

# Protective Role of Pulmonary Nitric Oxide in the Acute Phase of Endotoxemia in Rats

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**Abstract**—We present for the first time direct continuous assay of NO concentration (porphyrinic sensor) in the lung parenchyma of Sprague-Dawley rats in vivo during endotoxemia. Intravenous infusion of lipopolysaccharide (LPS, 2 mg · kg<sup>-1</sup> · min<sup>-1</sup> for 10 minutes) stimulated an acute burst of NO from constitutive NO synthase (NOS) that peaked 10 to 15 minutes after the start of LPS infusion, mirroring a coincident peak drop in arterial pressure. NO concentration declined over the next hour to twice above pre-LPS infusion NO levels, where it remained until the rats died, 5 to 6 hours after LPS infusion. The chronic drop in arterial pressure observed from 70 minutes to 6 hours after the start of LPS infusion was not convincingly mirrored by a chronic increase in NO concentration, even though indirect NO assay (Griess method, assaying NO decay products NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup>) showed that NO production was increasing as a result of continuous NO release by inducible NOS. A NOS inhibitor, N<sup>ω</sup>-nitro-L-arginine (L-NNA, 10 mg/kg IV) injected 45 minutes before LPS infusion, resulted in sudden death accompanied by macroscopically/microscopically diagnosed symptoms similar to acute respiratory distress syndrome <25 minutes after the start of LPS infusion. Pharmacological analysis of this L-NNA+LPS model by replacing L-NNA with 1-amino-2-hydroxy-guanidine (selective inhibitor of inducible NOS) or by pretreatment with S-nitroso-N-acetyl-penicillamine (NO donor), camonagrel (thromboxane synthase inhibitor), or WEB2170 (platelet-activating factor receptor antagonist) indicated that in the early acute phase of endotoxemia, LPS stimulated the production of cytoprotective NO, cytotoxic thromboxane A<sub>2</sub>, and platelet-activating factor. (*Circ Res.* 1998;82:819-827.)

**Key Words:** lung ■ microcirculation ■ shock

It has been observed in rats in vivo that LPS-stimulated/induced hypotension is composed of an early acute phase, peaking at 15 minutes, and a late chronic phase, beginning 70 minutes after LPS injection until death several hours later.<sup>1</sup> EDK mediates the early phase of LPS hypotension, and PAF mediates both phases.<sup>1</sup> Furthermore, on the basis of mammalian endothelial cell culture studies, it has been suggested that the LPS-stimulated release of EDK and the subsequent stimulation of cNOS and constitutive COX (COX-1) result in enhanced production of NO and PGI<sub>2</sub>, which may ultimately result in the early phase of endotoxin-stimulated hypotension in intact mammals.<sup>2</sup>

In addition to contributing to the early phase of endotoxin-stimulated hypotension, intermittent bursts of NO generated by cNOS are essential for the maintenance of vascular tone.<sup>1</sup> A continuous blast of NO generated by iNOS is important for nonspecific cytostatic host defense against tumor cells and pathogens and may be responsible for the late chronic phase of endotoxemic hypotension.<sup>1</sup> The signal to transcribe/translate the gene sequence for iNOS can be transmitted by several endotoxins, including LPS.<sup>3</sup> In rodents, iNOS expression occurs mainly in macrophages, but it also occurs in several other cell types, including endothelial cells.<sup>4</sup>

Clinically, L-NNA or a number of other L-arginine analogues, which are unable to differentiate between the inhibition of iNOS and cNOS, were used for the treatment of septic shock patients with encouraging results.<sup>5</sup> Soon, it became apparent that the timing of NOS-inhibitor treatment was critically important; LPS-induced vascular injury could be enhanced if NOS-inhibitor treatment was followed by LPS treatment or diminished if the order of treatment was reversed.<sup>6</sup> Taken together, these experimental and clinical studies are forming a new but still incomplete picture of the pathogenesis of endotoxemia, with the possibility of an important role for cNOS as well as iNOS.

We hypothesized that one of the crucial factors in the development of endotoxemia and septic shock is the dysfunction of the endothelium. A dysfunctional endothelium can generate reactive species like superoxide (O<sub>2</sub><sup>-</sup>), which can rapidly consume NO.<sup>7,8</sup> Therefore, the net NO concentration (that portion of the total amount of NO generated that can freely diffuse to targets that are several cell diameters from its source) may be significantly different from the concentration of NO measured indirectly, depending on NO decay products (NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup>) or NOS coproduct (L-citrulline) production.<sup>9</sup> In

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**Selected Abbreviations and Acronyms**

AGD	= 1-amino-2-hydroxy-guanidine
ARDS	= acute respiratory distress syndrome
cNOS	= constitutive NOS
COX	= cyclooxygenase
EDK	= endothelium-derived kinin
iNOS	= induced NOS
L-NNA	= <i>N</i> <sup>ω</sup> -nitro-L-arginine
LPS	= lipopolysaccharide
MAP	= mean arterial blood pressure
MAPTAM	= 1,2- <i>bis</i> -methylaminophenoxy ethane- <i>N,N,N',N'</i> -tetraacetoxymethyl acetate
NOS	= NO synthase
PAF	= platelet-activating factor
PGI <sub>2</sub>	= prostacyclin
SNAP	= <i>S</i> -nitroso- <i>N</i> -acetyl-penicillamine
SSCE	= silver/silver chloride electrode
TMHPPNi	= nickel (II) tetrakis(3-methoxy-4- hydroxyphenyl)porphyrin
TxA	= thromboxane A

the present study, we report for the first time the direct continuous measurement of the local change in NO concentration in rat lung parenchyma during endotoxemia. All measurements were performed *in vivo*, which eliminates the approximations of *in vitro* measurement in cultured endothelial cells or *ex vivo* tissue samples used in most of the previous studies.

To further investigate why the early acute phase of hypotension is perfectly mirrored by simultaneous acute increases of NO concentration (as expected) but, paradoxically, the late chronic phase of endotoxemia is not, we resorted to a pharmacological analysis of our experimental model of endotoxemia. We separately studied eight different known enzyme inhibitors, antagonists, or products suspected to be involved in the pathogenesis of endotoxemia and observed their effects on mean survival time, MAP, and the development of microscopically/macrospectically observable lung injury.

**Materials and Methods****Animals**

Male Sprague-Dawley rats (Charles River, Portage, Mich; 180 to 250 g) were maintained at (22±1°C) with a 12/12-hour light/dark cycle and were allowed water and standard rat chow *ad libitum*.

