Endothelin-1 and nitric oxide synthase in short rebound reaction to short exposure to inhaled nitric oxide

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1Department of Clinical Physiology and 2Department of Anesthesiology and Intensive Care, University Hospital, S-75185 Uppsala; and 3Department of Urology and 4Department of Physiology and Pharmacology, Karolinska Institute, S-17176 Stockholm, Sweden

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Chen, Luni, Hao He, Enrique Fernandez Mondejar, Filip Fredén, Peter Wiklund, Kjell Alving, and Göran Hedenstierna. Endothelin-1 and nitric oxide synthase in short rebound reaction to short exposure to inhaled nitric oxide. Am J Physiol Heart Circ Physiol 281: H124–H131, 2001.—On withdrawal of inhalation of nitric oxide (INO) administered after lung injury, pulmonary artery pressure (PAP) and arterial oxygen tension (PaO2) may deteriorate more than before INO (rebound response). In this study, we investigated the possible roles of endothelin (ET)-1 and nitric oxide (NO) synthase (NOS) activity in the short rebound reaction to short-term inhalation of NO. Twenty-six anesthetized mechanically ventilated piglets were given endotoxin infusion. Twelve animals then received INO (30 parts per million) for two 30-min periods. Nine controls were not given INO. Measurements were made of blood gases and hemodynamic parameters, lung tissue ET-1 expression and NOS activity, and plasma ET-1 concentration. INO decreased PAP and arterial oxygen tension (PaO2) but INO withdrawal caused a short rebound reaction with an increase in PAP. Lung tissue expression and plasma concentration of ET-1 increased during INO, and plasma ET-1 increased further after its withdrawal. Activity of constitutive NOS decreased during INO, whereas that of inducible NOS was unchanged. Upregulation of ET-1 and downregulation of NOS activity may have influenced the short rebound reaction to short-term INO.

PULMONARY HYPERTENSION AND HYPOXEMIA are commonly observed in various forms of acute lung disease. Therapeutic inhalation of nitric oxide (INO) is considered to dilate pulmonary blood vessels selectively in ventilated areas, thereby reducing pulmonary vascular resistance and improving arterial oxygenation by redistribution of blood flow to ventilated areas of the lung. These changes occur within minutes after the initiation of nitric oxide (NO) therapy. However, on withdrawal of INO, both arterial oxygen tension (PaO2) and pulmonary artery pressure not only may be reversed to baseline (1, 19, 21, 26) but also may deteriorate to a greater extent than before the NO administration (8, 21, 20). Such a rebound response has been observed in patients with acute respiratory distress syndrome (ARDS) and severe hypoxemic respiratory failure and in infants that develop pulmonary hypertension after cardiac surgery (1, 8, 19–21, 26). Sudden withdrawal may lead to life-threatening hemodynamic instability, and deaths have been reported (8, 20). The mechanisms responsible for the rebound response are not fully understood. Knowledge of these mechanisms could lead to its prevention, which could have important implications for patients with pulmonary hypertensive disorders when interruption of NO inhalation is necessary or accidental. Such knowledge could also add to our understanding of the regulation of vascular tone.

It has been hypothesized that NO inhalation, through a negative feedback mechanism, reduces endogenous NO synthesis by downregulation of NO synthase (NOS) activity (1, 20, 21). This hypothesis has been tested in cell and tissue cultures and in animal experiments in vivo, but the results have been conflicting and inconclusive (2, 6, 15, 18, 25, 28, 32). Also, Horstman and co-workers (7) and Frank and co-workers (11) have found that 3 wk of inhaled NO did not decrease endogenous NO synthesis. Christou and co-workers (5) reported that INO did not decrease but increased the concentration of the circulating vasoconstrictor endothelin (ET)-1. We therefore hypothesized that a compensatory increase in the endogenous production of a vasoconstrictor, e.g., ET-1, may be a further mechanism underlying the rebound phenomenon in acute lung injury.

The purposes of the present study were 1) to mimic the rebound phenomenon found in humans by using an animal model in which a short-term rebound reaction occurs on discontinuation of short-term NO inhalation (here called “short rebound”) and 2) to analyze the role of endogenous NO and ET-1 in the short rebound reaction.

