

*Inhaled Nitric Oxide**A Selective Pulmonary Vasodilator of Heparin-Protamine Vasoconstriction in Sheep*Marie-Dominique Fratacci, M.D.,* Claes G. Frostell, M.D.,* Tong-Yen Chen, M.D.,* John C. Wain, Jr., M.D.,†
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Nitric oxide (NO) has recently been discovered to be an important endothelium-derived relaxing factor and produces profound relaxation of vascular smooth muscle. To learn if NO could be a potent and selective pulmonary vasodilator, NO was inhaled by 16 awake lambs in an attempt to reduce the increase in pulmonary artery pressure (PAP) and pulmonary vascular resistance (PVR) induced by either the infusion of an exogenous pulmonary vasoconstrictor (the thromboxane analog U46619) or the endogenous release of thromboxane that occurs during the neutralization of heparin anticoagulation by protamine sulfate. Inhaling ≥ 40 ppm of NO during a continuous U46619 infusion returned the PAP to a normal value, without affecting systemic blood pressure or vascular resistance. Pretreatment with the cyclooxygenase inhibitor indomethacin before infusing U46619 did not reduce the pulmonary vasodilatory effect of inhaled NO, and we conclude that the dilatory effect of NO on the lung's circulation is independent of cyclooxygenase products such as prostacyclin. Continuously inhaling NO at 180 ppm did not significantly reduce the mean peak thromboxane B₂ concentration at 1 min after protamine injection; however, the mean values of pulmonary hypertension and vasoconstriction at 1 min were markedly reduced below the levels in untreated heparin-protamine reactions. Breathing NO at lower concentrations (40-80 ppm) did not decrease the mean peak PAP and PVR at 1 min after protamine but decreased the PAP and PVR values at 2, 3, and 5 min below those of control heparin-protamine reactions. Intravenous infusion of nitroprusside completely prevented the transient increase of PAP and PVR during the heparin-protamine reaction; however, marked concomitant systemic vasodilation occurred. Inhaled NO is a selective pulmonary vasodilator that can prevent thromboxane-induced

pulmonary hypertension during the heparin-protamine reaction in lambs and can do so without causing systemic vasodilation. (Key words: Blood, coagulation: heparin-protamine. Heart: vascular pressures. Hormones: thromboxane A₂. Lung: vascular resistance. Muscle, smooth: endothelium-derived relaxing factor. Nitric oxide.)

FURCHGOTT AND ZAWADZKI demonstrated that acetylcholine-elicited relaxation of isolated arteries is dependent on the presence of an intact endothelial cell layer.¹ The major factor released from endothelium that caused smooth muscle relaxation was subsequently named endothelium-derived relaxing factor (EDRF).² Ignarro *et al.*,³ Palmer *et al.*,⁴ and Furchgott⁵ subsequently reported that EDRF had many of the properties of the nitrogen free radical, nitric oxide (NO). Among other features, NO and EDRF both were rapidly inactivated by hemoglobin and methylene blue⁶ and exerted their dilatory effect by activating soluble guanylate cyclase intracellularly, the receptor being the heme-molecule.⁷ S-nitrosothiols, hydroxylamine, or anisothiol may comprise other potent forms of EDRF.⁸ Thus, the specific identity of EDRF remains controversial. The dilatory effects of EDRF and NO are of short duration, with a half-life of 3-5 s.⁹ These features characterize EDRF as a short-acting local vasodilator that is first released from the endothelium and then diffuses to subjacent smooth muscle cells. NO has been shown to dilate isolated precontracted rings of pulmonary vessels.¹⁰

Frostell *et al.* recently reported that in awake lambs inhalation of a gas mixture containing 40-80 parts per million (ppm) NO could reverse the acute pulmonary vasoconstriction induced by severe hypoxia.¹¹ A dose-dependent pulmonary vasodilator effect of inhaled NO was also observed during a steady-state infusion of the potent pulmonary vasoconstrictor 5Z 9 α ,13E,15S-11,9-(epoxy-methano)prosta-5,13-dien-1-oic acid (U46619), an endoperoxide analog of thromboxane. Breathing 80 ppm NO for 1 h produced sustained pulmonary vasodilation without evidence of tolerance. Since NO is rapidly inactivated by combination with hemoglobin,⁶ the vasodilating effect of inhaled NO is selectively exerted on the pulmonary vasculature, and both systemic arterial pressure and vascular resistance are unchanged. Inhaling NO at 80 ppm did not alter pulmonary artery pressure (PAP)