**Reagents**

The endotoxin LPS (*Escherichia coli* serotype 0127:B8, Sigma Chemical Co) was dissolved in saline. Then, guided by previously described<sup>2,6</sup> LPS dose-response curves, we administered LPS as an intravenous infusion (2 mg · kg<sup>-1</sup> · min<sup>-1</sup> for 10 minutes; total dose, 20 mg/kg). The nonspecific cNOS or iNOS inhibitor L-NNA was purchased from Sigma and dissolved in 0.1 mol/L phosphate buffer (pH 7.4). Then, guided by previously described<sup>6</sup> L-NNA dose-response curves, we injected L-NNA as an intravenous bolus (10 mg/kg). An iNOS specific inhibitor, AGD, was a gift from Prof C. Thiemermann (London, England); AGD was dissolved in saline and injected as an intravenous bolus (15 mg/kg).

The NO donor SNAP was synthesized from *N*-acetyl-penicillamine (Sigma), as previously described,<sup>10</sup> in our laboratory by Dr Z. Michalska; SNAP was dissolved in saline and intravenously infused over 30 minutes (10 μg · kg<sup>-1</sup> · min<sup>-1</sup>). Camonagrel, a thromboxane

synthase inhibitor, was a gift from Ferrer International, Barcelona, Spain; camonagrel dissolved in saline was injected as an intravenous bolus (25 mg/kg). Meloxicam, a specific COX-2 inhibitor, was a gift from Boehringer, Ingelheim, Germany; meloxicam was dissolved in 0.1 mol/L sodium bicarbonate, adjusted to pH 7.4, and injected as an intravenous bolus (1 or 5 mg/kg). WEB2170, a PAF receptor antagonist, was a gift from Boehringer, Ingelheim; WEB2170 was dissolved in a 1:20 (vol/vol) mixture of 1 mol/L HCl and saline, adjusted to pH 7.4, and injected as an intravenous bolus (5 mg/kg). Iloprost, a stable PGI<sub>2</sub> receptor agonist, was purchased from Schering AG; iloprost was dissolved in saline and intravenously infused over 30 minutes (0.3 μg · kg<sup>-1</sup> · min<sup>-1</sup>). Acetylsalicylic acid (aspirin), a COX-1 and -2 inhibitor, was bought from Polfa; aspirin was dissolved in 0.1 mol/L sodium bicarbonate, adjusted to pH 7.4, and injected as an intravenous bolus (50 mg/kg).

Ca<sup>2+</sup> chelators EGTA (Sigma) and MAPTAM (Molecular Probes) were used to confirm Ca<sup>2+</sup> dependence. *Ex vivo* lung sections were immersed in HBSS (Sigma) at pH 7.4 and 37°C containing EGTA and MAPTAM, both at a concentration of 200 μmol/L.

**Surgery and Instrumentation**

Under thiopentone anesthesia (Thiopental, 120 mg/kg IP, Vuab) rats were intubated intratracheally and ventilated with room air (Ugo Basile 7025 rodent ventilator; tidal volume, 10 mL/kg at 50 breaths/min). Polyurethane catheters were inserted into the left jugular and left femoral veins for drug administration. Another catheter was placed in the right carotid artery and connected to a pressure transducer for continuous recording of MAP. A Ventlon-2 22-gauge catheter was inserted into the pulmonary vein for intermittent blood sampling. Cardiac output measurements were obtained using a Doppler flow probe (Transonics). A thoracotomy was performed to place the probe at the aortic root. A standard method using a gravity-fed constant-flow setup was used to calibrate the signal from the Doppler flow probe. After these procedures were complete, animals were allowed at least 15 minutes to stabilize their MAPs.

**Experimental Protocol**

For pharmacological analysis, 66 rats were used. The rats were divided randomly into 11 groups (n=6 rats each). Then each group was subjected to one of the following intravenous regimens: LPS group, LPS infusion alone; LPS+L-NNA group, injection of L-NNA 45 minutes after the start of LPS infusion; L-NNA+LPS group, injection of L-NNA 45 minutes before the start of LPS infusion; AGD+LPS group, injection of AGD 45 minutes before the start of LPS infusion; L-NNA+SNAP+LPS group, infusion of SNAP for 30 minutes starting 30 minutes after the injection of L-NNA and 15 minutes before the start of LPS infusion; L-NNA+aspirin+LPS group, injection of aspirin 30 minutes after L-NNA and 15 minutes before LPS infusion; L-NNA+meloxicam+LPS group, two-dose injection of meloxicam (see "Reagents") using the same time regimen as described for aspirin; L-NNA+camonagrel+LPS group, injection of camonagrel as described for aspirin; L-NNA+WEB2170+LPS group, injection of WEB2170 as described for aspirin; and L-NNA+iloprost+LPS group, infusion of iloprost over the same time regimen as for SNAP.

In the above groups, the early (10-minute) and the late (3-hour) phases of hypotensive response to LPS and the survival times after the infusion of LPS were evaluated and compared statistically with those for the LPS group or the L-NNA+LPS group.

**Pathological Examination**

Macroscopic/microscopic analysis was performed in 24 rats. Three rats were decapitated 20 minutes after LPS infusion (early LPS shock). In due course, 6 rats were allowed to die 5 to 6 hours after LPS infusion (lethal LPS shock), 6 rats died 5 to 6 hours after LPS+L-NNA administration (lethal LPS shock treated with L-NNA), and 6 rats died 20 to 30 minutes after L-NNA+LPS administration (experimental ARDS). Three rats that received L-NNA alone and were decapitated 65 minutes later served as controls. Subsequent routine pathological examination of internal

organs of experimental ARDS rats revealed that the most significant changes occurred in the lung. Accordingly, in all other groups ( $n=3$  in each group), detailed histological examination was restricted to the lung.

### Morphological Evaluation

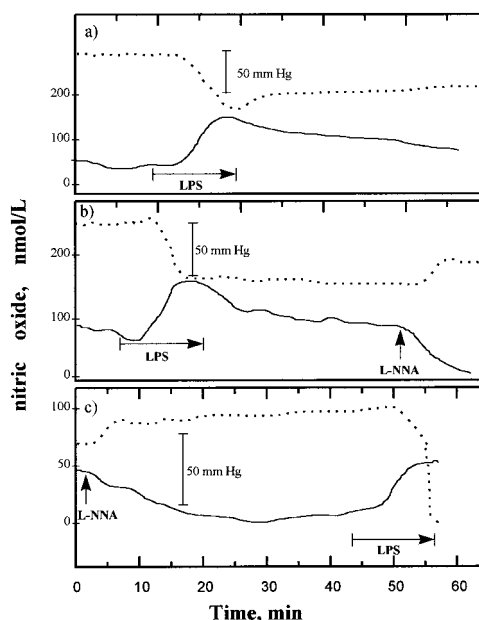
Lungs were fixed in 10% buffered formalin (pH 7.5) overnight at room temperature and then washed for 2 hours, dehydrated through a graded ethanol series (50% for 30 minutes, 60% for 60 minutes, 80% for 2 hours, 96% for 30 minutes, and 100% for 30 minutes), placed in xylene (three times for 15 minutes), embedded in paraffin (58°C for 3 hours), and cast into blocks. Tissue samples were cut into 3- to 5- $\mu\text{m}$ -thick sections by microtome and placed on glass slides. Before staining, paraffin slides were heated to 60°C for 30 to 45 minutes, paraffin was removed by placing the slides in fresh xylene (three times for 10 minutes), and then the slides were rehydrated following standard procedures (ethanol at 100% twice for 5 minutes, at 70% for 2 minutes, and at 50% for 2 minutes) and finally dipped into PBS (pH 7.4). Routine hematoxylin and eosin stainings were performed. Weigert staining was used to investigate fibrin deposition in the sections.<sup>11</sup> Light-microscopic examination was performed with photographic documentation.

