Short-term exposure to cigarette smoke induces endothelial dysfunction in small intrapulmonary arteries: analysis using guinea pig precision cut lung slices

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Wright JL, Churg A. Short-term exposure to cigarette smoke induces endothelial dysfunction in small intrapulmonary arteries: analysis using guinea pig precision cut lung slices. J Appl Physiol 104: 1462–1469, 2008. First published March 20, 2008; doi:10.1152/japplphysiol.00520.2007.—The pathogenesis of cigarette smoke-induced pulmonary hypertension is not understood. We have previously shown that smoke rapidly and persistently, but discoordinate manner, upregulates gene expression of mediators that control vascular constriction, vasoproliferation, and vasorelaxation in small intrapulmonary arteries. To investigate the possibility that smoke also induces endothelial dysfunction, a finding common to other forms of pulmonary hypertension, we exposed guinea pigs to smoke or air (control) daily for 2 wk and then prepared precision-cut lung slices. After exposure to endothelin-1, a vasoconstrictor, intracinar arteries in lung slices derived from smoke-exposed animals constricted more rapidly (greater constriction at a given concentration of endothelin) than did vessels from air-exposed animals. To examine relaxation responses, arteries were constricted with the vasoconstrictor U-46619 and then relaxed with progressively increasing doses of acetylcholine. Vessels from smokers had a delayed response to acetylcholine compared with vessels from controls. The NO synthase inhibitor N0-nitro-L-arginine methyl ester reduced relaxation in both control and smoke-exposed arteries, whereas the NO donor sodium nitroprusside increased relaxation of the smoke-exposed arteries, confirming that endothelial dysfunction with decreased effective NO production is present. These findings show that precision cut lung slices can be used to examine the physiological effects of cigarette smoke on intra-acinar pulmonary arteries and indicate that even relatively short-term exposure to smoke produces endothelial dysfunction with a resulting tendency to earlier constriction and later relaxation in cigarette smokers. These changes may be important in the development of pulmonary hypertension.

PULMONARY HYPERTENSION is a relatively common, although frequently unrecognized, form of cigarette smoke-induced lung disease. Pulmonary hypertension develops in ~6% of subjects with chronic obstructive pulmonary disease (COPD) but is present in ~40% of patients with an forced expiratory volume in 1 s of <1 liter (6, 23, 24, 44). Although usually relatively mild in degree, its presence is considered an important complication since it has been shown to be a significant predictor of mortality and is a major cause of morbidity in patients with COPD (9, 12, 42).

The pathogenesis of pulmonary hypertension in patients with COPD is not understood. The usual claims of pathogenesis, namely that pulmonary hypertension is secondary to either hypoxia or loss of vascular bed secondary to emphysema, are contradicted by a considerable body of evidence (reviewed in Ref. 47). We have suggested instead (47, 49) that pulmonary hypertension is caused by direct effects of cigarette smoke on the intrapulmonary vessels. In Hartley strain guinea pigs, chronic cigarette smoke exposure produces an ~25% increase in pulmonary arterial pressure (46, 49), and we have previously demonstrated, using laser-capture microdissection of small intrapulmonary arteries, that cigarette smoke exposure acts on the small pulmonary arteries of guinea pigs to induce discoordinate upregulation of mRNA and immunohistochemically detectable protein for endothelin, endothelial NO synthase (eNOS), and vascular endothelial growth factor (48), substances that control vascular contractility, relaxation, and cell proliferation. We were also able to demonstrate that there were significant correlations between either gene expression or immunohistochemical levels of these vasoactive mediators and pulmonary arterial pressure and vascular remodeling in animals exposed to smoke for 6 mo (49).

Endothelial dysfunction is defined as a physiological alteration of the normal biochemical processes carried out by the endothelium (7). The characteristic feature of dysfunction is the inability of the arteries to dilate fully in response to exercise, acetylcholine, or increases in flow. Although endothelial dysfunction may also be associated with an increased production of vasoconstrictors, its presence can ultimately lead to a chronic insufficiency in the production of vasodilators, thus allowing constriction to be either progressive or maintained.

