Vasoactive mediators and pulmonary hypertension after cigarette smoke exposure in the guinea pig

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Submitted 8 March 2005; accepted in final form 20 September 2005

Wright, Joanne L., Hsin Tai, and Andrew Churg. Vasoactive mediators and pulmonary hypertension after cigarette smoke exposure in the guinea pig. J Appl Physiol 100: 672–678, 2006.—The pathogenesis of pulmonary hypertension in patients with chronic obstructive pulmonary disease is not understood. We have previously shown increased levels of mediators that control vasoconstriction (endothelin-1), vascular cell proliferation (endothelin-1 and vascular endothelial growth factor), and vasodilation (endothelial nitric oxide synthase) in the intrapulmonary arteries of animals exposed to cigarette smoke. To determine whether these mediators could be implicated in the structural remodeling of the arterial vasculature and increased pulmonary arterial pressure caused by chronic cigarette smoke exposure, guinea pigs were exposed to daily cigarette smoke for 6 mo. Pulmonary arterial pressures were measured. Intrapulmonary artery structure was analyzed by morphometry, artery mediator protein expression by immunohistochemistry, and artery mediator gene expression by laser capture microdissection and real-time RT-PCR. We found that the smoke-exposed animals developed increases in pulmonary arterial pressure and increased muscularization of the small pulmonary arteries. Gene expression and protein levels of all three mediators were increased in both the muscularized and partially muscularized pulmonary arteries. We conclude that chronic smoke exposure produces increased vasoactive mediator expression in the small intrapulmonary arteries and that these mediators are associated with vascular remodeling as well as increased pulmonary arterial pressure. These findings support the idea that hypertension in chronic obstructive pulmonary disease is a result of direct cigarette smoke-mediated effects on the vasculature and suggest that interference with endothelin and VEGF production and activity or augmentation of nitric oxide levels may be beneficial.

endothelin; nitric oxide synthase; vascular endothelial growth factor

METHODS

The research protocol was approved by the University of British Columbia.

Smoke exposure, vascular physiology, and tissue collection. We exposed four male Hartley strain guinea pigs to the whole smoke of seven 2R1 research cigarettes (University of Kentucky) 5 days/wk for 6 mo using our previously published exposure protocol (33), with an additional group of three guinea pigs exposed to room air as a control population. At 6 mo, the animals were anesthetized using urethane anesthesia (0.5 g/kg ip) and intubated, and a catheter was placed into the pulmonary artery via the jugular vein (41). After a period of stabilization on the ventilator, with animals breathing room air, pulmonary arterial pressure was measured, and the animal was killed by anesthesia overdose followed by exsanguination. The lungs were removed and inflated with cold 100% ethanol. This type of fixation is needed to preserve RNA in guinea pigs, a species with very high levels of endogenous ribonucleases. Slices of lung were embedded in paraffin, and multiple 5-μm-thick sections were cut onto diethyl pyrocarbonate-treated (Sigma-Aldrich, Oakville, ON, Canada) water and collected on cleaned slides. One section was deparaffinized and stained with hematoxylin and eosin to serve as a dissection guide. The remaining sections were deparaffinized and stained by using the Arcturus (Arcturus, Mountain View, CA) stain and protocol for subsequent laser capture microdissection.

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Laser capture microdissection, mRNA extraction, and reverse transcription reaction. We utilized the Arcturus PixCell II (Arcturus) laser capture microdissection apparatus and separately collected the muscularized vessels adjacent to the membranous or respiratory bronchioles and the small, partially muscularized vessels adjacent to the alveolar ducts onto laser capture microdissection caps as per our previous protocol (44). The caps were placed on Eppendorf (Mississauga, ON, Canada) 500-μl tubes and stored at −80°C until the RNA extraction and isolation procedure. We used the PicoPure RNA Isolation kit (Arcturus) for RNA extraction and isolation as described by us previously (44), combining all samples from an individual animal as one data point for each of the respective sites. First-strand cDNA was synthesized by using superscript II RNase H Reverse Transcriptase (Invitrogen, Burlington, ON, Canada), and real-time RT-PCR reactions were carried out in LightCycler glass capillaries using a reaction mixture with LightCycler-FastStart DNA Master SYBR Green I (Roche, Mannheim, Germany). Each set of PCR reactions included water as a negative control and five dilutions of standards that were created by cloning part of the transcript of interest into a cloning vector (Invitrogen, Carlsbad, CA). β-Actin was used as a housekeeping gene.