### In Vivo Measurement of NO in Rat Lung Parenchyma

To reduce bleeding and piezoelectric noise and to maximize the NO signal from tissues, a catheter protecting the porphyrinic NO sensor was constructed from the needle of an 0.8-mm-gauge 2.5-mm-length intravenous catheter/needle unit (Angiocath, Becton Dickinson).<sup>12</sup> The needle was roughened around the length of the shaft, truncated near the tip, and then polished flat so that it was 4 mm shorter than its 0.8-mm-gauge protective catheter. A bundle of seven carbon fibers (6- $\mu\text{m}$  diameter, 55 mm long, 12  $\Omega$  cm, Amoco Performance Products) was threaded inside the hollow, truncated, 0.8-mm-gauge needle. The fiber bundle was protruded 5 mm from the tip with the aid of a 0.1-mm-diameter 100-mm-long copper wire coated with silver-conducting epoxy (A.I. Technology) inserted into the opposite end. The exterior of the abraded and truncated needle was coated with nonconductive epoxy (2-TON Clear Epoxy, Devcon), then rolled to ensure a uniform thin coat, and allowed to dry 2 hours. This insulating procedure was repeated three times. After it was cured for 24 hours, the protruding carbon fiber bundle was covered with conductive polymeric film and Nafion (Aldrich) as described below.

The carbon fiber tip was made more sensitive to NO and less sensitive to potential interference by the cyclic voltammetric deposition (for 10 cycles,  $-0.20$  to  $1.00$  V and back, at  $100$  mV/s) of a highly conductive polymeric porphyrin from a solution of monomeric  $0.25$  mmol/L TMHPPNi in  $0.1$  mol/L NaOH, all under nitrogen.<sup>13</sup> The multiple scanning of potentials from  $-0.20$  to  $1.00$  V (versus SSCE) was used for uniform deposition of several monolayers (surface coverage,  $2$  to  $5 \times 10^{-9}$  mol/cm<sup>2</sup>) of polymeric TMHPPNi. Dip coating of the carbon fiber tip with dried polymeric TMHPPNi (three times for 5 seconds) in 1% (wt/vol) Nafion (Aldrich) in ethanol, after drying in a vacuum oven for 1 hour at  $40^\circ\text{C}$ , produced a thin anionic film. This anionic film repelled or retarded charged species while allowing small neutral and hydrophobic molecules like NO access to the underlying electroactive surface of the polymeric porphyrin. In vivo measurement of NO was performed using amperometry (current change monitored in time at a constant potential of  $0.65$  V versus SSCE). Amperometry and cyclic voltammetric deposition (see above) were performed using a Princeton Applied Research model 273 voltammetric analyzer interfaced with an IBM AT 80486 computer plus data-acquisition and control software; a three-electrode mode, consisting of a porphyrinic sensor (working electrode), a platinum wire (auxiliary electrode), and an SSCE (reference electrode), was used.

In order to implant the porphyrinic NO sensor in the lung, the tip of the left inferior lobe (posterior basal) was pierced with a standard 0.8-mm-gauge angiocatheter needle (sheathed by its catheter, with  $4 \times 50$ - $\mu\text{m}$  ventilation holes near the tip). The catheter/needle unit was advanced 3 to 5 mm into the lung parenchyma. The catheter



**Figure 1.** Direct in vivo measurement of NO release in lung parenchyma (solid line) with simultaneous measurement of the change of MAP (dotted line) after infusion of  $2 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  LPS for 10 minutes (a), after infusion of LPS followed by a bolus of  $10 \text{ mg/kg}$  L-NNA (b), and after a bolus of L-NNA followed by an infusion of LPS (c).

position was secured, and the placement needle was removed and quickly replaced by an NO sensor. Since the lung formed a seal around the catheter and since the sensor diameter was the same size as the internal diameter of the catheter, pneumothorax was prevented during in vivo measurements of NO in the lung parenchyma. A platinum wire counterelectrode and SSCE were placed in contact with adjacent tissue. The porphyrinic sensor had a response time of 0.1 milliseconds at micromolar NO concentrations and 10 milliseconds at the detection limit of  $1$  nmol/L. Linear calibration curves were constructed for each sensor from  $2 \times 10^{-9}$  to  $2 \times 10^{-5}$  mol/L NO, before and after in vivo measurements, using aliquots of saturated NO prepared as described.<sup>14</sup> For comparison, the NO production was indirectly estimated by sampling the pulmonary vein intermittently and assaying for the NO decay products  $\text{NO}_2^-/\text{NO}_3^-$  in blood plasma by the Griess method.<sup>9</sup>

### Statistics

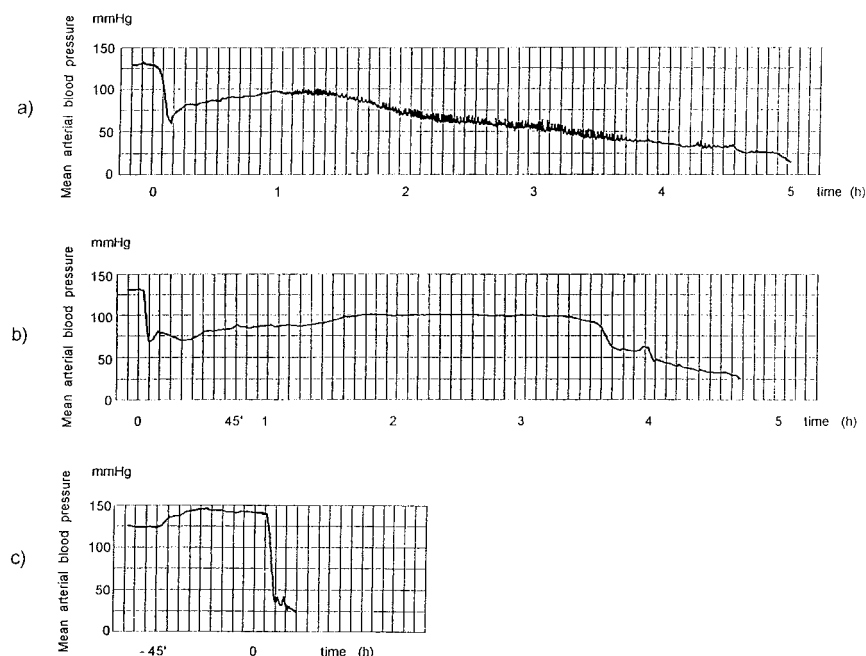
MAP (mm Hg) and survival time (minutes) are expressed as arithmetic mean  $\pm$  SD. Differences between the studied groups and reference group (ie, LPS group) were evaluated by one-way ANOVA with Bartlett's test for equal variances followed by the Dunnett multiple comparison test (GraphPad Prism Statistical Package). Two-tailed paired and unpaired Student  $t$  tests, survival probability, and graphs were done with Sigma Plot 3.06 for Windows (A00517, Jandel Scientific GmbH). Survival curves were plotted using the method of Kaplan and Meier, and comparisons of two survival curves were done by two-tailed nonparametric log-rank test.<sup>15-17</sup> A value of  $P < .05$  was considered significant.

