Cyclooxygenase inhibitor blocks rebound response after NO inhalation in an endotoxin model

LUNI CHEN, HAO HE, ENRIQUE FERNANDEZ MONDEJAR, AND GÖRAN HEDENSTIerna
Department of Medical Sciences, Clinical Physiology, University Hospital, SE-751 85 Uppsala, Sweden

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Chen, Luni, Hao He, Enrique Fernandez Mondejar, and Göran Hedenstierna. Cyclooxygenase inhibitor blocks rebound response after NO inhalation in an endotoxin model. Am J Physiol Heart Circ Physiol 284: H290–H298, 2003; 10.1152/ajpheart.00535.2002.—This study addressed the possible role of cyclooxygenase (COX) and its products in the rebound response to inhaled nitric oxide (INO). Anesthetized, mechanically ventilated piglets were exposed to endotoxin alone, endotoxin combined with INO, or endotoxin with INO plus the COX inhibitor diclofenac (3 mg/kg iv) (n = 8 piglets/group). A control group of healthy pigs (n = 6) was also studied. Measurements were made of blood gases, hemodynamic parameters, lung tissue COX expression, and plasma concentrations of thromboxane B2 (TxB2), PGF2α, and 6-keto-PGF1α. Endotoxin increased lung inducible COX (COX-2) expression and circulating prostanoids concentrations. Inhalation of NO during endotoxemia increased the constitutive COX (COX-1) expression, and the circulating TxB2 and PGF2α increased further after INO withdrawal. The combination of COX inhibitor with INO blocked all these changes and eliminated the rebound reaction to INO withdrawal, which otherwise was seen in endotoxemic piglets given INO only. We conclude that the rebound response to INO discontinuation is related to COX products.

INHALED NITRIC OXIDE (INO) is a selective pulmonary vasodilator in the treatment of pulmonary hypertension. However, life-threatening hemodynamic instability, reduced oxygenation, and even death have been observed during attempts to withdraw INO (1, 10, 17). These phenomena are referred to as the rebound response to INO withdrawal. Stepwise reduction of the NO dose implies prolongation of the NO therapy and may still not eliminate the rebound response (10).

The mechanisms responsible for the rebound response are not fully understood. NO stimulates the release of soluble guanylate cyclase from the tissues, with a consequent increase in cGMP. INO reduces the endogenous NO production as a negative feedback mechanism, and this mechanism is assumed to be related to the rebound reaction to INO withdrawal (1, 2, 9). We have previously found that the production and/or release of the vasoconstrictor peptide endothelin-1 (ET-1) and possibly of other vasoconstrictors is also related to the rebound, and this may be even more important than the downregulation of endogenous NO production by INO (5).

ET-1 and some prostanoids, in particular thromboxane A2 (TxA2) and PGF2α, are important vasoconstrictor mediators in primary and secondary pulmonary hypertension (6, 22, 23). In addition, ET-1-stimulated secondary release of TxA2 is one of the main modes of signal transduction in ET-1-induced vasoconstriction (21). PGI2 and TxA2, and PGF2α, are important mutually antagonistic vasodilator and vasoconstrictor products, respectively, of arachidonic acid. They are synthesized via a cyclooxygenase (COX)-dependent pathway. A PGI2 analog has been reported to mitigate the rebound response to INO withdrawal in a case study (14).

On the basis of these observations, we hypothesized that the rebound response to INO withdrawal is related to COX-derived vasoconstrictor products such as TxA2 and PGF2α. The purpose of the present study was to determine whether a COX inhibitor could prevent a rebound reaction to INO withdrawal in a porcine endotoxin shock model.

MATERIALS AND METHODS

Animal Preparation

The Animal Research Ethics Committee of Uppsala University approved the study. Thirty piglets of Swedish country breed, weighing 24–29 kg, were used. Anesthesia was induced with intramuscular atropine (0.04 mg/kg), tiletamine-zolazepam (6 mg/kg, Zoletid, Virbac Laboratories), and xylazine chloride (2.2 mg/kg, Rompun, Bayer) and maintained with a continuous infusion of a hypnotic, clorazethoate (400 mg/h, Heminevrin, Astra; Södertälje, Sweden), pancuronium (2 mg/ h), and fentanyl (150 μg/h) (5). Prewarmed (38°C) isotonic saline (10–20 ml·kg⁻¹·h⁻¹) was given intravenously in endotoxin-exposed piglets to prevent dehydration and maintain a stable intravascular volume; 5–10 ml·kg⁻¹·h⁻¹ saline was given intravenously in the healthy controls. The animals were placed in the supine position for the remainder of the study.

