Anesthesiology
1999; 91:1744–53

Congenital NOS2 Deficiency Protects Mice from LPS-induced Hyporesponsiveness to Inhaled Nitric Oxide

Jörg Weimann, M.D., Kenneth D. Bloch, M.D.,† Masao Takata, M.D., Ph.D.,‡ Wolfgang Steudel, M.D.,* Warren M. Zapol, M.D.§*

Background: **In animal models, endotoxin (lipopolysaccharide) challenge impairs the pulmonary vasodilator response to inhaled nitric oxide (NO). This impairment is prevented by treatment with inhibitors of NO synthase 2 (NOS2), including glucocorticoids and L-arginine analogs. However, because these inhibitors are not specific for NOS2, the role of this enzyme in the impairment of NO responsiveness by lipopolysaccharide remains incompletely defined.**

Methods: **To investigate the role of NOS2 in the development of lipopolysaccharide-induced impairment of NO responsiveness, the authors measured the vasodilator response to inhalation of 0.4, 4, and 40 ppm NO in isolated, perfused, and ventilated lungs obtained from lipopolysaccharide-pretreated (50 mg/kg intraperitoneally 16 h before lung perfusion) and untreated wild-type and NOS2-deficient mice. The authors also evaluated the effects of breathing NO for 16 h on pulmonary vascular responsiveness during subsequent ventilation with NO.**

Results: **In wild-type mice, lipopolysaccharide challenge impaired the pulmonary vasodilator response to 0.4 and 4 ppm NO (reduced 79% and 45%, respectively, *P* < 0.001), but not to 40 ppm. In contrast, lipopolysaccharide administration did not impair the vasodilator response to inhaled NO in NOS2-deficient mice. Breathing 20 ppm NO for 16 h decreased the vasodilator response to subsequent ventilation with NO in lipopolysaccharide-pretreated NOS2-deficient mice, but not in lipopolysaccharide-pretreated wild-type, untreated NOS2-deficient or untreated wild-type mice.**

Conclusions: **In response to endotoxin challenge, NO, either endogenously produced by NOS2 in wild-type mice or added to the air inhaled by NOS2-deficient mice, is necessary to impair vascular responsiveness to inhaled NO. Prolonged NO breathing, without endotoxin, does not impair vasodilation in response to subsequent NO inhalation. These results suggest that NO, plus other lipopolysaccharide-induced products, are necessary to impair responsiveness to inhaled NO in a murine sepsis model. (Key words: Gene deletion; lipopolysaccharide; mouse; pulmonary circulation.)**

INHALED nitric oxide (NO) is a selective vasodilator of human[1–3] and animal pulmonary vessels.[4–7] NO inhalation improves oxygenation in patients with acute respiratory distress syndrome (ARDS) by redistributing blood flow toward better ventilated lung areas, thereby reducing intrapulmonary shunting.[3] However, 30–40% of ARDS patients do not respond to inhaled NO.[1,2] Krafft *et al.*[8] reported a high incidence of nonresponders (60%) in patients with sepsis-associated ARDS. Similarly, Manktelow *et al.*[9] noted that ARDS patients with septic shock were less likely to respond to inhaled NO than were ARDS patients without septic shock. These reports suggest that endotoxemia or sepsis syndrome might impair responsiveness to inhaled NO.

Vasodilatation produced by NO is mediated primarily by stimulating soluble guanylate cyclase (sGC) to produce cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP). Cyclic GMP activates cGMP-dependent protein kinase, which phosphorylates several intracellular targets, resulting in smooth muscle relaxation (for review see Lincoln and Cornwell[10]). Cyclic GMP is catabolized to GMP by phosphodiesterases.

Nitric oxide is produced by NOS through the conversion of L-arginine to L-citrulline in the presence of oxygen (reviewed in Knowles and Moncada[11] and Moncada

* Research Fellow, Department of Anesthesia and Critical Care.

† Associate Professor of Medicine, Cardiovascular Research Center of the Department of Medicine.

‡ Visiting Assistant Professor of Anesthesia, Department of Anesthesia and Critical Care.

§ Reginald Jenney Professor of Anaesthesia, Department of Anesthesia and Critical Care.

Received from the Department of Anesthesia and Critical Care Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts. Submitted for publication January 22, 1999. Accepted for publication June 22, 1999. Supported by the National Institutes of Health, National Heart, Lung and Blood Institute, (USPHS HL 42397 to Dr. Zapol; USPHS HL 55377 to Dr. Bloch). Supported by research fellowships from the Deutsche Forschungsgemeinschaft (WE 2114/1-1 to Dr. Weimann and STE 835/1-2 to Dr. Steudel), Bonn, Germany. Presented in part at the 10th European Congress of Anaesthesiology, Frankfurt, Germany, June 30–July 4, 1998.

Dr. Bloch is an Established Investigator of the American Heart Association, Dallas, Texas.

The Massachusetts General Hospital has been granted patents on the inhalation of nitric oxide and the authors have a right to receive royalties.

Address reprint requests to Dr. Zapol: Reginald Jenney Professor of Anesthesia Department of Anesthesia and Critical Care, Massachusetts General Hospital, 32 Fruit Street, Boston, Massachusetts 02114. Address electronic mail to: WZapol@etherdome.mgh.harvard.edu

Table 1. Mice Treatment Groups and Studies

Treatment Groups		Number of Mice	Vasodilator (Studied in Isolated-perfused Lungs)
Wild-type mice			
Control		7	0.4, 4, and 40 ppm inhaled NO
LPS		7	
Control	+ 16 h NO exposure (20 ppm)	5	
LPS	+ 16 h NO exposure (20 ppm)	5	
Control		5	4 ppm inhaled NO, 2 and 20 μM 8-pCPT-cGMP
LPS		5	
NOS2-deficient mice			
Control		7	0.4, 4, and 40 ppm inhaled NO
LPS		7	
Control	+ 16 h NO exposure (20 ppm)	5	
LPS	+ 16 h NO exposure (20 ppm)	5	
LPS	+ 16 h NO exposure (0.2 ppm)	5	
LPS	+ 16 h NO exposure (2 ppm)	5	
Control		5	4 ppm inhaled NO, 2 and 20 μM 8-pCPT-cGMP
LPS		5	

Wild-type and NOS2-deficient mice were injected with 50 mg/kg LPS intraperitoneally 16 h before lung perfusion experiments. Groups of untreated mice served as controls. In some experiments, mice were additionally subjected to 16 h of NO breathing. In lung perfusion experiments, the vasodilator response produced by either 0.4, 4, and 40 ppm inhaled NO, or produced by 4 ppm inhaled NO followed by perfusion with 2 and 20 μM 8-pCPT-cGMP was measured.