MATERIALS AND METHODS

Animal Preparation

The Animal Research Ethics Committee of Uppsala University approved the study. Twenty-six piglets of Swedish...
country breed, weighing 24–29 kg, were used in the piglets. Before they were transported from the farm, the piglets were sedated with a neuroleptic, azaperone (40 mg im; Stresnil, Janssen). Anesthesia was induced with 0.04 mg/kg im atropine, 6 mg/kg tiletamine-zolazepam (Zoletil, Virbac Laboratories), and 2.2 mg/kg xylazine chloride (Rompun, Bayer AG). After induction, a cannula was inserted in an ear vein and an opioid, fentanyl (5 μg/kg; Antigen Pharmaceuticals; Roscrea, Ireland), was injected. Muscle relaxation was achieved with 0.2 mg/kg pancuronium (Pavulon, Organon Technika; Göteborg, Sweden). Anesthesia was maintained with a continuous infusion of a hypnotic, clomethiazol (400 mg/h; Heminevrin, Astra; Södertälje, Sweden), and 2 mg/h pancuronium and 150 μg/h fentanyl. Repeated doses of fentanyl (200–500 μg/h) were given as necessary. The depth of anesthesia was considered to be adequate when skin incisions did not cause any changes in heart rate (HR) or blood pressure. Prewarmed (38°C) isotonic saline (500 ml/h) was given for hydration. The animals were placed in the supine position for the remainder of the study.

A tracheotomy was performed after induction of anesthesia, and auffed tracheal tube (inner diameter, 6 mm) was inserted. Mechanical ventilation was provided in volume-controlled mode (Servo 900 C, Siemens-Elema; Lund, Sweden) at a respiratory frequency of 20 breaths/min, an inspiratory-to-expiratory ratio of 1:1, an end-inspiratory pause of 5%, and a positive end-expiratory pressure of 5 cmH2O. The inspired fraction of O2 was 0.5. The minute ventilation was adjusted to obtain an end-tidal CO2 tension of 33–45 mmHg (4.4–6.0 kPa) in the initial control situation and was then kept constant throughout the experiment. The mean tidal volume was 10 ± 1.4 ml/kg.

Catheterizations for Blood Sampling and Pressure Measurements

A triple-lumen balloon-tipped catheter (Swan-Ganz 7-Fr) was introduced into the pulmonary artery via the right external jugular vein for blood sampling and pressure recordings. A large-bore catheter was inserted into the contralateral jugular vein for infusion purposes, with its tip in the superior vena cava. The right carotid artery was cannulated for blood sampling and recording of arterial blood pressure.

The arterial, central venous, and pulmonary artery catheters were connected to the appropriate pressure transducers (Sorenson Transpac transducers, Abbott Critical Care Systems), and pressures were recorded on a Marquette 7010 monitor (Marquette Electronics). Mean arterial pressure, mean pulmonary artery pressure (MPAP), HR, central venous pressure, pulmonary capillary wedge pressure, and cardiac output were recorded. Airway pressures were registered from the ventilator. Vascular pressures were averaged over the whole respiratory cycle, and the midthorax was used as the zero reference level.

Cardiac output was measured by thermodilution; 10 ml of ice-cold isotonic saline was injected as a bolus and cardiac output was computed (cardiac output computer Marquette 7010, Marquette Electronics). At least three injections were given for each measurement, and the mean was calculated. The injections were evenly distributed over the respiratory cycle. The expired minute volume was recorded at each measurement.

Mixed venous and arterial blood samples were collected for blood gas analysis (ABL 3, Radiometer; Copenhagen, Denmark) and determination of oxygen saturation and hemoglobin concentration (OSM 3, Radiometer). Hemoximeter data were corrected for pig blood. Blood samples were collected at the same time for biochemical analysis (see below).