After the induction of anesthesia, a tracheotomy was performed and auffed tracheal tube was inserted. Mechanical ventilation was provided in the volume-controlled mode (Ser-

Address for reprint requests and other correspondence: G. Hedenstierna, Dept. of Medical Sciences, Clinical Physiology, Univ. Hospital, SE-751 85 Uppsala, Sweden (E-mail: goran.hedenstierna@medsci.uu.se).

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vo 900 C, Siemens-Elema; Lund, Sweden) at a respiratory frequency of 21 ± 2 breaths/min, an inspiratory-to-expiratory ratio of 1:2, and an end-inspiratory pause of 5% of the respiratory cycle, with oxygen in nitrogen. The minute venti-
lization 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. A positive end-
spiratory pressure of 5 cmH₂O was applied. The inspired fraction of oxygen (FIO₂) was 0.5. Further details are given in

A triple-lumen balloon-tipped catheter (Swan Ganz No. 7F) was introduced into the pulmonary artery for blood
sampling and pressure recording. The contralateral jugular vein and right carotid artery catheters were also introduced
for pressure recording, blood sampling, and infusion. Mean arterial pressure, mean pulmonary arterial pressure
(MPAP), heart rate (HR), central venous pressure, pulmonary
capillary wedge pressure, and cardiac output (Qc) were recorded. For further details, see a previous report by our
group (5).

NO Administration

NO, 1,000 ppm in N₂, was added to a mixture of O₂–N₂ and
administered through 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. FIO₂ was checked after the
addition of NO and kept stable at the pre-INO level.

Protocol

Thirty minutes after surgery, baseline measurements of
hemodynamic parameters were made and blood samples
were drawn. Blood gas analysis was performed, and plasma
was collected for subsequent biochemical analysis. In 24 animals, a septic model of acute lung injury was
created. This was achieved by an intravenous infusion of
endotoxin (LPS, Escherichia coli 0111:B4, Sigma; St. Louis,
MO) at a dose of 25 μg·kg⁻¹·h⁻¹ for 3 h, followed by a
maintenance dose of 10 μg·kg⁻¹·h⁻¹. The hemodynamic
and gas exchange responses were measured 30, 60, 120, 150,
and 180 min after the start of the endotoxin infusion. To test the
effects of endotoxins and INO, and the potential protective
effect of a COX inhibitor against a rebound response after
INO withdrawal, the piglets were allocated to one of the
following groups: 1) endotoxin, 2) endotoxin + INO, or 3)
endotoxin + INO + COX inhibitor. In addition, another six
piglets were studied as healthy controls (group 4).

Group 1: endotoxin (n = 8). These animals received the
endotoxin infusion at the dose mentioned above and mechan-
ic ventilation as described above for all animals for a 5-h
period, with intermittent recordings of hemodynamics, gas
exchange, and blood sampling. This was done to check for the
reaction to endotoxin per se.

Group 2: endotoxin + INO (n = 8). After 3 h of endotoxin
infusion, inhalation of NO (30 ppm) was started as described
above and maintained for 30 min (Fig. 1). Before INO was
discontinued and 5, 10, 15, and 30 min after its withdrawal,
hemodynamic parameters were measured, and blood was
sampled for gas exchange and biochemical analyses. The
purpose of this group was to check for the effectiveness of
INO per se and for any rebound reaction to INO withdrawal.

NO inhalation was administered for another 30 min (i.e.,
from 4 h after the start of endotoxin infusion) and withdrawn
again. The results were studied at the same time points as in
the first INO test. This was done to see whether any rebound
response was stronger than that after the first occasion.

The protocol design was based on the results of a previous
study (5) and preliminary experiments. These had shown
that the increase in MPAP and fall in arterial PO₂ (PaO₂)
had been most marked during the first 4 h after the start of
endotoxin infusion and that the increase in MPAP and fall in
arterial PO₂ (PaO₂) had reached a maximum 2.5 h after the start of endotoxin
injection; the values then remained comparatively stable for a few hours.

