Vascular remodeling and ET-1 expression in rat strains with different responses to chronic hypoxia

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Chronic exposure to hypoxia leads to pulmonary hypertension due, in part, to pulmonary vasoconstriction but also to increased blood viscosity and structural remodeling of pulmonary vessels. The vascular remodeling is characterized by hypertrophy of the vascular media and the extension of smooth muscle to previously unvascularized pulmonary arterioles (9).

Variation in susceptibility to hypoxia-induced pulmonary hypertension is well recognized in humans (24) and other species (11, 13, 23) and appears to have a genetic basis (23). Wilkins et al. (25) have reported on two rat strains, Fischer 344 (F-344) and Wistar-Kyoto (WKY), that differ in their hypoxic vasoconstrictor response: the pressor response to acute hypoxia (2% O2) in the F-344 rat lung is ~50% of that seen in the WKY rat lung. Furthermore, this difference is sustained during chronic exposure of the whole animal to normobaric hypoxia (10% inspired O2 fraction) for 3 wk and is paralleled by a lesser degree of right ventricular hypertrophy in the F-344 strain. The two strains develop a similar rise in hematocrit, but it has not been established whether the two strains differ in their pulmonary vascular anatomy before chronic hypoxic exposure and whether the different hemodynamic effects of chronic hypoxia are accompanied by differences in pulmonary vascular remodeling.

The mechanisms underlying hypoxia-induced pulmonary hypertension and remodeling are poorly understood. One candidate that may have a role in the pathogenesis of the condition is endothelin (ET)-1, a potent vasoconstrictor with trophic properties (19). Consistent with this thesis, hypoxia has been shown to elevate ET-1 levels in cultured endothelial cells (12) and circulating levels are raised in rats with hypoxia-induced pulmonary hypertension (3, 14, 26). Furthermore, ET-1-receptor antagonists have been shown to inhibit hypoxia-induced pulmonary hypertension in animal models (1, 2, 17, 21, 26). We conducted a detailed anatomic study of the pulmonary vasculature of F-344 and WKY rats before and after chronic exposure to hypoxia and determined whether the changes in pulmonary vascular structure are accompanied by changes in plasma and lung levels of ET-1 peptide and lung preproET-1 mRNA expression.

Methods

Animals. Male F-344 rats were obtained from OLAC (Blackthorn, Bicester, UK), and male WKY rats were obtained from Charles River (Margate, UK). The animals were fed standard rat chow, allowed free access to food and water, and studied at 10–14 wk of age.

Chronic hypoxia. The rats were exposed to hypoxia (10% inspired O2 fraction) for 21 days in a specially constructed environmental chamber described previously (28). Humidity and temperature were controlled and matched to normal laboratory conditions. Control animals were housed in the same room but outside the chamber.

Preparation of plasma and lung tissue. At the specified time point, the rats were killed with an overdose of pentobarbital sodium (100 mg/kg ip). Hypoxic rats were killed within 1 h of removal from the chamber or within 5 min if the animals...
were being used for assay of ET-1 mRNA or peptide to avoid rapid degradation of preproET-1 mRNA on return to normoxia.

When the lungs were used for morphological analysis, the pulmonary circulation was flushed with 10 ml of phosphate-buffered saline (PBS) via a cannula in the main pulmonary artery. The heart and lungs were removed en bloc (n = 6 rats/group). A mixture of barium sulfate and gelatin at 60°C was then infused into the pulmonary artery at a pressure of 76 mmHg over 5 min as previously described (9). The trachea was then cannulated, and the lungs were fixed at a constant inflation pressure of 36 cmH2O with 10% Formalin for 24 h. The Formalin-fixed tissues were then transferred to 70% ethanol before being embedded in paraffin and sectioned. Lung sections (4 μm) were cut parallel to the axial artery of the left lung and stained with hematoxylin and eosin and elastic Van Gieson stains. Lungs from some animals were fixed in inflation and processed as above except that no barium-gelatin was infused. These lungs (n = 4 rats/group) were used for immunohistochemical localization of ET-1. In addition, animals (n = 4–6/group) were acidified with an equal volume of 0.1% trifluoroacetic acid (TFA), centrifuged at 3,000 g for 15 min at 4°C, and applied to Sep-Pak C18 columns (Waters Associates, Milford, MA). The columns were prepared in RIA buffer and subjected to RIA with the use of a rabbit anti-ET-1 antiserum. According to the manufacturer, the anti-ET-1 antiserum has 98% cross-reactivity with ET-2 and ET-3 and 17% cross-reactivity with Big ET-1.