## Results

### NO Release

Figure 1 shows typical amperometric curves (current calibrated as NO concentration versus time) measured continuously in vivo with a porphyrinic sensor placed 2 to 3 mm in the rat lung parenchyma. Changes in MAP were monitored simultaneously. A short time ( $2.2 \pm 0.2$  minutes) after starting the infusion of LPS ( $2 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  for 10 minutes), an





**Figure 2.** Tracings of MAP in rats subjected to lethal LPS shock (a), in rats subjected to lethal LPS shock that were treated with L-NNA (b), and in rats that demonstrated symptoms quite similar to ARDS, which developed in response to LPS after blocking NO synthesis by L-NNA (c).

increase of NO concentration from its basal level of  $60 \pm 22$  nmol/L was observed (Figure 1a). The average rate of NO concentration increase was  $17.1 \pm 3$  nmol  $\cdot$  L $^{-1}$   $\cdot$  min $^{-1}$ . The concentration of NO reached a peak of  $174 \pm 34$  nmol/L at  $8.8 \pm 0.7$  minutes after the start of LPS infusion, which persisted for  $50 \pm 10$  seconds and then decayed at a rate of  $2.7 \pm 0.2$  nmol  $\cdot$  L $^{-1}$   $\cdot$  min $^{-1}$ . The changes of NO concentration mirrored changes of MAP. The maximal drop of pressure ( $68 \pm 8$  mm Hg) was observed at the peak concentration of NO. Administration of L-NNA 45 minutes after the start of the infusion of LPS caused rapid decrease of NO concentration (Figure 1b). In the L-NNA+LPS group, infusion of L-NNA decreased basal NO concentration by  $45 \pm 15$  nmol/L, with a concomitant increase of MAP. LPS, administered 42 minutes after L-NNA, increased NO concentration by  $47 \pm 12$  nmol/L (which is only  $\approx 41\%$  of that released in the absence of L-NNA) and brought it approximately to the initial basal level (Figure 1c). LPS decreased MAP by  $78 \pm 8$  mm Hg.

It is interesting to note that LPS stimulates a release of NO in the very early stage during its infusion, and this release is presumably stimulated by a Ca $^{2+}$ -dependent cNOS. Ca $^{2+}$ -dependent NO release stimulated by LPS, in the early acute phase of endotoxemia, was confirmed in separate experiments by ex vivo measurement of NO in lung tissue incubated with Ca $^{2+}$ -chelating agents. [Ca $^{2+}$ ] $_o$  and [Ca $^{2+}$ ] $_i$  were blocked by spiking the perfusate with the [Ca $^{2+}$ ] $_o$  chelator EGTA and the membrane-permeable [Ca $^{2+}$ ] $_i$  chelator MAPTAM. In the presence of EGTA and MAPTAM, the release of NO stimulated by LPS decreased by 95% (from  $174 \pm 43$  to  $9 \pm 3$  nmol/L in the absence and presence of Ca $^{2+}$  chelating agents, respectively).

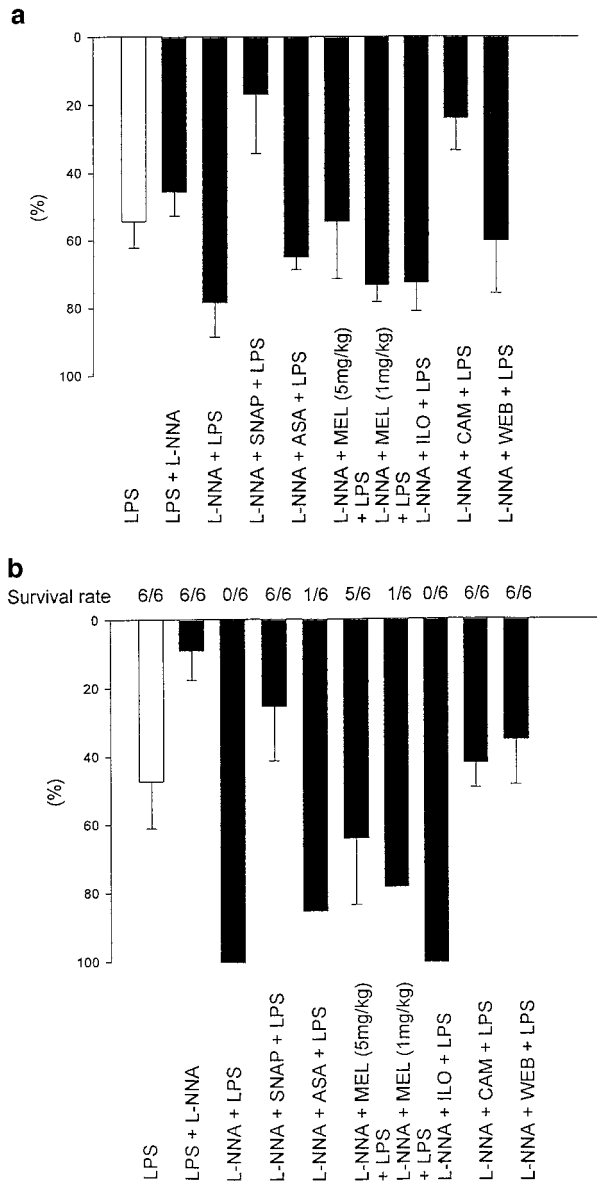
At the start of the late chronic phase of endotoxemia ( $\approx 1$  hour after the start of the LPS infusion), the NO concentration stabilized at a level similar to that observed after the end of the early acute phase of endotoxemia, and only slight changes of this NO concentration were observed during the next 5 hours of

endotoxemia. These NO concentrations were  $60 \pm 4$ ,  $68 \pm 3$ ,  $70 \pm 4$ ,  $67 \pm 4$ , and  $65 \pm 3$  nmol/L above the basal level for 1, 2, 3, 4, and 6 hours, respectively. However, the estimated concentration of NO measured indirectly by NO $_2^-$ /NO $_3^-$  assay in blood plasma by the Griess method<sup>9</sup> increased linearly within this time interval ( $1.3 \pm 0.1$ ,  $1.7 \pm 0.2$ ,  $2.4 \pm 0.2$ ,  $3.6 \pm 0.3$ , and  $5.8$   $\mu$ mol/L for 1, 2, 3, 4, and 6 hours, respectively).