LPS = lipopolysaccharide.

et al.[12]). Three different NOS isoforms have been characterized. Neuronal (NOS1) and endothelial (NOS3) NOSs are expressed constitutively and produce NO in response to an increased intracellular calcium concentration.[12] Transcription of the inducible NOS isoform (NOS2) is increased in response to endotoxin and cytokines, such as tumor necrosis factor α, interleukin-1β, and interleukin-6, and leads to accelerated production of NO.[11,12] Excessive NO synthesis has been suggested as an important mechanism that causes systemic hypotension during septic shock.[13] Moreover, NOS2 is capable of generating not only NO, but also superoxide radicals.[14] NO and superoxides combine rapidly to form peroxynitrite, which can cause nitrosative injury.[15]

Several authors have described alterations of NO–cGMP-mediated vasorelaxation after endotoxin treatment.[16–18] Holzmann *et al.*[18] demonstrated impaired vasorelaxation to inhaled NO in isolated, perfused, and ventilated lungs obtained from lipopolysaccharide-treated rats and noted that inhibition of NOS enzyme activity by N^G-nitro-L-arginine methyl ester (L-NAME) or aminoguanidine during the 16 h after lipopolysaccharide challenge maintained the pulmonary vasodilator responsiveness to inhaled NO. These data suggest a critical role for NOS2 in the development of endotoxin-induced hyporesponsiveness to inhaled NO.

However, these studies are limited because available NOS2 enzyme inhibitors are incompletely isoform specific.[19] Furthermore, it is unknown whether NO, or other molecules produced by NOS2, such as superoxide,[15] contributes to the lipopolysaccharide-induced hyporesponsiveness to inhaled NO. In a novel approach to understanding the effects of lipopolysaccharide-mediated NOS2 induction on pulmonary vascular responsiveness to inhaled NO, we studied mice with a congenital deficiency of the *NOS2* gene. We report that lipopolysaccharide induces hyporesponsiveness to inhaled NO in wild-type mice, but does not produce hyporesponsiveness in NOS2-deficient mice. Furthermore, we provide evidence that NO, either endogenously produced by NOS2 or added to the ambient air inhaled by NOS2-deficient mice, is necessary to produce pulmonary vascular hyporesponsiveness to inhaled NO in the mouse undergoing endotoxin challenge.

Materials and Methods

These investigations were approved by the Subcommittee for Research Animal Care of the Massachusetts General Hospital. A total of 78 adult male mice weighing 20–35 g were studied, as listed in table 1 and outlined herein. NOS2-deficient mice[20] were generously provided by Dr. Carl Nathan. Mice of the same background (F1-generation of the parental strains SV129 and C57 Black/6) were used as wild-type mice.[21]

Isolated, Perfused, and Ventilated Mouse Lung Model

Mice were killed by an intraperitoneal injection of pentobarbital sodium (200 mg/kg body weight) and placed in a 37°C water-jacketed chamber (Isolated Perfused Lung Size 1 Type 839; Hugo-Sachs Elektronik, March-Hugstetten, Germany). The trachea was isolated and intubated, and the lungs were ventilated with 21% O_2, 6% CO_2 and 73% N_2 using a volume-controlled ventilator (model 687; Harvard Apparatus, South Natick, MA) at a ventilatory rate of 85 breaths/min and 2 cm H_2O end-expiratory pressure. The tidal volume was adjusted to provide a peak inspiratory pressure of 10 cm H_2O throughout each study. The lungs were exposed *via* a midline sternotomy, and a ligature was placed around the aorticopulmonary outflow tract. After injection of 10 IU heparin into the right ventricle, the pulmonary artery was cannulated with a stainless steel cannula (1 mm ID) *via* the right ventricle. The pulmonary venous effluent was drained *via* a stainless steel cannula (1 mm ID) placed through the apex of the left ventricle across the mitral valve and into the left atrium. Left atrial pressure was maintained at 2 mmHg. Lungs were perfused at a constant flow (50 ml · kg body weight^{-1} · min^{-1}; Ismatec Reglo-Analogue roller pump; Laboratoriumstechnik GmbH, Wertheim-Mondfeld, Germany) with a nonrecirculating system at 37°C. The perfusate used was Hanks' Balanced Salt Solution (GibcoBRL, Grand Island, NY) containing 1.26 mM $CaCl_2$, 5.33 mM KCl, 0.44 mM KH_2PO_4, 0.50 mM $MgCl_2$, 0.41 mM $MgSO_4$, 138.0 mM NaCl, 4.0 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, and 5.6 mM glucose. Bovine serum albumin, 5%, and dextran, 5% (both from Sigma Chemical Co., St. Louis, MO), were added to the perfusate to prevent pulmonary edema, as previously described in the isolated, perfused, and ventilated rat lung.[17] Indomethacin, 30 mM (Sigma Chemical Co.), and 1 mM L-NAME (Sigma Chemical Co.) were added to the perfusate to inhibit endogenous prostaglandin and NO synthesis, respectively. Sodium bicarbonate was added to adjust the perfusate pH to 7.34–7.43. Lungs were included in this study if they had a homogenous white appearance without signs of hemostasis or atelectasis and showed a stable perfusion pressure less than 10 mmHg during the second 5 min of an initial 10-min baseline perfusion period. Using these two criteria, approximately 15% of lung preparations from each group were discarded before study.

Pulmonary artery pressure (PAP) and left atrial pressure were measured *via* saline-filled membrane pressure transducers (Argon, Athens, TX) connected to a side port of the inflow and outflow cannulae, respectively. Airway pressure (Paw) was measured using a differential pressure transducer (model MP-45-32-871; Validyne Engineering Corp., Northridge, CA) connected to the inspiratory limb just before the Y piece. Pressure transducers were connected to a biomedical amplifier (Hewlett Packard 7754B, Andover, MA), and data were recorded at 150 Hz on a personal computer using an analog-to-digital interface with a data acquisition system (DI-220; Dataq Instruments, Akron, OH). The system was calibrated before each experiment.

For NO inhalation, NO gas (800 or 80 ppm NO in nitrogen, Airco, Murray Hill, NJ) was blended (Oxygen Blender; Bird Corporation, Palm Springs, CA) with oxygen, carbon dioxide, and nitrogen to achieve a final concentration of 21% O_2, 6% CO_2, and the desired NO concentration. NO and higher oxidative states of NO (NO_x; CLD 700 AL; Eco Physics, Dürnten, Switzerland), oxygen (Hudson Ventronics Division, Temecula, CA), and carbon dioxide (Datex CO2 monitor; Puritan-Bennett Corporation, Los Angeles, CA) concentrations were monitored continuously.