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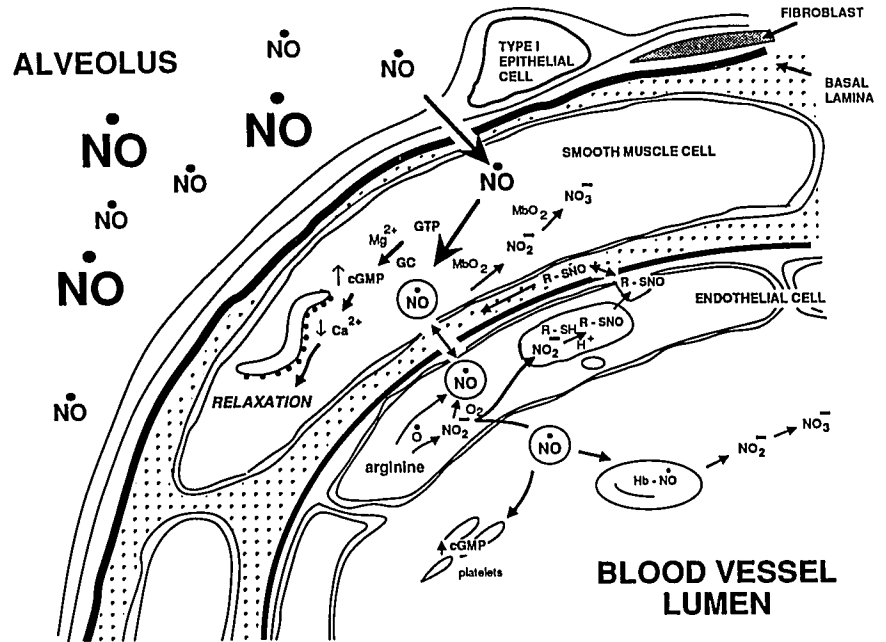
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FIG. 1. A schematic of the proposed routes of nitric oxide (NO) uptake and mechanism for pulmonary vasodilation. Hb = hemoglobin; Mb = myoglobin; cGMP = cyclic guanosine monophosphate; GC = guanyl cyclase; SH = sulfhydryl. Modified by Roberts JD after Ignarro L,⁶ with permission.



or pulmonary vascular resistance (PVR) when a vasoconstrictor was not given. A schematic diagram of our present understanding of the mechanism of inhaled NO vasodilation is presented in figure 1.

Severe acute pulmonary artery hypertension in lambs can be produced by the infusion of the exogenous thromboxane analog U46619 as well as by the endogenous release of thromboxane during the neutralization of heparin anticoagulation with protamine sulfate. The latter represents a repeatable laboratory model of the sporadic and often severe clinical hemodynamic adverse reaction. We therefore examined in awake lambs whether inhalation of NO during the heparin-protamine reaction could reduce the pulmonary vasoconstrictor response. In several lambs we compared the vasodilation produced by NO inhalation with that caused by intravenously infused nitroprusside, a vasodilator that acts by releasing NO within the smooth muscle cell to activate guanylate cyclase (*i.e.*, an endothelium-independent vasodilator). To examine the mechanism of NO vasodilation we studied whether the vasodilator effect of inhaled NO is mediated by another endothelium-derived relaxing factor, the potent pulmonary vasodilator prostacyclin (prostaglandin I₂). Therefore, during the infusion of the thromboxane analog, we examined the dose response to inhaled NO before and after pretreatment with a cyclooxygenase inhibitor.

Materials and Methods

All lamb studies were approved by the Massachusetts General Hospital Subcommittee on Animal Studies.

