Effects of ET-A receptor blockade on eNOS gene expression in chronic hypoxic rat lungs

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Blumberg, Friedrich C., Konrad Wolf, Michael Arzt, Cornelia Lorenz, Günter A. J. Riegger, and Michael Pfeifer. Effects of ET-A receptor blockade on eNOS gene expression in chronic hypoxic rat lungs. J Appl Physiol 94: 446–452, 2003. First published October 4, 2002; 10.1152/japplphysiol.00239.2002.—We tested the hypothesis that pulmonary endothelial nitric oxide synthase (eNOS) gene expression is primarily regulated by hemodynamic factors and is thus increased in rats with chronic hypoxic pulmonary hypertension. Furthermore, we examined the role of endothelin (ET)-1 in this regulatory process, since ET-1 is able to induce eNOS via activation of the ET-B receptor. Therefore, chronic hypoxic rats (10% O2) were treated with the selective ET-A receptor antagonist LU-135252 (50 mg·kg−1·day−1). Right ventricular systolic pressure and cross-sectional medial vascular wall area of pulmonary arteries rose significantly, and eNOS mRNA levels increased 1.8- and 2.6-fold after 2 and 4 wk of hypoxia, respectively (each P < 0.05). Pulmonary ET-1 mRNA and ET-1 plasma levels increased significantly after 4 wk of hypoxia (each P < 0.05). LU-135252 reduced right ventricular systolic pressure, vascular remodeling, and eNOS gene expression in chronic hypoxic rats (each P < 0.05), whereas ET-1 production was not altered. We conclude that eNOS expression in chronic hypoxic rat lungs is modified predominantly by hemodynamic factors, whereas the ET-B receptor-mediated pathway and hypoxia seem to be less important.

chronic hypoxic pulmonary hypertension is characterized by pulmonary vasoconstriction, increased blood viscosity due to polycythemia, and pulmonary vascular remodeling. Several lines of evidence suggest that alterations in the production and release of endothelial-derived vasoactive factors such as nitric oxide (NO) and endothelin (ET)-1 contribute significantly to the development of the disease.

NO is a major endothelium-derived inhibitor of platelet aggregation, smooth muscle cell proliferation, and vasoconstriction (7, 23). In the pulmonary vasculature, NO is formed from L-arginine by the endothelial NO synthase (eNOS) (21). Although there are conflicting data regarding the endogenous production of NO in patients with severe pulmonary hypertension, since decreased, unchanged, and increased pulmonary eNOS expression have all been reported (8, 32, 34), numerous studies clearly demonstrate that lung eNOS gene expression, protein content, and activity are increased in rats with chronic hypoxic pulmonary hypertension (16, 28). However, the mechanisms for the upregulation of eNOS gene expression in chronic hypoxic rat lungs are not completely understood.

Resta et al. (24) reported maintained upregulation of pulmonary eNOS gene expression in rats during recovery from chronic hypoxia, indicating that factors other than hypoxia, such as posthypoxic persistent pulmonary hypertension or sustained polycythemia, are crucial for this gene regulation. In contrast, Le Cras et al. (15) demonstrated that surgical left pulmonary artery stenosis reduced left pulmonary blood flow and pulmonary vascular remodeling in rats exposed to chronic hypoxia, whereas the increase in left pulmonary eNOS gene expression was not altered by the procedure, suggesting that low oxygen tension induced eNOS gene expression independently of hemodynamic factors in these experiments. Since it has been shown that ET-1 production is increased in rats with chronic hypoxic pulmonary hypertension (1, 17), and the substance, in addition to its ET-A receptor-mediated vasoconstrictive and proliferative actions on vascular smooth muscle cells, is able to stimulate the formation of NO through activation of the endothelial ET-B receptor (3, 35, 36), it might be that the upregulation of eNOS gene expression is mediated by ET-1. Finally, it might be that hypoxia-induced polycythemia attenuates the negative feedback of NO on eNOS gene expression due to the possibly greater NO-scavenging properties of polycythemic blood in these animals (24).

However, because eNOS gene expression is selectively enhanced in the pulmonary arteries of chronic hypoxic rats, but not in the veins (25), we hypothesized that hemodynamic factors play a pivotal role in the regulation of pulmonary eNOS gene expression. To test this hypothesis and to further investigate the role of...
ET-1, hypoxia, and polycythemia in this regulatory process, we determined right ventricular systolic pressure (RVSP), hematocrit, pulmonary vascular remodeling, pulmonary ET-1 mRNA expression, circulating ET-1 levels, and pulmonary eNOS mRNA expression in normoxic and chronic hypoxic rats in the presence or absence of the highly selective ET-A receptor antagonist LU-135252 (LU), which has previously been shown to attenuate experimental pulmonary hypertension in rats (13).