The morphological and molecular changes in the pulmonary arterial tree in cigarette smokers affect relatively small intrapulmonary vessels. Although morphological studies, or studies using laser-capture microdissected vessels of this size can be used to assess changes in mediator production, it is more difficult to evaluate physiological functional changes. In the present study, we utilized a precision-cut lung explant system to test the hypothesis that exposure to cigarette smoke is associated with evidence of endothelial dysfunction in the small intrapulmonary arteries. We examined the dose response of the small intra-acinar pulmonary arteries to the contractile effects of endothelin-1. Endothelium-dependent vasorelaxation effect was tested using acetylcholine, and endothelium-independent effects tested using sodium nitroprusside (SNP) each with or without the NO synthase (NOS) inhibitor N0-nitro-L-arginine (L-NAME).

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METHODS

Animals. These studies used 20 Hartley female guinea pigs. Two of the animals were utilized for initial time course experiments. In the first experimental study, which tested endothelin-1 contraction and acetylcholine relaxation responses, a group of five animals was randomly selected for cigarette smoke exposure and another group of five for control (sham smoke-air) exposure. The second experimental study tested endothelium-dependent and endothelium-independent relaxation responses with or without addition of the NOS inhibitor L-NAME and utilized four smoke-exposed and four sham-exposed animals. Smoke exposure consisted of a 2 wk exposure to five 2R1 Kentucky Research cigarettes each day, 5 days/wk using a nose-only exposure apparatus (4), whereas control animals were handled but not exposed to smoke. We chose the 2-wk time period since our previous studies (48) showed consistent upregulation of vasoactive mediators in the small pulmonary arteries at this point. The animals were housed on paper pellet bedding and allowed free access to guinea pig chow. The experimental protocol was approved by the Animal Care Committee of the University of British Columbia.

Solutions. Hanks solution was formulated as (in g) 0.14 CaCl2, 0.40 KCl, 0.06 KH2PO4, 0.10 MgCl2 · 7H2O, 8.00 NaCl, 0.35 NaHCO3, 0.048 NaHPO4, 1.00 n-glucose in 1 liter of deionized water, supplemented with antibiotics/antimycotics. The final solution was filtered and had a final pH of 7.4. HEPES-buffered culture medium was formulated using minimal essential medium powder with Earle’s salts and L-glutamine, supplemented with amino acid solution, sodium pyruvate, vitamin solution, HEPES, and antibiotic/antimycotic agents. All materials were purchased from GIBCO (Grand Island, NY) and Sigma-Aldrich (Oakville, Ontario, Canada).

All drugs were purchased from Sigma-Aldrich and were used in the following concentrations: 10−6 M U-46619 (9,11-dideoxy-11α, 9α-epoxymethanoprostaglandin F2α), 10−11 to 10−4 M SNP, 10−11 to 10−6 M endothelin-1, 10−11 to 10−4 M acetylcholine, and 10−4 M 1,10-phenanthroline. Hanks’ solution was used as a diluent.

Explant selection, vascular identification, and image acquisition. Twenty-four hours after initial smoke or sham-smoke exposure, the guinea pigs were anesthetized using intraperitoneal urethane (0.5 g/kg). The trachea was cannulated and the animals exsanguinated by severing the abdominal aorta. After opening the chest, the right ventricle was cannulated, and 40 ml of 37°C heparinized Hanks solution was slowly instilled to flush the vasculature. The lungs were then filled with 2% low melting point agarose and cooled, these agarose-inflated lung samples were removed and inflated (ECV100) with 100 ml of 37°C Hanks solution, a baseline image was obtained, and the liquid was removed. The test vasoconstrictor solution was then substituted, and the vessel was imaged every 30 s for 3 min and every minute thereafter for a final duration of 20 min. Although U-46619 contracted the vessels earlier than did endothelin, contraction was well established by 10 min and did not change thereafter. Although we imaged each explant at 1, 3, 5, and 10 min to determine progression of contraction (or relaxation for the relaxation studies described below), we chose 10 min as the final duration of the incubation period.

