No redistribution of lung blood flow by inhaled nitric oxide in endotoxemic piglets pretreated with an endothelin receptor antagonist

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Trachsel S, Hambraeus-Jonzon K, Bergquist M, Martijn C, Chen L, Hedenstierna G. No redistribution of lung blood flow by inhaled nitric oxide in endotoxemic piglets pretreated with an endothelin receptor antagonist. J Appl Physiol 118: 768–775, 2015. First published December 30, 2014; doi:10.1152/japplphysiol.00591.2014.—Inhaled nitric oxide (INO) improves ventilation-perfusion matching and alleviates pulmonary hypertension in patients with acute respiratory distress syndrome. However, outcome has not yet been shown to improve, and nonresponse is common. A better understanding of the mechanisms by which INO acts may guide in improving treatment with INO in patients with severe respiratory failure. We hypothesized that INO may act not only by vasodilation in ventilated lung regions, but also by causing vasoconstriction via endothelin (ET-1 in atelectatic, nonventilated lung regions. This was studied in 30 anesthetized, mechanically ventilated piglets. The fall in oxygenation and rise in pulmonary artery pressure during a sepsislike condition (infusion of endotoxin) were blunted by INO 40 ppm. Endotoxin infusion increased serum ET-1, and INO almost doubled the ratio between mRNA expression of endothelin receptor A (mediating vasoconstriction) and B (mediating vasodilation and clearance of ET-1) in the lung regions. INO caused a shift in blood flow away from atelectatic lung regions in the endotoxic piglets, but not during ET receptor antagonism. We conclude that INO in short-term experiments, in addition to causing selective pulmonary vasoconstriction in ventilated lung regions, increases the ET-A/ET-B mRNA expression ratio in lung tissue. This might augment the vasoconstriction in atelectatic lung regions, enhancing the redistribution of pulmonary blood flow to ventilated lung regions which are reached by INO. Such vasoconstriction may be an important additional factor explaining the effect of INO.

inhaled nitric oxide; endothelin-1; endothelin receptor; lung perfusion; hypoxemia

Nitric oxide (NO) is a most potent vasodilator, whether endogenously produced in the endothelium or given as a drug, e.g., nitroglycerine, and those who discovered this were awarded the Nobel Prize in 1998 (10, 15, 19). It was later shown that inhalation of NO (INO) dilated pulmonary vessels without affecting systemic vessels (9, 23). This selective effect was attributed to the short half-life of the NO molecule, and made INO a “rescue therapy” in acute respiratory failure with severe hypoxemia (25), although with varying success on arterial oxygenation and clinical outcome (1, 4, 6, 7, 14, 18). A clear understanding of how INO blunts hypoxemia has not yet been achieved, although there is general agreement on its main mechanisms of action. Thus INO has been shown to dilate vessels in ventilated lung regions by stimulating the release of the second messenger guanosine 3′,5′-cyclic monophosphate (cGMP) (21). This vasodilation shifts blood flow away from atelectatic, nonventilated lung regions and improves oxygenation. However, the increase of cGMP in plasma during INO does not correlate with the clinical response to INO (33). Moreover, the nonventilated regions in acute respiratory failure are located mainly in the lower, posterior lung regions (11), why a shift of blood flow must occur upward, against gravity. Reduced pulmonary vascular pressure as, e.g., by INO, redirects blood flow primarily to lower lung regions (32) that would increase shunt and worsen oxygenation of blood if posterior lung regions are atelectic. Thus other mechanisms behind the redistribution of lung blood flow by INO may also be involved. Endothelin 1 (ET-1) appears to be involved in rebound pulmonary hypertension after INO withdrawal (5, 22). ET-1 activity is mediated through two receptors: ET-A, mediating vasoconstriction, and ET-B, mediating vasodilatation and clearance of ET-1. A decrease in pulmonary artery pressure and an increase in serum ET-1 have been described when ET receptors are blocked in endotoxemic piglets (30). This effect is mainly achieved by ET-B receptor blockade, but a further decrease in pulmonary artery pressure is obtained by nonspecific ET-A/B receptor blockade (30).