Pulmonary Vascular Response to Inhaled NO after Lipopolysaccharide Challenge

Wild-type and NOS2-deficient mice were injected intraperitoneally with 50 mg/kg body weight *Escherichia coli* 0111:B4 lipopolysaccharide (LPS; Difco Laboratories, Detroit, MI) dissolved in saline 16 h before isolated lung perfusion. This time point was chosen based on our previous studies in rats.[17] Untreated wild-type and NOS2-deficient mice served as controls.

After an initial 10-min baseline perfusion period, pulmonary vasoconstriction was induced by continuous infusion of the thromboxane A_2 analog U-46619 (Cayman Chemicals, Ann Arbor, MI). The infusion rate was adjusted to provide a stable increase in PAP of 5 or 6 mmHg. Then, a dose–response curve to inhaled NO was obtained by sequentially ventilating the lungs with 0.4, 4, and 40 ppm NO for 5 min each. After each period of NO ventilation, the PAP was allowed to return to the pre-NO elevated baseline. U-46619 infusion was readjusted if the PAP was not within a range of ± 10% of the pre-NO value at 5 min after discontinuation of NO inhalation. The vasodilator response to inhaled NO (ΔPAP) was measured as the change in PAP produced by inhaled NO (PAP after 5 min of NO inhalation minus PAP pre-NO) as a percentage of the increase in PAP induced by U-46619 (PAP pre-NO minus PAP at initial baseline).

Effect of NO Exposure on Pulmonary Vascular Response to Inhaled NO

Four groups of mice breathed 20 ppm NO for 16 h. One group of wild-type mice and one group of NOS2-deficient mice were injected with 50 mg/kg lipopolysaccharide intraperitoneally immediately before NO exposure. Additional wild-type and NOS2-deficient mice groups were exposed to NO inhalation without receiving lipopolysaccharide. After 16 h of NO exposure, the lungs were isolated and perfused as described previously. Pulmonary vasoconstriction was induced by infusion of U46619, and the vasodilator response to 0.4, 4, and 40 ppm NO was measured.

During ambient-pressure NO exposure, animals were maintained in 40-l acrylic chambers. NO and NO_x concentrations were controlled carefully using soda lime[22] at a high fresh gas flow rate of NO (10,000 ppm NO in nitrogen; Airco, Murray Hill, NJ), air, and oxygen, as previously described.[23]

Two additional groups of NOS2-deficient mice were treated with lipopolysaccharide (50 mg/kg intraperitoneal) and then exposed to 0.2 and 2 ppm NO inhalation, respectively. Sixteen hours later, isolated lung perfusion studies measuring the degree of pulmonary vasodilatation produced by 0.4, 4, and 40 ppm inhaled NO were performed.

Pulmonary Vascular Response to 8-pCPT-cGMP

Nitric oxide synthase 2–deficient and wild-type mice were injected with lipopolysaccharide intraperitoneally, and, 16 h later, isolated lung perfusion was initiated as described previously. Other groups of wild-type and NOS2-deficient mice were studied without receiving lipopolysaccharide. U46619 was used to increase the baseline PAP by 5 or 6 mmHg. Lungs were then ventilated with 4 ppm NO for 5 min to evaluate vascular responsiveness to inhaled NO. After the PAP was allowed to increase to the baseline pressure, lungs were perfused sequentially with 2 and 20 mM 8-(4-chlorophenylthio)-guanosine-3′:5′-cyclic monophosphate (8-pCPT-cGMP; Biolog Life Science Institute, La Jolla, CA) for 10 min. 8-pCPT-cGMP was diluted with perfusate to reach the desired concentrations in two additional reservoirs before each experiment. This allowed immediate switching between perfusion with or without 8-pCPT-cGMP without discontinuing the flow of perfusate.

Wet-to-dry Lung Weight Ratio

At the end of each experiment, both lungs, excluding hilar structures, were excised and weighed (wet weight). Thereafter, the lungs were dried in a microwave oven for 60 min, as previously described,[17] and then reweighed (dry weight). Wet-to-dry lung weight ratios were calculated by dividing the wet weight by the dry weight.

Statistical analysis

All data are expressed as the mean ± standard error (SE). To compare groups, a two-way analysis of variance was performed. When significant differences were detected by analysis of variance, a *post hoc* least significant difference test for planned comparisons was used (Statistica for Windows; StatSoft, Inc., Tulsa, OK). Statistical significance was assumed at a P value < 0.05.

Results

Infusion of U46619 caused a stable increase of the PAP at a constant perfusate flow, which was reversible after discontinuing U46619 at the end of the experiment. The dose of U46619 necessary to increase the PAP by 5 or 6 mmHg did not differ in lipopolysaccharide-pretreated and untreated wild-type and NOS2-deficient mice.

Mice injected with intraperitoneal lipopolysaccharide had piloerection, diarrhea, and lethargy to a similar degree in both wild-type and NOS2-deficient mice. The mortality rate 16 h after lipopolysaccharide injection was approximately 15% and did not differ between the two mouse strains.

Pulmonary Vascular Response to Inhaled Nitric Oxide

Inhalation of NO decreased the PAP in a dose-dependent manner in all groups. A representative example of an original recording of PAP and left atrial pressure from an isolated–perfused mouse lung is provided in figure 1.

In the isolated–perfused lungs of wild-type mice that underwent lipopolysaccharide challenge, PAP decreased 79% and 45% less in response to 0.4 and 4 ppm inhaled NO, respectively, compared with untreated animals ($P <$ 0.001; fig. 2A). The pulmonary vasodilator response to 40 ppm NO did not differ between these groups.

Response to inhaled NO in untreated NOS2-deficient mice did not differ from that of untreated wild-type mice. In contrast, lungs obtained from lipopolysaccharide-challenged NOS2-deficient mice showed greater vasodilatation to inhaled NO than the lungs of lipopolysaccharide-treated wild-type mice ($P < 0.001$ at each NO dose; fig. 2B). Moreover, NO-induced vasodilatation was

