Inhalation of the ET$_A$ receptor antagonist LU-135252 selectively attenuates hypoxic pulmonary vasconstriction

Bodil Petersen,¹ Maria Deja,²* Roland Bartholdy,¹ Bernd Donaubauer,¹ Sven Laudi,¹ Roland C. E. Francis,² Willehad Boemke,² Udo Kaisers,¹ and Thilo Busch¹

¹Department of Anesthesiology and Intensive Care Medicine, University of Leipzig Medical Faculty, Leipzig; and ²Department of Anesthesiology and Intensive Care Medicine, Charité-University Medical Center, Berlin, Germany

Submitted 11 October 2007; accepted in final form 9 December 2007

PULMONARY ARTERIAL HYPERTENSION (PAH) can appear in a chronic, progressive idiopathic form, or as a consequence of acute cardiopulmonary decompensation, such as pulmonary embolism. In addition, PAH occurs together with chronic obstructive lung disease or chronic high-altitude exposure (23). A main pathophysiological mechanism for PAH consists of an imbalance between endogenously produced vasodilating and constricting mediators (14). In particular, increased concentrations of the potent constricting peptide endothelin (ET)-1 (11) have been identified as a major characteristic for PAH. The effects of ET-1 to increase pulmonary vascular tone are mediated by ET$_A$ and ET$_B$ receptors on smooth muscle cells, while ET$_B$ receptors at the pulmonary endothelium cause vasodila-

tion (2). Consequently, blockade of pulmonary ET$_A$ receptors is an important option for the treatment of PAH, and the use of the dual ET$_A$/ET$_B$ receptor antagonist bosentan has been proven to induce clinically relevant improvements with respect to pulmonary hemodynamics and exercise capacity (22). Currently, the selective ET$_A$ receptor antagonists sitaxsentan and ambrisentan are evaluated for the treatment of PAH (1, 10).

A serious disadvantage of any orally or intravenously applied vasodilator in PAH consists of the systemic vasodilation, which parallels the beneficial pulmonary effects. This may not only reduce exercise capacity, but will become deleterious in hemodynamically unstable patients and has to be avoided by dose titration. To overcome these difficulties, it is necessary to achieve a direct delivery of drugs to the pulmonary vasculature. This has prompted the concept of selective pulmonary vasodilation with inhaled nitric oxide (iNO) for the treatment of PAH (20). Hereby, iNO affects exclusively vascular smooth muscle cells in the ventilated lung areas and induces vasodila-
tion by an increase in cyclic guanosine monophosphate; there are no relevant systemic hemodynamic effects of iNO, since most of the gas is inactivated by immediate reaction with hemoglobin (16). Similarly, inhalation of the prostacyclin analog iloprost significantly improved cardiopulmonary hemo-
dynamics in patients with PAH, whereas systemic side effects were minimized using a low dose (19).

In analogy to the benefits of iNO and inhaled iloprost, we hypothesized that inhalation of an ET$_A$ receptor antagonist in pulmonary hypertension might restrict vasodilation predomi-
nantly to the pulmonary circulation without relevant systemic effects. Our hypothesis was tested in an animal model of hypoxic pulmonary vasconstriction (HPV).

METHODS

General experimental procedure. This study was approved by the Berlin Animal Protection Committee in accordance with the German Animal Protection Law, and conforms to the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services, Public Health Service, National Institutes of Health Publication no. 85–23). Sixteen piglets with a body weight of 25 ± 1 kg were studied. After intramuscular premedication with azaperone (5 mg/kg) and atropine (0.05 mg/kg), anesthesia was induced with an intravenous bolus of thiopental (10 mg/kg) and fentanyl (5 µg/kg), followed by a continuous infusion of thiopental (0.13 mg·kg$^{-1}$·min$^{-1}$) and fentanyl (0.05–0.08 µg·kg$^{-1}$·min$^{-1}$). The animals were orally intu-
bated (inner diameter 6.5 mm), and muscle relaxation was obtained with pancuronium bromide (a bolus of 0.15 mg/kg followed by 2.5

* B. Petersen and M. Deja contributed equally to this work.