The peptides were extracted from lung tissue by boiling in 10% (wt/vol) 1 M acetic acid for 10 min. The samples were then centrifuged and centrifuged at 1,500 g for 10 min at 4°C. Aliquots (2 ml) of supernatant were applied to Sep-Pak C18 columns prepared as above. The peptides were eluted from the column as above except that the column was washed with 1 M acetic acid before 10% acetonitrile. Lung peptide extracts were then evaporated to dryness and subjected to RIA as above.

Northern analysis of preproET-1 mRNA. Total RNA was extracted from rat lung with TRIzol Reagent following the manufacturer’s instructions (Life Technologies). RNA concentration and relative purity were determined by measuring absorbance at 260 nm and the ratio of the absorbance at 260 nm relative to that at 280 nm in a spectrophotometer. Ratio values of 1.9 were accepted as consistent with satisfactory RNA extraction. Total RNA (50 μg/lane) was size-fractionated by electrophoresis through a 1% agarose-9% formaldehyde gel, then transferred to a nylon membrane (Hybond-N+, Amersham) with 10× saline-sodium citrate (SSC; 1× SSC is 180 mM NaCl and 15 mM sodium citrate, pH 7.0). Nucleic acids were immobilized by ultraviolet irradiation. The membranes were prehybridized at 65°C for 2 h in buffer containing 5× SSC, 5× Denhardt’s solution, 0.5% sodium dodecyl sulfate (SDS), and 100 μg/ml of denatured salmon sperm DNA. The blots were hybridized at 65°C overnight in fresh buffer without salmon sperm DNA but with [32P]-labeled specific rat preproET-1 cDNA. The preproET-1 probe was a 0.7-kb cDNA for the 3’-noncoding region designed to be specific for ET-1 and generously provided by Dr. Masashi Yanagisawa (Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX). The blots were then washed with 2× SSC-0.1% SDS at room temperature for 10 min, then in 0.1% sodium azide in PBS. The antigen was visualized with the avidin-biotinylated peroxidase technique (Vectastain ABC, Vector Laboratories, Peterborough, UK) with 3,3′-diaminobenzidine substrate. Slides were batched (25–50 sections) and processed together to attain consistency in the degree of substrate development. The development of 3,3′-diaminobenzidine was halted by immersion in water after 5 min. The specificity of immunostaining was demonstrated by the absence of signal in sections incubated with control mouse IgG (Sigma). In sections processed after omission of the primary antibody, and after cold competition by coincubation with excess ET-1. The sections were then counterstained lightly with hematoxylin, dehydrated, cleared in xylene, mounted (Pertex, CellPath, Hemel Hempstead, UK), and examined by light microscopy.

Measurement of plasma and lung immunoreactive ET-1. Samples were assayed for ET-1 immunoreactivity with a specific RIA (Peninsula Laboratories, Belmont, CA) similar to that described previously (3, 14). Briefly 750 μl of rat plasma (n = 5 rats/group) were acidified with an equal volume of 0.1% trifluoroacetic acid (TFA), centrifuged at 3,000 g for 15 min at 4°C, and applied to Sep-Pak C18 columns (Waters Associates, Milford, MA). The columns were prepared in RIA buffer and subjected to RIA with the use of a rabbit anti-ET-1 antiserum. According to the manufacturer, the anti-ET-1 antiserum has 9% cross-reactivity with ET-2 and ET-3 and 17% cross-reactivity with Big ET-1.

