Inhalation of the $\text{ET}_\alpha$ receptor antagonist LU-135252 selectively attenuates hypoxic pulmonary vasoconstriction

Bodil Petersen a*, Maria Deja b*, Roland Bartholdy a, Bernd Donaubauer a, Sven Laudi a, Roland C. E. Francis b, Willehad Boemke b, Udo Kaisers a, Thilo Busch a

Departments of Anesthesiology and Intensive Care Medicine,

a University of Leipzig Medical Faculty, Leipzig, Germany,

and

b Charité - University Medical Center, Campus Virchow-Klinikum and Campus Mitte, Berlin, Germany

* these authors contributed equally to this study

Corresponding author and address for reprints:

Udo Kaisers, MD PhD
Klinik und Poliklinik fuer Anaesthesiologie und Intensivtherapie
Universitaetsklinikum Leipzig
Liebigstr. 20
D-04103 Leipzig
Germany

Tel.: +49-341-971-7700
Fax.: +49-341-971-7709
e-mail: udo.kaisers@medizin.uni-leipzig.de

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ABSTRACT

Endogenous endothelin-1 (ET-1) modulates hypoxic pulmonary vasoconstriction (HPV). Accordingly, intravenously applied ET\textsubscript{A} receptor antagonists reduce HPV, but this is accompanied by systemic vasodilation. We hypothesized that inhalation of an ET\textsubscript{A} receptor antagonist might act selectively on the pulmonary vasculature and investigated the effects of aerosolized LU-135252 in an experimental model of HPV. Sixteen piglets (weight: 25±1 kg) were anesthetized and mechanically ventilated at an inspiratory oxygen fraction (FiO\textsubscript{2}) of 0.3. After 1 h of hypoxia at FiO\textsubscript{2} 0.15, animals were randomly assigned to either receive aerosolized LU-135252 as bolus (0.3 mg/kg for 20 min) (n=8, LU group), or to receive aerosolized saline (n=8, Controls). In all animals, hypoxia significantly increased mean pulmonary artery pressure (MPAP: 32±1 vs. 23±1 mmHg; p<0.01; mean±SE) and increased arterial plasma ET-1 (0.52±0.04 vs. 0.37±0.05 fmol/ml; p<0.01) when compared to mild hyperoxia at FiO\textsubscript{2} 0.3. Inhalation of LU-135252 induced a significant and sustained decrease in MPAP compared to Controls (LU group: 27±1 mmHg; Controls: 32±1 mmHg; values at 4 h of hypoxia; p<0.01). In parallel, mean systemic arterial pressure and cardiac output remained stable and were not significantly different from Controls. Consequently, in our experimental model of HPV the inhaled ET\textsubscript{A} receptor antagonist LU-135252 induced selective pulmonary vasodilation without adverse systemic hemodynamic effects.

**Key words:** Inhalation, ET\textsubscript{A} receptor antagonist, hypoxic pulmonary vasoconstriction, selective pulmonary vasodilation.
INTRODUCTION

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 in an imbalance between endogenously produced vasodilating and constricting mediators (14). In particular, increased concentrations of the potent constricting peptide endothelin 1 (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 vasodilation (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 in 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 vasodilation 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). Similar, inhalation of the prostacyclin analogue iloprost significantly improved cardiopulmonary hemodynamics in patients with PAH while 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<sub>A</sub> receptor antagonist in pulmonary hypertension might restrict vasodilation predominantly to the pulmonary circulation without relevant systemic effects. Our hypothesis was tested in an animal model of hypoxic pulmonary vasoconstriction.

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 (DHHS, PHS, NIH Publication No. 85-23). Sixteen piglets with a body weight of 25 ± 1 kg were studied. After intramuscular premedication with azaperone (5 mg·kg<sup>-1</sup>) and atropine (0.05 mg·kg<sup>-1</sup>), anesthesia was induced with an intravenous bolus of thiopental (10 mg·kg<sup>-1</sup>) and fentanyl (5 µg·kg<sup>-1</sup>), followed by a continuous infusion of thiopental (0.13 mg kg<sup>-1</sup>·min<sup>-1</sup>) and fentanyl (0.05-0.08 µg kg<sup>-1</sup>·min<sup>-1</sup>). The animals were orally intubated (inner diameter 6.5 mm) and muscle relaxation was obtained with pancuronium bromide (a bolus of 0.15 mg·kg<sup>-1</sup> followed by 2.5 µg·kg<sup>-1</sup>·min<sup>-1</sup> for continuous infusion). Neither fentanyl nor thiopental 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<sup>-1</sup>, respiratory rate 16 min<sup>-1</sup>, FiO<sub>2</sub> 0.3, inspiratory - expiratory ratio 1:1, PEEP 8 cm H<sub>2</sub>O) using a Servo 300A ventilator (Siemens-Elema, Solna, Sweden). Throughout the experiments no inotropic or vasoactive drugs were administered.

