Increased pulmonary prostacyclin synthesis in rats with chronic hypoxic pulmonary hypertension

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Abstract

Objective: The regulation of pulmonary prostacyclin synthesis is not completely understood. We tested the hypothesis that prostacyclin production is predominantly stimulated by hemodynamic factors, such as increased shear-stress, and is thus increased in rats with chronic hypoxic pulmonary hypertension. Methods: To this end, we determined pulmonary prostacyclin synthase (PGIS) gene expression, circulating levels of the stable prostacyclin metabolite 6-keto prostaglandin F (6-keto-PGF), pulmonary endothelin (ET)-1 gene expression, and ET-1 plasma levels in rats exposed to 4 weeks of hypoxia (10% O) in the presence or absence of either the nitric oxide (NO) donor molsidomine (MD, 15 mg/kg/day) or the ET-A receptor antagonist LU135252 (LU, 50 mg/kg/day). Results: Right ventricular systolic pressure (RVSP), the cross-sectional medial vascular wall area of pulmonary arteries, and ET-1 production increased significantly during hypoxia. PGIS mRNA levels increased 1.7-fold, and 6-keto-PGF plasma levels rose from 8.2±0.8 to 12.2±2.2 ng/ml during hypoxia (each P<0.05 vs. normoxic controls). MD and LU reduced RVSP and pulmonary vascular remodeling similarly (each P<0.05 vs. hypoxia), but only MD inhibited pulmonary ET-1 formation (P<0.05 vs. hypoxia). Nevertheless, both drugs attenuated the increase in PGIS gene expression and plasma 6-keto-PGF levels (each P<0.05 vs. hypoxia). Conclusion: Our data suggest that prostacyclin production in hypertensive rat lungs is predominantly increased by hemodynamic factors while hypoxia, NO and ET-1 per are less important stimuli, and that this increase may serve as a compensatory mechanism to partially negate the hypoxia-induced elevation in pulmonary vascular tone.

Keywords: Endothelial factors; Endothelins; Hypoxia/anoxia; Prostaglandins; Pulmonary circulation

1. Introduction

Chronic pulmonary hypertension is characterized by an elevation in pulmonary vascular tone and the development of pulmonary vascular remodeling resulting in increased pulmonary vascular resistance, often leading to right ventricular failure and death. A growing body of evidence suggests that alterations in the production of endothelial-derived vasoactive factors, such as nitric oxide (NO), endothelin (ET)-1 and prostacyclin contribute significantly to the development of the disease.

Prostacyclin, a major arachidonic acid metabolite in the vascular wall, is a potent endogenous inhibitor of platelet aggregation, smooth muscle cell proliferation and vasoconstriction [1,2]. The final committed step in the synthesis of prostacyclin, the conversion of prostaglandin H2 to prostacyclin, is catalyzed by the membrane-bound enzyme prostacyclin synthase (PGIS), which is constitutively expressed in vascular endothelial and smooth muscle cells [3]. Continuous intravenous infusion of prostacyclin has been shown to be effective in the treatment of various forms of pulmonary hypertension [4]. In addition, it has been demonstrated that transgenic mice with an over-expression of pulmonary PGIS are protected against the development of chronic hypoxic pulmonary hypertension.
[5], and that augmentation of pulmonary prostacyclin production by tracheal gene transfer of human PGIS attenuates pulmonary hypertension in monocrotaline-treated rats [6].

Only limited data exist regarding the endogenous production of prostacyclin in pulmonary hypertension. In humans with severe pulmonary hypertension, prostacyclin production seems to be decreased as indicated by reduced 24-h urinary excretion of the stable prostacyclin metabolite 2,3-dinor-6-keto prostaglandin F, and reduced pulmonary PGIS gene expression [7,8]. In accordance with these reports in advanced disease, Badesch et al. reported declined prostacyclin synthesis in neonatal calves with severe pulmonary hypertension [9].

However, in contrast, Shaul et al. demonstrated that prostacyclin production is enhanced in endothelial and smooth muscle cells of pulmonary arteries derived from rats with chronic hypoxic pulmonary hypertension [10]. Moreover, Voelkel et al. [11] described an increase in prostacyclin formation in isolated rat lungs during vasoconstriction, corroborating the finding that shear-stress is an important stimulus for both prostacyclin formation and PGIS gene expression in cultured endothelial cells [12].