The peptides were extracted from lung tissue by boiling in 10% (wt/vol) 1 M acetic acid for 10 min. The samples were then centrifuged and centrifuged at 1,500 g for 10 min at 4°C. Aliquots (2 ml) of supernatant were applied to Sep-Pak C18 columns prepared as above. The peptides were eluted from the column as above except that the column was washed with 1 M acetic acid before 10% acetonitrile. Lung peptide extracts were then evaporated to dryness and subjected to RIA as above.
0.2× SSC-0.1% SDS at 65°C for 1 h. Finally, the membranes were wrapped incling film and exposed to film (Kodak X-AR) at –80°C with intensifier screens. To control for variation in RNA loading, the membranes were then rehybridized with a 32P-labeled 18S rRNA-specific oligonucleotide probe (5'-ACGTTATCTGATCGTCTTCGAACC-3') after being stripped in 0.1% SDS at 60°C for 1 h.

Quantitation of specific mRNA transcripts was performed by analysis of the integrated absorbance over background absorbance with a PhosphorImager 445 SI system and ImageQuant image-analysis software (Molecular Dynamics, Kemsing, UK). Data are expressed as the densitometric intensity of the hybridization signal for preproET-1 mRNA relative to that obtained with the 18S rRNA-specific oligonucleotide probe.

Statistical methods. Results are expressed as means ± SE. Changes in serum and lung ET-1 peptide and lung mRNA were analyzed with one-way analysis of variance for multiple comparisons (Microsoft Excel 97, Microsoft). A P value of <0.05 was taken to indicate that conventional significance had been achieved.

RESULTS

Pulmonary artery morphometry. The percentages of muscularized and nonmuscularized vessels at the level of the terminal bronchiolar, respiratory bronchiolar, alveolar duct, and alveolar wall from the F-344 and WKY rats exposed to normal air and hypoxia are shown in Table 1. There was no significant difference between the two strains in the degree of muscularization of pulmonary vessels in the normal adult rat. Both strains showed a significant increase in the degree of peripheral muscularization after exposure to chronic hypoxia. This process was significantly more pronounced in the WKY strain than in the F-344 strain such that significantly fewer arteries remained nonmuscular at the level of the alveolar duct and alveolar wall in the WKY strain compared with F-344 strain.

The percentage wall thickness at each anatomic level is shown in Table 2. There were no significant differences between the two rat strains with regard to the medial thickness of muscularized arteries at the level of the terminal and respiratory bronchioles in the normal adult animals. During chronic hypoxia, there was a significant increase in the medial thickness of arteries accompanying extra-acinar, terminal, and respiratory bronchioles. Although there was a trend toward a greater increase in medial wall thickness in the WKY strain compared with the F-344 strain, this did not achieve significance.

Immunohistochemistry. In normoxic rats of both strains, ET-1 immunoreactivity was observed in scattered epithelial cells of small airways (bronchiolus, terminal bronchiole, and respiratory bronchiole), with little staining of the vascular endothelium (Fig. 1). Vascular ET-1 expression in the normoxic rats was confined to occasional endothelial cells around the vascular lumen. Exposure to hypoxia was associated with a marked increase in ET-1 immunoreactivity in bronchiolar epithelial cells such that almost all cells lining the small airways stained positively (Fig. 1). This change was most prominent in the WKY strain. Furthermore, chronic hypoxia was associated with the appearance of intense ET-1 staining in small peripheral pulmonary arteries, again most pronounced in the WKY strain. Interestingly, in the small peripheral arteries of the hypoxic WKY rats, ET-1 expression appeared not to be confined to the endothelium but also involved the subjacent vascular smooth muscle.

Plasma immunoreactive ET-1 levels. During normoxia, there were no differences in lung or plasma ET-1 levels between the two strains (Fig. 2). However, after 3 wk of hypoxia, there was a significant increase in lung and plasma ET-1 in WKY rats. There was a small rise in plasma ET-1 in the hypoxic F-344 rats, but this did not achieve significance (P = 0.07). In hypoxic rats, the levels of lung and plasma ET-1 were significantly greater in the WKY strain compared with those in the F-344 strain.

Northern analysis of preproET-1 mRNA expression. The specificity of the labeled preproET-1 cDNA probe was confirmed by hybridization to a single 2.3-kb band on Northern blots (Fig. 3). Within strains, the hybridization signal appeared to be increased in hypoxic compared with normoxic rats. Rehybridization of the blots with labeled 18S rRNA oligonucleotide confirmed a similar loading of lanes. Densitometric analysis of blots with the phosphorimager revealed a 2.8-fold increase in lung preproET-1 mRNA relative to 18S rRNA in hypoxic WKY rats compared with that in normoxic control rats. A 1.7-fold increase in lung preproET-1 mRNA was seen in hypoxic F-344 rats. In hypoxic rats, the preproET-1-to-18S rRNA ratio was higher in the WKY strain than in the F-344 strain (P < 0.05).