### Early Response of MAP

Fifteen minutes after the surgery, when MAP stabilized, control values of MAP did not differ significantly between the 11 groups studied, varying from  $112 \pm 12$  to  $129 \pm 12$  mm Hg. However, differences between groups appeared in both the early phase ( $\approx 10$  minutes) and late phase (3 hours) of hypotension after the infusion of LPS. The early-phase MAP fell after LPS stimulation by 54.8% ( $P < .01$ ) and slowly evolved into the late irreversible and vasoplegic phase (Figure 2a). After the treatment with L-NNA (the LPS+L-NNA group) had prevented the early transition of both phases of hypotension (but only up to a certain point,  $\approx 3.5$  hours), then lethal hypotension developed abruptly (Figure 2b). Pretreatment with L-NNA (the L-NNA+LPS group) resulted in an expected moderate rise in MAP from  $126 \pm 17$  to  $148 \pm 23$  mm Hg ( $P < .001$ , Figure 2c). Subsequent LPS treatment produced a sudden lethal fall in MAP ( $78 \pm 10\%$ ,  $P < .01$  versus the LPS group) accompanied by symptoms similar to ARDS. L-NNA pretreatment caused a 30% drop in cardiac output (from  $52 \pm 3$  to  $36 \pm 2$  mL/min,  $n=6$ ). Administration of AGD instead of L-NNA did not cause a significant change in MAP (from  $124 \pm 20$  to  $122 \pm 16$  mm Hg) and had no significant effect on cardiac output.

The profound early-phase drop of MAP in the L-NNA+LPS group could not be effectively prevented by additional treatment (Figure 3a) with aspirin (a  $65 \pm 4\%$  fall in MAP,  $P=NS$ ), with meloxicam at 1 mg/kg (a  $73 \pm 5\%$  fall in



**Figure 3.** Effect of treatment with SNAP ( $10 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  for 30 minutes), acetylsalicylic acid (ASA, 50 mg/kg), meloxicam (MEL, 1 mg/kg and 5 mg/kg), iloprost (ILO,  $0.3 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  for 30 minutes), camonagrel (CAM, 25 mg/kg), or WEB2170 (WEB, 5 mg/kg) on the fall of MAP in rats with experimental ARDS that received a 10-minute infusion of LPS ( $2 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) after the pretreatment with L-NNA ( $10 \text{ mg/kg}$ ). The experimental groups ( $n=6$ ) are indicated at the bottom of each figure. Columns represent percent fall in MAP; bars, SD. a, MAP was measured at the end of LPS infusion. b, MAP was measured 3 hours after the LPS treatment. Figures at the top ( $x/6$ ) represent survival rate at the same time.

MAP,  $P<.05$ ) or 5 mg/kg (a  $54 \pm 17\%$  fall in MAP,  $P=\text{NS}$ ), with iloprost (a  $72 \pm 8\%$  fall in MAP,  $P=\text{NS}$ ), or with WEB2170 (a  $60 \pm 15\%$  fall in MAP,  $P=\text{NS}$ ). The drugs that prevented the LPS-stimulated early-phase hypotension aggravated by L-NNA were SNAP and camonagrel.

Additional treatment with camonagrel diminished the early hypotensive response to LPS (MAP fell only by  $24 \pm 10\%$  [ $P<.01$ ]; Figure 3a). Additional treatment with SNAP appeared to be even more effective; however, because of the

hypotensive action of SNAP, interpretation of the result is difficult (Figure 4). During the infusion of SNAP, MAP fell from  $142 \pm 15$  to  $69 \pm 31$  mm Hg. LPS did not really add much to this hypotension (fall to  $57 \pm 24$  mm Hg); however, the cessation of SNAP infusion immediately brought MAP to  $115 \pm 22$  mm Hg; ie, the fall in MAP appeared to be only  $17 \pm 17\%$  ( $P<.01$ , Figure 3a).

### Late Responses of MAP and Survival Time

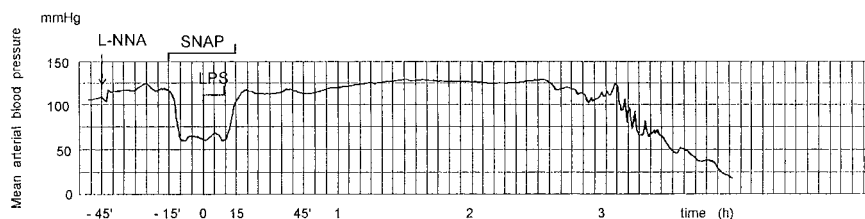
Three hours after the infusion of LPS, all 6 rats of the LPS group were still alive, although their MAP fell by  $47 \pm 14\%$  (Figures 2a and 3b), and their responses to hypertensive action of noradrenaline ( $0.5 \mu\text{g/kg}$  IV) were reduced by 55% (data not shown). The posttreatment with L-NNA prevented this delayed hypotensive effect of LPS (Figures 2b and 3b): MAP fell by  $9 \pm 9\%$  ( $P<.01$ ), and cardiac output decreased by  $14 \pm 2\%$  ( $n=6$ ). However, the survival time showed only a slight increase compared with the LPS group, ie,  $314 \pm 77$  versus  $278 \pm 49$  minutes ( $P=\text{NS}$ , Figures 3b and 5a).

In contrast to the LPS+L-NNA group, in the L-NNA+LPS group the survival time shortened from  $278 \pm 49$  to  $22 \pm 5$  minutes ( $\chi^2=12.09$ ,  $df=1$ , and  $P<.0005$ ; Figure 5a). However, pretreatment with AGD, instead of L-NNA, significantly increased mean survival time ( $\chi^2=4.147$ ,  $df=1$ , and  $P<.05$  versus the LPS group). The L-NNA+iloprost+LPS group was similar to the L-NNA+LPS group, with a mean survival time of  $48 \pm 41$  minutes. Iloprost (stable  $\text{PGI}_2$  receptor agonist) showed only a slight initial hypotensive effect of its own, only lowering MAP from  $140 \pm 12$  to  $133 \pm 12$  mm Hg (Figure 3b).

Cyclooxygenase inhibitors such as aspirin and meloxicam at two dose levels inhibited the L-NNA+LPS-induced mortality only partially (Figure 5b). Three hours after the LPS administration, survival rate in the aspirin and meloxicam ( $1 \text{ mg/kg}$ )–pretreated groups was 1/6. In the surviving rats (one in each group), MAP decreased  $\approx 80\%$  compared with the control value. In the meloxicam ( $5 \text{ mg/kg}$ )–pretreated group, survival rate at 3 hours was 5/6; however, MAP was lower than its initial value by  $\approx 70\%$  ( $P<.05$ , Figures 3b and 5b).