Based on these latter observations, we speculated that pulmonary prostacyclin production is primarily regulated by hemodynamic factors, and is thus increased in rats with chronic hypoxic pulmonary hypertension. To test this hypothesis and to examine the role of NO and ET-1 in this regulatory process, we determined pulmonary PGIS mRNA and circulating 6-keto prostaglandin F, (6-keto-PGF,) levels, as well as pulmonary ET-1 mRNA and ET-1 plasma levels in chronic hypoxic rats in the presence or absence of either the NO donor molsidomine (MD) or the ET-A receptor antagonist LU135252 (LU), both of which have previously been shown to ameliorate experimental pulmonary hypertension in rats [13,14].

2. Methods

2.1. Experimental groups and chronic hypoxia

Adult male Wistar rats (Charles River Labs., Sulzfeld, Germany; 250–300 g) were randomly assigned to one of the following groups: 4 weeks of normoxia (Norm, n=10), 4 weeks of hypoxia (4wHyp, n=10), or 4 weeks of hypoxia receiving either LU (50 mg/kg/day; Knoll, Mannheim, Germany, 4wHyp+LU, n=10) or MD (15 mg/kg/day; Hoechst Marion Roussel, Bad Soden, Germany, 4wHyp+MD, n=10) in the drinking water. Rats were exposed to normobaric hypoxia (10% O,–rest N,) in separate transparent plastic chambers as previously described [15]. Relative humidity within the chamber was kept at <70% with anhydrous CaSO,. Boric acid was used to keep NH, levels in the chamber at a minimum. The percentage of CO, in the chamber was measured daily and did not exceed 0.3%. 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 Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and German laws on the protection of animals.

2.2. 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 (BP monitor TSE 210 000-2T, Technical and Scientific Equipment, Kronberg, Germany).

At the end of the study period, right ventricular systolic pressure (RVSP) was measured in all rats with a closed-chest technique, as previously described [15]. For this purpose, each rat was taken from its cage and immediately anesthetized with thiopental (50 mg/kg, i.p.). Via a nose mask the lungs were artificially ventilated with room air using a rodent respirator (Animal Respirator, Technical and Scientific Equipment, Kronberg, Germany). 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 (1000 I.U./ml). After reaching a stable hemodynamic condition, RVSP was measured using a pressure transducer (P23Db, Statham Labs., Hatorey, Puerto Rico).

2.3. Organ sampling

Immediately following 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. For morphological measurements, the left lung was fixed in the distended state by the infusion of 10% buffered formalin into the pulmonary artery and trachea at 10 and 25 cm H,O perfusion pressure, respectively, for 3 min and kept in 10% buffered formalin for at least 24 h. Lungs were then embedded in paraffin and 5-µm sections were stained with Van Gieson’s stain.

2.4. Light microscopic analysis of pulmonary arteries

Microscopic slices were analyzed using a computerized morphometric system (ANALYSIS, Soft-Imaging Software, Münster, Germany). Total vessel area was defined as the area within the elastica externa. Medial area was defined as the area between the lamina elastica externa and the lamina elastica interna. Pulmonary arteries with an external diameter ranging between 30 and 100 µm were examined. Thirty arteries per animal were measured. The average of
three measurements obtained from each artery was used for calculations as described previously [13]. Slides were analyzed by two observers who were blinded for the modality of treatment. Variability was assessed by performing repeated analyses and was calculated as 4% (intra-observer) and 6% (interobserver).

2.5. RNA extraction

After homogenization of the tissue in solution D [guanidine thiocyanate (4 M) containing 0.5% N-laurylsarcosinate, 10 mM EDTA, 25 mM sodium citrate, 700 mM β-mercaptoethanol] 1/10 vol. 2 M sodiumacetate (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 diethylpyrocarbonate treated water and stored at −80 °C until further processing [16].