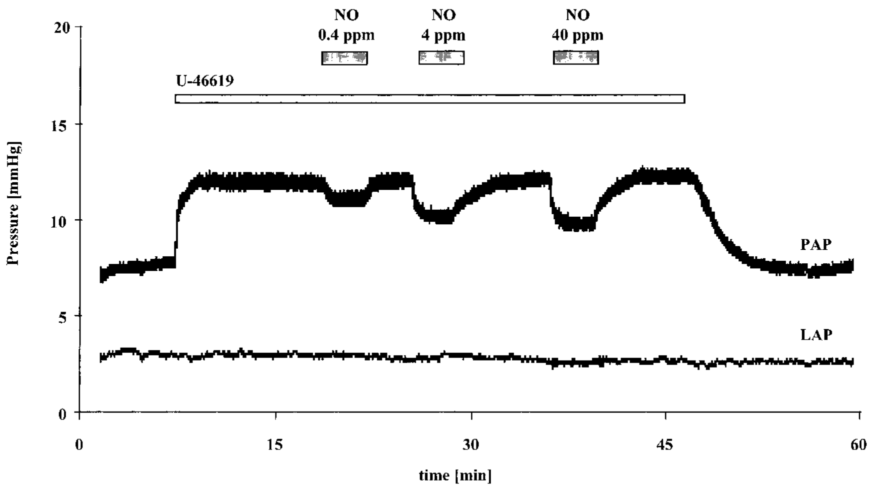

Fig. 1. A tracing of a representative experiment measuring pulmonary artery pressure (PAP; equivalent to perfusion pressure) and left atrial pressure (LAP) in an isolated–perfused lung of an untreated wild-type mouse. The stable thromboxane A_2 analog U46619 was infused to increase PAP by 5 or 6 mmHg. Varying doses (0.4, 4, and 40 ppm) of inhaled NO were administered for 5 min each. After each dose, PAP was allowed to return to the pre-NO level.

enhanced in lipopolysaccharide-treated NOS2-deficient mice, compared with untreated NOS2-deficient or wild-type mice ($P < 0.05$, respectively, at each NO dose; fig. 2B).

Pulmonary Vascular Response to Inhaled NO after Inhaled NO Exposure

To investigate the role of molecular NO in the development of hyporesponsiveness to inhaled NO, we studied lipopolysaccharide-treated and untreated NOS2-deficient and wild-type mice that breathed air supplemented with 20 ppm NO for 16 h. Previous NO inhalation exposure did not alter the responsiveness to subsequently inhaled NO in perfused lungs obtained from untreated wild-type or NOS2-deficient mice or in lipopolysaccharide-pretreated wild-type mice. In contrast, the pulmonary vasodilator response to inhaled NO was decreased in lipopolysaccharide-pretreated NOS2-deficient mice exposed to ambient NO for 16 h, compared with non-NO–exposed lipopolysaccharide-pretreated NOS2-deficient mice. In isolated–perfused lungs from NOS2-deficient mice exposed to 20 ppm ambient NO for 16 h, the subsequent vasodilator responsiveness to inhaled NO was impaired after pretreatment with lipopolysaccharide (*vs.* untreated controls) at 0.4 (ΔPAP $-24 \pm 4\%$ *vs.* $-42 \pm 4\%$; $P < 0.05$) and 4 ppm NO (ΔPAP $-39 \pm 5\%$ *vs.* $-58 \pm 4\%$; $P < 0.01$), but not at 40 ppm NO (ΔPAP $-55 \pm 3\%$ *vs.* $-62 \pm 5\%$; P = not significant; fig. 3). Similar to animals without previous NO inhalation exposure, NO-induced vasodilation was reduced in lipo-

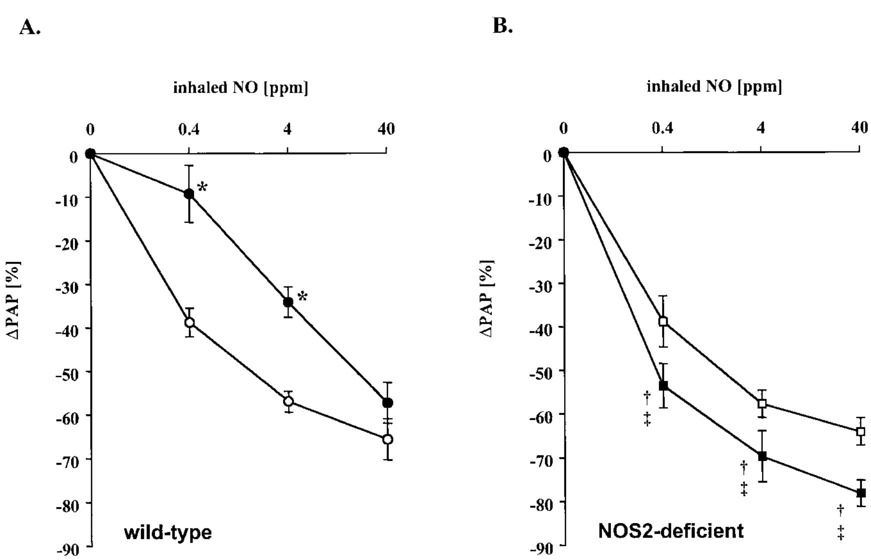

Fig. 2. (*A*) Dose–response curves to inhaled NO in lipopolysaccharide-pretreated (closed circles) and untreated (open circles) wild-type mice. (*B*) Dose–response curves to inhaled NO in lipopolysaccharide-pretreated (closed squares) and untreated (open squares) nitric oxide synthase 2 (NOS2)–deficient mice. The response to inhaled NO was impaired in lipopolysaccharide-pretreated compared with untreated wild-type mice (P* < 0.001). Lipopolysaccharide-treated NOS2-deficient mice had a greater response to inhaled NO when compared with untreated NOS2-deficient mice (†*P* < 0.05) and lipopolysaccharide-pretreated wild-type mice (‡*P* < 0.001). Data are expressed as the mean ± SE. ΔPAP = change in pulmonary artery pressure as a percent of its U46619-induced increase.**

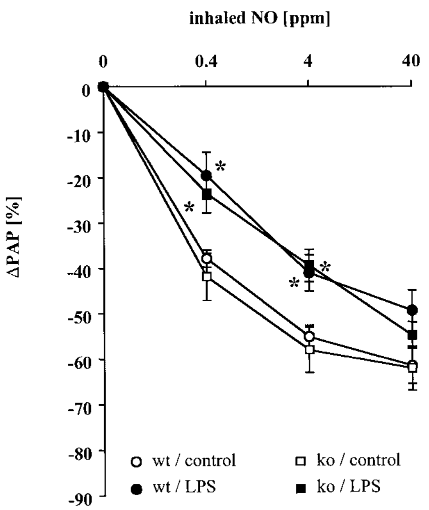

Fig. 3. Vasodilation by short-term nitric oxide (NO) inhalation in isolated–perfused lungs from lipopolysaccharide-pretreated (wild-type/lipopolysaccharide) and untreated (wt/control) wild-type mice and in isolated–perfused lungs from lipopolysaccharide-pretreated (ko/lipopolysaccharide) and untreated (ko/control) NOS2-deficient mice previously exposed to 20 ppm NO for 16 h in ambient air. After prolonged NO exposure, lipopolysaccharide-pretreated NOS2-deficient mice were less responsive to short-term NO inhalation than were NOS2-deficient mice that did not receive lipopolysaccharide (*P < 0.05). Similarly, after prolonged NO exposure, lipopolysaccharide-pretreated wild-type mice were less responsive to short-term NO inhalation than were wild-type mice that did not receive lipopolysaccharide (*P < 0.05). Data are expressed as the mean ± SE.

polysaccharide-pretreated wild-type mice, compared to untreated wild-type mice that had breathed 20 ppm NO for 16 h before lung perfusion experiments (fig. 3).

