabbits randomized to non-SHS groups (L-arginine exposure and normal rabbits) were placed in separate cages in the same type of exposure chamber in another room without a smoking machine. Rabbits randomized to the L-arginine groups (SHS/L-arginine exposure and L-arginine exposure) received L-arginine in their drinking water (2.25% weight/volume, ad libitum). After 10 weeks of exposure to SHS (or control conditions), rabbits were killed by lethal injection with IV administration of pentobarbital (130 mg/kg of body weight). Ring segments of the left or right first branch of the extrapulmonary pulmonary artery (2 mm in diameter, 5 mm in length, distal to the main pulmonary artery bifurcation) for organ bath studies were rapidly excised. The main pulmonary artery segment (4 to 6 mm in diameter and 7 to 10 mm long) was excised, immersed in chilled (4°C) Krebs solution, and kept for elution of endothelial cells.

**Vascular Reactivity Studies**

Each ring was suspended horizontally between two parallel stainless steel wires for the measurement of isometric tension, in individual organ baths containing Krebs solution, as previously described.9 The isometric force generated by the ring segment was measured and recorded continuously, as previously described.9

Ring segments were stabilized at 1 g of resting tension for 60 min before the study was begun. To measure responsiveness to phenylephrine and to calculate the dose needed for precontraction, phenylephrine in increasing concentrations (from 10-9 to 10-4 mol/L) was added to each ring/bath.14 For each ring, the effective concentration of phenylephrine needed to achieve half-maximal contraction (EC50) was calculated. Indomethacin (10-5 mol/L) was added to the organ baths 20 min before phenylephrine to stabilize contraction, and to eliminate the influence of prostaglandin synthesis.15 After the phenylephrine contraction series, the baths were washed out three times with fresh Krebs solution and the rings were allowed to stabilize over an hour.

To examine endothelium-derived nitric oxide-mediated vasorelaxation, pulmonary artery rings were exposed first to a series of increasing doses of acetylcholine (from 10-9 to 10-4.5 mol/L) following precontraction by the EC50. At the end of the acetylcholine series, the baths were washed out twice with fresh Krebs solution and the rings allowed to stabilize at baseline tension. To examine endothelium-dependent relaxation via a nonreceptor-dependent mechanism, pulmonary artery rings were exposed to calcium ionophore in increasing doses (from 10-8 to 10-7 mol/L) following precontraction by the EC50.

Finally, to examine endothelium-independent relaxation,16 a single maximal dose of nitroglycerin (10-5 mol/L) was added to the organ baths at the end of the calcium ionophore series. Vascular reactivity experiments were performed by an investigator who was blinded to the rabbit treatment group.

**Determination of Oxygen Saturation:** To determine whether 10-week SHS exposure reduced oxygen saturation, saturation was measured at the ear by pulse oximetry (NPB-190; Nellcor Puritan-Bennett; Freemont, CA) in 20 rabbits, at the completion of an identical protocol (same arginine diet and duration, and same SHS exposure and duration).

**NOS Activity Assay**

To determine the effects of SHS on endothelial constitutive NOS activity, inducible NOS activity, and arginase activity, pulmonary artery segments from eight control rats and from eight SHS-exposed (10 weeks) rats were studied using the titrated arginine to citrulline conversion assay.

Tissue samples of pulmonary arteries were homogenized using a 3:1 volume of homogenizing buffer (10 mmol/L piperazine-ethanesulfonic acid; 0.32 mol/L sucrose; 0.1 mmol/L ethylenediaminetetraacetic acid; 1 mmol/L dihydrothreitol; 10 μg/mL leupeptin, 2 μg/mL aprotinin; 1 mg/mL phenylmethylsulfonyl fluoride; final pH 7.2). The homogenized samples were centrifuged at 12,000 revolutions per minute at 4°C for 15 min, and the supernatants were removed and placed on ice.