Table 1. Muscularization of terminal bronchiolar, respiratory bronchiolar, alveolar duct, and alveolar wall arteries in normoxic and hypoxic WKY and F-344 rats

<table>
<thead>
<tr>
<th></th>
<th>Terminal Bronchiolar</th>
<th>Respiratory Bronchiolar</th>
<th>Alveolar Duct</th>
<th>Alveolar Wall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>PM</td>
<td>NM</td>
<td>M</td>
</tr>
<tr>
<td>WKY normoxic</td>
<td>78.5 ± 7.9*</td>
<td>21.4 ± 8.0</td>
<td>0</td>
<td>56.7 ± 8.9*</td>
</tr>
<tr>
<td>F-344 normoxic</td>
<td>84.5 ± 2.0</td>
<td>15.4 ± 2.0</td>
<td>0</td>
<td>62.0 ± 7.1*</td>
</tr>
<tr>
<td>WKY hypoxic</td>
<td>58.4 ± 11.7</td>
<td>33.8 ± 9.6</td>
<td>7.7 ± 3.9</td>
<td>9.8 ± 3.6</td>
</tr>
<tr>
<td>F-344 hypoxic</td>
<td>40.4 ± 12.3</td>
<td>49.7 ± 11.6</td>
<td>9.7 ± 5.6</td>
<td>3.9 ± 1.5</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 rats/group. WKY, Wistar-Kyoto; F-344, Fischer 344; M, muscularized; PM, partially muscularized; NM, nonmuscularized. Significant difference (P < 0.05): *from corresponding normoxic rat strain; †for hypoxic WKY rats compared with hypoxic F-344 rats.
DISCUSSION

This study has demonstrated that the pulmonary vasculature of normal adult F-344 and WKY rats does not differ significantly in degree or pattern of muscularization as judged by the thickness of the muscle coat in muscularized vessels or percentage muscularization of distal vessels. Exposure to moderate hypoxia, however, leads to more extensive muscularization of distal vessels in the WKY than in the F-344 rat as evidenced by fewer nonmuscular arteries at the level of the alveolar duct and wall. This increased capacity for vascular...
remodeling is accompanied by a greater increase in circulating and lung ET-1 and lung preproET-1 mRNA in the WKY strain compared with that in the F-344 strain, suggesting a role for ET-1 in the different sensitivities of these strains to hypoxia-induced pulmonary hypertension.

Previous studies with isolated perfused lung preparations have shown that the pressor response to acute hypoxia in the F-344 rat pulmonary circulation is ~50% of that produced in the WKY rat (25) and markedly reduced compared with outbred Sprague-Dawley rats (8). The F-344 rat pulmonary vascular response to other pressor stimuli such as angiotensin II, however, is similar to that observed in the WKY rat (25), and others (8) have found that pulmonary artery rings from F-344 rats contract as well as those from Sprague-Dawley rats to phorbol 12-myristate 13-acetate, a protein kinase C activator. Taken together with our histological findings, the attenuated response of the F-344 rat to hypoxic pulmonary vasoconstriction does not appear to be due to a relative paucity of pulmonary vascular smooth muscle. Likewise, the comparable rise in hematocrit in F-344 and WKY rats during chronic exposure to hypoxia (25) would argue against a generalized abnormality of perception of oxygen tension.