Address for reprint requests and other correspondence: U. Kaisers, Klinik und Poliklinik fuer Anaesthesiologie und Intensivtherapie, Universitaetsklini-
kum Leipzig, Liebgrstr. 20 D-04103 Leipzig, Germany (e-mail: udo.kaisers
@medizin.uni-leipzig.de).

http://www.ajpregu.org 0363-6119/08 $8.00 Copyright © 2008 the American Physiological Society

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
µg·kg⁻¹·min⁻¹ for continuous infusion). Neither fentanyl nor thio-
pental influence HPV (3, 4). The relaxant pancuronium bromide has a
vagolytic effect and balanced the slight bradycardia induced by
fentanyl. After tracheotomy, a tubus with an inner diameter of 8.0 mm
and fitted with a heat moisture exchanger was inserted. The animals
were placed in a supine position and mechanically ventilated in a
volume-controlled mode at mild hyperoxia to secure oxygen supply and
to establish cardiopulmonary stabilization [tidal volume 10 ± 1
ml/kg, respiratory rate 16 breaths/min, inspiratory oxygen fraction
(FIO₂) 0.3, inspiratory-to-expiratory ratio 1:1, positive end-expiratory
pressure 8 cmH₂O] using a Servo 300A ventilator (Siemens-Elema,
Solna, Sweden). Throughout the experiments, no inotropic or vaso-
active drugs were administered.

In each pig, a pulmonary arterial catheter (model 93A-431-7.5Fr,
Baxter Healthcare, Irvine, CA) was inserted via the femoral vein, and
an arterial line (18 G; Vygon, Ecouen, France) was placed into the
femoral artery. These catheters served for blood sampling and hemo-
dynamic measurements. Heart rate, mean arterial pressure, and mean
pulmonary arterial pressure (MPAP) were recorded using a Hewlett-
Packard monitoring system (model 66 S, Boblingen, Germany). Car-
diac output (CO) was determined using the thermodilution technique
and is expressed as the mean of four measurements during different
phases of the respiratory cycle. Pulmonary vascular resistance and
systemic vascular resistance were calculated according to standard
formulas from CO and pulmonary capillary wedge pressure (PCWP)
or central venous pressure (CVP), respectively. Blood samples for
blood-gas analysis were collected anaerobically and analyzed imme-
diately (ABL 520, Radiometer, Copenhagen, Denmark). Arterial frac-
tion of oxygenated hemoglobin was measured by spectrophotometry
with the analyzer calibrated for pig blood (OSM 3 Hemoximeter,
Radiometer). Quantitative determination of ET-1 plasma concentra-
tion was performed with an enzyme immunoassay (BI-20052, Bio-
medica, Vienna, Austria); detection limit 0.05 fmol/ml, intra-assay
coefficient of variation <5%, interassay coefficient of variation
<10%. Samples were collected in aprotonin-coated, cooled plastic
vials. After centrifugation, plasma was stored in uncoated vials at
−20°C until analysis. According to the manufacturer’s instruction for
the handling of plasma samples from animals, a precipitation reaction
to reduce nonidentifying interfering substances was performed before
measuring ET-1.

Experimental protocol. Following the preparation, the animals
were mechanically ventilated at mild hyperoxia (FIO₂ 0.3) for 1 h.
Hypoxia was then induced by decreasing the FIO₂ to 0.15, and
respiratory frequency was adjusted to maintain arterial PCO₂ at 30 Torr
to approximate physiological hyperventilation. After 1 h of hypoxia
(measurement point hypoxia baseline), animals were randomly as-
signed to either receive the aerosolized ETA receptor antagonist
LU-135252 (Knoll AG, Ludwigshafen, Germany) as bolus (0.3 mg/kg
for 20 min; n = 8, LU group), or receive no treatment (n = 8,
controls). In the LU group, 10 mg of LU-135252 were dissolved in
7 ml distilled water containing 250 µl 1 M NaOH and titrated to a pH
of 7.4 with 1 M HCl, followed by nebulization of 0.3 mg/kg over
20 min, using an ultrasonic nebulizer (Servo Ultra Nebulizer 345;
Siemens-Elema, Solna, Sweden). Controls received nebulized saline
buffer. The nebulizer was placed in the inspiratory limb of the
ventilator tubing. According to the manufacturer’s description, 80%
of the aerosol produced consisted of particles with diameters between
0.5 and 5 µm. Parameters of gas exchange and hemodynamics were
measured at mild hyperoxia and during the following 4 h of hypoxia.
In addition, blood samples for later analysis of ET-1 plasma levels
were taken at hourly intervals.