ANIMAL PREPARATION

After tracheal intubation and mechanical ventilation and during general anesthesia (halothane 1–4% in oxygen [O₂]), Suffolk lambs weighing 25–35 kg were surgically instrumented using sterile techniques. The femoral artery was cannulated with a polyvinyl chloride catheter (3 mm outside diameter, 1.2 mm inside diameter) advanced 30 cm into the aorta for intermittent blood sampling and mean arterial pressure (MAP) monitoring. A pulmonary artery flow-directed thermodilution catheter (model 93A-131H-7F, Edwards Laboratory, Santa Ana, CA) was inserted percutaneously *via* an 8-Fr sheath (Cordis Corporation, Miami, FL) in the right external jugular vein and advanced into the pulmonary artery to measure mean PAP and pulmonary artery wedge pressure at end expiration. Central venous pressure was measured *via* a side hole of this catheter in the right atrium. The pulmonary artery catheter remained in place for the duration of the series of studies. In five lambs a left thoracotomy was performed to insert a left atrial catheter (19-G Intracath, Deseret Medical, Inc., Sandy, UT) to monitor mean left atrial pressure. All lambs underwent tracheostomy (7 mm, Portex, Wilmington, MA). The catheters were flushed with normal saline containing heparin (1,000 units/ml), and antibiotics (Combiotic, Pfizer, New York, NY) were injected intramuscularly daily.

HEMODYNAMIC MEASUREMENTS

MAP, PAP, central venous pressure, and pulmonary artery wedge pressure were monitored using calibrated

Hewlett-Packard (Palo Alto, CA) 1280C pressure transducers with the zero reference level at midthoracic level in the standing position. Pressures were continuously recorded on a four-channel recorder (model 7754B, Hewlett-Packard), and mean values at the various time intervals were measured at end expiration. Cardiac output (CO) was recorded as the average of two determinations by thermodilution using an Edwards Laboratory computer (model 9520A) and injecting 5 ml of 0° C lactated Ringer's solution. PVR and systemic vascular resistance (SVR) were calculated using standard formulas. Left atrial pressure was used to calculate PVR when available; otherwise, pulmonary artery wedge pressure was used.

MEASUREMENT OF LEUKOCYTES AND EICOSANOIDS

Arterial blood samples (4 ml) were withdrawn into plastic syringes and transferred to glass test tubes containing 0.05 ml 15% EDTA and 100 µg indomethacin; 40 µl was removed for determination of leukocyte concentration with a Coulter cell counter (model ZF, Coulter Electronics, Hialeah, FL); and the remainder was immediately transferred to ice. Blood samples were then centrifuged at 1,250 g at 4° C for 10 min, aspirated, and stored in polypropylene tubes at -20° C until assayed. Plasma levels of thromboxane B₂ (TXB₂) were determined by radioimmunoassay as described previously,¹² using antisera provided by Dr. L. Levine, Brandeis University (Waltham, MA). The assays were standardized with TXB₂ obtained from Sigma Chemical Company (St. Louis, MO).

ADMINISTRATION OF NITRIC OXIDE

Each lamb was connected *via* the tracheostomy to a nonbreathing circuit consisting of a 5-l reservoir bag and a one-way valve to isolate inspired from expired gas. The residence half-time of NO in the gas reservoir was 30 s or less, with a fresh gas flow of 10 l/min. Expired gas was scavenged and discarded. The inspired gas was a precise mixture of O₂ and nitrogen (N₂) immediately diluted with NO to produce the correct inspired concentration of NO. Using volumetrically calibrated flowmeters, varying quantities of NO mixed with N₂ were substituted for pure N₂ to obtain the desired inspired NO concentration at a constant inspired O₂ concentration (FI_{O₂}). NO was obtained from Air Products and Chemicals, Inc. (Allentown, PA), as a mixture of 235 ppm in pure N₂. Chemiluminescence analysis¹³ demonstrated less than 5 ppm nitrogen dioxide (NO₂) in this mixture. During the studies NO and NO₂ concentrations of the breathing mixtures were chemically analyzed using Matheson-Kitagawa toxic gas detectors (model 8014-400A, Matheson Gas Products, Montgomeryville, PA).