Endothelin-1 contraction dose response curves. After an initial incubation in Hanks solution for 10 min, a baseline image was taken, the liquid removed and 10−11 M endothelin-1 added, incubated for 10 min, and the vessel re-imaged. The liquid was then removed, and the next concentration was added. The concentrations used were 10−11 to 10−6 M. Thus each vessel was analyzed over 70 min.

Endothelium-dependent or -independent mediated relaxation dose response curves. After an initial incubation in Hanks solution for 10 min, a baseline image was taken, the liquid removed and U-46619 added, incubated for 10 min, re-imaged. The liquid was then removed, the explant washed with 1 ml of Hanks solution, and 10−11 acetylcholine or 10−11 M SNP (with or without L-NAME) solution added, followed by a 10-min incubation before re-imaging. The liquid was then removed and the next concentration added. Concentrations were 10−11 to 10−4 M. Thus each vessel was analyzed over 100 min.

Image analysis. The photographic images were analyzed using the Image Pro (MediaCybernetics, Silver Spring MD) system. We made the assumption that a change in vessel shape with reduced luminal area represented contraction, and increased luminal area indicated relaxation. The luminal area was outlined in each image, with the value of the baseline image defined as 100%; maximal contraction was defined as the luminal area after 10−6 M endothelin-1 or U-46619. Percent maximal contraction was calculated as 100 × (area at selected endothelin concentration)/baseline area − area at maximal contraction. Thus, for this calculation, 100% is representative of full contraction, and 0% indicates no contraction. Percent return to baseline was calculated as 100 × (area at selected acetylcholine concentration − area at maximal contraction)/baseline area − area at maximal contraction). Thus, for this calculation, 100% indicated a complete return to baseline, whereas 0% indicated persistence of maximal contraction.

Histology. After the completion of the dose-response curves, the explants were fixed in 100% alcohol. To provide confirmation of the images, we stained some of the explants with hematoxylin and eosin. For standard histology, we obtained a sample from the original lung tissue, which remained after the explant sectioning was performed. This was fixed in formalin, processed to paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin.

Statistics. For each of the contraction and relaxation dose-response curves, we examined at least three explants containing between three and six vessels in each animal. The mean value per animal at each concentration was calculated, and a repeated analysis of variance was first performed to determine whether the two curves deviated. This was followed by an analysis of variance used to determine differences between the control and cigarette smoke-exposed groups at each concentration of endothelin or acetylcholine. The concentration needed to reach 50% maximal contraction or relaxation was calculated (EC50) by curve fitting, and an analysis of variance was used to determine differences between control and smoke-exposed groups.
RESULTS

Vascular selection. Selection of vessels for contraction and relaxation curves was performed on the unstained precision cut lung slices, but for the purposes of illustration, Fig. 1A shows an hematoxylin and eosin stained 225-μm lung slice, and the circle identifies the morphological features used to select the vessels seen at a higher magnification in Fig. 1B. These consist of a transversely sectioned artery situated adjacent to a terminal membranous bronchiole or immediately subtending alveolar duct. Figure 1C shows the 5-μm histological counterpart of the airway-vessel complex, which we include here to clearly illustrate the size of the vessels selected.

Vascular contraction. Figure 2 demonstrates that the small arteries of the control and the smoke-exposed animals contracted to a similar degree regardless of whether endothelin-1 or U-46619 was the agonist.