We assumed that better understanding of the mechanisms by which INO acts may guide toward more effective use and improved outcome. The primary objective of the present study was, accordingly, to determine whether INO affects the mRNA expression of the ET-A and ET-B receptors in the lung, and the secondary objective was to determine whether a shift of lung blood flow to better aerated and ventilated upper (anterior) lung regions is blunted by administering an ET receptor antagonist. This was tested in a piglet model of systemic endotoxemia. The piglet is a frequently used animal for studies of sepsislike conditions, e.g., endotoxemia. INO doses that have been used experimentally and clinically range from a few parts per million to 80 ppm (18, 12). In this study we used 40 ppm.

MATERIALS AND METHODS

Ethics Statement

The Animal Research Ethics Committee of Uppsala University, Uppsala, Sweden, approved the study (nr C 69/10; approval date 26 March 2010).

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Animal Preparation

A total of 30 healthy piglets of Swedish country breed, weighing 28 kg (24–33 kg) (median and range), were brought from the farm to the lab in the morning of the experimental day, premedicated with an intramuscular injection of tiletamine/zolazepam (Zoletil Forte, Virbac Laboratories) 6 mg/kg, and xylazine chloride (Rompun, Bayer AG, Germany) 2.2 mg/kg, which also served as induction of anesthesia. Anesthesia was then maintained with an intravenous (iv) infusion of midazolam 0.1 mg·kg⁻¹·h⁻¹, ketamine vet 30 mg·kg⁻¹·h⁻¹, pancuronium 0.3 mg·kg⁻¹·h⁻¹, and fentanyl 3 μg·kg⁻¹·h⁻¹. Oxygen saturation and inspiratory and end-tidal concentrations of oxygen (O₂) and carbon dioxide (CO₂) were monitored continuously (Datex AS/3 Anesthesia Monitor, Datex Ohmeda, Helsinki, Finland) throughout the experiments. Warm buffered Ringer’s solution (Fresenius Kabi AB, Uppsala, Sweden) (10–15 ml·kg⁻¹·h⁻¹) was infused, and a suprapubic catheter was inserted for urinary output. The piglets were studied lying supine on a heating mattress, enclosed in warm blankets to maintain a normal and stable body temperature.

Ventilation

After induction of anesthesia, a 7-mm ID orotracheal tube was inserted. The lungs were mechanically ventilated by a Servo 300B NO/A (Siemens Elema AB, Lund, Sweden), set at volume-controlled ventilation, a tidal volume of 8 ml/kg, 20 breaths per min, and an inspired fraction of oxygen (FIO₂) of 0.5. A positive end-expiratory pressure of 5 cm H₂O was applied and an inspiration/expiration ratio of 1:2. Prior to baseline measurements, the minute ventilation was adjusted, if needed, to obtain an arterial CO₂ tension (PaCO₂) of 30–40 mmHg (5.3 kPa) and was then kept constant throughout the experiment.

Hemodynamics

Catheters were inserted in a central vein, the pulmonary artery, and a systemic artery (Criti Cath No. 7F, Ohmeda Pte, Singapore) for blood sampling, pressure recording, and measurement of cardiac output (Q̇ₐ) by thermodilution (mean of three bolus injections of cold saline) and temperature (Datex AS/3 Anesthesia Monitor, Datex Ohmmeda, Helsinki, Finland) (Siemens SC7000 Monitor Siemens Medical Solutions, Solna, Sweden).

Blood Gases

Arterial and mixed venous blood were collected for analysis of O₂ tension (PaO₂, PvO₂), PaCO₂, and pH (ABL 625, Radiometer, Copenhagen, Denmark), and arterial and mixed venous O₂ saturation and hemoglobin concentration (OSM 3, Radiometer, Copenhagen, Denmark). Pulmonary shunt (or venous admixture) was calculated according to the standard shunt equation. Five-milliliter blood samples were collected at the same time. The plasma was separated by centrifugation at 4°C and kept at ~−70°C prior to biochemical analysis.