Primer sequences.

β-actin: 116 bp
  Forward: 5′/TTG TTA CCA ACT GGG ACG ACA TG 3′
  Reverse: 5′/GGG TCA TCT TCT CAC GGT TGG 3′
  VEGF: 226 bp
  Forward: 5′/ATG GCA GAA GGA GAG CAG AAG CC 3′
  Reverse: 5′/TGCG ATG GTG TTG AAC TCC TC 3′
  Endothelin-1: 251 bp
  Forward: 5′/CTT GGC AGA AAT TCC AGC ACT TCT 3′
  eNOS: 222 bp
  Forward: 5′/CTT GAA GAG TGT GGG CCA GGA GCC 3′
  Reverse: 5′/CCA GTT CTT CAC TCG AGG GAA CTT 3′
  iNOS: 215 bp
  Forward: 5′/AGA CAC ACT GCA CACC 3′
  Reverse: 5′/CGG TTT CCA CTC TGCC 3′

where iNOS is inducible nitric oxide synthase.

Immunohistochemistry and morphometric analysis. Muscularization of the small vessels adjacent to the alveolar ducts was determined by immunohistochemical staining with monoclonal mouse anti-human α-smooth muscle actin (catalog M0851, DAKO, Mississauga, ON, diluted 1:200) using standard immunohistochemical procedures with appropriate positive and negative controls. In each animal, we examined 25 vessels adjacent to alveolar ducts identified by random light microscopic fields using a ×20 objective and ×10 eyepieces. Each vessel was categorized into quartiles, depending on the percentage of the vessel circumference surrounded by muscle cells. To develop a final animal score, we then weighted each quartile by 1–4, respectively, and summed the values.

Immunohistochemical evaluation of VEGF, endothelin, and eNOS was carried out as previously described (44), using standard technique with appropriate controls. The slides were coded to prevent bias. Anti-eNOS and anti-VEGF were obtained from R&D Systems (Minneapolis, MN) and diluted to 10 μg/ml and 15 μg/ml, respectively; anti-endothelin was obtained from Biodesign (Saco, ME) and diluted 1:100. To evaluate staining, we utilized a grading system based on extent and intensity of staining reaction compared with background.

Each vessel of the appropriate size in the histological section was examined and assigned a grade. Grade 0 was defined as no staining, whereas grade 3 was defined as intense and diffuse vessel staining. A final animal grade was obtained by summation of all grades and expressed as a percentage of the maximum possible grade for that number of vessels.

Data analysis and statistics. By using the crossing point curves values generated by the LightCycler and the standard curves, relative concentrations of each RNA of interest were determined and corrected for loading with the corresponding actin value. We compared the mRNA data from control animals and smoke-exposed animals by analysis of variance. Because the immunohistochemical staining data were not normally distributed, relationships between these data and pulmonary arterial pressure or the mRNA levels were determined by the Spearman rank correlation test.

RESULTS

The pulmonary arterial pressure (Ppa) in the smoke-exposed guinea pigs was significantly increased compared with that of the control animals [smoke exposed (mean ± SD) 9.8 ± 1.3 vs. control 7.0 ± 1.2 cmH2O, P = 0.04]. There was considerable variation in Ppa in the smoke-exposed animals, with a range of 8.7 to 11.7 cmH2O, compared with 5.7 to 7.7 in the control animals. The muscularization score index of the partially muscularized arteries (Fig. 1) was (mean ± SD) 72 ± 5 in the smoke-exposed animals compared with 54 ± 5 in the control animals (P = 0.005).

Figure 2 shows the relative levels of gene expression for the animals in each group separated into partially muscularized and muscularized vessels. For ease of interpretation and to allow for visualization of the range of the data points in each group, the data are presented with the mean control animal level for each time, vessel size, and gene normalized to a value of 1.0 and other values adjusted accordingly. However, all statistical analyses were performed from the raw data sets. The controls showed relatively little animal-to-animal variation in gene expression levels; however, gene expression levels for all three mediators were significantly, but variably, increased in the smoke-exposed animals. In the partially muscularized vessels from the smoke-exposed animals, endothelin was increased on average ~10-fold, VEGF was increased ~12-fold, and eNOS was increased ~4-fold over control levels. In the muscularized arteries, endothelin and VEGF were increased about eightfold, and eNOS was increased fourfold compared

Fig. 1. Muscularization index of the usually partially muscularized vessels (PMV) adjacent to the alveolar ducts. Values are means ± SD. Smoke exposure is associated with a significant increase in the degree of muscularization of these vessels (*P = 0.005).
with the levels in the control animals. There was no difference in the level of iNOS between control and smoke-exposed animals.