To determine whether the inhalation of a lower level of NO for 16 h would impair vasoreactivity to short-term NO inhalation during lung perfusion, lipopolysaccharide-treated NOS2-deficient mice were exposed to 0.2 and 2 ppm NO inhalation. Breathing 0.2 ppm NO for 16 h after lipopolysaccharide administration did not cause subsequent hyporesponsiveness to short-term inhaled NO in NOS2-deficient mice. However, breathing 2 ppm NO for 16 h decreased the vasodilator response to 0.4 ppm NO inhalation, compared with the response in control mice ($P < 0.05$; fig. 4), but there was no reduction of vasodilator responsiveness with 4 and 40 ppm inhaled NO.

Pulmonary Vascular Response to 8-pCPT-cGMP

We investigated whether the altered pulmonary vasodilator response to inhaled NO after lipopolysaccharide challenge is associated with an impaired vasodilator response to cGMP. The vasodilator effect of the membrane-permeable, phosphodiesterase-resistant cGMP analog 8-pCPT-cGMP was studied in wild-type and NOS2-deficient mouse lungs with and without 16 h of previous lipopolysaccharide challenge. In preliminary studies, it was observed that 10 min of lung perfusion with 8-pCPT-cGMP was necessary to achieve stable vasodilation (data not shown).

The vasodilation produced by perfusing isolated lungs with 2 and 20 mM 8-pCPT-cGMP was reduced 63 and 32%, respectively, in lipopolysaccharide-pretreated wild-type mice as compared to untreated wild-type mice ($P <$ 0.05; fig. 5A). In contrast, in NOS2-deficient mice, exposure to lipopolysaccharide did not alter 8-pCPT-cGMP–induced vasorelaxation (fig. 5B). Moreover, after lipopolysaccharide challenge, the pulmonary vasodilator response to 8-pCPT-cGMP was greater in NOS2-deficient mice (fig. 5B) than in wild-type mice (fig. 5A; $P < 0.05$ at both 2 and 20 mM).

Wet-to-dry Lung Weight Ratios

The absence of pulmonary edema was confirmed by unchanged wet-to-dry lung weight ratios after perfusion. There was no difference between lipopolysaccharide-pretreated wild-type (wet weight–dry weight: 4.3 ± 0.2) and NOS2-deficient (4.8 ± 0.1) mice, or untreated wild-type (4.6 ± 0.1) and untreated NOS2-deficient (4.6 ± 0.1) mice. Exposure to NO inhalation for 16 h did not alter the wet-to-dry lung weight ratios in lipopolysaccha-

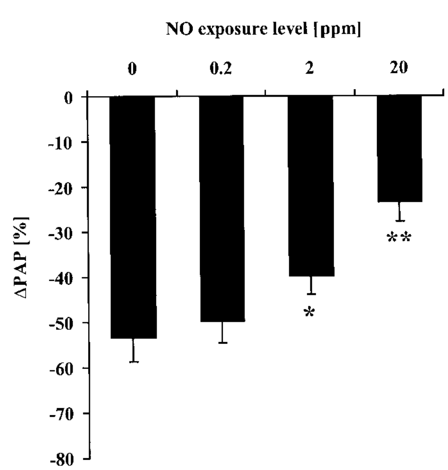

Fig. 4. Effect of breathing 0, 0.2, 2, and 20 ppm nitric oxide (NO) for 16 h after lipopolysaccharide challenge during subsequent short-term vasodilatation in response to 0.4 ppm inhaled NO in isolated–perfused lungs from NOS2-deficient mice. Exposure to 2 and 20 ppm inhaled NO decreased the pulmonary vasodilatory response to inhaled NO. Data are expressed as the mean ± SE. *P < 0.05, **P < 0.001 *versus* 0 ppm NO exposure for 16 h.

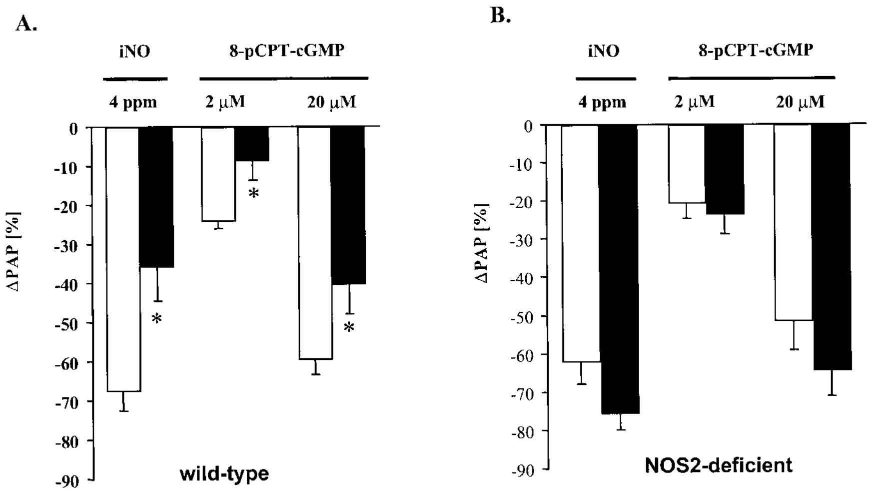

Fig. 5. The pulmonary vasodilator response to 4 ppm inhaled nitric oxide (NO) and perfusion with 2 and 20 mM 8-pCPT-cGMP in lipopolysaccharide-pretreated (closed bars) and control (open bars) wild-type mice (*A*) and NOS2-deficient mice (*B*). Vasodilatation in response to inhaled NO (iNO), and to 8-pCPT-cGMP, was impaired in lipopolysaccharide-pretreated wild-type mice compared with control wild-type mice (* $P < 0.05$ *vs.* control) but was not different between lipopolysaccharide-pretreated and control NOS2-deficient mice. Data are expressed as the mean ± SE.

ride-pretreated wild-type (4.9 ± 0.1) and NOS2-deficient (5.1 ± 0.2) mice or in untreated wild-type (4.5 ± 0.3) and untreated NOS2-deficient (4.8 ± 0.1) mice, compared with unexposed mice. Wet-to-dry lung weight ratios did not correlate with the vasodilator response to inhaled NO.