In each pig a pulmonary artery catheter (model 93A-431-7.5Fr, Baxter Healthcare Corporation, Irvine, CA, USA) 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 hemodynamic measurements. Heart rate (HR), mean arterial pressure (MAP), and mean pulmonary artery pressure (MPAP) were recorded using a Hewlett-Packard monitoring system (Model 66 S, Böblingen, Germany). Cardiac 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 or central venous pressure, respectively. Blood samples for blood gas analysis were collected anaerobically, and analyzed immediately (ABL 520, Radiometer, Copenhagen, DK). Arterial fraction of oxygenated hemoglobin (HbaO₂) was measured by spectrophotometry with the analyzer calibrated for pig blood (OSM 3 Hemoximeter, Radiometer). Quantitative determination of ET-1 plasma concentration was performed with an enzyme immunoassay (BI-20052, Biomedica, Vienna, Austria): detection limit 0.05 fmol/mL, intra-assay coefficient of variation < 5%, interassay coefficient of variation < 10%. Samples were collected in aprotinin-coated, cooled plastic vials. After centrifugation plasma was stored in uncoated vials at -20 °C until analysis. According the manufacturer’s instruction for the handling of plasma samples from animals, a precipitation reaction to reduce non-identified 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 one hour. Hypoxia was then induced by decreasing the FiO₂ to 0.15 and respiratory frequency was adjusted to maintain PaCO₂ at 30 mm Hg to approximate physiological hyperventilation. After one hour of hypoxia (measurement point hypoxia baseline), animals were randomly assigned to either receive the aerosolized ET₄ receptor antagonist LU-135252 (Knoll AG, Ludwigshafen, Germany) as bolus (0.3 mg/kg for 20 min) (n=8, LU group), or to receive no treatment (n=8, Controls). In the LU group, 10 mg LU-135252 was 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⁻¹ over 20 min, using an ultrasound 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 hours 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 mean ± SEM. Statistical analysis was performed using SPSS for Windows 9.0 (SPSS Inc., Chicago Illinois, USA). Normal distribution of the variables was verified with the Kolmogorov-Smirnov test. Differences between groups were evaluated 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 pre-study conditions. Averaged over all animals, induction of hypoxia decreased PaO₂ from 151 ± 5 mm Hg to 60 ± 2 mm Hg 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). Mean pulmonary artery pressure increased from 23 ± 1 % to 32 ± 1 % (p<0.05; Table 1). Systemic arterial blood pressure and cardiac output 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 fmol/ml 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 mm Hg at hypoxia baseline following short term inhalation of LU-1352525 after one hour to 25 ± 1 mm Hg and remained low at 27 ± 1 mm Hg after 3 hours. These values were significantly different from the control group, in which MPAP continued to stay at a high level of 32 ± 1 mm Hg at three hours after hypoxia baseline (Figure 1A). Concomitantly, systemic MAP remained stable during hypoxia and measured values were not significantly different between groups (Figure 1B). Central venous pressure (CVP) and pulmonary capillary wedge pressure (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 mm Hg, n.s.; PCWP: 11 ± 1 vs. 10 ± 1 mmHg, n.s.; LU group vs. Controls). Cardiac output 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; n.s.). Accordingly, there were no significant relative changes in cardiac output from hypoxia baseline during the protocol in both groups (Figure 2). Inhalation of LU-135252 induced a significant decrease in pulmonary vascular resistance when relative changes from hypoxia baseline were compared to those in Controls (p<0.05 at 1-3 h after hypoxia baseline; Figure 3A). Relative changes in systemic vascular resistance were not different between groups (Figure 3B). Inhalation of LU-135252 did not influence arterial oxygenation; at 3 h after hypoxia baseline PaO₂ values were 50 ± 3 mm Hg and 51 ± 4 mm Hg in the LU group and in Controls, respectively (n.s.). 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 (Figure 4). Mean airway pressure (MIP) of volume controlled mechanical ventilation was not influenced by inhalation of LU-135252 and measured values remained stable in both groups (LU group: ΔMIP 0.3 ± 0.1%; Controls 0.4 ± 0.2%; relative changes from hypoxia baseline after 3 hours).
DISCUSSION

In an experimental model of hypoxic pulmonary vasoconstriction we found that the inhalation of LU-1352525 at a dose of 0.3 mg/kg over 20 min induced a significant decrease in pulmonary arterial blood pressure for more than 3 hours without affecting the systemic circulation. This demonstrates sustained selective pulmonary vasodilation due to the inhaled ET\textsubscript{A} receptor antagonist.