Figure 3 shows the data from the immunohistochemical score analyses, and Fig. 4 illustrates representative immunohistochemistry images for the various mediators. There was a small but consistent increase in the staining score for eNOS in both the partially muscularized and muscularized vessels. Endothelin scores were approximately tripled in the partially muscularized vessels and doubled in the muscularized vessels. Staining scores for VEGF were approximately doubled in both vessel sizes. Table 1 shows that gene expression levels of endothelin and eNOS correlated with the muscularization score and that even stronger correlations were found for immunohistochemically graded protein levels of all three mediators and muscularization scores.

Table 2 documents the correlations between Ppa and the levels of mRNA and immunoreactive protein. There were positive correlations with the gene expression levels of all mediators in the muscularized vessels and levels of both VEGF and eNOS in the partially muscularized vessels; correlations with endothelin approached but did not achieve significance in the muscularized arteries. Correlations between Ppa and immunoreactive protein grade scores were significant for all three mediators. The Ppa also correlated with the muscularization score in the partially muscularized vessels ($R = 0.74$, $P = 0.03$).

**DISCUSSION**

In this study we have shown that chronic exposure to cigarette smoke produces pulmonary hypertension in guinea pigs and that the level of Ppa elevation correlates with increases in production of vasoactive mediators as well as increases in muscularization in the small, normally partially muscularized, vessels. It is important to recognize that in guinea pigs with chronic cigarette smoke exposure (41, 43), as in humans with COPD (16), the increases in Ppa tend to be small. However, because even these small increases in Ppa appear to be a separate and significant factor in morbidity and mortality, it is useful to understand the factors that may be responsible for the vascular alterations.

In previous studies (44, 45), our laboratory has shown that cigarette smoke can induce rapid (within a few hours) gene upregulation of the vasoactive mediators endothelin, VEGF, and eNOS in small intrapulmonary arteries. Gene upregulation was not transient but persisted with smoke exposures of up to 3 mo (44) and was associated with increases in immunoreactive protein in the muscularized and partially muscularized vessels during this time. The present study shows that continued upregulation is present for as long as 6 mo of smoke exposure. Most importantly, it demonstrates that there are significant correlations between the Ppa and mRNA and protein levels of these mediators in both the muscularized and partially muscularized vessels. Furthermore, correlations are present between the Ppa and the degree of vascular remodeling of the partially muscularized arteries.

It is now known that endothelial cells have the ability to modulate their own function through the synthesis and release of a number of bioactive substances, including endothelin, VEGF, and nitric oxide, which act on the underlying smooth muscle cells and also act on the endothelial cells in a feedback
mechanism (reviewed in Ref. 13). However, the relationships among these mediators are complex. It appears that there is an intimate association between endothelin and nitric oxide, with each modulating the production of the other in an autocrine fashion and the actions of the other in a paracrine fashion (13). Endothelin stimulates production and release of nitric oxide (46), and nitric oxide production dissociates endothelin from its receptors and interferes with the pathway for calcium mobilization (27), thus inhibiting end responses to endothelin (9). Nitric oxide decreases expression of both VEGF and VEGF-receptor (36), similar to its effect on endothelin (19). Conversely, VEGF appears to augment nitric oxide release from the endothelium (30). Finally, VEGF has been found to activate eNOS through phosphorylation at serine 1177 (23). These complicated relationships help to explain our results.