NO Administration

NO was provided from a gas cylinder at a concentration of 1,000 ppm (AGA Gas AB, Lidingö, Sweden). The NO delivery was adjusted by a calibrated NO meter, yielding an inspiratory concentration of 40 ppm (Siemens NO/NO₂ monitor, Siemens Elema, AB Sweden).

cGMP

For quantification of cGMP, lung tissue lysates were analyzed with a commercially available enzyme immunoassay(EIA) kit (Detect X Direct Cyclic GMP, Arbor Assays, MI), according to manufacturer’s instructions. Optical density was read at 450 nm, which was corrected at 570 nm with a Tecan Sunrise instrument (Tecan Nordic AB, Mölndal, Sweden) with Magellan software. For normalization, the protein concentration in the tissue lysates was determined with a commercially available protein assay kit based on the Bradford assay (Coomassie Plus Assay Kit, Thermo Scientific, IL).

Endothelin-1

Plasma samples were extracted on C₁₈ columns (ISOLUTE, 200 mg, Sorbent, Sollentuna, Sweden) with one column volume (CV) 100% methanol (MeOH), followed by one CV of ultrapure water and one CV 10% MeOH. The serum sample was acidified with an equal volume of 20% acetic acid and centrifuged at 3,000 × g for 10 min, 4°C. The supernatant was applied to the column and washed once with one CV of 10% acetic acid, and twice with one CV ethyl acetate. The sample was eluted with 3 ml 100% MeOH/0.05 M ammonium bicarbonate (80:20 vol/vol). The eluted sample was evaporated to dryness with a speedvac system (Speedvac ISS110, Thermo Savant, Holbrook, NY) and stored at ~−20°C until analysis. For quantification of ET-1, a commercially available EIA kit was used (Assay Designs, MI). The extracted samples were reconstituted with 25 μl assay buffer and applied to the EIA plate in duplicate as instructed by the manufacturer. Samples were incubated on the plate over night at 4°C. Optical density was read as described above for cGMP.

Inflammatory Markers

The inflammatory markers TNFα and IL-10 were quantified in porcine serum samples with commercially available ELISA (Quantikine, R&D Systems, Abingdon, UK). All serum samples were applied to the ELISA plates in duplicate and assayed according to the manufacturer’s recommendations. Optical density was read at 450 nm and correction wavelength 540 nm with a Tecan Sunrise instrument with the Magellan software.

ET- Receptor A and B mRNA Expression

RNA isolation and reverse transcription. Total RNA was extracted from ~30 mg of lung tissue from dependent and nondependent parts of the lung (see protocol). The tissue was homogenized with an IKA D118 basic homogenizer (IKA labortechnik, Staufen, Germany) in 600 μl RTL buffer (RNeasy kit), and total RNA was extracted with the RNeasy Mini kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions. Purity and integrity of the RNA was assessed on the Agilent 2100 bioanalyzer with the RNA 6000 Nano LabChip reagent set (Agilent Technologies, Böblingen, Germany). The RNA was quantified by spectrophotometry and then stored at ~−70°C. First-strand cDNA synthesis was performed with iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA).

Real-time quantitative PCR. RT-qPCR reactions were carried out with TaqMan gene expression assays (Applied Biosystems) for porcine ET-A (ss03394412), ET-B (ss03379833), and TATA box binding protein (TBP) (ss03377123_u1) as a reference gene in each sample on a Stratagene Mx3005P QPCR System (Agilent Technologies). In each 25-μl TaqMan reaction, 4 μl cDNA (corresponding to the cDNA reverse transcribed from ~200 ng RNA) was mixed with 1.5 μl TaqMan Gene Expression Assay and 12 μl TaqMan Fast Advanced Master Mix (Applied Biosystems) and 7.5 μl H₂O. This allowed for the consistent use of standardized thermal cycling conditions: 50°C for 2 min, 95°C for 20 s, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Three technical replicas were performed, and the mean values were used. PCRs for each gene fragment were performed alongside standard dilution curves. The qPCR efficiencies were determined with standard dilution curves in triplicate and were >90% for ET-A and ET-B primers.

The gene expression of ET-A and ET-B was normalized against TBP in each sample. Data was analyzed using the MxPro Mx3005P software.