Group 3: endotoxin + INO + COX inhibitor (n = 8). After
~150 min of endotoxin infusion, a nonselective COX inhibi-
tor, diclofenac (Lot 117H0326, Sigma) in saline (3 mg/kg),
was given as an intravenous injection (Fig. 1). Inhalation of
NO (30 ppm) was started 30 min after the diclofenac
injection, 3 h after the start of endotoxin infusion. The protocol
was thus the same as in the endotoxin + INO group, except
that the COX inhibitor diclofenac was given.

Diclofenac was chosen because it is effective, its action is
rapid, and it inhibits both constitutive COX (COX-1) and
inducible COX (COX-2). The dose of diclofenac was decided
on the basis of our preliminary tests and a previous study by
other authors (28).

Group 4: healthy controls (n = 6). These animals did not
receive endotoxin infusion, but otherwise the protocol was
the same as in the endotoxin group.

Finally, all piglets were killed with an intravenous injec-
tion of KCl via the central venous catheter. A thoracotomy
was performed, and a piece of lung tissue was cut off from the
left middle lobe. The lung tissue samples were cut into blocks
of ~0.5 × 0.5 × 0.3 cm, which were snap-frozen with liquid
nitrogen and kept at −70°C pending Western blot measure-
ments. The total study time, including anesthesia, prepara-
tion, and baseline measurements, was ~7 h.

Lung Tissue COX Analysis

The total protein contents of the lung tissue was extracted
by homogenization (UltraTurrax, Janke and Kunkel, IKA
Laborteknik; Staufen, Germany) in 5 vol of ice-cold 0.05 M
Tris buffer (pH 7.4) containing 0.5 mM phenylmethylsulfonyl
fluoride to inhibit proteinolysis. The supernatant was collected
and stored at −70°C until analyzed.
The concentration of whole protein in the supernatant was determined by the method of Lowry, and the protein was then fractionated by SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. The blot was blocked with 5% BSA in Tris-buffered saline (TBS) at 4°C overnight. It was then incubated with anti-COX-1 (1:500, Catalog No. 161018, Cayman Chemical) and anti-COX-2 (1:1,000, Catalog No. 360120, Cayman Chemical) in TBS containing 1% BSA at 4°C overnight. After being washed with TBS five times, the blot was incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2,500 dilution, Vector Laboratories) for COX-1 and COX-2 detection. The blot was then again washed five times in TBS, after which the antigen-antibody complex was detected on photographic film using enhanced chemiluminescence reagent (Amersham; Arlington Heights, IL). All the experiments were carried out three times, and the bands from each experiment were analyzed using the NIH Image 1.6 C program for statistical analysis.

**Plasma Prostaglandin Analysis**

Tx$_A_2$ and PG$_L_2$ converted to their stable metabolites TxB$_2$ and 6-keto-PGF$_{1α}$, respectively, within a minute after they had been released. We therefore measured plasma TxB$_2$ and 6-keto-PGF$_{1α}$ as indicators of Tx$_A_2$ and PG$_L_2$ release. PGF$_{2α}$, TxB$_2$, and 6-keto-PGF$_{1α}$ concentrations were measured with commercially available enzyme immunoassay kits (prostaglandin F$_{2α}$ ELISA kit, 516011; thromboxane B$_2$ ELISA kit, 519031; and 6-keto-prostaglandin F$_{1α}$ ELISA kit, 515211; Cayman Chemical).

To ensure that all samples were free from organic solvents, the serum was purified according to the instructions of Cayman Chemical before it was added to the assay. Enzyme immunoassays were performed in duplicate by mixing 50 µl antiserum on each sample with 50 µl Horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2,500 dilution, Vector Laboratories) for COX-1 and COX-2 detection.

**Statistical Analysis**

Means ± SD were calculated for all variables under all study conditions. Two-way ANOVA for repeated measurements on one factor was applied to disclose any differences within groups and also any differences between groups (endotoxin + INO + COX inhibitor group compared with endotoxin + INO group, and both compared with endotoxin and healthy control groups). The $P$ values were corrected for multiple comparisons in the least-significance difference test, and the least-significant difference test was used for post hoc tests. Differences were regarded as significant at a $P$ level of <0.05.