Thromboxane synthase inhibition (L-NNA+camonagrel +LPS group) or PAF receptor blockage (L-NNA+WEB2170+LPS group) resulted in the elongation of survival time to  $298 \pm 68$  and  $311 \pm 19$  minutes, respectively, which was close to that of the LPS group ( $278 \pm 49$  minutes). Furthermore, these pharmacological interventions “normalized” late hypotensive responses (after 3 hours) within the L-NNA+LPS group to the level of the LPS group (Figure 5c). Indeed, there was a  $42 \pm 7\%$  fall in MAP in the L-NNA+camonagrel+LPS group ( $P=\text{NS}$ ) and a  $35 \pm 13\%$  fall in MAP in the L-NNA+WEB2170+LPS group ( $P=\text{NS}$ ) compared with a  $47 \pm 14\%$  fall in MAP in the LPS group (Figures 3b and 5c).

Interestingly, the substitution of endogenous NO with exogenous NO released from SNAP produced the most pronounced protection against the late hypotensive action of LPS in the L-NNA+LPS system. In the L-NNA+SNAP+LPS group, the fall in MAP, 3 hours after the administration of LPS, was  $25 \pm 16\%$  ( $P=\text{NS}$ , Figure 3b). At the same time, SNAP elongated the survival time from 22.5 minutes in the



**Figure 4.** Tracing of MAP in a rat that, after injection of L-NNA ( $10 \text{ mg/kg IV}$ ), was infused with SNAP ( $10 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  for 30 minutes). The prominent hypotensive effect of SNAP hardly allowed the superimposed infusion of LPS ( $2 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  for 10 minutes) to show its early hypotensive phase of action. It probably did not appear at all, since withdrawal of the SNAP infusion caused an immediate leveling of MAP to its preinfusion value for the next 2.5 hours.

L-NNA+LPS group to  $287 \pm 45$  minutes in the L-NNA +SNAP+LPS group (Figures 4 and 5d).

### Morphological Findings

Lungs from rats subjected to early LPS shock did not differ macroscopically from control lungs; however, congestion and accumulation of granulocytes in alveolar capillaries and small vessels were seen microscopically (Figure 6a). In rats that were allowed to die in due course, 5 to 6 hours after LPS, autopsies revealed patchy hemorrhages on the surface of their lungs (Figure 6b). Histological analysis showed features of advanced inflammation with marked congestion of the alveolar capillaries. Weigert staining revealed fibrin deposition inside the small vessels and in the perivascular edema. Fibrin-rich edema fluid was focally distributed in the interstitium. Some of alveolar walls were thickened, particularly in the subpleural region. There was also evidence of patchy alveolar edema.

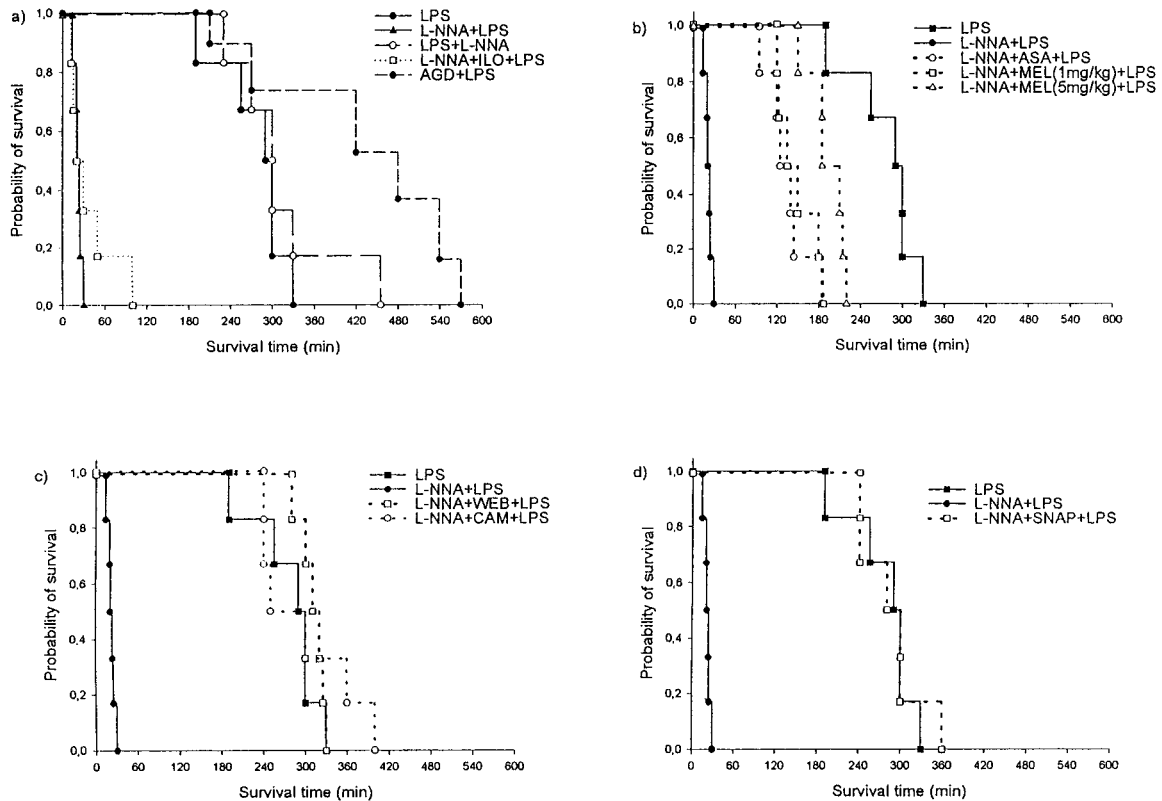
The lungs of rats treated with L-NNA after lethal LPS shock did not differ from those without L-NNA treatment. The lungs of rats that were pretreated with L-NNA before administration of LPS presented a picture typical of fulminant respiratory distress syndrome (Figure 6c and 6d). Their lungs were heavy, firm, red, and boggy. After the removed lung was cut, a large amount of bloody and foamy exudate appeared, in contrast with focal pathological changes that were observed in the lungs of rats described above (Figure 6a and 6b). The lungs of experimental ARDS rats were characterized by widespread edema, diffused hemorrhages, massive congestion of capillaries, and "ring-shaped" hemorrhages in the perivascular space of nonmuscular vessels. The perivascular edema dotted with erythrocytes was found in nonmuscular and in some muscular vessels. Diffuse hemorrhages into the interstitium, subpleural connective tissue, and alveoli were constantly seen. Significant accumulation and margination of granulocytes in pulmonary blood vessels were widespread. Moreover, capillary plugging by neutrophils was common. Intra-alveolar granulocytes, monocytes, and macrophages were rare. In Weigert staining, fibrin strands in small vessels were seen. The alveolar walls were thicker than in the healthy lungs, and a pale pink-stained edematous area was seen in the air-exchange space of the lung. This picture of uniformly hemorrhagic edematous lung with the obturated pulmonary vessels differs very much from that of the rats with lethal LPS shock (nontreated or treated with L-NNA). The lungs of rats with early LPS shock constitute a direct reference for the lungs of experimental ARDS rats, since the timing is the same

and the only difference is NO deficiency in the experimental ARDS rats. Figure 6 shows this difference. Pretreatment with L-NNA followed by SNAP (NO donor), camonagrel (thromboxane synthase inhibitor), or WEB2170 (PAF receptor antagonist) mollified macroscopically/microscopically observable lung damage stimulated by subsequent LPS infusion to the level of LPS infusion alone.