Inhalation of LU-135252 reduced pulmonary vascular resistance during hypoxia only by about 40%. The attenuation of HPV in our study is in line with the results of Holm and colleagues who applied the ET\textsubscript{A} receptor antagonist BMS-182874 in pigs at a dose of 30 mg/kg intravenously during hypoxia at FiO\textsubscript{2} 0.1 and reported a decrease in MPAP from 38 ± 4 mm Hg to 30 ± 5 mm Hg, a reduction being similar in magnitude as 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\textsuperscript{2+} concentration in pulmonary artery smooth muscle cells via a membrane depolarisation (18).

Our measurements of ET-1 plasma levels revealed a steady increase over 4 hours of hypoxia with the main change of 80% during the first two hours and the timing of the inhalation has most likely influenced the response to the ET\textsubscript{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 hours of isocapnic hypoxia (9). Both investigations reveal the necessity for sufficient duration of study protocols when aiming to investigate effects of ET\textsubscript{A} blockade at hypoxia, which could be otherwise underestimated.
The findings of our current study may be compared to 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 a doses of 0.3 mg/kg and 3 mg/kg induced similar effects as inhaled nitric oxide; however MPAP was prevented to increase rather than being reduced when compared to untreated controls (6, 7, 15). The main effect inhaled LU-135252 in experimental acute lung injury was a significant increase in PaO2/FiO2 ratio due to a redistribution of blood flow from unventilated shunt areas towards ventilated lung regions, which was analogous to the action of inhaled nitric oxide reported in patients with acute respiratory distress syndrome (21). Application of the inhaled ETA receptor antagonist during hypoxia in the current 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 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 tenfold higher dose in experimental acute lung injury tended to increase systemic vascular resistance and to decrease cardiac output rather than causing systemic vasodilation (7); these effects were possibly a consequence of increased ET-1 plasma levels due to a partial ETB blockade at the higher concentrations of LU-135252. Whether this may also be associated with the inhalation of LU-135252 at doses greater than 0.3 mg /kg during hypoxia remains to be investigated in future studies. Additionally, it might be argued that pulmonary selectivity could be attenuated by a spill over 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 below 0.9 µmol/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 found a significant systemic hypotensive effect of LU-135252 only at plasma concentrations of 200 and 400 µmol/mL but not at values between
50 and 100 μmol/mL (5). This implicates 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 consist of ETB receptors. Consequently, in humans and guinea-pigs the ETA receptor antagonist BQ-123 antagonized ET-1 induced contraction of the pulmonary artery but had no effect on bronchoconstriction which was on the other hand markedly enhanced by application of the ETB 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 endothelin levels. Therefore, inhaled ETₐ receptor antagonists offer a new therapeutic option for PAH adding to inhaled nitric oxide and the inhaled prostacyclin analogue iloprost (19) while acting on a different pharmacological pathway (14). Since the inhalation of ETₐ receptor antagonists targets the pulmonary vasculature directly it allows to reduce the effective dose when compared to 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 anaemia, as have been reported following prolonged oral application of bosentan (8), or of the novel ETₐ 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 artery 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 artery pressure during hypoxia without influencing systemic hemodynamics. Our results suggest the potential of inhaled ET\textsubscript{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.

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GRANTS

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REFERENCES


LEGENDS FOR FIGURES

**Figure 1:** Mean pulmonary artery pressure (MPAP, panel A) and mean systemic arterial blood pressure (MAP, panel B) during hypoxia in animals receiving aerosolized LU-135252 (LU group, solid circles, n=8) in comparison with Controls (open circles, 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 while systemic MAP remained unchanged; *: p<0.05 vs. Controls.

**Figure 2:** Relative Change in cardiac output (CO) from hypoxia baseline in animals receiving aerosolized LU-135252 (LU group, solid circles, n=8) in comparison with Controls (open circles, 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.

**Figure 3:** Relative changes in pulmonary vascular resistance (PVR, panel A) and in systemic vascular resistance (SVR, panel B) among animals receiving aerosolized LU-135252 (LU group, solid circles, n=8) in comparison with Controls (open circles, 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.

**Figure 4:** Plasma concentration of Endothelin-1 (ET-1) during hypoxia in animals receiving aerosolized LU-135252 (LU group, solid circles, n=8) in comparison with Controls (open circles, 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 ET-1 plasma levels.
<table>
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<th>Parameter</th>
<th>Group</th>
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<th>Hypoxia Baseline (FiO₂ 0.15)</th>
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Hypoxia baseline: measurement point after one hour of hypoxia; PaO₂: partial pressure of oxygen in arterial blood; PaCO₂: partial pressure of carbon dioxide in arterial blood; HF: heart frequency; MAP: mean systemic arterial blood pressure; MPAP: mean pulmonary artery pressure; CVP: central venous pressure; PCWP: pulmonary capillary occlusion pressure; CO: cardiac output; Plasma ET-1: plasma concentration of endothelin-1; §: p<0.05 vs. mild hyperoxia.
Hypoxia Baseline

Relative Change in CO [%]

Hypoxia 1 h 2 h 3 h

-60 -40 -20 0 20 40