**RESULTS**

**Baseline Variables and Effects of Endotoxin-Induced Lung Damage**

In the animals of the four groups, the baseline hemodynamics and arterial oxygenation were similar to those observed in healthy piglets and in previous experiments from our laboratory (5), and there were no differences between the groups. In the healthy control piglets, no change was seen in any variable during the 5-h study period.

In the animals exposed to endotoxin alone (endotoxin group), MPAP was increased more than twofold after 150 min of endotoxin infusion. Pa$_O_2$ was significantly reduced, to less than one-half the baseline value. HR increased and $Q_b$ decreased. There were no significant differences between the values obtained 3, 4, and 5 h after the commencement of the endotoxin infusion (Table 1). There was no significant difference in the increase in MPAP and decrease in Pa$_O_2$ in response to endotoxin infusion in the endotoxin, endotoxin + INO, and endotoxin + INO + COX inhibitor groups before INO and the COX inhibitor were administered.

**NO Inhalation and Discontinuation**

In the endotoxin + INO group, inhalation of NO (30 ppm) after 3 h of endotoxin infusion resulted in a significant decrease in MPAP and a significant increase in Pa$_O_2$. When, after 30 min, the NO inhalation was discontinued, MPAP increased rapidly (within 5 min) to a level that was 24% ($P < 0.05$) higher than before INO; thus a rebound response had occurred (Fig. 2A). Pa$_O_2$ decreased rapidly to the pre-INO value ($P > 0.05$) but did not display a clear rebound phenomenon (Fig. 2C).

When the NO inhalation was repeated 4 h after endotoxin infusion, the MPAP was significantly decreased, but the increase in Pa$_O_2$ was small and no longer significant. Five minutes after discontinuation of NO inhalation, MPAP had again increased significantly.

**Table 1. Effect of endotoxin on hemodynamics and blood gases**

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<td>Pa$_O_2$, mmHg</td>
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<td>Healthy</td>
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<td>76</td>
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<tr>
<td>Endotoxin</td>
<td>80</td>
<td>36</td>
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Values are means ± SE; $n = 6$ healthy control piglets and 8 piglets exposed to endotoxin only. HR, heart rate; MAP, mean arterial pressure; MPAP, mean pulmonary arterial pressure; $Q_b$, cardiac output; Pa$_O_2$ and Pa$_CO_2$, arterial Pa$_O_2$ and Pa$_CO_2$, respectively. *$P < 0.05$ vs. time 0; †$P < 0.05$ vs. healthy controls.
cantly to above the pre-INO level ($P < 0.05$) and $\text{PaO}_2$ had fallen significantly to below the pre-INO level ($P < 0.05$). Thus distinct rebound hypoxemia had occurred (Fig. 2, B and D).

In the endotoxin + INO + COX inhibitor group, pretreatment with diclofenac tended to lower MPAP ($P = 0.06$) and to reduce oxygenation ($P = 0.08$). INO significantly decreased MPAP and increased $\text{PaO}_2$. On discontinuation of INO, no rebound was observed in either MPAP or $\text{PaO}_2$. Similarly, after a second INO challenge, no rebound response was recorded (Fig. 2).

INO had no significant effect in HR, system blood pressure, and $Q_t$ (Table 2).

Expression of COX-1 and COX-2

COX-1 expression in the lung tissue exposed to endotoxin alone (endotoxin group) did not differ compared with that in the healthy lung (Fig. 3A), but COX-2 expression in the endotoxin group was significantly higher than that in the healthy controls (Fig. 3B). The results indicate that COX-2 is upregulated by endotoxin exposure, but that COX-1 did not change.

The expression of COX-1 was twice as high in the endotoxin + INO group compared with that in the endotoxin group ($P < 0.05$; Fig. 4A), whereas the expression of COX-2 was of the same magnitude in the endotoxin and endotoxin + INO groups (Fig. 4B). This shows that COX-1 expression is upregulated by INO under endotoxemia.

In the endotoxin + INO + COX inhibitor group, both COX-1 and COX-2 expression were significantly lower than in the endotoxin and endotoxin + INO groups (Fig. 4, A and B), indicating that both COX-1 and COX-2 expression are downregulated by diclofenac.

Concentration of Plasma $\text{TxB}_2$

The plasma $\text{TxB}_2$ concentration did not differ between the four groups at baseline and remained stable...
in the healthy controls (Fig. 5). Thirty minutes after the start of endotoxin infusion, this concentration had increased dramatically and then decreased to about four to five times the baseline level 2.5 h after the onset of endotoxin infusion, with no difference between the endotoxin-exposed groups. The plasma TxB$_2$ then increased again throughout the study period.