EXPERIMENTAL PROCEDURE

Lambs were allowed 24 h to recover from anesthesia and cannulation. Catheters were aspirated before each study and continuously flushed (2 ml/h) with lactated Ringer's solution without heparin during the experiments. Lambs were studied while awake and spontaneously breathing in the standing position. Water and food were given *ad libitum*. Experiments were performed if the animal met all of the following criteria: rectal temperature less than 40.1° C, circulating white blood cell concentration between 4,000 and 12,000 cells/mm³, and mean PAP less than 20 mmHg.

Nitric Oxide Dose-Response Curves with U46619 Infusion and the Effect of Cyclooxygenase Inhibition

Five lambs were included in this study. On day 1, the stable endoperoxide analog U46619 (Upjohn Co., Kalamazoo, MI) was infused at a rate of 0.4–0.8 µg · kg⁻¹ · min⁻¹ to increase the mean PAP to 30 mmHg. After a stable period of pulmonary hypertension, lambs breathed a series of increasing NO/N₂ mixtures containing 5, 10, 20, 40, and 80 ppm NO for 6 min at FI_{O₂} 0.6–0.7. Each level of NO exposure was followed by 6 min of breathing the O₂ mixture without NO at the same FI_{O₂}. Then a second exposure to 5, 10, and 20 ppm NO was examined for similar periods. Hemodynamic measurements were performed at each 3- and 6-min time period and were repeated after ceasing U46619 infusion. Maximal changes of PAP and PVR were calculated as follows:

$$\text{PAP}(\text{Max}) = 100 \times (\text{PAP}_{\text{ht}} - \text{PAP}_{\text{NO}}) / (\text{PAP}_{\text{ht}} - \text{PAP}_{\text{c}}) \quad (1)$$

where PAP_{max} is the change of PAP induced by NO inhalation, expressed as a percent of the maximal change of PAP induced by infusion of U46619; PAP_{ht} is the PAP recorded 6 min after infusing U46619; PAP_{NO} is the PAP recorded during NO inhalation and infusion of U46619; and PAP_c is the value recorded at baseline before starting U46619 infusion.

$$\text{PVR}(\text{Max}) = 100 \times (\text{PVR}_{\text{ht}} - \text{PVR}_{\text{NO}}) / (\text{PVR}_{\text{ht}} - \text{PVR}_{\text{c}}) \quad (2)$$

where PVR_{max} is the change of PVR induced by NO inhalation, expressed as a percent of the maximal change of PVR induced by infusion of U46619; PVR_{ht} is the PVR obtained 6 min after infusing U46619; PVR_{NO} is the PVR obtained during inhalation of NO and infusion of U46619; and PVR_c the value of PVR obtained at baseline before starting U46619 infusion. The formula (PAP – left atrial pressure)/CO was used to calculate PVR.

On day 2, each of the five lambs was pretreated with a cyclooxygenase inhibitor (indomethacin, 10 mg/kg), infused intravenously over 30 min. Indomethacin (Sigma, St. Louis, MO) was dissolved in 1 ml ethanol, which was then added to lactated Ringer's solution (400 ml) neu-

tralized to pH 7.3–7.6 with sodium bicarbonate. Cyclooxygenase inhibition was confirmed by measuring the pulmonary vascular response during a brief infusion of arachidonic acid (AA). AA (Sigma) first was dissolved in a small amount of 90% ethanol and then diluted in sterile saline to a final concentration of 150 $\mu\text{g}/\text{ml}$, and was infused intravenously at a constant rate ($100 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) for 5 min while pulmonary and systemic hemodynamic measurements and plasma eicosanoid samples were obtained. After AA challenge the animals rested for 30 min, and then an infusion of U46619 was begun and the previously described protocol (inhalation of increasing concentrations of NO) was repeated.