Endothelin-1-induced vascular contraction dose response. Figure 3 illustrates a sample vessel at baseline and at three concentrations of endothelin-1 to show progressive constriction. Figure 4 shows the means ± SD data for the five guinea pigs in each of the control and smoke-exposure groups. The bar plot of the arteries from the smoke-exposed animals is significantly shifted from that of the control animals (P < 0.02). Analysis of the individual endothelin-1 concentrations shows that the arteries from the smoke-exposed animals contracted to a greater percentage of their maximum at lower concentrations of endothelin-1 compared with the arteries from the control guinea pigs (individual P values: 10⁻¹¹ M = 0.004; 10⁻¹⁰ M = 0.002; 10⁻⁹ M = 0.001; 10⁻⁷ M = 0.001). The EC₅₀ of endothelin was 8.5 ± 0.5 in the control animals compared with 10.0 ± 0.4 in the smoke-exposed animals (P = 0.001).

Acetylcholine-induced vascular relaxation. Figure 5 illustrates a sample vessel at baseline, after maximum contraction by U-46619, and at two concentrations of acetylcholine to show progressive relaxation. Figure 6 shows the means ± SD data for the two guinea pig groups. The bar plot of the arteries in the smoke-exposed group is significantly different from that of the control animals (P = 0.05). Although both groups relaxed to baseline, the arteries from the smoke-exposed animals had a delayed response compared with those from the control guinea pigs (individual P values: 10⁻¹¹, 10⁻¹⁰, and 10⁻⁹ M = 0.05). The EC₅₀ measured 8.6 ± 1.0 in the control animals compared with 7.0 ± 0.6 in the smoke-exposed animals (P = 0.02).

Table 1 summarizes the data for the endothelium-dependent and -independent relaxation curves. The acetylcholine endothelial-dependent driven relaxation data recapitulate that from the first part of the experiment, with differences in the response between the control and smoke-exposed animals (P = 0.001). With the addition of L-NAME, the acetylcholine response is markedly diminished as assessed by the EC₅₀ (P = 0.05 for control and 0.02 for smoke exposed). For the control animals, the SNP-driven endothelium-independent response is similar to that of the endothelium-dependent response. However, in the smoke-exposed animals, SNP produced a greater response than did acetylcholine as assessed by the EC₅₀ (P = 0.01). As would be expected, L-NAME did not alter the SNP response.

DISCUSSION

The studies reported here shed light on the mechanisms by which cigarette smokers may develop pulmonary hypertension, namely alteration of the normal balance between vasoconstriction and vasorelaxation. Human cigarette smokers appear to have both abnormal functional levels of the vasorelaxant NO...
and an abnormal vascular response to NO, although there is controversy in the literature about the exact effects of smoke on NO production. Smokers have a decreased amount of exhaled NO (20, 26, 32, 36), and smoke appears to be able to alter the metabolism of NO by facilitating its oxidation and thus increasing oxidative products (3). Most importantly, long-term smoking appears to be associated with a decreased NO response (21), with impaired endothelium-dependent relaxation of the main pulmonary artery found in patients with COPD (10, 11). Reduced immunohistochemical expression of NOS has been found in the vessels of patients who had pulmonary hypertension due to a variety of etiologies (14). Interestingly, in patients with pulmonary hypertension, inhaled NO response appears able to predict a long-term response to therapeutic oral vasodilators (27).

The issue of what effects smoke has on vascular NO production and the vascular responses to NO is complicated, and the literature is somewhat contradictory. It has been suggested...
from work using an in vitro endothelial cell culture system that smoke extract inhibits eNOS mRNA transcription (40). However, our previous study using laser-capture microdissection of the small pulmonary arteries of smoke-exposed mice demonstrated upregulation of mRNA for eNOS, although this upregulation was delayed compared with endothelin and VEGF. However, even if translated into protein, as suggested by the immunohistochemical staining data, the upregulation of mRNA might not have resulted in an increased functional level of NO.