Discussion

The principal finding of this study is that the congenital absence of the gene encoding for *NOS2* in mice completely prevents the development of lipopolysaccharide-induced hyporesponsiveness of the pulmonary vasculature to inhaled NO. In NOS2-deficient mice, the addition of 2 or 20 ppm inhaled NO to ambient air for 16 h after lipopolysaccharide challenge decreased pulmonary vasodilator responsiveness to short-term inhaled NO. Therefore, in mice, the NO molecule, either produced endogenously by NOS2 or added exogenously to ambient air, is necessary to produce the lipopolysaccharide-induced impairment of pulmonary vascular responsiveness to inhaled NO.

Hyporesponsiveness to Inhaled NO

Inhaled NO decreases pulmonary vascular resistance and improves oxygenation in patients with ARDS. Unfortunately, approximately 30–40% of patients with ARDS do not respond to inhaled NO therapy.[1–3] The precise mechanisms responsible for this variability in the clinical therapeutic response to inhaled NO are unknown. Manktelow *et al.*[9] reported that patients with ARDS associated with sepsis are less likely to respond to inhaled NO than are patients with ARDS associated with other disease processes. Studies of isolated rat pulmonary artery ring preparations[16] and isolated-perfused rat lungs[17] have showed that the administration of lipopolysaccharide can impair NO-mediated vasodilatation. In this study, we observed that pretreatment with lipopolysaccharide impairs vasodilator responsiveness to inhaled NO in an isolated-perfused mouse lung model (fig. 2A). In contrast to previous studies in rats,[18] this impaired response is reflected by a rightward shift of the inhaled NO dose–pulmonary vasodilator response curve, rather than by a change in maximal effectiveness because the vasodilatory response to 40 ppm inhaled NO did not differ, with or without lipopolysaccharide-treatment.

Nitric Oxide Synthase 2 and Hyporesponsiveness to NO

Studies of isolated aortic rings[24] and isolated-perfused lungs[18] showed that lipopolysaccharide-mediated vasodilator hyporesponsiveness to NO can be partially prevented by agents that inhibit lipopolysaccharide-induced NOS2-synthesis, such as cycloheximide[24] and dexamethasone,[18,24] and by an inhibitor of NOS2 enzyme activity, aminoguanidine.[18] These studies suggest a role for NOS2 in the development of hyporesponsiveness to inhaled NO. Definition of the precise mechanism is limited by the lack of specificity of the inhibitory drugs for NOS2.[19] Therefore, we studied mice with a congenital absence of the *NOS2* gene[20] and developed an isolated, perfused, and ventilated mouse lung model to investigate the role of NOS2 in the development of lipopolysaccharide-induced hyporesponsiveness to inhaled NO.

Nitric oxide synthase 2 deficiency prevented lipopolysaccharide-induced hyporesponsiveness to inhaled NO (fig. 2A and B). Therefore, the expression of *NOS2* is necessary for the production of lipopolysaccharide-mediated hyporesponsiveness to inhaled NO. Moreover, there was greater vasodilatation in response to inhaled NO in lipopolysaccharide-pretreated NOS2-deficient mice compared with untreated NOS2-deficient and wild-type mice (fig. 2). The mechanism responsible for the lipopolysaccharide-induced vasodilator hyper-responsiveness to inhaled NO in NOS2-deficient mice is unknown and needs further investigation.

Hinder *et al.*[25] reported that 40 ppm NO inhalation was an effective pulmonary vasodilator in septic sheep administered L-NAME. This sheep study did not evaluate lower doses of inhaled NO and the effect of sepsis on NO responsiveness. Therefore, it cannot be directly contrasted with our murine studies.

Pulmonary Vascular Response to Inhaled NO after 16 h of Ambient NO Exposure

Because NOS2 was necessary for the development of lipopolysaccharide-induced hyporesponsiveness to inhaled NO, we considered the possibility that a product of NOS2, other than NO, could impair NO pulmonary vasodilator responsiveness. For example, superoxide generation by NOS2 has been reported in cells depleted of L-arginine.[15] To learn whether NO itself is an essential factor in the mechanism leading to impaired responsiveness to inhaled NO, NOS2-deficient and wild-type mice, with and without lipopolysaccharide-pretreatment, breathed 20 ppm NO in air for 16 h before lung perfusion studies. Sixteen hours of NO inhalation did not affect the vasodilator response to short-term NO inhalation in untreated wild-type and NOS2-deficient mice, nor did it induce any further decrease of responsiveness to inhaled NO in lipopolysaccharide-pretreated wild-type mice (fig. 3). These results are consistent with the observations of other investigators, which showed that prolonged NO inhalation does not alter the pulmonary vasodilatation produced by administration of an NO donor compound[26,27] or by inhalation of NO.[28] In contrast, vasodilator response to short-term NO inhalation markedly decreased in lipopolysaccharide-pretreated NOS2-deficient mice breathing[20] ppm NO in air for 16 h (fig. 3). This effect was dose-dependent: breathing 0.2 ppm NO for 16 h did not alter NO-induced vasodilatation, whereas breathing 2 ppm NO for 16 h decreased the short-term pulmonary vasodilator response to a subsequent challenge with 0.4 ppm NO, but not with 4 and 40 ppm (fig. 4). Therefore, it is clearly NO, and not another product of NOS2, that is a cofactor in pulmonary vasodilator hyporesponsiveness associated with lipopolysaccharide challenge. If we can extrapolate the known data from mice to humans, our observations suggest that, in some patients with septic ARDS, NO inhalation at 2 to 20 ppm may impair pulmonary vascular responsiveness to inhaled NO. The finding that a low concentration of inhaled NO did not decrease NO-responsiveness in NOS2-deficient mice supports the wide-spread clinical practice of using the lowest concentration of inhaled NO necessary to achieve the desired therapeutic effect.[29]

Our data show that NO production by NOS2 is necessary for the development of lipopolysaccharide-induced hyporesponsiveness to NO. However, the observation that prolonged inhalation of NO does not alter the pulmonary vasodilator response to short-term NO inhalation in mice not treated with lipopolysaccharide suggests that NO alone does not account for the development of NO hyporesponsiveness in lipopolysaccharide-treated animals. Therefore, in addition to the induction of NO synthesis, other lipopolysaccharide-associated factors appear to be necessary for the development of lipopolysaccharide-mediated NO hyporesponsiveness. Ungureanu-Longrois *et al.*[30] observed in isolated ventricular myocytes that induction of NOS2 was necessary, but was not sufficient, to cause cytokine-induced contractile dysfunction. NO-independent mechanisms that may contribute to lipopolysaccharide-mediated hyporesponsiveness to inhaled NO include the induction of cytokines and of enzymes responsible for superoxide radical production, such as xanthine oxidase or NADPH oxidase.[31] It is possible that the reaction of NO with superoxide, by leading to the production of highly reactive peroxynitrite, may impair pulmonary vascular NO responsiveness. Future therapeutic approaches to improve pulmonary vascular responsiveness to inhaled NO should include the identification and modulation of these NOS2–NO-independent lipopolysaccharide-induced factors.