Statistical analysis. Results are expressed as means ± SE. Statisti-
cal analysis was performed using SPSS for Windows 9.0 (SPSS,
Chicago, IL). Normal distribution of the variables was verified with
the Kolmogorov-Smirnov test. Differences between groups were eval-
uated with Student’s t-test for unpaired samples (2-tailed). Covariance
analysis with respect to differences in parameters at hypoxia baseline
was performed with simple factorial ANOVA. Data at mild hyperoxia
and hypoxia baseline within groups were compared with Student’s
t-test for paired samples (2-tailed). A P value <0.05 was considered
to be significant.

RESULTS

The groups were comparable with regard to body weight and
prestudy conditions. Averaged over all animals, induction of
hypoxia decreased arterial PO2 (PaO₂) from 151 ± 5 to 60 ± 2
Torr and reduced the arterial oxyhemoglobin fraction from
96 ± 0.1 to 89 ± 1% (P < 0.05; values for single groups are presented in Table 1). MPAP increased from 23 ± 1 to 32 ±
1% (P < 0.05; Table 1). Systemic arterial blood pressure and
CO remained stable at 94 ± 3 mmHg and 4.8 ± 0.3 l/min,
respectively. In all animals, ET-1 plasma levels increased
significantly from mild hyperoxia to hypoxia baseline (0.37 ±
0.04 vs. 0.52 ± 0.04 fmol/ml, P < 0.05; Table 1).

Inhalation of the ETA receptor antagonist LU-135252 at a
dose of 0.3 mg/kg for 20 min induced a substantial and
sustained improvement in pulmonary hemodynamics during
hypoxia. In the LU group, MPAP decreased from 33 ± 2
mmHg at hypoxia baseline following short-term inhalation of
LU-135252 after 1 h to 25 ± 1 mmHg and remained low at 27 ± 1
mmHg after 3 h. These values were significantly different from
those of the control group, in which MPAP continued to stay at
a high level of 32 ± 1 mmHg at 3 h after hypoxia baseline
(Fig. 1A). Concomitantly, systemic mean

Table 1. Effects of hypoxia on gas exchange, hemodynamics,
and ET-1 plasma levels

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mild Hyperoxia (FIO₂ 0.15)</th>
<th>Hypoxia Baseline (FIO₂ 0.15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PaO₂, Torr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LU group</td>
<td>150±7</td>
<td>59±3*</td>
</tr>
<tr>
<td>Controls</td>
<td>151±8</td>
<td>62±2*</td>
</tr>
<tr>
<td>PaCO₂, Torr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LU group</td>
<td>36±1</td>
<td>30±1*</td>
</tr>
<tr>
<td>Controls</td>
<td>37±1</td>
<td>29±1*</td>
</tr>
<tr>
<td>HF, beats/min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LU group</td>
<td>101±6</td>
<td>106±5</td>
</tr>
<tr>
<td>Controls</td>
<td>93±4</td>
<td>97±4</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LU group</td>
<td>101±4</td>
<td>94±4</td>
</tr>
<tr>
<td>Controls</td>
<td>90±4</td>
<td>93±5</td>
</tr>
<tr>
<td>MPAP, mmHg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LU group</td>
<td>24±1</td>
<td>33±2*</td>
</tr>
<tr>
<td>Controls</td>
<td>22±1</td>
<td>30±1*</td>
</tr>
<tr>
<td>CVP, mmHg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LU group</td>
<td>11±1</td>
<td>13±1</td>
</tr>
<tr>
<td>Controls</td>
<td>10±1</td>
<td>11±1</td>
</tr>
<tr>
<td>PCWP, mmHg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LU group</td>
<td>12±1</td>
<td>11±1</td>
</tr>
<tr>
<td>Controls</td>
<td>11±1</td>
<td>11±1</td>
</tr>
<tr>
<td>CO, l/min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LU group</td>
<td>5.7±0.5</td>
<td>5.3±0.4</td>
</tr>
<tr>
<td>Controls</td>
<td>4.6±0.2</td>
<td>4.3±0.2</td>
</tr>
<tr>
<td>Plasma ET-1, fmol/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LU group</td>
<td>0.37±0.07</td>
<td>0.54±0.04*</td>
</tr>
<tr>
<td>Controls</td>
<td>0.36±0.06</td>
<td>0.50±0.06*</td>
</tr>
</tbody>
</table>