Lung Blood Flow and Atelectasis

Single photon emission computerized tomography (SPECT) measurements were performed after intravenous bolus injection of

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Fig. 1. The protocols for the two studies. A: Endothelin (ET) receptor study. [T0 (baseline) to T5 (time in hours)]. The two control groups (indicated by +/- Endogen; n = 5 in each) were healthy throughout the study, one group receiving inhaled nitric oxide (INO) 1 h after baseline measurements and the other group did not (indicated by +/- INO). The two endotoxin-exposed groups (n = 5 in each) received endotoxin by infusion immediately after baseline measurements and then throughout the study. One group received INO 1 h later, immediately after time point 1, and then throughout the study; the other group did not. B: ET receptor antagonist and lung blood flow study. Two groups (n = 5 in each) received endotoxin by infusion throughout the study. One group (INO-first protocol) received INO after 4 h that continued throughout the study, and an ET antagonist (tezosentan) at 4.5 h. The other group (ET antagonist-first protocol) received an ET antagonist (tezosentan) at 3 h and INO at 4 h. Measurements were made as indicated in the figure [γ + CT: gamma camera (SPECT) and CT measurement].

99mTechnetium-labeled macro aggregated albumin (99mTc-MAA) (Pulmocis, CIS bio international, France). Two or three SPECT measurements were made, and the injected activity was ~25 MBq 99mTc-MAA for the first SPECT, 80 MBq for the second, and 200 MBq for the last measurement. Images were acquired on a dual-head gamma camera (Millennium VG, General Electric Systems, Milwau-kee, WI) equipped with all-purpose low-energy collimators. The SPECT acquisitions were performed in 60 projections (30 per head) and stored in a 128 × 128 matrix. A transmission computed tomography scan (CT; 140 keV; 3 mAs) was performed before the acquisitions to correct for attenuation. The activity in 0.442-cm thick slices from posterior to anterior in the gravitational direction and from base to apex was calculated. These activity profiles corresponded to the perfusion distribution.

The CT scans were also used to evaluate the amount of atelectasis. A cut 1 cm above diaphragm was analyzed by creating a region of interest (ROI) covering the dense area in dependent lung but excluding big vessels. Atelectasis was defined as the area of all voxels in the ROI with −100 < HU < 100, and the area was related to the lung area in that cut.

Protocols

ET receptor study. The piglets were randomized to receive endotoxin or serve as healthy controls. Two experiments were run simultaneously with INO given to one but not the other animal (Fig. 1A).

For the control group (n = 5), the piglets were ventilated for 5 h without intervention. For the INO group (n = 5), INO 40 ppm was started 1 h (T1) after baseline measurements and continued for 4 h throughout the experiment. For the endotoxin group (n = 5), an endotoxin infusion (LPS) (E. coli 0111:B4, Sigma Chemicals, St Louis, MO) was started after baseline measurements (T0) and continued for 2 h at 40 μg·kg⁻¹·h⁻¹, followed by 10 μg·kg⁻¹·h⁻¹ for the following 2 h until end of study. For the endotoxin + INO group (n = 5), endotoxin was given as above. INO (40 ppm) was started 1 h (T1) after baseline measurements and continued throughout the experiment.

The piglets were euthanized with an iv injection of potassium chloride, and the left lung was harvested through a lateral thoracotomy. Tissue blocks 0.5 × 0.5 × 0.3 cm from dependent (collapsed lung in endotoxin piglets) and nondependent regions were snap-frozen by liquid nitrogen and kept at −70°C, pending biochemistry.

ET receptor antagonist and lung blood flow study. The dual endothelin receptor antagonist tezosentan (Actelion, Allschwil, Switzerland) was infused at 1 mg·kg⁻¹·h⁻¹ (Fig. 1B). This dose has been used in previous studies (16, 17) and was also tolerated in own pilot experiments.