Mechanisms of Endotoxin-induced Hyporesponsiveness to Inhaled NO

Lipopolysaccharide-induced hyporesponsiveness to inhaled NO is correlated with an impaired pulmonary vasodilator responsiveness to the phosphodiesterase (PDE)–resistant cGMP analog 8-pCPT-cGMP (fig. 5). This suggests that the effects of lipopolysaccharide on NO signal transduction in isolated–perfused mouse lungs are, at least in part, independent of changes in cGMP synthesis or metabolism.

Our results differ from those of Fullerton *et al.,*[16] who found that, in pulmonary artery rings isolated from rats exposed to lipopolysaccharide for 6 h, the vasodilator response to the PDE-sensitive cGMP analog 8-Bromo-cGMP was preserved, whereas vasorelaxation in response to an NO donor compound was impaired. These investigators suggested that the lipopolysaccharide-induced impairment of NO-dependent vasodilatation was attributable to decreased NO-stimulated cGMP synthesis. Holzmann *et al.*,[17] in an isolated–perfused rat lung model, reported that lipopolysaccharide pretreatment decreased the pulmonary vasodilator response to PDE-sensitive 8-Bromo-cGMP, but not to PDE-insensitive 8-pCPT-cGMP, suggesting that lipopolysaccharide-induced NO hyporesponsiveness was caused by increased pulmonary cGMP–PDE activity. Possible explanations for these differing results include differences in the species studied, the dose of lipopolysaccharide administered, the duration of exposure to lipopolysaccharide, and the experimental technique (*i.e.*, isolated vessel preparation *vs.* whole-organ perfusion).

It has been proposed that neutrophils mediate the endotoxin-induced impairment of the pulmonary vasodilator response to NO.[32] Kristof *et al.*[33] recently reported that endotoxin-induced pulmonary injury was reduced in NOS2-deficient mice compared with wild-type mice. They observed that, in wild-type mice, intraperitoneal injection of lipopolysaccharide (25 mg/kg) induced interstitial leukocyte infiltration, air-space cellularity, and exudation, associated with increased wet-to-dry lung weight ratios and increased nitrotyrosine immunostaining (reflecting peroxynitrite production). These changes were less marked or were absent in lipopolysaccharide-treated NOS2-deficient mice. In contrast, Hickey *et al.*[21] found that, in response to a lower dose of lipopolysaccharide (30 μg/kg intravenous), recruitment of leukocytes into the lungs of NOS2-deficient mice was greater than into the lungs of wild-type mice. Differences in the findings reported in these two studies may be attributable to differences in the dose of lipopolysaccharide used. The finding that the administration of high doses of lipopolysaccharide induced interstitial swelling and exudation into air spaces raises the possibility that impaired responsiveness to inhaled NO was caused by decreased diffusion of NO from the alveoli to the pulmonary vasculature. Although we used a higher dose of lipopolysaccharide in our study (50 mg/kg intraperitoneal), wet-to-dry lung weight ratios did not differ in lipopolysaccharide-treated wild-type and NOS2-deficient mice from untreated mice of either genotype, suggesting the absence of lipopolysaccharide-induced pulmonary edema. Differences in the degree of endotoxin-induced pulmonary injury between our study and that of Kristof *et al.*[33] may reflect differences in the strain of *Escherichia coli* lipopolysaccharide used. We observed that lipopolysaccharide from differing sources and lot numbers vary dramatically in the ability to induce pulmonary *NOS2* gene expression (unpublished data). Moreover, the observation that vasodilation in response to the addition of 8-pCPT-cGMP to the perfusate was impaired in the lungs of lipopolysaccharide-treated wild-type mice provides additional evidence that hyporesponsiveness to inhaled NO is unlikely to be solely attributable to reduced diffusion of gaseous NO into the vasculature.

It is also unlikely that lipopolysaccharide-induced hyporesponsiveness to inhaled NO in wild-type mice is caused by nonspecific dysfunction of the pulmonary vascular contractile apparatus because lipopolysaccharide did not reduce the ability of U46619 to induce pulmonary vasoconstriction. Moreover, discontinuation of U46619 infusion resulted in prompt vasorelaxation of isolated–perfused lungs obtained from lipopolysaccharide-treated and untreated wild-type and NOS2-deficient mice.

Conclusions

Lipopolysaccharide-mediated development of pulmonary vascular hyporesponsiveness to inhaled NO was prevented in mice by targeted disruption of the *NOS2* gene. Breathing 20 ppm NO in ambient air for 16 h in lipopolysaccharide-pretreated NOS2-deficient mice impaired NO-mediated vasodilation, suggesting that NO produced by NOS2 (or inhaled as a supplement in air) is essential for producing lipopolysaccharide-induced NO hyporesponsiveness. Prolonged NO inhalation alone did not produce NO hyporesponsiveness and must be accompanied by another lipopolysaccharide-mediated, NOS2–NO-independent inflammatory mediator or byproduct to produce hyporesponsiveness to inhaled NO.

The authors thank Jonathan Hromi for technical help, and YuChiao Chang, Ph.D., Medical Practice Evaluation Center, Massachusetts General Hospital, for statistical advice.

References

1. Bigatello LM, Hurford WE, Kacmarek RM, Roberts JD Jr, Zapol WM: Prolonged inhalation of low concentrations of nitric oxide in patients with severe adult respiratory distress syndrome. ANESTHESIOLOGY 1994; 80:761–70

2. Dellinger RP, Zimmerman JL, Taylor RW, Straube RC, Hauser DL, Criner GJ, Davis KJ, Hyers TM, Papadakos P: Effects of inhaled nitric oxide in patients with acute respiratory distress syndrome: Results of a randomized phase II trial. Crit Care Med 1998; 26:15–23

3. Rossaint R, Falke KJ, Lopez F, Slama K, Pison U, Zapol WM: Inhaled nitric oxide for the adult respiratory distress syndrome. N Engl J Med 1993; 328:399–405

4. Frostell C, Fratacci MD, Wain JC, Jones R, Zapol WM: Inhaled nitric oxide. A selective pulmonary vasodilator reversing hypoxic pulmonary vasoconstriction. Circulation 1991; 83:2038–47