**MATERIALS AND METHODS**

**Experimental groups and chronic hypoxia.** Adult male Wistar rats (250–300 g; Charles River Laboratories, Sulzfeld, Germany) were randomly assigned to one of the following groups: normoxia (Norm; n = 10), normoxia receiving LU (Norm+LU; 50 mg·kg⁻¹·day⁻¹, n = 10; Knoll, Mannheim, Germany), 2 (2wHyp; n = 10) and 4 wk of hypoxia (4wHyp; n = 10), and 4 wk of hypoxia receiving LU (4wHyp+LU; n = 10). Rats were exposed to normobaric hypoxia (10% O₂, balance N₂) in transparent plastic chambers, as described previously (1). Normoxic rats were housed in identical cages adjacent to the chambers in the same room while breathing room air. Animal experiments were conducted according to the National Institute of Health Guide for the Care and Use of Laboratory Animals and German laws on the protection of animals.

**Hemodynamic measurements.** Systolic blood pressure was measured in all rats the day before the end of the study period under prewarmed and normoxic conditions by the tail-cuff method (blood pressure monitor TSE 210 000-2T, Technical and Scientific Equipment, Kronberg, Germany).

At the end of the study period, RVSP was measured in all rats with a closed-chest technique as a surrogate parameter for the development of pulmonary hypertension, as previously described (1). For this purpose, each rat was taken from its cage and was immediately anesthetized with thiopental (50 mg/kg ip). The lungs were artificially ventilated with room air via a nose mask and with the use of a rodent respirator (Animal Respirator, Technical and Scientific Equipment). The right jugular vein was cannulated, and a catheter was introduced into the right ventricle. The system was filled and flushed with <2 ml of heparin solution (1,000 IU/ml). After a stable hemodynamic condition was reached, RVSP was measured by using a pressure transducer (P23Db, Statham Laboratories, Hatorey, Puerto Rico).

**Organ sampling.** Immediately after the hemodynamic measurements, the animals were killed by decapitation. Blood was collected from the carotid arteries. Heart and lungs were removed, and the pulmonary artery was perfused with 0.9% saline until the obtained solution was clear. The right lung was dissected and frozen in liquid nitrogen until RNA extraction. After homogenization of the tissue in solution D (4 M guanidine thiocyanate containing 0.5% N-laurylsarcosinate, 10 mmol/l EDTA, 25 mmol/l sodium citrate, 100 mmol/l β-mercaptoethanol), 1/10 vol 2 M sodium acetate (pH 4), 1 vol phenol (water saturated), and 1/5 vol chloroform were added sequentially to the homogenate. After cooling on ice for 15 min, samples were centrifuged at 10,000 g for 15 min at 4°C. RNA in the supernatant was precipitated with an equal volume of isopropanol at −20°C for at least 1 h. The resulting RNA pellets were resuspended in 0.5 ml of solution D, again precipitated with an equal volume of isopropanol at −20°C. Pellets were finally dissolved in diethyl pyrocarbonate-treated water and stored at −80°C until further processing (2). For verification of quality of isolated total RNA, 1 µg of each sample was separated on an ethidium bromide gel and controlled for integrity of 28S and 18S rRNA bands. Approximately 100 µg of total RNA were obtained from parts of the right lung of each animal. Thus it was not necessary to pool the RNA.

**Quantification of ET-1, eNOS and glyceraldehyde-3-phosphate dehydrogenase mRNAs by RNase protection assay.** ET-1 mRNA was measured by RNase protection assay, as described previously (26). The plasmid containing the antisense sequence of ET-1 was a friendly gift of Peter Ratcliffe, Oxford, United Kingdom. It yields a 154-bp protected fragment in the RNase protection assay. Twenty micrograms of total lung RNA were used for ET assays.

The used polymerase chain reaction primers for eNOS (accession no. U02534) were as follows: we used forward 5′-CGGGATCCCTGCTGCCCGAGATATCTT-3′ and reverse 5′-ggattggtggtctgctgta-3′ primers yielding a 174-bp antisense transcript. Polymerase chain reaction inserts were cloned into the EcoRI/BamH I cloning site of the transcription vector pSP73 according to standard protocols. Linearization with HindIII followed by vitro transcription with SP6 RNA polymerase yields the respective antisense transcripts plus 50-bp polylinker sequences of the pSP73 vector. As negative control, 30 µg of yeast tRNA were analyzed in each assay. Moreover, an aliquot of the undigested probe was analyzed on a separate lane to confirm correct length of the protected fragments. Thirty micrograms of total lung RNA were used for the eNOS assay.