NO Administration and Recording of NO in Respiratory Air

NO [1,000 parts per million (ppm) in N2] was added to a mixture of O2-N2 and connected to the low-flow inlet of the ventilator. The inspired gas was passed through a canister containing soda lime to absorb any NO2. The inhaled NO was set to 30 ppm, and the concentration of inspired NO2 was always <0.5 ppm. The concentrations of inspired NO and NO2 were measured continuously by chemiluminescence (9841 NOx, Lear Siegler Measurement Controls; Englewood, CO) in the inspiratory limb of the ventilator tubing. The inspired fraction of O2 was checked after the addition of NO and kept stable at the pre-NO level.

The NO concentration in exhaled air was measured intermittently during the experiment by chemiluminescence (chemiluminescence NO-NO2-NOx analyzer, model 42, Thermo Environmental Instruments, Franklin, MA).

Protocols

Thirty minutes after surgery, baseline measurements of hemodynamic parameters and blood samples were taken. Blood gas analyses were performed, and blood was collected for subsequent biochemical analysis. A septic model of acute lung injury was created by an intravenous infusion of endotoxin (lipopolysaccharide, Escherichia coli 0111:B4, Sigma; St. Louis, MO), which was given at a dose of 25 μg·kg–1·h–1 for 3 h and then maintained at 10 μg·kg–1·h–1. The hemodynamic and gas exchange responses were measured 30, 60, 120, and 180 min after the start of the endotoxin infusion.

The piglets were then allocated to one of the following three groups:

INO group (n = 12). After ~180 min of endotoxin infusion, inhalation of 30 ppm NO was started. After 15 min of INO and before its discontinuation after 30 min, further measurements of hemodynamic parameters and gas exchange were performed, and blood was sampled for biochemical analyses. INO was then withdrawn, and the measurements were repeated after 5, 10, 15, and 30 min to check for evidence of a short rebound reaction. Thereafter, INO was given for another 30 min and withdrawn again (from 240 to 270 min), and the same measurements were made over the next 30 min at the same time points as after the first INO challenge. This was done to see whether any short rebound reaction occurred and was stronger after NO inhalation after an additional hour of lung damage.

Lung tissue group (n = 5). To measure the NOS activity and the ET-1 expression in vivo during inhalation and after withdrawal of NO, lung tissue samples were collected in vivo in five additional piglets. A median sternotomy was performed, and the left pleura was opened. The incision was covered with plastic film to prevent drying of the lung surface. The piglet was ventilated for 1 h before baseline measurements were made. The protocol was the same as in the INO piglet experiments except that NO was administered for only one 30-min period and, in addition, a piece of lung tissue was taken on four occasions (after 3 h of endotoxin infusion, after 30 min of NO inhalation, and 15 and 30 min after NO withdrawal). With a pair of forceps, a small part of the left middle lobe was clamped, and a piece of lung tissue was cut off. For each new sampling, another pair of forceps was used to clamp a piece of tissue in the left middle lobe. The lung tissue sample was cut into blocks of ~0.5 × 0.5 × 0.3 cm, snap-frozen with liquid nitrogen, and kept at −80°C until NOS activity and Western blot measurements were made.

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Control group (n = 9). The control group received the same dose of endotoxin as the INO group. The setup of the mechanical ventilation was also the same, with O₂ in N₂, but no NO was inhaled. The control piglets were studied at baseline and at time points coinciding with the beginning and end of the two INO challenges in the INO group and also at the end of the experiment, 5 h after commencement of the endotoxin administration. This was done to check for the reaction to endotoxin per se.

Finally, all piglets were killed with an intracardiac injection of KCl. The total study time, including anesthesia, preparation, and baseline measurements before endotoxin infusion, was ~7 h. Two piglets died 10 to 15 min after INO withdrawal in the second trial of NO inhalation in the INO group.

Plasma ET-1

Blood was collected in prechilled tubes containing EDTA (10 mM final concentration) and centrifuged (for 10 min at 4°C) to separate the plasma. Acid ethanol was added to precipitate the protein. The precipitate was finally analyzed for ET-1-like immune reactivity by radioimmunoassay using an antiserum (E1) raised against ET-1 in rabbits. The detection limit of the assay was 1.91 pM (17). The cross-reactivity for ET-1 detection. The blot was then washed five times in TBS, after which the antigen-antibody complex was detected on photographic film using enhanced chemiluminescence reagent (Amersham; Arlington Heights, IL). According to the manufacturer, the cross-reactivity of the ET-1 antiserum was as follows: ET-1, 100%; ET-2, 9%; ET-3, 8%; and Big ET-1, 0.14%. The level of the plasma ET-1 was expressed as picomoles per milliliter of plasma.