Heparin-Protamine Reaction

On day 3, lambs received 200 units/kg porcine intestine-derived heparin (Elkins-Sinn, Inc., Cherry Hill, NJ) as an intravenous bolus through the right atrial catheter. Five minutes later 2 mg/kg protamine sulfate (Elkins-Sinn) was injected through the right atrial catheter over a period of 10 s. Hemodynamic measurements and blood samples were obtained at -6, -1, 1, 2, 3, and 5 min relative to the injection of protamine sulfate at time zero. During the heparin-protamine reactions three groups of lambs were studied:

Group 1: Seven lambs were allowed to breathe NO mixtures at either 40 ppm ($n = 3$) or 80 ppm ($n = 4$) with FI_{O_2} 0.6–0.7. NO inhalation began 5 min before protamine injection.

Group 2: Nine lambs breathed 180 ppm NO at FI_{O_2} 0.2–0.4. NO inhalation began 5 min before protamine injection.

Group 3: In six lambs after both a control and NO-inhalation heparin-protamine reaction were studied, a third heparin-protamine reaction was examined during an infusion of sodium nitroprusside (SNP). Freeze-dried SNP (Elkins-Sinn) was dissolved in 5% dextrose to produce a final concentration of 100 $\mu\text{g}/\text{ml}$ and infused at a dose of $40 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, beginning 5 min before heparin injection and lasting until 5 min after protamine injection. This SNP dose was chosen in order to achieve the same pulmonary vasodilation induced by 40 ppm inhaled NO during an infusion of U46619 (data not shown). In these pilot studies during U46619 infusion, SNP was given in incremental doses from 1 to $40 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$. Plasma cyanide concentrations were sampled and measured at the end of each SNP study.

Heparin-protamine reactions were performed in a random order for group 1 and 2 lambs. In all lambs heparin-protamine reactions were separated by at least 3 h. Montalescot *et al.*¹⁴ demonstrated that the course and the magnitude of plasma thromboxane increase and pulmonary hypertension were not altered when heparin-protamine challenges were repeated at 3-h intervals.

Control Heparin-Protamine Reactions

In each group 1 and 2 lamb, a control heparin-protamine reaction was studied in random order with the NO inhalation studies. In group 3 lambs SNP infusion was always studied last to avoid any prolonged cyanide toxicity. The FI_{O_2} was 0.6–0.7 in group 1 and 0.3–0.4 in groups 2 and 3 to produce the appropriate inhaled NO levels.

STATISTICAL ANALYSIS

All results are expressed as mean \pm standard error of the mean. A two-way analysis of variance (ANOVA, version 5.16, SAS Institute, Cary, NC) was used to analyze dose-response curves. Data before and after pretreatment by indomethacin were compared at each inhaled NO dose using a paired *t* test. Values from both the early and late trials of the 5, 10, and 20 ppm NO doses were averaged to minimize any effect of time.

For the heparin-protamine reactions, measurements were compared at each time period using a paired *t* test. The eicosanoid data were statistically analyzed after being subjected to logarithmic transformation; this provided a better approximation to a normal distribution at the various time intervals.

Results

CYCLOOXYGENASE INHIBITION

The pulmonary vasodilator effects of NO inhalation during U46619 infusion were similar to those previously reported,¹¹ with marked dilatation of the constricted pulmonary vascular bed at ≥ 40 ppm NO. Systemic vascular resistance was not altered by inhalation of NO at 5–80 ppm. Indomethacin pretreatment completely blocked the increase of thromboxane ($n = 3$) and PAP ($n = 4$) after injection of AA (data not shown). After pretreatment with indomethacin and infusion of U46619, there was no reduction of the profound vasodilator effect of breathing 5–80 ppm NO on the PAP and PVR (figs. 2 and 3).