For normalization of the ET-1 and eNOS values, we constructed a transcription vector producing a 341-bp antisense RNA fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (31). To minimize discrepancies due to RNA quantity and quality, each assayed probe was coanalyzed for GAPDH mRNA. The vector producing the GAPDH antisense fragment used in RNase protection assay was cloned as follows. The polymerase chain reaction-derived fragment resulting from amplification with the upstream (5′-acctgaaggggtgtgc-3′; binding at bp 356–373) and downstream primers (5′-cagctctgggatgacct-3′; binding at bp 680–697) was cloned in the transcription vector pGEM 4Z (Pharmacia, Heidelberg, Germany). One microgram of total lung RNA was used for GAPDH assays. Correctness of the constructed plasmids was confirmed by sequence analysis done by Fa (Sequiseve, Vaterstetten, Germany).
For RNase protection assay transcripts were continuously labeled with \[^{32}P]\)uridine triphosphate (1,000–3,000 Ci/mmol; Amersham Biosciences) and purified on a Sephadex G50 spin column. For hybridization, total RNA was dissolved in a buffer containing 80% formamide, 40 mmol/l piperazine-N,N’-bis(2-ethanesulphonic acid), 400 mmol/l NaCl, 1 mmol/l EDTA (pH 8), and RNA (20 µg for ET-1; 30 µg for eNOS) and hybridized in a total volume of 50 µl at 60°C overnight with 5 \times 10^6 counts/min radiolabeled probe. RNase digestion with RNase A and T1 was carried out at room temperature for 30 min and terminated with proteinase K digestion for 30 min at 37°C (0.1 mg/ml containing 0.4% SDS). After phenol/chloroform extraction and ethanol precipitation, protected fragments were separated on a 8% polyacrylamide gel, the gel was dried for 2 h, signals were quantitated in a phosphoimager (Instant Imager 2024, Canberra-Packard, Dreieich, Germany), and autoradiographs were performed at −80°C for 1–3 days.

**Measurement of ET-1 plasma levels.** Plasma ET-1 levels were measured with a commercially available radioimmunoassay (Amersham International, Amersham, UK). Plasma extraction was performed with a standard technique, as described elsewhere (9).

**Statistical analysis.** All values are presented as means ± SD if not stated otherwise. ANOVA followed by Bonferroni post hoc testing was used for comparisons between the different study groups. A value of \(P < 0.05\) was considered significant.

**RESULTS**

**Hematocrit and hemodynamic measurements.** Hematocrit was significantly elevated in hypoxic animals and was not changed by LU (Table 1). Chronic hypoxia resulted in pulmonary hypertension, as indicated by a significant increase in RVSP, but did not alter systolic blood pressure (Table 1). LU significantly reduced RVSP in hypoxic animals compared with rats exposed solely to 4 wk of hypoxia but had no effect on RVSP in normoxic animals (Table 1). Systolic blood pressure was not significantly affected by the drug (Table 1).

**Morphological studies.** Medial cross-sectional vascular wall area of the small pulmonary arteries increased significantly in chronically hypoxic rats (Table 1). LU attenuated the medial vascular wall hypertrophy during hypoxia, although it has no effect on the morphology of pulmonary arteries in normoxic animals (Table 1).

**ET-1 plasma levels.** Exposure to 4 wk of hypoxia was accompanied by a significant increase in ET-1 plasma levels, which were not altered by LU (Table 1). LU had no effect on ET-1 plasma levels in normoxic animals (Table 1).

**Pulmonary GAPDH, ET-1, and enOS mRNA expression.** Pulmonary GAPDH mRNA expression did not change significantly in the different study groups (Fig. 1). After 2 and 4 wk of hypoxia, levels for pulmonary eNOS mRNA increased 1.8- and 2.6-fold, respectively (Figs. 2 and 3). Pulmonary ET-1 mRNA levels increased 2.7-fold after 4 wk of hypoxia (Figs. 4 and 5). LU reduced lung eNOS gene expression in hypoxic animals significantly (Figs. 2 and 3) but did not influence pulmonary ET-1 gene expression (Figs. 4 and 5).