It is quite possible that cigarette smoke is interfering with the function of eNOS. Smokers have serum and tissue evidence of oxidative damage to many proteins (reviewed in Ref. 25), and tetrahydrobiopterin (BH4), a required co-factor for eNOS activity, can be altered to its inactive BH2 form by oxidants, and in this situation switches production from NO to superoxide (O2−) (15, 34). Peroxynitrite, a highly reactive molecule, is produced in a near diffusion-limited rate as a reaction between O2− and NO. Peroxynitrite can oxidatively inactivate eNOS, thus decreasing NO synthesis, and in addition peroxynitrite catalytically disrupts eNOS, resulting in increased O2− production by the eNOS dimers (50). Furthermore, cigarette smoke has been shown to inhibit production of tetrahydrobiopterin (15), disrupt the active eNOS dimers, and abnormally phosphorylate eNOS, producing an inhibitory state, all of which are

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**Fig. 5.** A sample vessel at baseline, after precontraction with U-46619 and at two concentrations of acetylcholine. Note the progressive relaxation response to acetylcholine. The measured area (in μm²) of the vessel lumen is indicated in parentheses after the molar acetylcholine concentration.

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**Table 1. Vascular contraction and endothelium-dependent and -independent relaxation**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Smoke Exposed</th>
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</thead>
<tbody>
<tr>
<td>% Maximal contraction</td>
<td>46.5±11.3</td>
<td>45.4±9.8</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>43.5±10.4</td>
<td>54.3±4.9</td>
</tr>
<tr>
<td>SNP</td>
<td>50.6±12.3</td>
<td>44.1±8.9</td>
</tr>
<tr>
<td>SNP + L-NAME</td>
<td>44.1±5.0</td>
<td>47.0±10.9</td>
</tr>
<tr>
<td>Estimated negative log concentration to produce 50% relaxation from maximal contraction</td>
<td>9.2±0.5</td>
<td>7.2±0.3</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>6.0±2.5*</td>
<td>5.2±1.2*</td>
</tr>
<tr>
<td>SNP</td>
<td>8.6±0.9</td>
<td>8.7±1.0*</td>
</tr>
<tr>
<td>SNP + L-NAME</td>
<td>9.6±0.8</td>
<td>8.6±0.4</td>
</tr>
</tbody>
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Values are means ± SD. L-NAME, N^6^-nitro-L-arginine methyl ester; SNP, sodium nitroprusside. *Significant difference from acetylcholine response (actual P values in RESULTS).
alterations that would reduce NO bioavailability (43). Finally, the endothelial response to NO appears to be attenuated by endothelin-1 (18).

Thus eNOS protein production would not have a one-to-one relationship to function (18), which might explain the impaired endothelium-dependent relaxation found in the pulmonary arteries of patients with COPD (1, 10, 31). This conclusion is supported by work in which endothelial cells were treated with serum from cigarette smokers (5); although the treated cells had increased amounts of eNOS protein, there was decreased eNOS activity and decreased production of NO.

Smoke also exerts a number of other effects on eNOS production. TNF-α, which is increased in the sputum of human smokers with COPD (19) and in the plasma of animal models of COPD (8), destabilizes eNOS mRNA (22, 28, 37). Smoke also contains large concentrations of O₂⁻, which, as described above, can reduce functional levels of NO by converting it to peroxynitrite.

The question arises as to how smoke exerts its effects on the vasculature. In humans, some eNOS gene variants are over represented in patients with COPD and are associated with increases in the lipid peroxidation product malonaldehyde (2), thus suggesting that smoke induces a oxidative effect. A recent report on the vascular effects of an acute exposure to diesel exhaust provides further evidence of the importance of oxidative stress in inducing endothelial dysfunction, at least in the systemic vasculature (41). It is known that NO itself can be inactivated by reactive oxygen, and the above clinical findings suggest that inhalation of oxidant-generating agents can produce endothelial dysfunction. We have not specifically addressed the role of oxidants in the present study.

Early studies have demonstrated the usefulness of lung explants to examine the reactions of the pulmonary vasculature (38, 39). However, these studies were limited to examination of the larger vessels because the explants were hand-sectioned, ~1 mm in thickness, and small vessels could not be visualized. Precision-cut lung explants, which allow sections of 250 μm or less, have recently proven to be useful in studying the responses of the airways and pulmonary vasculature to various agents or interventions. Airways examined using this technique have been shown to maintain the characteristics of the whole lung as determined by comparison to isolated perfused physiological measurements (16). In particular, a recent study showed that precision-cut lung explants from the guinea pig are an excellent model of human pharmacological reactions, at least in the airways, and in fact are superior to rats or mice (35).