2.6. Quantification of ET-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and PGIS mRNA

Endothelin-1 mRNA was measured by RNase protection assay as described previously [17]. The plasmid containing the antisense sequence of ET-1 was a friendly gift of Mr. Peter Ratcliffe, Oxford, UK. It yields a 154-bp long expression and plasma levels. Data were analyzed by an aprotected fragment in the RNase protection assay. A 20-phosphate dehydrogenase

Quanti®cation of ET

2.6. Plasma 6-keto-PGF1α levels were measured with a commercially available enzyme immunoassay kit (Assay Designs, Ann Arbor, MI, USA; cross-reactivities: 6-keto-PGF1α 100%; 2,3-dinor-6-keto-PGF1α 3.17%; PGF1α 1.67%; PDG2 0.60%; PGF1α 0.60%; PGF2α 0.20%; 2,15-diketo-13,14-dihydro-PGF1α 0.12%; other prostaglandins <0.01%). ET-1 levels were measured with a commercially available radioimmunoassay (Amersham, UK). Plasma extraction was performed with a standard technique as described elsewhere [19].

2.8. Statistical analysis

All values are presented as means±S.D. if not stated otherwise. We compared the variations among the different study groups in respect to hematocrit, hemodynamics, pulmonary vascular remodeling, pulmonary PGIS gene expression, 6-keto-PGF1α plasma levels, lung ET-1 gene expression and plasma levels. Data were analyzed by an a priori ANOVA (for planed comparisons) using spss 10.0 software (SPSS, Chicago, IL, USA). A value of P<0.05 was considered significant.

3. Results

3.1. Hematocrit and hemodynamic measurements

Hematocrit was signi®cantly elevated in hypoxic animals and was not changed by LU or MD (Table 1). Chronic hypoxia resulted in pulmonary hypertension as indicated by a signi®cant increase in RVSP, but did not alter systolic blood pressure. LU and MD signi®cantly reduced RVSP as compared to rats exposed solely to hypoxia. Systolic blood pressure was not signi®cantly affected by the drugs (Table 1).

3.2. Morphological studies

Medial cross-sectional vascular wall area of the small pulmonary arteries increased signi®cantly in chronically hypoxic rats. LU and MD attenuated the medial vascular wall hypertrophy during hypoxia (Table 1).
### Table 1

Hematocrit, hemodynamic measurements, medial vascular wall area of pulmonary arteries, and 6-keto-PGF\(_{1\alpha}\) and ET-1 plasma levels in all study groups

<table>
<thead>
<tr>
<th></th>
<th>Normoxia</th>
<th>4 weeks of hypoxia</th>
<th>4 weeks of hypoxia + LU</th>
<th>4 weeks of hypoxia + MD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit (%)</td>
<td>43±2</td>
<td>74±3*</td>
<td>74±3*</td>
<td>74±3*</td>
</tr>
<tr>
<td>SAP (mmHg)</td>
<td>134±5</td>
<td>138±6</td>
<td>137±7</td>
<td>134±6</td>
</tr>
<tr>
<td>RVSP (mmHg)</td>
<td>21±2</td>
<td>47±5*</td>
<td>36±5†</td>
<td>35±3†</td>
</tr>
<tr>
<td>Medial area (%)</td>
<td>20±3</td>
<td>38±4*</td>
<td>30±3†</td>
<td>28±3†</td>
</tr>
<tr>
<td>6-Keto-PGF(_{1\alpha}) (ng/ml)</td>
<td>8±1</td>
<td>12±2*</td>
<td>9±2†</td>
<td>9±1†</td>
</tr>
<tr>
<td>ET-1 (pg/ml)</td>
<td>9±1</td>
<td>13±2*</td>
<td>9±2†</td>
<td>9±1†</td>
</tr>
</tbody>
</table>

Values are means±S.D.; \(n=10\) rats/group. LU, LU135252; MD, molsidomine; SAP, systolic arterial pressure; RVSP, right ventricular systolic pressure; Medial area, ratio of the medial vascular wall area to lumen area×100; 6-keto-PGF\(_{1\alpha}\), 6-keto prostaglandin F\(_{1\alpha}\); ET-1, endothelin-1.
* \(P<0.05\) vs. normoxic control; † \(P<0.05\) vs. 4 weeks of hypoxia.