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**Table 1. Hematocrit, hemodynamic measurements, medial vascular wall area of pulmonary arteries, and ET-1 plasma levels in all study groups**

<table>
<thead>
<tr>
<th></th>
<th>Normoxia</th>
<th>Normoxia + LU</th>
<th>2 wk of Hypoxia</th>
<th>4 wk of Hypoxia</th>
<th>4 wk of Hypoxia + LU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit, %</td>
<td>42.5 ± 2.4</td>
<td>43.8 ± 3.1</td>
<td>64.1 ± 3.4*</td>
<td>73.6 ± 3.3*</td>
<td>74.4 ± 2.7*</td>
</tr>
<tr>
<td>SAP, mmHg</td>
<td>134 ± 5</td>
<td>141 ± 7</td>
<td>132 ± 6</td>
<td>138 ± 6</td>
<td>137 ± 7</td>
</tr>
<tr>
<td>RVSP, mmHg</td>
<td>21 ± 2</td>
<td>20 ± 4</td>
<td>36 ± 4*</td>
<td>47 ± 5*</td>
<td>36 ± 5*†</td>
</tr>
<tr>
<td>Medial area, %</td>
<td>19.5 ± 2.5</td>
<td>22.2 ± 2.9</td>
<td>31.0 ± 3.0*</td>
<td>37.8 ± 4.1*</td>
<td>29.5 ± 3.4*‡</td>
</tr>
<tr>
<td>ET-1, pg/ml</td>
<td>8.6 ± 0.7</td>
<td>9.1 ± 0.8</td>
<td>13.0 ± 1.6*</td>
<td>12.7 ± 2.0*</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD; \(n = 10\) rats/group. LU, LU-135252; SAP, systolic arterial pressure; RVSP, right ventricular systolic pressure; medial area, ratio of the medial vascular wall area to lumen area × 100; ET, endothelin. *\(P < 0.05\) vs. normoxia group. †\(P < 0.05\) vs. 4 wk of hypoxia group.

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**Fig. 1. Autoradiograph of a representative RNase protection assay showing pulmonary glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression in normoxic controls (Norm), in rats exposed to 2 wk (2wHyp) and 4 wk (4wHyp) of hypoxia, and in rats exposed to 4 wk of hypoxia that received LU-135252 (4wHyp+LU). One microgram of total lung RNA was used for the assay.**

**Fig. 2. Autoradiograph of a representative RNase protection assay showing pulmonary endothelial nitric oxide synthase (eNOS) mRNA expression in Norm, 2wHyp, 4wHyp, and 4wHyp+LU rats. Thirty micrograms of total lung RNA were used for the assay.**
4 and 5). In normoxic animals, LU had no effect on pulmonary eNOS or ET-1 mRNA levels (Figs. 6–9).

**DISCUSSION**

The main finding of the present study is that treatment of pulmonary hypertension with a highly selective ET-A receptor antagonist reduced pulmonary eNOS gene expression in chronic hypoxic rats despite continuing hypoxic exposure. This suggests that hemodynamic factors associated with the development of pulmonary hypertension, as indicated by the increase in RVSP and pulmonary vascular remodeling, are crucial for the induction of eNOS gene expression in chronic hypoxic rat lungs, whereas the ET-B receptor-mediated pathway and other factors such as hypoxia or polycythemia seem to be less important.

The pivotal role of hemodynamics is, furthermore, strengthened by the finding that the gradual increase in lung eNOS gene expression observed during hypoxic exposure parallels the progression of pulmonary hypertension in our experiments. In addition, it has been shown that shear-stress augments NOS gene expression in endothelial cells (18) and that eNOS expression is selectively enhanced in the hemodynamically stressed pulmonary arteries of chronic hypoxic rats and mice, but not in the veins or the aorta, and that eNOS gene expression is also increased in rats with posthypoxic persistent and monocrotaline-induced pulmonary hypertension (6, 24, 25, 30).

Although it seems that the increase in eNOS gene expression is not accompanied by an increase in NO synthesis during hypoxic exposure (27), eNOS-deficient mice develop more severe chronic hypoxic pulmonary hypertension than wild-type controls (5, 29). Moreover, inhaled NO causes selective pulmonary vasodilatation in patients with pulmonary hypertension, and prolonged inhalation of NO as well as the administration of NO donors attenuates experimental pulmonary hypertension in rats (1, 14, 20, 22). These observations suggest that the increase in eNOS gene expression may serve as a compensatory mechanism to partially negate the development of pulmonary hypertension.