Five piglets received endotoxin, and after 4 h INO (40 ppm) was administered for 30 min, and then during continuing INO, tezosentan was infused for 1 h (INO-first protocol). SPECT and CT were done at 4, 4.5, and 5.5 h, allowing 30 min for any effect by INO and 1 h for the ET receptor antagonist. In five other animals, the sequence of INO and tezosentan was inverted (tezosentan-first protocol). Thus 3 h after onset of endotoxin, infusion of tezosentan was started. One hour later, INO was concomitantly administered for 30 min (tezosentan-first protocol). SPECT and CT were done at 3, 4, and 4.5 h. Thus the effect of INO was studied at the same time points in both protocols. Every second animal was allocated to the INO-first protocol and every second one to the tezosentan-first protocol. The investigators of the CT and gamma camera images were blinded to group allocation.

Statistical Analysis

The complexity of the study limited the number to five animals in each group, with all animals surviving the study and with no missing data. Results are presented as median and range.

ET receptor study. Within-group comparisons were made between baseline and final, 5-h data, and significance tests were performed with the Wilcoxon matched-pairs signed-rank test. Group compar-

Table 1. Hemodynamic parameters in healthy and endotoxin-exposed piglets with and without INO

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Time point</th>
<th>HR, bpm</th>
<th>MaP, mmHg</th>
<th>Qr, l/min</th>
<th>PVR, dyn/s</th>
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<td>T5</td>
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<td>T5</td>
<td>Baseline</td>
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<tr>
<td>Group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>76; 68–110</td>
<td>81; 67–94</td>
<td>84; 81–91</td>
<td>75; 56–82</td>
</tr>
<tr>
<td>Control + INO</td>
<td></td>
<td>83; 74–114</td>
<td>78; 73–90</td>
<td>81; 67–120</td>
<td>66; 60–93</td>
</tr>
<tr>
<td>Endotoxin</td>
<td></td>
<td>84; 68–92</td>
<td>108; 96–133</td>
<td>83; 69–103</td>
<td>67; 53–91</td>
</tr>
<tr>
<td>Endotoxin + INO</td>
<td></td>
<td>99; 58–110</td>
<td>98; 67–147</td>
<td>72; 61–82</td>
<td>75; 43–92</td>
</tr>
</tbody>
</table>

Values are median and range. INO, inhaled nitric oxide; HR, heart rate; MaP, mean arterial pressure; QT, cardiac output; PVR, pulmonary vascular resistance. *Significant difference compared to baseline (P = 0.007; Wilcoxon matched-pairs signed-rank test); †Significant difference between endotoxin and endotoxin + INO (P = 0.014; exact Wilcoxon-Mann-Whitney test with Bonferroni correction). MaPa and PaO₂ are illustrated in Fig. 1.

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The ET-A and ET-B receptor mRNA expressions were made between the two control groups with and without INO and between the two endotoxin groups with and without INO. The exact Wilcoxon-Mann-Whitney test was used for significance testing between groups. Bonferroni-Holm correction was made for multiple tests (two comparisons between groups: baseline and end of study).

**ET receptor antagonist and lung blood flow study.** Comparisons were made between the two study groups (INO followed by ET receptor antagonist vs. ET receptor antagonist followed by INO), and any difference between the groups was analyzed for significance by the exact Wilcoxon-Mann-Whitney test. A probability of \( P = 0.05 \) was accepted as a statistically significant result. All calculations were made with SAS version 9.3 and R (version 3.0) (26a).

**RESULTS**

**ET Receptor Study**

**Central hemodynamics and gas exchange.** At baseline MaP, mean pulmonary artery pressure (MPaP), QT, pulmonary vascular resistance, and PaO2 were all within the normal range (17, 27, 29) (Table 1 and Fig. 2, A and B). In the control group respiratory and hemodynamic parameters remained stable over time whether INO was given or not.

After 3 h of endotoxin infusion MPaP had more than doubled and remained high until the end of the experiment (\( P = 0.007 \)) (Fig. 2A). PaO2 decreased continuously over time, to reach 30% of the baseline value (\( P = 0.007 \)) (Fig. 2B).

The addition of INO during ongoing infusion of endotoxin attenuated the increase in MPaP, resulting in a lower MPaP than in the endotoxin group (\( P = 0.007 \)) and was no longer significantly different from the baseline value (Fig. 2A). INO prevented the oxygenation impairment, keeping PaO2 stable over time, with a significant difference compared with endotoxin alone (Table 1 and Fig. 2B).