5. Fratacci MD, Frostell CG, Chen TY, Wain JC Jr, Robinson DR, Zapol WM: Inhaled nitric oxide. A selective pulmonary vasodilator of heparin-protamine vasoconstriction in sheep. ANESTHESIOLOGY 1991; 75:990–9

6. Shah NS, Nakayama DK, Jacob TD, Nishio I, Imai T, Billiar TR, Exler R, Yousem SA, Motoyama EK, Peitzman AB: Efficacy of inhaled nitric oxide in a porcine model of adult respiratory distress syndrome. Arch Surg 1994; 129:158–64

7. Ogura H, Offner PJ, Saitoh D, Jordan BS, Johnson AA, Pruitt BA Jr, Cioffi WG Jr: The pulmonary effect of nitric oxide synthase inhibition following endotoxemia in a swine model. Arch Surg 1994; 129:1233–9

8. Krafft P, Fridrich P, Fitzgerald RD, Koc D, Steltzer H: Effectiveness of nitric oxide inhalation in septic ARDS. Chest 1996; 109:486–93

9. Manktelow C, Bigatello LM, Hess D, Hurford WE: Physiologic determinants of the response to inhaled nitric oxide in patients with acute respiratory distress syndrome. ANESTHESIOLOGY 1997; 87:297–307

10. Lincoln TM, Cornwell TL: Intracellular cyclic GMP receptor proteins. FASEB J 1993; 7:328–38

11. Knowles RG, Moncada S: Nitric oxide synthases in mammals. Biochem J 1994; 298:249–58

12. Moncada S, Palmer RM, Higgs EA: Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991; 43:109–42

13. Petros A, Bennett D, Vallance P: Effect of nitric oxide synthase inhibitors on hypotension in patients with septic shock. Lancet 1991; 338:1557–8

14. Xia Y, Zweier JL: Superoxide and peroxynitrite generation from inducible nitric oxide synthase in macrophages. Proc Natl Acad Sci U S A 1997; 94:6954–8

15. Xia Y, Dawson VL, Dawson TM, Snyder SH, Zweier JL: Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. Proc Natl Acad Sci U S A 1996; 93:6770–4

16. Fullerton DA, McIntyre RC Jr, Hahn AR, Agrafojo J, Koike K, Meng X, Banerjee A, Harken AH: Dysfunction of cGMP-mediated pulmonary vasorelaxation in endotoxin- induced acute lung injury. Am J Physiol 1995; 268:L1029–35

17. Holzmann A, Bloch KD, Sanchez LS, Filippov G, Zapol WM: Hyporesponsiveness to inhaled nitric oxide in isolated, perfused lungs from endotoxin-challenged rats. Am J Physiol 1996; 271:L981–6

18. Holzmann A, Manktelow C, Bloch KD, Zapol WM: Inhibition of inducible nitric oxide (iNOS) prevents hyporesponsiveness to inhaled NO in lungs of lipopolysaccharide (LPS)-treated rats. ANESTHESIOLOGY 1999; 91:215–21

19. Southan GJ, Szabo C: Selective pharmacological inhibition of distinct nitric oxide synthase isoforms. Biochem Pharmacol 1996; 51:383–94

20. MacMicking JD, Nathan C, Hom G, Chartrain N, Fletcher DS, Trumbauer M, Stevens K, Xie QW, Sokol K, Hutchinson N: Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. Cell 1995; 81:641–50

21. Hickey MJ, Sharkey KA, Sihota EG, Reinhardt PH, MacMicking JD, Nathan C, Kubes P: Inducible nitric oxide synthase-deficient mice have enhanced leukocyte-endothelium interactions in endotoxemia. FASEB J 1997; 11:955–64

22. Weimann J, Hagenah JU, Motsch J: Reduction in nitrogen dioxide concentration by soda lime preparations during simulated nitric oxide inhalation. Br J Anaesth 1997; 79:641–4

23. Steudel W, Scherrer-Crosbie M, Bloch KD, Weimann J, Huang PL, Jones RC, Picard MH, Zapol WM: Sustained pulmonary hypertension and right ventricular hypertrophy after chronic hypoxia in mice with congenital deficiency of nitric oxide synthase 3. J Clin Invest 1998; 101:2468–77

24. Tsuchida S, Hiraoka M, Sudo M, Kigoshi S, Muramatsu I: Attenuation of sodium nitroprusside responses after prolonged incubation of rat aorta with endotoxin. Am J Physiol 1994; 267:H2305–10

25. Hinder F, Stubbe HD, van Aken H, Waurik R, Booke M, Meyer J: Role of nitric oxide in sepsis-associated pulmonary edema. Am J Resp Crit Care Med 1999; 159:252–7

26. Combes X, Mazmanian M, Gourlain H, Herve P: Effect of 48 hours of nitric oxide inhalation on pulmonary vasoreactivity in rats. Am J Respir Crit Care Med 1997; 156:473–7

27. Oka M, Ohnishi M, Takahashi H, Soma S, Hasunuma K, Sato K, Kira S: Altered vasoreactivity in lungs isolated from rats exposed to nitric oxide gas. Am J Physiol 1996; 271:L419–24

28. Roos CM, Frank DU, Xue C, Johns RA, Rich GF: Chronic inhaled nitric oxide: Effects on pulmonary vascular endothelial function and pathology in rats. J Appl Physiol 1996; 80:252–60

29. Gerlach H, Pappert D, Lewandowski K, Rossaint R, Falke KJ: Long-term inhalation with evaluated low doses of nitric oxide for selective improvement of oxygenation in patients with adult respiratory distress syndrome. Intensive Care Med 1993; 19:443–9

30. Ungureanu-Longrois D, Balligand JL, Simmons WW, Okada I, Kobzik L, Lowenstein CJ, Kunkel SL, Michel T, Kelly RA, Smith TW: Induction of nitric oxide synthase activity by cytokines in ventricular myocytes is necessary but not sufficient to decrease contractile responsiveness to beta-adrenergic agonists. Circ Res 1995; 77:494–502

31. DeLeo FR, Renee J, McCormick S, Nakamura M, Apicella M, Weiss JP, Nauseef WM: Neutrophils exposed to bacterial lipopolysaccharide upregulate NADPH oxidase assembly. J Clin Invest 1998; 101: 455–63

32. Sheridan BC, McIntyre RC, Agrafojo J, Meldrum DR, Meng X, Fullerton DA: Neutrophil depletion attenuates endotoxin-induced dysfunction of cGMP-mediated pulmonary vasorelaxation. Am J Physiol 1996; 271:L820–8

33. Kristof AS, Goldberg P, Laubach V, Hussain SNA: Role of inducible nitric oxide synthase in endotoxin-induced lung injury. Am J Respir Crit Care Med 1998; 158:1883–9