Samples were assayed in duplicate to measure the conversion of 14C-arginine to 14C-citrulline. Twenty microliters of supernatant was incubated in 100 μL of NOS activity buffer to measure...
for total NOS activity and also in the presence of either 24 mmol/L ethyleneglycoltetra-acetic acid (for inducible NOS activity) or 24 mmol/L ethyleneglycoltetra-acetic acid plus 24 mmol/L nitro-l-arginine methyl ester (no activity). Samples were incubated for 60 min at 37°C. After incubation, the reaction was terminated with the addition of 750 μL of cold stop buffer (50 mmol/L piperazine-ethanesulfonic acid, 5 mmol/L ethylenediaminetetra-acetic acid, pH 5.5). The samples were then applied to a 1-nL column of Dowex (Ft. Pierce, FL) AG 50 W-X8 (Na+ form) and then eluted using 4 mL of stop buffer. 14C-Citrulline in the eluent was measured by liquid scintillation spectroscopy and expressed as picomoles per milligram of protein per minute. Baseline tension), EC50, and slope (calculated by the Hill equation).

Endothelial l-arginine levels were measured by eluting the endothelial layer of segments of pulmonary arteries, and assaying the eluted solution for l-arginine in four animals of each group. After careful removal of adipose tissue, the main pulmonary artery was infused over 4 to 5 s with 3 mL of distilled water containing 1% Triton X detergent (Sigma; St. Louis, MO) to hydrolyze the endothelial cell layer, as has been previously described. Histologic examination of pulmonary artery segments that had been infused with hypotonic Triton X-containing solution revealed absence of most of the endothelium. The recovered elute was immediately frozen and later assayed chromatographically using a Beckman 6300 Amino Acid Analyzer (Fullerton, CA), which detects the colored ninhydrin derivatives of most amino acids at 570. Blood for biochemical analysis was obtained when the rats were killed. Total serum cholesterol, triglyceride, high-density lipoprotein (HDL) cholesterol concentrations, as well as carbon monoxide, cotinine, and nicotine were measured, as previously described. Endothelial l-arginine levels were measured by eluting the endothelial layer of segments of pulmonary arteries, and assaying the eluted solution for l-arginine in four animals of each group. After careful removal of adipose tissue, the main pulmonary artery was infused over 4 to 5 s with 3 mL of distilled water containing 1% Triton X detergent (Sigma; St. Louis, MO) to hydrolyze the endothelial cell layer, as has been previously described. Histologic examination of pulmonary artery segments that had been infused with hypotonic Triton X-containing solution revealed absence of most of the endothelium. The recovered elute was immediately frozen and later assayed chromatographically using a Beckman 6300 Amino Acid Analyzer (Fullerton, CA), which detects the colored ninhydrin derivatives of most amino acids at 570.

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Statistical Analysis
All results are expressed as mean ± SEM. Relaxation of aortic rings is expressed as percentage change of net developed tension (measured tension – baseline tension)/precontracted tension – baseline tension), EC50, and slope (calculated by the Hill equation). A curve of best fit was calculated for each ring, using the equation for the Hill coefficient (Kaleidagraph, version 3.0, Synergy Software; Reading, PA), which calculated the EC50 and slope. The response to phenylephrine was expressed as change in tension in grams (from baseline) and recorded and analyzed as above.

The effects of SHS and l-arginine on vascular reactivity were evaluated using a general linear model (GLM) analysis of variance (ANOVA) [GLM procedure, MINITAB Version 10.2; MINITAB Statistical Software; State College, PA], which is a suitable model to analyze a balanced two-by-two design. This form of ANOVA allows for statistical description of the principal effects of two (or more) factors; in our study, these were SHS (present or absent) and l-arginine (present or absent). GLM ANOVA also allows for determination of the interaction of the factors (SHS and l-arginine).19 Testing the significance of the interaction term (SHS × l-arginine interaction) specifically permitted us to test whether the effect of SHS exposure was modified by the presence of l-arginine, beyond purely additive effects. l-Arginine ingestion, arginase activity, and inducible nitric oxide activity were compared using a t test. SHS exposure parameters (air particulate matter, carbon monoxide) in the two SHS-exposed groups and in the non-SHS-exposed groups were compared by a one-way ANOVA. Nitric oxide activity was compared using a repeated-measures ANOVA. The relationship of maximal acetylcholine-induced relaxation and maximal calcium-ionophore-induced relaxation was analyzed using a Pearson correlation test (Primer of Biostatistics, v3.01; McGraw-Hill; New York, NY). The relationships of serum and endothelial l-arginine levels to endothelium-dependent relaxation were similarly analyzed using a Pearson correlation test correlating the log value of l-arginine with relaxation. A p value of < 0.05 was considered significant.