ET-1 Expression Assessed by Western Blotting

The lung tissue blocks were rinsed with PBS at 4°C. The total protein in the lung tissue was extracted by homogenization and stored at

**Statistical Analysis**

Means ± SD were calculated for all variables under all study conditions. Two-way ANOVA for repeated measurements was used for comparisons within and among groups. The least significant difference was used for post hoc tests. Differences were regarded as significant at a P level of <0.05. Simple regression analysis was used to analyze the correlation between changes in MPAP and PaO₂ during INO and after INO withdrawal.

**RESULTS**

**Endotoxin-Induced Lung Damage**

The baseline values for the studied hemodynamic parameters were similar to those observed in healthy piglets in a previous experiment (12) (Table 1).

### Table 1. Effect of endotoxin and INO on hemodynamics and PaCO₂

<table>
<thead>
<tr>
<th>Condition: Time:</th>
<th>Baseline</th>
<th>Pre-INO 3 h</th>
<th>INO 3 h</th>
<th>After INO 3 h 30 min</th>
<th>After INO 3 h 35 min</th>
<th>Pre-INO 4 h</th>
<th>INO 4 h</th>
<th>After INO 4 h 30 min</th>
<th>After INO 4 h 35 min</th>
</tr>
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<tbody>
<tr>
<td>HR, beats/min</td>
<td>104 ± 14</td>
<td>156 ± 16*</td>
<td>142 ± 25</td>
<td>150 ± 18</td>
<td>158 ± 21</td>
<td>158 ± 21</td>
<td>146 ± 22</td>
<td>154 ± 20</td>
<td>148 ± 32</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>107 ± 12</td>
<td>160 ± 27*</td>
<td>154 ± 20</td>
<td></td>
<td></td>
<td>165 ± 26</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CO, l/min</td>
<td>86 ± 15</td>
<td>66 ± 20*</td>
<td>71 ± 16</td>
<td>68 ± 26</td>
<td>67 ± 25</td>
<td>70 ± 19</td>
<td>69 ± 16</td>
<td>66 ± 29</td>
<td></td>
</tr>
<tr>
<td>CO₂, mmHg</td>
<td>3.7 ± 0.4</td>
<td>2.9 ± 0.5</td>
<td>3.2 ± 1.3</td>
<td>2.5 ± 1.6*</td>
<td>2.9 ± 1.2</td>
<td>3.1 ± 1.4</td>
<td>2.4 ± 1.9*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>34 ± 5</td>
<td>46 ± 4*</td>
<td>40 ± 5</td>
<td>45 ± 8</td>
<td>42 ± 8</td>
<td>49 ± 6*</td>
<td>50 ± 5</td>
<td></td>
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</tbody>
</table>

Values are means ± SD. INO, inhalation of nitric oxide; HR, heart rate; CO, cardiac output. *P < 0.05 compared with baseline; †P < 0.05 compared with values during INO.
After 3 h of endotoxin infusion, MPAP was increased more than twofold in both the control and INO groups. PaO₂ was significantly reduced to one-half the baseline value. The HR was increased, and cardiac output was decreased. There were no significant differences between values obtained at 3 and 4 h after commencement of the endotoxin infusion in the INO group or between those obtained after 3, 4, and 5 h in the control group.

**NO Inhalation and Discontinuation**

After 3 h of endotoxin infusion, inhalation of 30 ppm NO for 30 min resulted in a 26% decrease in MPAP (P < 0.05; Fig. 1A) and a 59% increase in PaO₂ (P < 0.05; Fig. 1C). When, after 30 min, the NO inhalation was discontinued, MPAP rapidly increased (within 5 min) to a level that was 23% (9 mmHg) higher than before the NO inhalation and significantly higher than in the control group (P < 0.05). Thus a short rebound reaction had occurred (Fig. 1A). PaO₂ decreased rapidly after withdrawal of INO (P < 0.05), but no clear short rebound was seen (Fig. 1C). During the next 30 min after NO withdrawal, MPAP and PaO₂ returned to their pre-NO inhalation levels.