Intuitively, one would expect, given the presence of vascular remodeling leading to thicker walled vessels with more muscle (see below) and increased Ppa (lack of vasodilatation) in the smokers, that endothelin and VEGF production should be upregulated, but eNOS should not. We suggest that, in our model, upregulation of endothelin and VEGF drives increased production of eNOS as well but that the levels and/or activity of eNOS in the smokers are insufficient (see below) to compensate for the proliferative and constrictive effects of increased endothelin and VEGF. Thus the levels of eNOS gene expression and protein probably serve here as surrogate markers of endothelin and VEGF activity, hence their correlation with vascular remodeling and Ppa. This explanation is supported by the close correlations in mRNA levels between eNOS and both VEGF (muscularized vessels $R = 0.89, P = 0.003$, partially muscularized vessels $R = 0.82, P = 0.01$) and endothelin (muscularized vessels $R = 0.96, P = 0.001$, partially muscularized vessels $R = 0.75, P = 0.03$).

Cigarette smoke contains large amounts of nitric oxide, which appears to decrease exhaled nitric oxide through down-regulation of NOS isoforms in the epithelium (17); our previous work, however, demonstrated acute upregulation of eNOS and iNOS in airway epithelium of rats exposed to cigarette smoke. In fact, the exact relationship of eNOS production and activity in animals or humans exposed to cigarette smoke is debated and certainly complicated. For example, Su and colleagues (33a), using a pulmonary artery endothelial cell culture model, found that smoke reduced eNOS activity by a reduction of protein levels and mRNA, and Barbera et al. (1) have shown decreased immunohistochemical expression of eNOS in the pulmonary arteries of smokers. However, Tuder et al. (37) found increased nitric oxide production by cultured epithelial cells after exposure to cigarette smoke condensate, and our laboratory (42) has found increased gene expression of eNOS by in situ hybridization in the pulmonary endothelium of rats exposed to smoke, as well as evidence of upregulated gene expression in isolated intrapulmonary arteries in rats and guinea pigs exposed to smoke (44, 45). Smoke also exerts a number of other effects on eNOS production. TNF-$\alpha$, which is increased in the sputum of human smokers with COPD (15) and in the plasma of animal models of COPD (2), downregulates eNOS protein levels by destabilizing eNOS mRNA (21). Smoke also inhibits production of tetrahydrobiopterin (10), a required cofactor for eNOS activity. In addition, the endothelial response to nitric oxide is attenuated by increased levels of endothelin (14).

Nitric oxide could also be produced by increases in iNOS. In rats, our laboratory has previously found (42) that smoke
transiently upregulates gene expression of iNOS, although it does not increase immunohistochemically detectable protein, and that iNOS gene expression increases do not persist over the long term. The present study indicates that long-term smoke exposure also does not alter iNOS gene expression in the guinea pig over the long term and implies that there is considerable selectivity to the genes that are upregulated in the vessels by cigarette smoke.

In theory, nitric oxide present in smoke should itself be able to act as a vasodilator; however, it is likely that the bioavailability of either endogenously or exogenously produced nitric oxide is low because it is consumed in the vessel wall or is not transported through the systemic circulation.

Table 1. Correlations of muscularization with gene expression and immunoreactive protein

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<tr>
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<th>Correlations between muscularization of partially muscularized arteries and gene expression levels</th>
<th>Correlations between muscularization of partially muscularized arteries and immunoreactive protein levels</th>
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<tr>
<td></td>
<td>$R = 0.79, P = 0.02$</td>
<td>$R = 0.82, P = 0.01$</td>
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<td>Endothelin</td>
<td>$R = 0.61, P = 0.07$</td>
<td>$R = 0.89, P = 0.003$</td>
</tr>
<tr>
<td>VEGF</td>
<td>$R = 0.36, P = 0.01$</td>
<td>$R = 0.95, P = 0.001$</td>
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<td>eNOS</td>
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Table 2. Correlations of pulmonary arterial pressure with gene expression and immunohistochemical protein