Hematologic and Biochemical Analysis
Animal Data
SHS and l-arginine did not influence body weight. l-Arginine ingestion by the two groups of arginine-supplemented animals was similar. SHS exposure reduced average food ingestion. l-Arginine supplementation did not affect food ingestion, but did block the SHS-induced reduction in food intake (Table 1).

SHS exposure markedly increased smoking-chamber carbon monoxide and particulate matter levels, and serum nicotine and cotinine levels. Neither SHS nor l-arginine affected levels of total cholesterol, serum HDL, or serum triglyceride concentrations. The arterial oxygen saturation of similarly prepared animals was > 98% for all four groups, and was not influenced by SHS exposure.

Vascular Reactivity Studies
Vasorelaxation: SHS reduced both maximal endothelium-dependent relaxation to acetylcholine and to calcium ionophore (Table 2). SHS reduced the slope of the acetylcholine dose-response curves, and similarly tended to reduce the slope of the curves to calcium ionophore, suggesting reduced sensitivity. l-Arginine increased maximal endothelium-dependent relaxation to acetylcholine and tended to increase calcium ionophore-induced relaxation (Fig 1). Neither SHS nor l-arginine increased sensitivity of endothelium-dependent relaxation (EC50) to acetylcholine or to calcium ionophore. Neither SHS nor l-arginine influenced maximal nitroglycerin-induced endothelium-independent relaxation.

Vasocontraction: Neither SHS nor l-arginine influenced phenylephrine-induced vasoconstriction.
L-Arginine levels and their relation to endothelium-dependent relaxation

L-Arginine supplementation increased both serum L-arginine levels and endothelial L-arginine content (Table 3). SHS did not reduce serum L-arginine levels, but did reduce endothelial L-arginine content (Fig 2). Maximal acetylcholine-induced relaxation correlated with maximal calcium ionophore-induced relaxation (Pearson correlation, 0.53; p = 0.03). A stronger correlation was seen between endothelial L-arginine levels and maximal acetylcholine-induced relaxation (Pearson correlation, 0.67; p = 0.03) and also with maximal calcium ionophore-induced relaxation (Pearson correlation, 0.67; p = 0.01; Fig 3).

Arginase and NOS activity

Endothelial constitutive NOS activity was reduced in SHS-exposed pulmonary artery tissue (0.29 ± 0.05 pmol/mg/min) compared to control tissue.