When the NO inhalation was repeated after 4 h of endotoxin infusion, the fall in MPAP and increase in PaO₂ (26%) were less pronounced than during the test 1 h earlier, and the increase in PaO₂ was no longer significant. Five minutes after discontinuation of the second 30 min of NO inhalation, MPAP had again increased significantly by 24% to a value above the pre-NO level (P < 0.05) and above that in the control group (P < 0.05; Fig. 1B). Meanwhile, PaO₂ had decreased to a level 31% lower than the pre-INO value (P < 0.05). Thus a clear short rebound hypoxemic reaction had occurred (Fig. 1D).

There was an inverse relationship between the improvement in PaO₂ during NO inhalation and the mag-
even more clearly the improvements in MPAP and PaO₂ achieved by the two INO challenges and the short rebound reaction on discontinuation of NO (Fig. 1).

**Plasma ET-1 Levels**

Under baseline conditions, the plasma ET-1 concentration was of the same magnitude in the INO and control groups. After 3 h of endotoxin infusion, it had increased ~10-fold (P < 0.05) and showed no significant difference between the two groups (Fig. 3). In the control group, there was no further change in the plasma ET-1 level during the subsequent 2 h. In the INO group, plasma ET-1 increased further during and after the two INO trials, and the increase was significant in the second INO trial compared with the value before the INO challenges and the control group (P < 0.05; Fig. 3).

**Effect of INO on Expression of ET-1 in Lung Tissue (Lung Tissue Group)**

The expression of ET-1 in the endotoxin-exposed lung increased more than threefold during NO inhalation compared with that before INO (P < 0.05). It then decreased after withdrawal of inhaled NO, and 30 min after cessation of INO, it had regained the pre-INO level (Fig. 4).

**NOS Activity (Lung Tissue Group)**

NO inhalation downregulated the activity of cNOS, nNOS, and eNOS (Fig. 5A) but not of iNOS (Fig. 5B). Fifteen minutes after NO withdrawal, the iNOS activity was upregulated. At the same time, the cNOS activity had increased, and 30 min after discontinuation of INO, it had returned to the pre-INO level.

**NO in Expired Air**

Expired NO was increased by 3 h of endotoxin infusion [from below 1 to ~4 parts per billion (ppb)] in both the INO and control groups (Fig. 6). There was no significant change in expired NO during the subsequent 2-h study period in the control group. In the INO group, expired NO was much higher during the first 5 min after NO discontinuation than before INO (80–100 compared with 4 ppb) and was still significantly higher (P < 0.05) than before NO therapy 10 min after cessation of INO. Thirty minutes after discontinuation of INO, expired NO had returned to the pre-INO level (Fig. 6).

**DISCUSSION**

**Endotoxin Lung Damage**

The endotoxin model triggers activation of a number of inflammatory markers (4) and has frequently been used as a model of septic acute lung injury. In this study, the exposure to endotoxin induced expression of iNOS, which plays a major role in NO production in inflammation, and the expired concentration of NO was also increased. We noted an improvement both in MPAP and arterial oxygenation on NO inhalation and
a short rebound reaction on discontinuation of a short-term inhalation of NO. The endotoxin exposure also induced a pronounced increase in the plasma ET-1 concentration, and this was increased further by the INO challenges.

ET-1 is a strong vasoconstrictor and a proinflammatory factor, acting via two subtypes of receptors (ET\textsubscript{A} and ET\textsubscript{B}) by direct receptor binding and/or by stimulating the release of thromboxane A2, another potent vasoconstrictor (22, 23). It has been reported that blockage of the ET\textsubscript{A} receptor abolishes the increase in MPAP that was noted 2 h after the start of endotoxin infusion (29) but not the early short-lasting increase 10–15 min after this start (31). This underscores the importance of ET-1 in the late phase of endotoxin-induced pulmonary hypertension.