In the present study, we utilized endothelin-1 to assess vasoconstriction because of the known relationships between endothelin and pulmonary hypertension and because of the putative association of endothelin with endothelial dysfunction (17, 33). We utilized acetylcholine as the vasorelaxant stimulus because acetylcholine is known to dilate blood vessels via activation of the endothelium to release NO (13), and thus may reveal evidence of endothelial dysfunction. To confirm the endothelial dependence of our results, we examined the response to the endothelium-independent vasorelaxant SNP, and to confirm the role of nitric oxide we examined the endothelial-dependent and endothelial-independent responses in the presence of a NOS inhibitor, L-NAME.

As opposed to studies on eNOS, there are very few animal models that have examined the effects of cigarette smoke on the vasculature itself. A study of the systemic vessels in the rat showed increased sensitivity to endothelin after in vivo smoke exposure (33). Interestingly, in that study, NOS inhibition by L-NAME, and thus reduction of NO, did not reduce the relaxation curve in the smokers to the same degree as the control group, a feature again suggestive of endothelial dysfunction, since it implies that the arteries in the smoke-exposed animals had a deficiency of functional NO. Similarly, in our study, the smoke-exposed animals had a greater response to SNP than to acetylcholine as assessed by the EC₅₀, suggesting that there is a lack of effective NO production in the vessels from the smoke-exposed animals. Thus, in the present experiments, the increased sensitivity of the arteries to endothelin combined with the delayed response to acetylcholine in our study suggests that cigarette smoke has induced endothelial dysfunction. Our results also indicate that NO does drive relaxation in this model.

We chose to examine the intra-acinar vessels for several reasons. First, we had previously found that these vessels become muscularized after long-term cigarette smoke exposure, and this muscularization correlates with both increased pulmonary arterial pressure and the level of mRNA of vasoactive mediators. Second, although the cross section of the vascular bed at this level is large, remodeling of these vessels may be associated with increased vascular resistance, much in the same way as alteration of the small airways in COPD increases resistance to airflow (30, 45). Finally, intra-acinar vessels have been shown to contract to hypoxic stimulus (29) and thus appeared to represent an appropriate approach to our investigations. Our study appears to be the first use of the intra-acinar arteries to provide dose response information.

However, our study does have limitations. First, we have interpreted a change in lumenal shape as evidence of constriction or relaxation. Although we believe that this assumption is warranted, it cannot be absolutely proven in our system. Second, it is possible that the vessels might be partially tethered by the adjacent alveolar parenchyma. Although this might affect constriction, we do not believe that it would alter vascular relaxation. Finally, we must note that other vascular mediators such as the prostaglandin system have the potential to be activated by, and may be important in, the overall vascular response to cigarette smoke.

In summary, we have utilized precision-cut lung explants to examine the dose response characteristics of the intra-acinar pulmonary arteries to endothelin-1 and acetylcholine. These arteries showed an increased sensitivity to endothelin and a delayed reaction to acetylcholine, both of which are suggestive of endothelial dysfunction. As demonstrated by the differing relaxation responses, when the vessels were exposed to SNP or when L-NAME was added to acetylcholine, the alterations in relaxation induced by cigarette smoke were shown to be endothelium dependent. This is the first time endothelial dysfunction has been demonstrated in these arteries and provides confirmation that cigarette smoke has a direct effect on the intrapulmonary vessels. The presence of endothelial dysfunction after a short-term smoke exposure provides new data on mechanisms by which pulmonary hypertension develops in cigarette smokers.
SMOKE-INDUCED ENDOTHELIAL DYSFUNCTION

GRANTS
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endothelial nitric oxide synthase phosphorylation: role of protein kinase C. 