Table 2—Vascular Reactivity Results

<table>
<thead>
<tr>
<th>Variables</th>
<th>SHS Group</th>
<th>SHS/Arginine Group</th>
<th>L-Arginine Group</th>
<th>Control Group</th>
<th>P-SHS</th>
<th>P-L-Arginine</th>
<th>P-SHS × L-Arginine</th>
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<tbody>
<tr>
<td>A23187-induced relaxation</td>
<td></td>
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<tr>
<td>Maximal response, %</td>
<td>-32 ± 5.7</td>
<td>-58 ± 9</td>
<td>-54 ± 5</td>
<td>-49 ± 5</td>
<td>0.02</td>
<td>0.08</td>
<td>0.42</td>
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<tr>
<td>EC50, mol/L</td>
<td>7.5 ± 10^-6 ±</td>
<td>1.25 ± 10^-6 ±</td>
<td>9.1 ± 10^-6 ±</td>
<td>3.4 ± 10^-6 ±</td>
<td>0.90</td>
<td>0.87</td>
<td>0.50</td>
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<tr>
<td>Slope</td>
<td>2.19 ± 0.6</td>
<td>1.14 ± 0.2</td>
<td>0.84 ± 0.1</td>
<td>1.01 ± 0.2</td>
<td>0.08</td>
<td>0.71</td>
<td>0.60</td>
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<td>Acetylcholine-induced relaxation</td>
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<tr>
<td>Maximal response, %</td>
<td>-27 ± 4.6</td>
<td>-60 ± 7.8</td>
<td>-49 ± 7</td>
<td>-49 ± 8</td>
<td>0.04</td>
<td>0.047</td>
<td>0.29</td>
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<tr>
<td>EC50, mol/L</td>
<td>1.26 ± 10^-7 ±</td>
<td>1.7 ± 10^-7 ±</td>
<td>4.1 ± 10^-7 ±</td>
<td>2.2 ± 10^-7 ±</td>
<td>0.51</td>
<td>0.76</td>
<td>0.76</td>
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<tr>
<td>Slope</td>
<td>1.6 ± 0.3</td>
<td>1.9 ± 1</td>
<td>1.7 ± 0.5</td>
<td>1.1 ± 0.1</td>
<td>0.01</td>
<td>0.02</td>
<td>0.18</td>
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<tr>
<td>Nitroglycerin-induced relaxation</td>
<td></td>
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<tr>
<td>Maximal response, %</td>
<td>-82 ± 7.1</td>
<td>-83 ± 5.2</td>
<td>-84 ± 2</td>
<td>-87 ± 5</td>
<td>0.57</td>
<td>0.75</td>
<td>0.53</td>
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<tr>
<td>Relaxation (maximal), Δ%</td>
<td></td>
<td></td>
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<tr>
<td>Phenylephrine-induced contraction</td>
<td>11.4 ± 3.0</td>
<td>13.9 ± 4.2</td>
<td>13.9 ± 3.9</td>
<td>12.9 ± 2.5</td>
<td>0.18</td>
<td>0.95</td>
<td>0.47</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SE. See Table 1 for definition of abbreviations.
Arginase activity was similar in SHS-exposed (0.60 ± 0.16 pmol/mg/min) and control tissue (0.43 ± 0.13 pmol/mg/min; p = 0.25; Fig 4, right). Inducible nitric oxide activity was similar in SHS-exposed and control tissue (0.04 ± 0.01 vs 0.01 ± 0.01 pmol/mg/min; p = 0.06).

**DISCUSSION**

This study demonstrates that (1) SHS reduces rodent conduit pulmonary artery endothelium-dependent relaxation, (2) SHS reduces rodent pulmonary artery endothelial constitutive nitric oxide activity, (3) a correlation between endothelial L-arginine content and endothelium-dependent relaxation exists, (4) SHS reduces endothelial L-arginine and long-term L-arginine supplementation increased endothelial L-arginine content, and (5) long-term L-arginine supplementation protects against SHS-induced endothelial dysfunction.

As maximal relaxation to both the muscarinic receptor-dependent vasodilator acetylcholine and to the receptor-independent vasodilator A23187 was impaired, our observations suggest that the SHS-induced abnormality of endothelium-dependent relaxation is mediated through impaired nitric oxide production. The observation of reduced endothelial constitutive NOS activity is consistent with this interpretation. Maximal endothelium-independent relaxation was not affected by SHS; however, an SHS-induced impairment of smooth-muscle sensi-

**Table 3—L-Arginine Levels**

<table>
<thead>
<tr>
<th>Variables</th>
<th>SHS Group</th>
<th>SHS/L-Arginine Group</th>
<th>L-Arginine Group</th>
<th>Control Group</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Arginine plasma, mmol/L</td>
<td>0.15 ± 0.01</td>
<td>0.36 ± 0.05</td>
<td>0.30 ± 0.05</td>
<td>0.12 ± 0.01</td>
<td>P-SHS &lt; 0.0005</td>
</tr>
<tr>
<td>L-Arginine endothelial, mmol/300-µL perfusate/cm²</td>
<td>0.06 ± 0.02</td>
<td>0.67 ± 0.20</td>
<td>1.01 ± 0.20</td>
<td>0.50 ± 0.13</td>
<td>0.04 &lt; 0.0005</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SE. See Table 1 for definition of abbreviations.*
activity to nitric oxide, or general impairment of sensitivity to vasodilators, cannot be excluded.

Tobacco smoke-induced impairment of endothelial nitric oxide activity has been suggested previously, although not of the pulmonary artery. Higman et al. demonstrated an impairment of NOS activity in saphenous vein segments that was attributable to an inadequate supply of the coenzyme tetrahydrobiopterin.