Fig. 3. Effect of endotoxin infusion on plasma endothelin (ET)-1 concentration and the effect of 30 min of INO and of its discontinuation. Two challenges with INO were made. The values in the control group without NO inhalation are shown for comparison. Values are means ± SD (INO group, \( n = 12 \); control group, \( n = 9 \)). *\( P < 0.05 \) vs. baseline; †\( P < 0.05 \) compared with value of pre-INO-1; ‡\( P < 0.05 \) compared with value of pre-INO-2; §\( P < 0.05 \) vs. control group.

Fig. 4. Results of Western blot determination of the protein expression of ET-1 in lung tissue of piglets submitted to INO and INO withdrawal using the enhanced chemiluminescence reagent technique. Top: blots from the samples from 1 piglet; bottom: expression in arbitrary units (AU). Values are means ± SD of 5 experiments. *\( P < 0.05 \) vs. pre-INO value.

Fig. 5. Activity of calcium-dependent nitric oxide (NO) synthase (cNOS; A) and calcium-independent NO synthase (iNOS; B) before and during INO and after its withdrawal. Values are means ± SD of 5 experiments. *\( P < 0.05 \) vs. pre-INO value.
Endogenous (3) but not exogenous NO (24) down-regulates ET-1 synthesis. We found that exogenous NO did not decrease the plasma ET-1 concentration but that it upregulated ET-1 production in lung tissue, and, in the INO group, the plasma ET-1 level increased continuously during and after withdrawal of NO inhalation. Similar observations have been made in human neonates with primary pulmonary hypertension that received INO therapy (5). This increase may have been caused by INO, as supported by the absence of a further increase in plasma ET-1 in the control group during the same period, between 3 and 5 h of the study.

It is reasonable to assume that exogenous NO interferes with the balance between ET-1 and endogenous NO and stimulates production of ET-1. The expression of ET-1 in lung tissue changed relatively quickly. ET-1 is converted from its proform, Big ET-1, by ET-converting enzyme (23). The ET-1 anti-serum used for this study is specific for ET-1 detection and has minimal cross-reaction with Big ET-1. We therefore conclude that the change in ET-1 expression could not have occurred at the transcriptional but at the posttranscriptional level. ET peptides are not stored within synthesizing cells but are produced constitutively and released to the extracellular medium at a steady rate (17). This makes it likely that ET peptides are mediators of slow physiological and pathological processes. However, a significant increase in the cellular expression of ET-1 mRNA and in the ET-1 concentration in culture media and bronchoalveolar lavage fluid has been seen shortly after stimulation (10, 13, 16, 30). This suggests that ET-1 may also mediate acute physiological effects, as suggested by our findings.

ET-1 expression was dramatically increased during NO inhalation. Its potential vasoconstrictive effect was obviously countered by the vasodilatory action of the inhaled NO. In addition, it has been shown that exogenous NO antagonizes ET-1 effects both at the level of direct ligand/receptor binding and by interference with the postreceptor pathway for calcium mobilization (14). This should also reduce the vasoconstrictor effect of ET-1. After withdrawal of INO, high concentrations of ET-1 were still circulating in the blood, coincident with the maximum short rebound reaction. Moreover, after NO withdrawal, the blockage of the signal transduction of the ETA receptor should have ceased. We therefore hypothesize that the increased concentration of the vasoconstrictor ET-1 and, possibly, increased availability of ETA receptor contributed to the short rebound reaction. The questions of whether prolonged NO inhalation would have enhanced this reaction and whether the mechanism of the clinical rebound phenomenon after several hours of INO is similar to that of the short rebound reaction were not tested in the present study, but they remain a possibility.
We also found that the weaker the response to INO, the greater the fall in PaO₂ when INO was discontinued. A similar observation has been made in the patients receiving long-term INO treatment (8). Whether a poor or modest response to INO in an ARDS patient also signals the risk of a rebound response remains to be shown in clinical trials.

In conclusion, we consider that the short rebound reaction occurring on discontinuation of short-term NO inhalation is influenced not only by a downregulation of NOS activity but also, and possibly more importantly, by an increase in the amount of the vasoconstrictor ET-1 in the lung and circulating blood. However, clinical studies are needed before any extrapolation of the present findings to human conditions can be made.

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