Values are means ± SE. FIO₂, inspiratory oxygen fraction; hypoxia baseline,
measurement point after 1 h of hypoxia; LU, LU-135252; PaCO₂, partial pressure
of oxygen in arterial blood; PaO₂, partial pressure of carbon dioxide in arterial
blood; HF, heart frequency; MAP, mean systemic arterial blood pressure;
MPAP, mean pulmonary arterial pressure; CVP, central venous pressure;
PCWP, pulmonary capillary wedge pressure; CO, cardiac output; plasma ET-1,
plasma concentration of endothelin-1. *P < 0.05 vs. mild hyperoxia.
arterial pressure remained stable during hypoxia, and measured values were not significantly different between groups (Fig. 1B). CVP and PCWP were not different between groups at hypoxia baseline (Table 1) and remained stable after 3 h of hypoxia [CVP: 12 ± 1 vs. 11 ± 1 mmHg, not significant (NS); PCWP: 11 ± 1 vs. 10 ± 1 mmHg, NS; LU group vs. controls]. CO tended to be lower in controls than the LU group already at mild hyperoxia and at hypoxia baseline (Table 1), but the difference was mainly unchanged throughout the protocol and did not reach statistical significance (LU group: 5.3 ± 0.4 l/min; controls: 4.1 ± 0.2 l/min; values 3 h after hypoxia baseline; NS). Accordingly, there were no significant relative changes in CO from hypoxia baseline during the protocol in both groups (Fig. 2). Inhalation of LU-135252 induced a significant decrease in pulmonary vascular resistance when relative changes from hypoxia baseline were compared with those in controls (P < 0.05 at 1–3 h after hypoxia baseline; Fig. 3A). Relative changes in systemic vascular resistance were not different between groups (Fig. 3B). Inhalation of LU-135252 did not influence arterial oxygenation; at 3 h after hypoxia baseline, PaO₂ values were 50 ± 3 and 51 ± 4 Torr in the LU group and controls, respectively (NS). During hypoxia, there were no significant differences in ET-1 plasma levels between groups. At 3 h after hypoxia baseline, an identical value of 0.62 fmol/ml was measured in both groups (Fig. 4). Mean airway pressure of volume-controlled mechanical ventilation was not influenced by inhalation of LU-135252, and measured values remained stable in both groups (LU group: change in mean airway pressure 0.3 ± 0.1%; controls 0.4 ± 0.2%; relative changes from hypoxia baseline after 3 h).

Fig. 1. Mean pulmonary arterial pressure (MPAP; A) and mean systemic arterial blood pressure (MAP; B) during hypoxia in animals receiving aerosolized LU-135252 (LU group, ●, n = 8) compared with controls (○, n = 8). Inhalation of LU-135252 at a dose of 0.3 mg/kg for 20 min immediately after hypoxia baseline induced a significant and sustained decrease in MPAP, whereas systemic MAP remained unchanged. *P < 0.05 vs. controls.