In the endotoxin/INO group, the plasma TxB$_2$ concentration did not change during NO inhalation.

### Table 2. Effects of INO on hemodynamics and blood gases

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<tr>
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<th>Baseline (1 h)</th>
<th>Pre-INO (3 h)</th>
<th>INO (3 h, 30 min)</th>
<th>Post-INO (3 h, 35 min)</th>
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<td>E + INO</td>
<td>89 ± 20</td>
<td>156 ± 16*</td>
<td>142 ± 25</td>
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<td>144 ± 16</td>
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<td>E + INO + COX-i</td>
<td>98 ± 14</td>
<td>149 ± 20*</td>
<td>153 ± 23</td>
<td>150 ± 22</td>
<td>158 ± 21</td>
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<td><strong>MAP, mmHg</strong></td>
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<tr>
<td>E + INO</td>
<td>86 ± 15</td>
<td>66 ± 20</td>
<td>71 ± 16</td>
<td>62 ± 25*</td>
<td>68 ± 26</td>
<td>70 ± 24</td>
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<td>E + INO</td>
<td>3.7 ± 0.4</td>
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<td>E + INO</td>
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<td>46 ± 4*</td>
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<td>44 ± 5</td>
<td>46 ± 5*</td>
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Values are means ± SE; $n = 8$ endotoxin-exposed piglets treated with inhaled nitric oxide (INO) only (E + INO) and 8 endotoxin-exposed piglets given INO plus the cyclooxygenase inhibitor (COX-i) diclofenac (E + INO + COX-i). Endotoxin was administered at time 0. *$P < 0.05$ vs. baseline; †$P < 0.05$ vs. the pre-INO value.

Fig. 3. Results of Western blot determination of the protein expression of constitutive COX (COX-1; A) and inducible COX (COX-2; B) in lung tissue of healthy controls ($n = 6$) and piglets exposed to endotoxin alone ($n = 8$) using the enhanced chemiluminescence reagent technique. Top: blots from the lung tissue samples from two piglets of each group; bottom: expression in each group (in arbitrary units (AU)). Values are means ± SD. *$P < 0.05$, difference between groups.

Fig. 4. Results of Western blot determination of the protein expression of COX-1 (A) and COX-2 (B) in lung tissue of piglets exposed to endotoxin alone ($n = 8$), endotoxin combined with INO ($n = 8$), and endotoxin combined with INO plus the COX inhibitor diclofenac ($n = 8$) using the enhanced chemiluminescence reagent technique. Top: blots from the lung tissue samples from three piglets of each group; bottom: expression in each group (in AU). Values are means ± SD. *$P < 0.05$, difference compared with the endotoxin group; †$P < 0.05$, difference between the E + INO and E + INO + COX inhibitor groups.
compared with that in the endotoxin control group (Fig. 5), but it was increased 5 min after INO withdrawal and peaked at 15 min after the withdrawal ($P < 0.05$). It then returned toward the same level as in the endotoxin controls. The pattern was similar when INO was withdrawn after the second trial (Fig. 5). In the endotoxin + INO + COX inhibitor group, the administration of diclofenac decreased the plasma $TxB_2$ concentration, and this decrease reached significance 1 h later (i.e., 4 h after onset of the endotoxin infusion) compared with the value in the endotoxin and endotoxin + INO groups (Fig. 5).

**Concentration of Plasma $PGF_{2\alpha}$**

There was no significant difference in plasma $PGF_{2\alpha}$ between the four groups at baseline, and the plasma level remained stable throughout the study period in the healthy controls (Fig. 6). The plasma $PGF_{2\alpha}$ concentration was increased five- to sixfold 30 min after the start of endotoxin infusion with a further rise over the following 4.5 h of the study. In the endotoxin + INO group, the plasma $PGF_{2\alpha}$ concentration did not change during NO inhalation compared with that in the endotoxin control group (Fig. 6), but it was increased 5 min after INO withdrawal and peaked at 15 min after the withdrawal ($P < 0.05$). It then returned toward the same level as in the endotoxin group. The pattern was similar when INO was withdrawn after the second trial (Fig. 6).