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### 3.4. Pulmonary GAPDH, PGIS and ET-1 mRNA expression

Pulmonary GAPDH mRNA expression did not change significantly in the different study groups (Fig. 1). After 4 weeks of hypoxia, levels for pulmonary PGIS mRNA

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### 3.3. 6-Keto-PGF\(_{1\alpha}\) and ET-1 plasma levels

Hypoxia was accompanied by an increase in 6-keto-PGF\(_{1\alpha}\) and ET-1 plasma levels. LU and MD both reduced 6-keto-PGF\(_{1\alpha}\) levels. In contrast, MD reduced ET-1 plasma levels, whereas LU did not modify this parameter (Table 1).
increased 1.7-fold (Figs. 2 and 3). LU and MD reduced lung PGIS gene expression in hypoxic animals (Figs. 2 and 3). Pulmonary ET-1 gene expression also increased significantly during hypoxia (Figs. 4 and 5). While MD significantly reduced ET-1 mRNA expression, LU did not influence this gene expression significantly (Figs. 4 and 5).

4. Discussion

The current study demonstrates for the first time that pulmonary PGIS gene expression and 6-keto-PGF$_{1\alpha}$ plasma levels are increased in rats with chronic hypoxic pulmonary hypertension, and that this increase is primarily the result of the hemodynamic changes associated with the development of pulmonary hypertension, while hypoxia, NO and ET-1 are less important factors for the upregulation of prostacyclin synthesis in chronic hypoxic rat lungs in vivo. Our findings corroborate and extend previous results by Shaul et al. who reported increased prostacyclin formation in lung endothelial and vascular smooth muscle cells of rats exposed to chronic hypoxia [10]. However, the mechanisms for the regulation of prostacyclin synthesis have not yet been completely elucidated.

Reduced oxygen tension has been described as an independent stimulus for the upregulation of prostacyclin production in dog lungs in vitro [20]. Nevertheless, the observation that the increase in prostacyclin formation in our experiments is ameliorated during the treatment of pulmonary hypertension, despite continuing hypoxic exposure, indicates that hemodynamic factors such as increased pulmonary artery pressure or shear-stress associated with hypoxia-induced pulmonary vasoconstriction, and not hypoxia per se are crucial for the induction of pulmonary PGIS gene expression in chronic hypoxic rat lungs in vivo.

This hypothesis is in accordance with the findings of Voelkel et al. [11] who demonstrated that the increase in prostacyclin synthesis in isolated rat lungs is linked to vasoconstriction and not to hypoxia, and Okahara et al. [12] who reported that shear-stress is a potent stimulus of PGIS gene expression and prostacyclin formation in vascular endothelial cells. Consistent with the finding that drug treatment did not completely prevent the development of pulmonary hypertension and vascular remodeling, lung PGIS mRNA as well as circulating 6-keto-PGF$_{1\alpha}$ levels tended to increase in both treatment groups as compared to normoxic controls. However, this increase did not reach statistic significance.

Nitric oxide has also been reported to induce prostacyclin formation in cultured endothelial cells in vitro [21,22]. Nevertheless, in contrast to these in vitro reports, treatment with MD, which has previously been shown to act primarily through the liberation of NO [23], reduced PGIS gene expression and 6-keto-PGF$_{1\alpha}$ plasma levels in our experiments, indicating that NO is not an important stimulus for prostacyclin synthesis in chronic hypoxic rat lungs in vivo. Nevertheless, although various studies demonstrated increased NO synthase gene expression and protein content in chronic hypoxic rat lungs [24,25], findings of Sato et al. suggest that pulmonary NO synthesis is impaired under hypoxic conditions [26]. Thus, it might be that prostacyclin serves as a compensatory mediator that counteracts the hypoxia-induced NO deficiency. Such a theory could also explain why exogenously administered NO reduces the formation of prostacyclin in these animals. However, we did not investigate these possibilities in the current study.