<table>
<thead>
<tr>
<th></th>
<th>Correlations between pulmonary arterial pressure and gene expression levels in muscularized arteries</th>
<th>Correlations between pulmonary arterial pressure and immunohistochemical protein levels in muscularized arteries</th>
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<tr>
<td>Endothelin</td>
<td>$R = 0.83, P = 0.01$</td>
<td>$R = 0.88, P = 0.004$</td>
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<tr>
<td>VEGF</td>
<td>$R = 0.69, P = 0.05$</td>
<td>$R = 0.74, P = 0.03$</td>
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<tr>
<td>eNOS</td>
<td>$R = 0.78, P = 0.02$</td>
<td>$R = 0.67, P = 0.05$</td>
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<tr>
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<th>Correlations between pulmonary arterial pressure and gene expression data in partially muscularized arteries</th>
<th>Correlations between pulmonary arterial pressure and immunohistochemistry data in partially muscularized arteries</th>
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<tr>
<td>Endothelin</td>
<td>$R = 0.63, P = 0.06$</td>
<td>$R = 0.74, P = 0.03$</td>
</tr>
<tr>
<td>VEGF</td>
<td>$R = 0.81, P = 0.01$</td>
<td>$R = 0.85, P = 0.008$</td>
</tr>
<tr>
<td>eNOS</td>
<td>$R = 0.88, P = 0.004$</td>
<td>$R = 0.76, P = 0.02$</td>
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cNOS, endothelial nitric oxide synthase.

Fig. 4. Photographic panel illustrating immunohistochemical staining for endothelin (A, control; B, smoke exposed), VEGF (C, control; D, smoke exposed), eNOS (E, control; F, smoke exposed), and smooth muscle actin (G, control; H, smoke exposed).
oxide can be impaired by other reactive oxygen species present in smoke, because superoxide anion and nitric oxide rapidly combine to form the highly reactive oxidant peroxynitrite (25).

Overall, these considerations suggest that eNOS protein production would not have a one-to-one relationship to function (10), thus helping to explain the impaired endothelium relaxation found in the pulmonary arteries of patients with COPD (4).

Our data imply that all three mediators are involved in cigarette smoke-induced vascular remodeling. Endothelin production or release is stimulated by vascular stress, airway inflammation, and oxidants (7, 12), and cigarette smoke is a highly concentrated source of oxidants (26). Cigarette smoke extract has been demonstrated to induce increased expression of endothelin in cultured pulmonary artery cells (20), and studies of patients with COPD have demonstrated an increased plasma level of endothelin (28, 34). As was true in the present experiment, increased expression of endothelin has been found in the lungs of patients with pulmonary hypertension (8) from a variety of etiologies, including COPD. Endothelin increases reactive oxygen species production in arterial smooth muscle cells and is able to decrease eNOS expression through a hydrogen peroxide-based mechanism (38). Increased oxidant stress is known to induce increased synthesis of VEGF from vascular smooth muscle cells, with subsequent enhancement of atherogenic vascular remodeling (18).

The importance of adequate levels of eNOS in preventing vascular remodeling has been demonstrated by Fagan et al. (6), who showed that eNOS null mice develop marked hypoxia-induced pulmonary hypertension, with a pronounced increase in the number of muscularized small arteries. These authors compared homozygous knockout to heterozygous knockout mice and found that at least 50% of wild-type NOS expression was required to maintain normal pulmonary vascular tone.

Increased expression of VEGF has been identified in the medial smooth muscle of arteries in the lungs of patients with pulmonary hypertension secondary to a variety of etiologies (11), but there are few data regarding the role of VEGF in remodeling of the pulmonary vasculature in COPD. Santos and colleagues (29) reported immunohistochemically increased VEGF protein in the pulmonary arteries of smokers with and without COPD. On the other hand, they (29) failed to find alterations of VEGF mRNA levels between smokers with and without COPD, but their measurements were made on bulk lung samples, and thus subtle differences may not have been identified.

In summary, the present study has demonstrated a positive relationship between levels of the vasoactive mediators endothelin, eNOS, and VEGF, and Ppa. To our knowledge, this is the first time such a relationship has been shown. Muscularization of the small pulmonary arteries is also positively related to these mediators, suggesting that they probably have a causative role in structural remodeling. Whether the increases in Ppa result from dynamic mediator activity or from mediator-induced vascular remodeling is not clear, and both processes probably play a role. These results must be extrapolated from laboratory animals to humans with caution. However, our study suggests that pulmonary hypertension associated with cigarette smoke is a direct consequence of the response of the vessels to the smoke and that interference with endothelin and VEGF production, or augmentation of nitric oxide production, might be beneficial in smokers with pulmonary hypertension.

GRANTS

This research was supported by Grant MOP62693 from the Canadian Institutes of Health Research.

DISCLOSURES

J. L. Wright and A. Churg have received funds from AstraZeneca to examine animal models of emphysema and test the effectiveness of various compounds under development.

REFERENCES