Fig. 2. Relative change in cardiac output (CO) from hypoxia baseline in LU animals (●, n = 8) compared with controls (○, n = 8). Inhalation of LU-135252 at a dose of 0.3 mg/kg for 20 min immediately after hypoxia baseline had no significant influence on CO.

Fig. 3. Relative changes in pulmonary vascular resistance (PVR; A) and in systemic vascular resistance (SVR; B) among LU animals (●, n = 8) compared with controls (○, n = 8). Inhalation of LU-135252 at a dose of 0.3 mg/kg for 20 min immediately after hypoxia baseline induced a significant and sustained decrease in PVR, while SVR remained unchanged, thus demonstrating pulmonary selectivity of vasodilation. *P < 0.05 vs. controls.
Inhaled ETA receptor antagonist during hypoxia

DISCUSSION

In an experimental model of HPV, we found that the inhalation of LU-135252 at a dose of 0.3 mg/kg over 20 min induced a significant decrease in pulmonary arterial blood pressure for more than 3 h without affecting the systemic circulation. This demonstrates sustained selective pulmonary vasodilation due to the inhaled ET\(_A\) receptor antagonist.

Inhalation of LU-135252 reduced pulmonary vascular resistance during hypoxia only by \(\sim40\%\). The attenuation of HPV in our study is in line with the results of Holm and colleagues (13), who applied the ET\(_A\) receptor antagonist BMS-182874 in pigs at a dose of 30 mg/kg intravenously during hypoxia at FIO\(_2\) 0.1 and reported a decrease in MPAP from 38 ± 4 to 30 ± 5 mmHg, a reduction similar in magnitude as what we found. In contrast to the inhaled treatment in our study, intravenous injection of BMS-182874 was, however, accompanied by a significant decrease in systemic blood pressure by 20\% (13). Although the effects of the inhaled treatment are expected to be dose dependent, the incomplete inhibition of HPV might also indicate that the measured increase in ET-1 during hypoxia represents a modulating factor rather than a causal effect of HPV. It is widely accepted that HPV is mainly evoked by low alveolar oxygen partial pressures, which increase Ca\(^{2+}\) concentration in pulmonary arterial smooth muscle cells via a membrane depolarization (18).

Our measurements of ET-1 plasma levels revealed a steady increase over 4 h of hypoxia, with the main change of 80\% during the first 2 h, and the timing of the inhalation has most likely influenced the response to the ET\(_A\) receptor antagonist. This is paralleled by the finding in humans that a short-term hypoxic challenge of 5–10 min in which MPAP appears to approach a plateau is followed by a slower second phase of HPV that reaches a maximum at 2 h of isocapnic hypoxia (9). Both investigations reveal the necessity for sufficient duration of study protocols when aiming to investigate effects of ET\(_A\) blockade at hypoxia, which could be otherwise underestimated.

The findings of our present study may be compared with results being reported from previous applications of inhaled LU-135252 in an experimental model of acute lung injury. In these studies, inhalation of LU-135252 at doses of 0.3 and 3 mg/kg induced similar effects as iNO; however, MPAP was prevented to increase rather than being reduced, compared with untreated controls (6, 7, 15). The main effect of inhaled LU-135252 in experimental acute lung injury was a significant increase in PaO\(_2\)-to-FIO\(_2\) ratio due to a redistribution of blood flow from unventilated shunt areas toward ventilated lung regions, which was analogous to the action of iNO reported in patients with acute respiratory distress syndrome (21). Application of the inhaled ET\(_A\) receptor antagonist during hypoxia in the present study did not improve gas exchange, most likely because the largely homogenous distribution of HPV without a relevant shunt fraction offers only minor occasion to redirect blood flow.