Endothelin is another powerful vasoactive factor which seems to play a pivotal role in the pathogenesis of various forms of pulmonary hypertension. In accordance with previous studies, we found increased pulmonary ET-1 mRNA and plasma levels in rats exposed to chronic hypoxia [13,27,28]. Nevertheless, the role of ET-1 in the pulmonary circulation is complicated by the fact that it acts through two distinct receptor subtypes mediating opposite effects. The vasoconstrictive and proliferative actions of ET-1 are mediated through activation of the ET-A receptor on the vascular smooth muscle cell [29,30], whereas stimulation of the endothelial ET-B receptor induces vasodilation via the release of NO and prostacyclin [31]. Both receptor subtypes are upregulated in chronic hypoxic rat lungs [28]. Therefore, it could be that the increase in prostacyclin synthesis observed in hypoxic animals is the result of an increased ET-1 mediated activation of the ET-B receptor. However, the finding that treatment of pulmonary hypertension with the highly selective ET-A receptor antagonist LU [14] reduced pulmonary prostacyclin synthesis despite increased ET-1 levels, makes such a mechanism rather unlikely.

Moreover, our findings are in accordance with studies using isolated rat lung and rabbit kidney [32], and isolated
rat aortic rings [33], demonstrating that the ET-1 induced increase in prostacyclin formation could be attenuated by ET-A receptor antagonists, whereas selective ET-B receptor agonists and ET-B receptor antagonists did not influence prostacyclin release. Since ET-A receptor antagonists also reduced the ET-1-induced vasoconstriction in the isolated organ preparations, and the ET-1-induced release of prostacyclin in isolated aortic rings was mediated by an influx of calcium, these studies are, furthermore, in good agreement with our hypothesis that an increase in vascular tone is crucial for the stimulation of prostacyclin formation.

Nevertheless, since it has been reported by Grunstein et al. that ET-1 reduced the acetylcholine-induced contraction of isolated tracheal muscle segments of the rabbit probably due to an ET-A receptor mediated increase in prostacyclin production [34], we cannot exclude that prostacyclin formation is in part stimulated by an ET-A receptor pathway independently from smooth muscle cell contraction. However, further study is needed to elucidate such possibilities.

Only limited data exist regarding the discrepancies and interactions between the effects of NO donors and ET-A receptor antagonists in pulmonary hypertension. We were recently able to show that the beneficial actions of MD on the pulmonary circulation are associated with a suppression of ET-1 [13]. Furthermore, it has previously been reported that ET-A receptor antagonists attenuate chronic hypoxic pulmonary hypertension in rats without altering ET-1 production [27]. Thus, it might be that ET-1 levels fall as a consequence of the hemodynamic improvement induced by MD, and that ET-A receptor antagonists exert a stimulatory effect that counterbalances the inhibitory effects on ET-1 production that would otherwise accompany the fall in RVSP. However, this needs to be investigated by further study.

Taken together, our results favor the importance of hemodynamic factors for the regulation of pulmonary prostacyclin synthesis, and thus, it might be that the upregulation of prostacyclin formation in hypertensive rat lungs serves as a compensatory mechanism to partially counteract the hypoxia-induced elevation in pulmonary vascular tone and the development of pulmonary vascular remodeling. This hypothesis is supported by the findings that inhibition of prostaglandin synthesis by indomethacin enhances hypoxic vasoconstriction in experimental animals [35], that pulmonary PGIS overexpression in transgenic mice inhibits the development of chronic hypoxic pulmonary hypertension [5], and that tracheal gene transfer of human PGIS ameliorates pulmonary hypertension in monocrotaline-treated rats [6].

Nevertheless, our study contrasts with the findings of Tuder et al. [8] who reported reduced PGIS gene expression in patients with pulmonary hypertension, and Badesch et al. [9] who described decreased prostacyclin production in neonatal calves with severe pulmonary hypertension. Whether these contradictory findings are species-dependent or due to differences in the pathogenesis or severity of the disease and the degree of endothelial destruction requires further investigation. However, our results show that pulmonary hypertension is not inevitably associated with reduced prostacyclin formation.

In conclusion, our findings demonstrate that pulmonary prostacyclin production is increased in rats with chronic hypoxic pulmonary hypertension predominantly due to hemodynamic factors, while hypoxia, NO and ET-1 per se seem to be less important stimuli, that the upregulation may serve as a compensatory mechanism to partially counteract the elevation in pulmonary vascular tone, and that both NO donors and ET-A receptor antagonists reduce the formation of prostacyclin probably through their beneficial effects on pulmonary hemodynamics.

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