### Discussion

As evident from the data obtained by direct and continuous *in vivo* measurement of NO, a rapid increase of NO concentration in the lung was observed soon after the administration of LPS. This increase of NO concentration appears to be generated mainly by  $\text{Ca}^{2+}$ -dependent cNOS in the endothelium and is mirrored by a coincident decrease in MAP during the early acute phase of endotoxemia. The kinetics (pattern) of NO release after the administration of LPS, especially the early acute phase, is similar to that observed after acute ischemia or acute hypoxia.<sup>18–20</sup> The high production of NO by the endothelium is observed only in the early acute phase of endotoxemia; however, it has a profound effect on the late chronic phase of endotoxemia, when iNOS becomes the main generator of NO. The porphyrinic NO sensor measured only free NO, ie, the net NO concentration that is not consumed in the extremely fast chemical reaction with  $\text{O}_2^-$  and other redox centers in the tissue.<sup>8</sup> This free NO, measured intermittently, remained approximately the same during the second chronic phase of endotoxemia, in spite of high NO production by iNOS, measured indirectly by assaying the accumulation of the NO decay products  $\text{NO}_2^-/\text{NO}_3^-$  in the blood plasma by the Griess method.<sup>9</sup> These data clearly indicate that the net concentration of NO is only two times higher during the late chronic phase of endotoxemia than its observed preendotoxemic basal concentration. This net concentration is much lower than the total NO produced during this period but is still sufficient to account for the coincident chronic hypotension observed during this period.

Induction of iNOS by bacterial toxins directly or through cytokines leads to generation of NO in rodent arterial walls and in macrophages.<sup>4</sup> This delayed NO production may contribute to arterial hypotension, vasoplegia, metabolic acidosis, and cytotoxic effects in the late chronic phase of endotoxemia and septic shock.<sup>1</sup> At this late stage of septic shock, the toxicity of NO at high concentrations may derive from peroxynitrite ( $\text{ONOO}^-$ ), which is formed in the near-diffusion limited reaction of  $\text{O}_2^-$  with NO ( $6.7 \times 10^9 \text{ L} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$ ) that is even faster than the reaction of  $\text{O}_2^-$  with



**Figure 5.** Probability of survival time (ordinate) for each of 6 rats within 11 studied groups; survival time is given in minutes (abscissa). Each panel (a to d) contains the data for rats with lethal LPS shock and for rats subjected to experimental ARDS (L-NNA+LPS). Additionally, panel a shows results of the cotreatment of L-NNA+LPS rats with iloprost (ILO,  $0.3 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  for 30 minutes), the treatment of rats with L-NNA (10 mg/kg) after LPS shock (LPS+L-NNA group), or the pretreatment of LPS rats with AGD (15 mg/kg). Panels b to d show the results after cotreatment of L-NNA+LPS rats with acetylsalicylic acid (ASA, 50 mg/kg) and meloxicam (MEL, 1 mg/kg and 5 mg/kg) (b), camonagrel (CAM, 25 mg/kg) and WEB2170 (WEB, 5 mg/kg) (c), and SNAP ( $10 \text{ g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  for 30 minutes) (d).

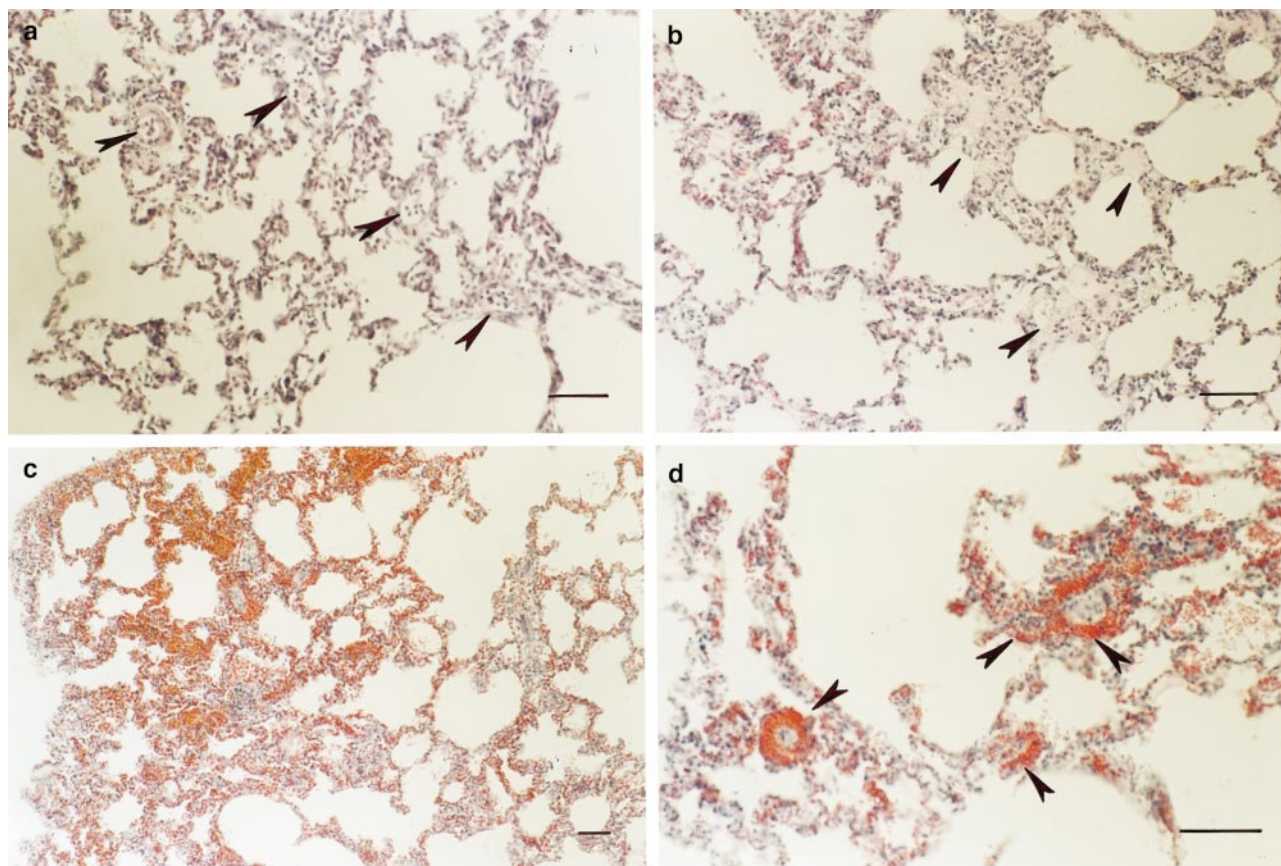
superoxide dismutase ( $2 \times 10^9 \text{ L} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$ ).<sup>8,21</sup> Normally, after protonation, the peroxyntrous acid (HOONO) formed rapidly ( $t_{1/2}$ , <1 second) is rearranged into hydrogen ion and  $\text{NO}_3^-$ .<sup>21</sup> However, after the induction of iNOS, HOONO concentration may reach a sufficient magnitude to ensure its efficient transport for several cell diameters, resulting in cleavage to highly damaging reactive oxygen species.<sup>21</sup>