**Inflammatory reactions.** At baseline, there were no measurable differences between the groups for TNF and IL-10 (Table 2). In the healthy controls, with or without INO, the inflammatory markers remained low, with no difference between the two groups. Endotoxin administration for 5 h caused a marked increase in the concentration of TNF (\( P = 0.007 \)) but only a small and insignificant increase in IL-10. INO had no effect on either TNF or IL-10.

**Tissue cGMP distribution.** Median values for cGMP in samples from the nondependent, aerated lung (nd) were similar to those in the dependent, collapsed lung (d) in all four groups (Fig. 3A). After pooling of data from nd and d lung, it was seen that endotoxin decreased cGMP (with or without INO) (\( P = 0.033 \)) and that INO increased cGMP in healthy controls (\( P = 0.047 \)) and without reaching significance in endotoxin-exposed animals (Fig. 3A).

**Serum ET-1.** Baseline serum ET-1 levels were similar to those in the dependent, collapsed lung (d) in all four groups (Fig. 3B). Five hours of endotoxin infusion caused 10-fold higher concentrations in ET-1 in serum than at baseline (\( P = 0.033 \)), with no significant difference between piglets with and without INO.

**mRNA expression and distribution of ET receptor A and B.** The ET-A and ET-B receptor mRNA expressions were

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**Table 2. Inflammatory markers and ET-A and ET-B receptor expression in lung tissue in healthy control and endotoxin-exposed piglets with and without INO**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Time</th>
<th>TNF, pg/ml</th>
<th>IL-10, pg/ml</th>
<th>ET-A Receptor</th>
<th>ET-B Receptor</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Nondependent</td>
<td>Nondependent</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>lung</td>
<td>lung</td>
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<td></td>
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<td>T5</td>
<td>Dependent lung</td>
<td>Dependent lung</td>
</tr>
<tr>
<td>Group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>99.3; 65.5–145</td>
<td>80.1; 73.5–613</td>
<td>0.09; 0.07–0.10</td>
<td>0.09; 0.09–0.09</td>
<td>0.95; 0.68–1.20</td>
</tr>
<tr>
<td>Control + INO</td>
<td>73.9; 72.0–95.7</td>
<td>84.3; 48.6–101</td>
<td>0.08; 0.07–0.09</td>
<td>0.08; 0.08–0.09</td>
<td>0.85; 0.76–1.50</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>76.8; 65.1–112</td>
<td>2.597; 2.462–3.448*</td>
<td>0.09; 0.08–0.10</td>
<td>0.14; 0.10–0.18</td>
<td>0.81; 0.66–1.23</td>
</tr>
<tr>
<td>Endotoxin + INO</td>
<td>85.1; 0–102</td>
<td>3.135; 2.779–4.750*</td>
<td>0.09; 0–0.09</td>
<td>0.12; 0–0.14</td>
<td>1.14; 0.96–1.74</td>
</tr>
</tbody>
</table>

Values are median and range. *Significant difference compared with baseline (\( P = 0.007 \); Wilcoxon matched-pairs signed-rank test). T5: end of study, 5 h of endotoxin.
similar in dependent and nondependent lung regions in healthy controls with or without INO (Table 2). Infusion of endotoxin had no significant effect on the ET-A and ET-B receptor mRNA expressions, although mean decreases were seen. When INO was given to endotoxemic piglets, higher levels were seen in ET-A receptor mRNA expression, both in nondependent (48%) and dependent (40%) lung regions, compared with endotoxin alone, but without reaching significance (P = 0.093 and 0.129 for each lung separately) (Table 2). No clear effect was seen on the ET-B receptor mRNA expression. However, the ratio of ET-A/ET-B receptor mRNA was increased and was almost twice as high in dependent lung regions in the endotoxemic piglets receiving INO compared with the endotoxin-exposed piglets with no INO (P = 0.033) (Fig. 4, A and B).