In the endotoxin + INO + COX inhibitor group, the plasma $PGF_{2\alpha}$ significantly decreased after administration of diclofenac compared with the values in the other two groups that received endotoxin ($P < 0.05$), and it remained at the lower level throughout the study period (Fig. 6).

**Concentration of Plasma 6-keto-$PGF_{1\alpha}$**

Under baseline conditions, the plasma concentration of 6-keto-$PGF_{1\alpha}$, a stable metabolite of $PGI_2$, was of the same magnitude in the four groups and remained stable in the healthy controls (Fig. 7). This concentration increased after 2 h of endotoxin infusion, and the increase reached significance at 3 h in the endotoxin and endotoxin + INO groups. In the endotoxin + INO + COX inhibitor group, the plasma 6-keto-$PGF_{1\alpha}$ level showed a decrease after the administration of diclofenac. This decrease reached significance at 4 h of endotoxin infusion compared with the values in the endotoxin and endotoxin + INO groups ($P < 0.05$), and it remained at the lower level throughout the study period (Fig. 7).

However, the increase of plasma 6-keto-$PGF_{1\alpha}$ was significantly lower than the increase in plasma $TxB_2$ and $PGF_{2\alpha}$ in response to endotoxin infusion (Fig. 8).

**DISCUSSION**

In this study, endotoxin infusion upregulated the expression of the inducible COX and caused a marked increase in the plasma concentrations of the prostaglandins $TxB_2$, $PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$. In addition, an upregulation of COX-1 expression by endotoxin and INO, and a further increase in plasma $TxB_2$ and $PGF_{2\alpha}$ after INO withdrawal during endotoxemia, were observed. The combination of INO and a COX inhibitor
eliminated the increase in prostanoids caused by endotoxin and INO withdrawal and blocked the rebound response. These results support our hypothesis that a short rebound response is related to products synthesized via a COX-dependent pathway.

Endotoxin Model and INO

The initial phase of endotoxin exposure (0–2 h) seemed to be linked to the release of COX-synthesized products, in particular TxA2, a finding in accordance with previous studies.

Fig. 6. Plasma PGF2α concentration in piglets exposed to endotoxin alone (n = 8), endotoxin combined with INO (n = 8), and endotoxin combined with INO plus the COX inhibitor diclofenac (n = 8) in the 5-h study period. Two challenges with INO were made both in the E + INO and E + INO + COX inhibitor groups. The values in healthy control piglets are shown for comparison. Values are means ± SD. *P < 0.05 vs. baseline; †P < 0.05 vs. the pre-INO value; ‡P < 0.05, difference between the E + INO + COX inhibitor and E + INO groups.

Fig. 7. Plasma 6-keto-PGF1α concentration in piglets exposed to endotoxin alone (n = 8), endotoxin followed by INO (n = 8), and endotoxin followed by INO plus the COX inhibitor diclofenac (n = 8) in the 5-h study period. Two challenges with INO were made both in the E + INO and E + INO + COX inhibitor groups. The values in healthy control piglets are shown for comparison. Values are means ± SD. *P < 0.05 vs. baseline; †P < 0.05, difference between the E + INO + COX inhibitor and E + INO groups.
with results of others (16, 28). We also observed a time-dependent increase in the plasma level of TxB2 and PGF2α, and a less pronounced increase in 6-keto-PGF1α (the metabolite of PGI2) on endotoxin exposure. This may reflect a time- and dose-dependent upregulation of COX-2 by endotoxin (4, 7, 25). Moreover, the findings suggest that the increased PGF2α, TxB2, and PGI2 participated in the second (2.5–5 h) prolonged increase in MPAP and decrease in PaO2 and in the fall in systemic blood pressure.

NO inhalation did not alter the plasma prostanoid levels, but TxB2 and PGF2α showed additional increases after INO withdrawal, suggesting that they may play a role in the rebound response on discontinuation of INO. It may also be hypothesized that the increase in prostanoids contributed to the poorer response to the second INO trial and seemingly to the stronger rebound reaction after the second INO withdrawal, but this requires further study.

**COX and Its Products**

COX-1 is constitutively expressed in virtually all tissues and is responsible for the basal production of prostanoids for the maintenance of normal renal and gastric function, vascular hemostasis, and the autocrine response to circulating hormones (7). COX-2 is triggered by many factors, including endotoxin and cytokines, to release large amounts of prostanoids in inflammatory states (4, 7), but it is also constitutively expressed in some organs at a low level (7).