Nevertheless, Le Cras et al. (15) described that surgical left pulmonary artery stenosis reduced ipsilateral pulmonary blood flow and pulmonary vascular remodeling in chronic hypoxic rats, whereas the increase in eNOS gene expression was not influenced by the procedure. Hence, the authors concluded that eNOS gene expression is primarily regulated by oxygen tension and not by hemodynamic factors. However, the hemodynamics distal to the stenosis have not been determined in this study, and it might be that the hypoxia-induced vasoconstriction behind the stenosis increased shear-stress sufficiently enough to induce eNOS gene expression.
expression. Nevertheless, further study is needed to elucidate this point.

Polycythemia has been discussed as an alternative and independent stimulus for eNOS gene expression because of the possibly greater NO-scavenging properties of polycythemic blood, resulting in a decreased negative feedback of NO on eNOS transcription (24). However, treatment of pulmonary hypertension reduced eNOS gene expression without changing hematocrit. Therefore, such a mechanism is rather unlikely.

Pulmonary ET-1 gene expression and circulating ET-1 levels are increased in chronic hypoxic rats (1, 17). Furthermore, because both the ET-A receptor on vascular smooth muscle cells, which mediates the vasoconstrictive and proliferative actions of ET-1, and the endothelial ET-B receptor, which induces the formation of NO (3), are upregulated in chronic hypoxic rat lungs (17), it may be that upregulation of eNOS gene expression is the result of increased ET-B receptor stimulation.

Indeed, Sato et al. (27) have shown that the increase in NO synthesis in rats exposed to chronic hypoxia can be blocked by short-term administration of the selective ET-B receptor antagonist BQ-788. However, this effect could only be observed during normoxic ventilation and not under hypoxic conditions. Furthermore, eNOS gene expression was not determined in this study, and chronic effects of ET-A or ET-B receptor blocker were not tested (27). Moreover, it has been reported that ET-B receptor-deficient rats exhibit decreased pulmonary eNOS protein levels under normoxic conditions (11), whereas they show enhanced eNOS protein levels after exposure to chronic hypoxia (12). These findings indicate that upregulation of eNOS in chronic hypoxic rat lungs is not primarily mediated by the ET-B receptor.

To further investigate the role of ET-1 in the regulation of eNOS gene expression, we used the highly selective ET-A receptor antagonist LU (13). This enabled us to create a situation in which ET-1 plasma levels remain unchanged and only the ET-A receptor but not the ET-B receptor is blocked (4, 13). On the assumption that LU exerts no effect on ET receptor expression, ET-B receptor stimulation should be unaffected or even more pronounced when chronic hypoxic rats are treated with LU. However, the observation that eNOS levels decreased during treatment provides further evidence that the ET-B receptor-mediated pathway plays no crucial role in the stimulation of pulmonary eNOS gene expression in chronic hypoxic rats in vivo.

Nevertheless, in theory it might be that the increased ET-B receptor stimulation indirectly downregulates eNOS gene expression via an NO-mediated negative feedback mechanism. However, because it has been shown that NO synthesis is reduced during hypoxic exposure despite elevated eNOS levels (27), such a negative feedback effect is rather unlikely. Moreover, Marsen et al. (19) reported diminished eNOS mRNA expression after ET-A receptor inhibition in human endothelial cells under normoxic conditions. Although we cannot exclude such an effect in hypoxic animals, we found no influence of LU on eNOS mRNA expression in normoxic controls. Finally, we cannot rule out that LU influenced eNOS expression by altering the redox state of the cells (10).

In addition to the increase in eNOS expression, increased pulmonary inducible NOS mRNA and protein expression have been found in experimental animals with pulmonary hypertension, including chronic hypoxic rats (16). However, studies on isolated hypertensive lungs of chronic hypoxic, monocrotaline-treated, and fawn-hooded rats exhibited increased perfusion pressure only in response to eNOS inhibitors but not in response to inducible NOS inhibitors, which suggests that upregulation of inducible NOS is of lower functional significance for the pulmonary circulation (27).
33. In the present study, we, therefore, focused on the regulation of eNOS. A potential limitation of the present study is that we solely measured mRNA levels for eNOS. However, because previous studies have clearly shown that upregulation of eNOS gene expression in chronic hypoxic rat lungs is located in the endothelium of small pulmonary arteries and is accompanied by an increase in protein content and enzyme activity, it is reasonable to determine eNOS mRNA levels in whole lung homogenates as a reliable parameter for the assessment of the changes in the pulmonary artery eNOS system (16, 25).

In conclusion, our study suggests that, in chronic hypoxic rats, pulmonary eNOS gene expression is increased predominantly by hemodynamic factors associated with the development of pulmonary hypertension, and that hypoxia, the elevation in hematocrit, and the ET-B receptor-mediated pathway are less important factors for this gene regulation.

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