Serum ET-1. The rise in serum endothelin-1 levels on endotoxin exposure was similar to the rise in our previous experiments. Serum ET-1 was 2.8 ± 1.8 at baseline, before LPS, and increased by exposure to LPS to 10.3 ± 4.1 pmol/ml. The ET receptor antagonist tezosentan at 1.0 mg·kg⁻¹·h⁻¹ increased the ET-1 levels more than fourfold in the endotoxemic piglets to 45.7 ± 3.7 pmol/ml (P < 0.001 compared with baseline and during endotoxia).

Hemodynamics. When either INO or tezosentan was given to the endotoxin-exposed piglets, a similar decrease in MPaP was seen (Fig. 5A). PaO₂ increased with INO but fell in the other animals given tezosentan, with a significant difference between the groups (P = 0.03) (Fig. 5B). Tezosentan blunted the INO-mediated increase in PaO₂, resulting in similarly low values regardless of whether tezosentan had been given first before INO or after INO (Fig. 5B). MaP was reduced by tezos-

Fig. 3. A: cGMP in dependent (posterior), collapsed lung (●) and nondependent (anterior), aerated lung tissue (○) with lung samples from each animal (n = 5 in all groups). Median values shown by horizontal bars. Endotoxin decreased (P = 0.033) and INO increased cGMP (P = 0.047) (Wilcoxon matched-pairs signed-rank test for both analyses) when data were pooled (*) [dependent (posterior) + nondependent (anterior) lung regions]. B: serum ET-1 at baseline (T0) and end of study (T5) in the four groups (median and range). Significantly higher ET-1 at T5 in the endotoxin group compared with baseline (P = 0.033; Wilcoxon matched-pairs signed-rank test) indicated by *. INO to the endotoxemic piglets had no significant effect compared with the endotoxin alone piglets. N = 5 in all groups.

Fig. 4. The ETA/ETB ratio in dependent, posterior (A) and nondependent, anterior lung tissue (B) at the end of study. Individual data (n = 5 in all groups) and median shown as a horizontal bar. INO induced a significant increase in the ETA/ETB ratio in the posterior, atelectatic lung in the endotoxin-exposed piglets indicated by * (P = 0.033; Wilcoxon matched-pairs signed-rank test). INO also induced a rise in the ETA/ETB ratio in the anterior, ventilated lung in endotoxemic piglets, but it did not reach significance.
Distribution of lung blood flow. SPECT measurements showed that INO caused a shift of blood flow away from dependent, collapsed lung regions to the upper parts of the lung in all five endotoxin-exposed piglets (posterior-anterior direction) (Fig. 6A and Fig. 7). No clear shift of blood flow along the isogravitational plane (basal-apical direction) was seen, and perfusion distributions are therefore not shown. Tezosentan during ongoing INO completely blunted the INO-induced shift of blood flow in the posterior regions. Thus perfusion of the collapsed, atelectatic region increased to the same level as before INO.

Tezosentan before INO had no clear effect on the perfusion of posterior lung regions, and the increase in cardiac output that tezosentan caused was only seen in the upper, anterior half of the lung (Fig. 6B). INO during tezosentan infusion had no effect on perfusion in posterior regions ($P = 0.03$ compared with the effect by INO before tezosentan; Fig. 7). The increase in cardiac output induced by the combined treatment (tezosentan and INO) caused a further increase in perfusion in anterior lung regions (Table 3 and Fig. 6). Perfusion along the isogravitational axis was almost the same as with tezosentan alone (not shown).

**DISCUSSION**

The major findings in the present study were that INO in endotoxemic piglets 1) almost doubled the ratio between endothelin receptor A and B mRNA in posterior, atelectatic lung regions, 2) caused a shift in blood flow away from these regions, and 3) the shift of blood flow was almost completely blunted by an ET receptor antagonist. This suggests that NO and ET interact and that their combined effect enhances redistribution of blood flow in the lung. INO causes dilation of pulmonary vessels in the ventilated lung regions, whereas an increased ET-A/ET-B ratio causes stronger ET-1-mediated vasoconstriction in atelectatic, nonventilated lung. A redistribution of blood flow, against gravity despite lowering of pulmonary vascular pressure (cf. 32), would be more easily comprehended if there is not only vasodilation in the anterior, nondependent regions but simultaneously a vasoconstriction in the posterior, nonventilated regions that are not accessible to INO. This fits with our findings and suggests that vasoconstriction in the posterior lung regions during INO augments the redistribution of the pulmonary blood flow toward the vasodilated anterior regions. An interaction between INO and ET-1 in the ventilated, anterior lung regions cannot be excluded, but if so, it is not evident since it is counteracted by the vasodilatory effect exerted by INO reaching these lung regions (3). The net
effect on pulmonary vascular pressure, and on oxygenation, will depend on the size of each region. The larger the atelectatic, nonventilated region, the greater the increase in PaO$_2$ and the smaller (if any) the fall in MPaP.