The dose of 0.3 mg/kg that we used in our study was chosen, since it induced an improvement in gas exchange and pulmonary hemodynamics without systemic vasodilation following inhalation in experimental acute lung injury (6). Interestingly, inhalation of a 10-fold higher dose in experimental acute lung injury tended to increase systemic vascular resistance and to decrease CO rather than cause systemic vasodilation (7); these effects were possibly a consequence of increased ET-1 plasma levels due to a partial ET\(_B\) blockade at the higher concentrations of LU-135252. Whether this may also be associated with the inhalation of LU-135252 at doses >0.3 mg/kg during hypoxia remains to be investigated. Additionally, it might be argued that pulmonary selectivity could be attenuated by a spillover of inhaled LU-135252 into the pulmonary circulation. Supplementary measurements using normal phase high-pressure liquid chromatography with ultraviolet detection in plasma samples of three animals having received nebulized LU-135252 in our study revealed LU-135252 plasma concentrations <0.9 \(\mu\)mol/l. These low levels, which are further attenuated by plasma binding, are not expected to influence systemic hemodynamics. Reporting measurements in dogs, Chernacek and colleagues (5) found a significant systemic hypotensive effect of LU-135252 only at plasma concentrations of 200 and 400 \(\mu\)mol/ml, but not at values between 50 and 100 \(\mu\)mol/ml. This implies that LU-135252 plasma levels of the intervention group in our study were insufficient to affect the systemic circulation.

Besides contributing to pulmonary vasoconstriction, ET-1 causes bronchoconstriction in intact airways, and ET receptor antagonism should induce vasodilation. However, the vast majority of the binding sites for ET-1 on bronchial smooth muscle cells consists of ET\(_B\) receptors. Consequently, in humans and guinea pigs, the ET\(_A\) receptor antagonist BQ-123 antagonized ET-1-induced contraction of the pulmonary artery, but had no effect on bronchconstriction, which was, on the other hand, markedly enhanced by application of the ET\(_B\) agonist sarafotoxin S6c (12). In line with these findings, there was no bronchodilating effect due to inhaled LU-135252 in our study; moreover, mean airway pressure of volume-controlled mechanical ventilation remained unchanged in both groups.

There is no reason why the pulmonary selective effect of inhaled LU-135252, as demonstrated in our study, should be restricted to hypoxia; moreover, a similar result can be expected in other forms of pulmonary vasoconstriction, which are accompanied by increased ET levels. Therefore, inhaled ET\(_A\) receptor antagonists offer a new therapeutic option for PAH, adding to iNO and the inhaled prostacyclin analog iloprost (19), while acting on a different pharmacological pathway (14). Since the inhalation of ET\(_A\) receptor antagonists targets the pulmonary vasculature directly, it allows the reduction of the
effective dose compared with intravenous or oral application. This way, inhaled treatment is also expected to reduce side effects like headache, nausea, nasal congestion, increased amino transferases, peripheral edema, and anemia, as have been reported following prolonged oral application of bosentan (8), or of the novel ET\(_A\) receptor antagonist sitaxsentan (1, 24). In particular, when bosentan has been orally applied in mountaineers at high altitude to prevent acute mountain sickness, the beneficial effects on the pulmonary arterial pressure were opposed by an increase in fluid retention, which might enhance the risk for pulmonary edema formation (17).

**Perspectives and Significance**

We demonstrated that inhaled LU-1352525 induced a significant and sustained reduction in pulmonary arterial pressure during hypoxia without influencing systemic hemodynamics. Our results suggest the potential of inhaled ET\(_A\) receptor antagonists as a future option for the selective treatment of pulmonary hypertension that may be already effective at lower doses and thus might contribute to reduce side effects of an alternative oral application.

**ACKNOWLEDGMENTS**

We thank Daniela Bayerl for excellent performance in the measurement of the ET-1 plasma levels, and Dr. Klaus Muenter from Knoll AG, Ludwigshafen, Germany, for kindly supplying LU-135252 and analyzing LU-135252 plasma levels.

This work was presented, in part, at the 10th International Conference on Endothelin, Bergamo, Italy, September 16–19, 2007.

Present address of B. Petersen: Dept. of Anesthesia and Critical Care, Massachusetts General Hospital, Harvard Medical School, Boston, MA.

**GRANTS**

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (KA 1212/4-1).

**REFERENCES**