Loss of NOS, the enzyme responsible for nitric oxide synthesis, is seen in patients with advanced pulmonary hypertension, but the relationship of loss of NOS to pulmonary hypertension is not fully understood. Chronic lung disease induced by both tobacco smoke and wood smoke is associated with pulmonary artery endothelial dysfunction. Our results suggest that SHS has direct effects on the pulmonary vasculature. It is possible then that loss of endothelium-dependent vasodilation function secondary to SHS may contribute to an increase in pulmonary artery tone and pulmonary artery pressure. In our study, SHS did not influence arterial oxygenation; however, additional alveolar hypoxia-induced pulmonary artery

![Figure 2](http://journal.publications.chestnet.org/pdfaccess.ashx?url=data/journals/chest/21970/)

**Figure 2.** L-Arginine. Top: L-arginine supplementation results in a several-fold increase in serum L-arginine levels (L-arginine effect). There is no SHS effect on serum levels of L-arginine. Bottom: L-arginine supplementation increases endothelial L-arginine levels (L-arginine effect), whereas SHS decreases endothelial L-arginine content (SHS effect). See Table 1 for definition of abbreviations.
vasoconstriction is likely to accentuate pulmonary hypertension, as in patients with COPD.

In this study, we observed a relationship between endothelial L-arginine content and endothelium-dependent relaxation to both vasodilator agents. This finding is supportive of the notion of substrate loading the NOS pathway to increase nitric oxide production. The increase in endothelial L-arginine content from long-term L-arginine supplementation may explain some of the observed benefit of long-term L-arginine supplementation on endothelial function in the presence of SHS. Other stimuli than SHS, such as hypoxia, have been demonstrated to inhibit L-arginine uptake by pulmonary artery endothelial cells. SHS-induced lowering of endothelial L-arginine does not appear explained by an increase in L-arginase.

Our observations suggest that loss of enzyme activity and reduced substrate availability underlie SHS-induced endothelial dysfunction. However, other mechanisms, such as oxidant stress, may contribute as well. Oxidant stress is reported to

**Figure 3.** Evidence for a direct relationship between the extent of endothelium-dependent relaxation induced by acetylcholine (ACh) and endothelial L-arginine levels.

**Figure 4.** Left: SHS exposure reduces endothelial constitutive NOS (ecNOS) activity. Right: SHS exposure does not influence L-arginase activity.
induce dysfunction of the pulmonary artery nitric oxide pathway.\textsuperscript{28} We have demonstrated that SHS increases superoxide anion generation by rabbit aorta and that antioxidant substances partially prevent SHS-induced endothelial dysfunction.\textsuperscript{29} These observations are consistent with those of Murohara et al.,\textsuperscript{30} who demonstrated that cigarette smoke causes superoxide-mediated contraction of porcine coronary arteries by degeneration of nitric oxide.

\textit{Limitations of this Study}

Since we studied only male rabbits, the results of this study cannot be applied to both genders. Studies in organ baths preclude examination of resistance vessels, and are limited to large conduit arteries. Therefore, the observations of this study do not apply directly to resistance vessels, which are involved in pulmonary vascular hypertension. However, the nitric oxide pathway is an important determinant of basal pulmonary vascular tone\textsuperscript{4} in both conduit and resistance arteries.\textsuperscript{1} Further studies of this nature in resistance arteries are required. Another limitation of the present study is that no histopathologic studies of the pulmonary arterial conductance and resistance vessels were performed to assess effects of SHS and L-arginine. The salutary effect of L-arginine on endothelial function has previously been demonstrated\textsuperscript{31} to be of finite duration. As the present study was designed to assess the effects of L-arginine after 10 weeks of treatment, the long-term effects of L-arginine need to be studied.

\textbf{CONCLUSION}

SHS exposure reduces rodent pulmonary artery endothelial NOS activity, lowers L-arginine content through a mechanism independent of L-arginase, and impairs pulmonary artery endothelium-dependent relaxation. Long-term dietary L-arginine supplementation increases serum and endothelial L-arginine content and prevents SHS-induced endothelial dysfunction. Although L-arginine supplementation addresses SHS-induced loss of substrate, the SHS-induced loss of NOS activity, if progressive, would not be expected to be remediable by L-arginine over the long term.

\textbf{REFERENCES}