The observed effect by INO on the ET receptor mRNA in posterior, nonventilated lung regions would require a distant effect of INO. We have shown this in experiments in which two piglets were ventilated independently by separate ventilators and blood was cross-circulated between them. When one pig received INO, the other one responded with a change in its endogenous NO production regardless of whether the piglets were healthy or endotoxemic (13, 20). This could only be explained by a blood-borne mediator, which, however, has not yet been identified.

We saw an increase in serum ET-1 by endotoxin, similar to previous results (30, 31), but no effect by INO. Varying results have been obtained in previous studies on INO with increase in ET-1 in some (5, 22) and decrease via a cGMP-dependent mechanism in others (for a review see Ref. 2). This variation may depend on experimental and methodological differences. A decrease in both ET-A and ET-B receptor mRNA during endotoxemia has been shown previously (8), and a decrease could explain or contribute to the increase in serum ET-1 with fewer receptors being available for binding and clearance of ET-1 (16, 26). An increased ET-A/ET-B mRNA ratio by INO, as in the present study, should reflect increased binding and reduced clearance; the net effect on serum ET-1 is difficult to predict.

Because a more severe inflammation might impede shift in lung blood flow and worsen the oxygenation of blood, we also tested the degree of inflammation, but did not see any differences in serum TNF and IL-10 between LPS animals with or without INO. Another issue is which inflammatory mediators to measure. At present, no marker has been considered superior to the others (24).

We studied an INO dose that is in the upper range of therapeutic concentrations. Whether we would get other responses with other doses remains to be shown. Moreover, to what extent our findings can be translated to human biology remains to be discussed. However, the piglet is frequently used in experimental research, not in the least with focus on sepsis-like conditions, and a huge amount of data has been gathered over the years, enabling comparison with previous studies (cf. 5, 8, 13, 20, 29, 31).

In conclusion, in short-term experiments INO acts not only through the c-GMP pathway but also by increasing the ratio between the ET-A and ET-B receptors in posterior, atelectatic lung. Thus two mechanisms of blood flow redistribution interact: vasodilation in anterior, ventilated regions induced by

![Figure 6](https://example.com/fig6.png)

**A** recordings made first during endotoxin infusion, then during INO, and, finally, during ET receptor antagonism (tezosentan) with ongoing INO. **B** recordings made first during endotoxin infusion, then after tezosentan administration, and, finally, during INO and ET receptor antagonism. Note the absence of shift of blood flow away from posterior regions by tezosentan and also by INO, when tezosentan was given prior to INO.

![Figure 7](https://example.com/fig7.png)

**Fig. 7.** The percentage distribution of blood flow to the posterior quarter of the lung distance (Qpulm). Individual data are shown. Panel at left: recordings made first during endotoxin infusion, then during INO, and, finally, during ET receptor antagonism (tezosentan) with ongoing INO. Panel at right: recordings made first during endotoxin infusion, then after tezosentan administration, and, finally, during INO and ET receptor antagonism. *Significant fall in blood flow by INO (left) and significant difference between the decrease in posterior blood flow by INO (left) and the absence of change with INO + tezosentan (right) (Wilcoxon matched-pairs signed-rank test) (P = 0.03; n = 5 in each group). Horizontal lines show median values under each condition.
INO and vasooconstriction in posterior, atelectatic regions induced by ET-1. This dual effect of INO explains why a vasodilator that lowers pulmonary vascular pressure can shift blood flow upward, against gravity.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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