We found that both COX-1 and COX-2 proteins are expressed in the healthy piglet lung and that endotoxin increases the COX-2 expression. This fits with previously demonstrated changes in mRNA (4, 18). Cross-talk between exogenous NO and COX has been observed, with exogenous NO activating COX-1, but the effects of NO on COX-2 are controversial (8, 24, 27). In the present study, INO during endotoxemia increased the COX-1 protein level compared with that in the healthy controls, but did not further increase the COX-2 protein level beyond that reached with endotoxin alone. As anticipated, strong coupling between the expression and activity of COX-2 has been shown (7), and we assume that this was also the case in the present study, although we did not measure COX activity.

Diclofenac is a nonselective, competitive, reversible inhibitor. It produces COX inhibition by competing with the substrate arachidonic acid for the active site of the enzyme (11, 15, 26). Our results show that it also decreases lung COX protein expression. Whether this decrease is caused by a downregulation of COX synthesis on a transcriptional or a posttranscriptional level, or by increased decomposition of COX protein, is not clear. We assume the decrease may be caused by increased breakdown of COX, after binding of its competitive inhibitor diclofenac.

Illogically, diclofenac also blocked the release of vasodilator prostanoids, but our results and those of others (16) indicate that the endotoxin-induced increases in the vasconstrictors TxA2 and PGF2α are much stronger than the increase in the vasodilator PGI2 (Fig. 8). This may suggest that, in this study, diclofenac mainly blocked the prostanoids that acted as vasoconstrictors.

**Rebound Phenomenon After INO Withdrawal**

The rebound phenomenon after INO withdrawal has frequently been assumed to be due to reduction of endogenous NO production by a negative feedback effect of NO inhalation (2, 5, 9). However, in a previous study (5) on endotoxic piglets, there were indications that besides the decrease in endogenous NO production, increased activity of the vasoconstrictor ET-1 and possibly of other vasoconstrictor substances might be important in the rebound, in agreement with findings in a lamb model (21). The present study also proved that prostanooids play a role in the rebound reaction to INO withdrawal in our model.

A clinical rebound phenomenon is reported after discontinuation of long-term NO inhalation (1, 10, 17). Whether the mechanisms of the clinical rebound are similar to those of the rebound reaction after a short period (30 min) of NO inhalation, as demonstrated here, were not tested in the present study but remains a possibility. Also, the study was performed in an endotoxin model, and whether a different lung damage model would yield the same results is not yet known.
The peak increase of plasma TxB\textsubscript{2} and PGF\textsubscript{2\alpha} levels after INO withdrawal appeared 10 min after the peak of the rebound reaction in the present study. Whether this was an effect of the time-dependent conversion of TxA\textsubscript{2} to the metabolite TxB\textsubscript{2} or reflected involvement of other vasoactive substances such as ET-1 is not clear. The conversion of TxA\textsubscript{2} to TxB\textsubscript{2} takes only about a minute (23). We therefore believe in the latter possibility and assume that the increases in TxB\textsubscript{2} and PGF\textsubscript{2\alpha} after INO withdrawal are related to the increase in ET-1 during NO inhalation (5).

ET-1 is an important releaser of TxA\textsubscript{2} and PGF\textsubscript{2\alpha}. When ET-1 binds to its receptors, it mediates vasoconstriction by two pathways: 1) it changes intracellular calcium; and 2) it stimulates secondary release of the vasoconstrictors TxA\textsubscript{2} and PGF\textsubscript{2\alpha} (21). This second pathway seems to be a major mechanism in the pulmonary vasoconstriction induced by ET-1 injection (13). We have previously shown that the lung tissue expression and plasma concentration of ET-1 increase during INO in endotoxic piglets (5). INO seems to antagonize ET-1 binding to its receptor (12), and this antagonistic effect might cease as soon as INO is discontinued. The increase in binding of the available ET-1 to its receptor thus mimics an ET-1 injection. Therefore, the rebound could be blocked both by a COX inhibitor and by a nonselective ET-1 antagonist (21).

We conclude that the combination of INO with a COX inhibitor blocks the rebound reaction to acute INO withdrawal during endotoxaemia. This may have important clinical implications.

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