The mechanisms underlying hypoxia-induced pulmonary vasoconstriction and remodeling are poorly understood. There is considerable interest in the role of locally produced vasoactive factors and, in particular, ET-1. This peptide is a potent vasoconstrictor with trophic properties (19) and is synthesized in vascular endothelial (27) and smooth muscle (18) cells, including those isolated from the rat pulmonary artery (20). Hypoxia stimulates its secretion from endothelial cells in culture (12), and it has previously been shown (3, 14, 21, 26) that circulating and lung levels are elevated in the whole animal exposed to hypoxia. Differences in ET-1 expression may underlie differences in acute hypoxic pulmonary vasoconstriction as well as in chronic vascular remodeling in the F-344 and WKY strains. In support of this, it has recently been shown (15) that ET-1 leads to inhibition of voltage-gated K⁺ channels, thereby potentiating hypoxic vasoconstriction. Furthermore, pretreatment with ET-receptor antagonists blocks hypoxia-induced increases in pulmonary arterial pressure in the rat, lamb, and dog (17, 22, 26). Chronic treatment with specific ETA- and nonselective ET-receptor antagonists has been reported to prevent hypoxia-induced pulmonary vascular remodeling and right ventricular hypertrophy in the rat and guinea pig (1, 2, 21). These observations strongly support a role for ET-1 in the regulation of pulmonary vascular tone.

Fig. 2. Lung content (A) and plasma levels (B) of ET-1 in WKY and F-344 strains (n = 4–6 rats/group). Under normoxic conditions, similar levels of ET-1 were found in both strains. However, after chronic hypoxia, ET-1 levels increased significantly in WKY strain only. *P < 0.05 compared with normoxia.

Fig. 3. A: Northern blot of rat lung total RNA (50 µg/lane) probed with radiolabeled 0.7-kb rat preproET-1 cDNA showing hybridization to a single 2.3-kb band. Density of signal was increased in hypoxic lungs (H) compared with normoxic lungs (N). Rehybridization of stripped blots with a radiolabeled oligonucleotide to 18S rRNA confirmed similar RNA loading between lanes. B: quantification of hybridization signal allowing comparison of relative abundance of preproET-1 mRNA to that of 18S rRNA (ppET-1/18S mRNA) in lungs of WKY and F-344 rats (n = 4/group). There was a significant increase in ppET-1/18S mRNA after hypoxic exposure in both strains, but increase was greater in WKY strain. *P < 0.01 compared with normoxia. †P < 0.05 compared with hypoxic F-344 strain.
ET-1 in acute and chronic hypoxia-induced pulmonary hypertension.

Despite the potential importance of ET-1 in the pathogenesis of hypoxia-induced pulmonary hypertension and evidence for increased ET-1 levels in serum and whole lung (3, 14), there are few data regarding the cellular localization of ET-1 in the lung and changes occurring during hypoxia (4). In the normal canine (6) and human (4, 7) lung, ET-1-like immunoreactivity is only rarely seen in vascular endothelial cells but is prominent in the epithelium. In patients with pulmonary hypertension (7) and dogs with postobstructive pulmonary vasculopathy (6), increased ET-1 immunoreactivity has been described in endothelial cells of remodeled peripheral vessels. In the present study, ET-1 immunoreactivity in the normal adult rat lung was prominent in bronchial epithelial cells, with relatively little staining of the pulmonary vasculature in either strain, consistent with previous reports in other species (4, 6, 7). Exposure to hypoxia led to a marked increase in immunoreactivity in the bronchial epithelium and increased ET-1 immunoreactivity in the endothelium and tunica media of peripheral pulmonary vessels, in keeping with a role for ET-1 in the pulmonary vascular response to chronic hypoxia. The novel finding of increased airway ET-1 expression in hypoxia raises the possibility that increased epithelial production of ET-1 may contribute to the vascular remodeling process. Furthermore, it is conceivable that ET-1 is involved in the process of airway remodeling, which is known to accompany the vascular changes during prolonged hypoxia (10). Consistent with the results of Northern analysis and RIA for lung ET-1, the increase in ET immunoreactivity in pulmonary arterioles and airways was considerably greater in the WKY rat compared with the F-344 rat.

In conclusion, we have shown that two strains of rat with differing susceptibilities to hypoxia-induced pulmonary hypertension are similar with respect to their baseline pulmonary artery anatomy and ET-1 expression during normoxia. However, during hypoxic exposure, the WKY strain exhibits a greater degree of muscularization of peripheral pulmonary arteries accompanied by greater increases in plasma and lung ET-1 compared with the F-344 strain. These results suggest that differences in the regulation of ET-1 expression by hypoxia might partly underlie the different sensitivities of the F-344 and WKY strains to hypoxia-induced pulmonary hypertension.

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