Figure 1c shows that L-NNA lowers NO in the lung below the basal level, whereas subsequent LPS administration seems to be able to raise it only to this basal level, but not higher. To have a protective effect against our experimental model of endotoxin-stimulated lung injury, pulmonary NO must rise well above the basal level, as shown in Figure 1a. Six different drugs were used to help elucidate the mechanism of our experimental endotoxin-stimulated lung injury. SNAP (NO donor) and iloprost (stable  $\text{PGI}_2$  receptor agonist) were administered at doses producing hypotensive effects (25% to 50% fall in MAP). Aspirin (nonselective COX-1/COX-2 inhibitor) was used at a high dose of 50 mg/kg, which totally inhibited  $\text{PGI}_2$  and  $\text{TxA}_2$  synthesis during a period of 3 hours. Camonagrel (thromboxane synthase inhibitor) was used at its highest, still tolerated, dose of 25 mg/kg, which inhibited  $\text{TxA}_2$  formation and increased the generation of endogenous  $\text{PGI}_2$  in pilot experiments. As a rule, we used an inhibitor at the highest, still tolerated, dose that produced the expected pharmacological effects. For SNAP, camonagrel,

and WEB2170 (PAF receptor antagonist), we obtained 100% of protection. For iloprost (stable  $\text{PGI}_2$  receptor agonist), there was no protection; however, we could not increase its dose because of lethal hypotension. For meloxicam (specific COX-2 inhibitor), we tried two dose levels for COX-1/COX-2 differentiation but still obtained only partial protection. The megadose of aspirin (nonselective COX-1/COX-2 inhibitor) was also only partially protective.

Pharmacological analysis of the above findings clearly point to the fact that during the early acute phase of endotoxemia NO is a powerful protective agent, at least in the lung. However, the effects of L-NNA and LPS are global, and the observed 30% fall in cardiac output is quite possibly why these rats do very poorly after L-NNA+LPS treatment, but we focused our attention on the lung because the greatest macroscopically/microscopically observable changes were noticed there. Inhibition of NOS by L-NNA before the LPS administration shortens by  $\approx 15$ -fold the period required for development of the lethal action of our experimental dose of LPS. Moreover, the sudden death that quickly follows the administration of LPS in the absence of endogenous NO is accompanied by massive hemorrhages, edema, and, most important, obturation of almost all pulmonary blood vessels, both by intraluminal deposition of fibrin and leukocytes and by perivascular "ring constriction" caused by hemorrhages and edema. This macroscopically/microscopically observable endotoxin-stimulated lung injury can be prevented





**Figure 6.** Microscopic examination (hematoxylin-eosin staining) of lungs from the following rats: a, Rat decapitated 20 minutes after infusion of LPS (early LPS shock). Arrows show pulmonary vessels with few granulocytes inside. Otherwise, lung appears normal (original magnification  $\times 330$ ). b, Rat that died 5 hours after the infusion of LPS. Arrows show edematous alveolar walls (original magnification  $\times 330$ ). c, Rat with lung injury, evoked by combined treatment with L-NNA followed by LPS. Massive hemorrhages are distributed almost equally across the whole lung. Marked congestion of alveolar wall is shown (original magnification  $\times 165$ ). d, The same rat as in panel c. Arrows show ring-shaped perivascular hemorrhages of small vessels and obturation of their lumen by fibrin clots and blood cells (original magnification  $\times 540$ ). Bars =  $100 \mu\text{m}$ .

by exogenous NO and also by inhibition of synthesis of  $\text{TxA}_2$ <sup>22</sup> or by an antagonist of the PAF receptor.<sup>23</sup> Interestingly, inhibition of cyclooxygenases has only moderate preventive action, whereas a  $\text{PGI}_2$  receptor agonist is ineffective. These findings correspond well with the observation of Weir et al<sup>24</sup> that other nonsteroid anti-inflammatory drugs show only partial protective effect in shock.

The results of our electrochemical, pharmacological, and morphological analyses fit a plausible scenario. The early hypotensive phase of the LPS action is associated with a partial obstruction of blood flow through the lung and with a subsequent decrease in cardiac output. This early acute phase of LPS-induced partial obstruction of blood flow through the lung is a net effect of the opposing actions of locally generated vasoconstrictor and proinflammatory  $\text{TxA}_2$  and PAF versus vasodilator and cytoprotective NO. Removal of the NO-defensive system gives way to develop the fully deleterious actions of  $\text{TxA}_2$  and PAF, culminating in sudden death accompanied by severe lung injury. These two lipid-derived substances may act independently, or PAF might increase  $\text{TxA}_2$  generation in the lungs.<sup>25,26</sup> The pneumotoxic action of L-NNA before LPS treatment can be greatly mollified by canagrel (thromboxane synthase inhibitor),

WEB2170 (PAF receptor antagonist), or by exogenous NO from SNAP. However, SNAP is able to do more than restore the “regular” early acute phase of hemodynamic response to LPS in NO-deficient rats: it cancels the early acute phase of hypotension due to LPS, and it also retards development of the late chronic hypotensive phase.

Of course, during the late chronic phase of LPS action, the appearance of NO mainly from iNOS coincides with the appearance of a plethora of lethal cytokines and reactive oxygen species. The relatively constant amounts of free NO measured throughout the late phase of endotoxemia are more than sufficient to account for the observed coincident chronic hypotension. However, it appears that most of the NO generated by iNOS during the late chronic phase of endotoxemia is consumed in rapid chemical reactions presumably with  $\text{O}_2^-$ , from many sources, to give rise to HOONO and its toxic cleavage products.<sup>21</sup> This high production of HOONO may be a major contributor to the cytotoxic effects of LPS, including shortened life span.<sup>27</sup> The significant elongation of survival time by AGD supports this view.<sup>28</sup> In this late chronic phase of endotoxemia, the role of NO is profoundly different from its defensive role during the early acute phase after the first exposure to LPS.



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