PULMONARY HEMODYNAMICS DURING HEPARIN-PROTAMINE REACTIONS

As previously described,¹⁵ in control heparin-protamine-reaction lambs, the maximum increase of PAP and PVR occurred 1 min after injection of protamine sulfate to reverse heparin anticoagulation. These hemodynamic variables rapidly returned toward baseline values during the next 4 min. Breathing 40–80 ppm NO did not significantly reduce the mean peak PAP at 1 min, although the subsequent 2-, 3-, and 5-min mean PAP values were significantly less than the control heparin-protamine reaction levels. Inhaling NO at 180 ppm before protamine infusion markedly reduced the mean peak PAP (*P*

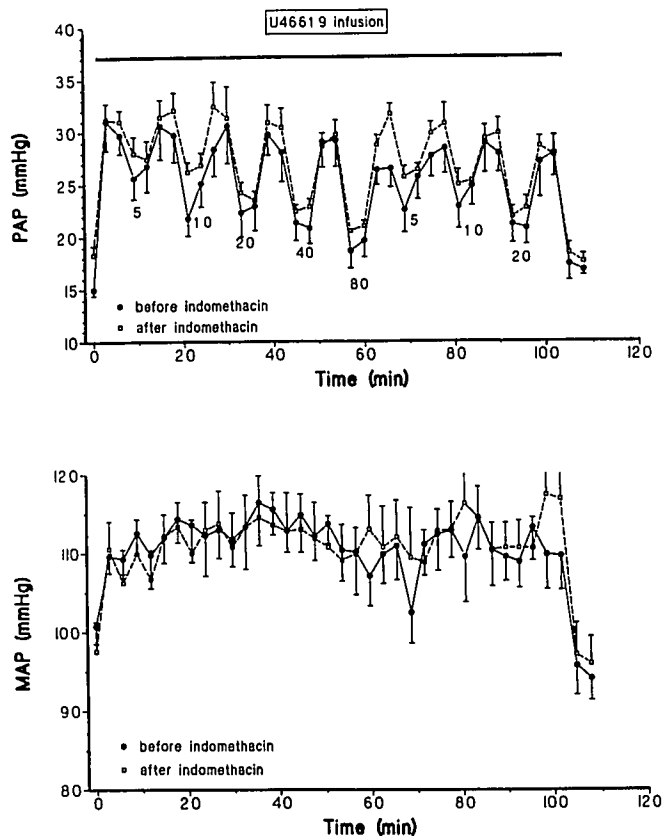


FIG. 2. Dose-response curves at varying inhaled nitric oxide (NO) concentration during the continuous infusion of U46619 on mean pulmonary arterial (PAP) and mean arterial (MAP) pressures before and after pretreatment by indomethacin ($n = 5$, mean \pm SE).

< 0.005). The increase of PVR at 1 min due to the heparin-protamine reaction was reduced by inhalation of both 40–80 ppm NO ($P < 0.01$) and 180 ppm NO ($P < 0.05$) (figs. 4 and 5). Baseline levels of PAP and PVR did not change significantly between these studies. Intravenous infusion of SNP increased baseline CO and reduced baseline PVR. SNP infusion at $40 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ reduced the increase of PAP and PVR due to the heparin-protamine reaction, as did inhalation of 180 ppm NO (fig. 6). Neither treatment completely blocked the increase of PAP from baseline levels.

ARTERIAL PLASMA THROMBOXANE B_2 CONCENTRATIONS

The results are summarized in table 1. In each group, 1 min after protamine infusion in control heparin-protamine studies, plasma TXB_2 concentrations significantly increased and then returned toward baseline values. In comparison with control heparin-protamine reactions, breathing NO at either 40–80 or 180 ppm did not sig-

nificantly reduce the mean peak plasma TXB_2 levels at 1 min after protamine neutralization of heparin. In group 3, infusion of SNP significantly reduced the plasma TXB_2 concentrations to below those of control heparin-protamine reactions at 1 and 3 min.

ARTERIAL WHITE BLOOD CELL CONCENTRATION

Mean white blood cell levels in control heparin-protamine studies decreased 1 and 3 min after protamine infusion (table 1) and remained significantly decreased for more than 5 min (data not shown). Breathing either 40–80 or 180 ppm NO or an SNP infusion did not modify the transient leukopenia. Baseline white blood cell levels did not differ significantly between these studies.

SYSTEMIC HEMODYNAMICS

In control lambs MAP and SVR did not change significantly; mean heart rate and CO also did not change significantly. None of these variables was altered by NO inhalation (40–80 or 180 ppm). However, SNP infusion

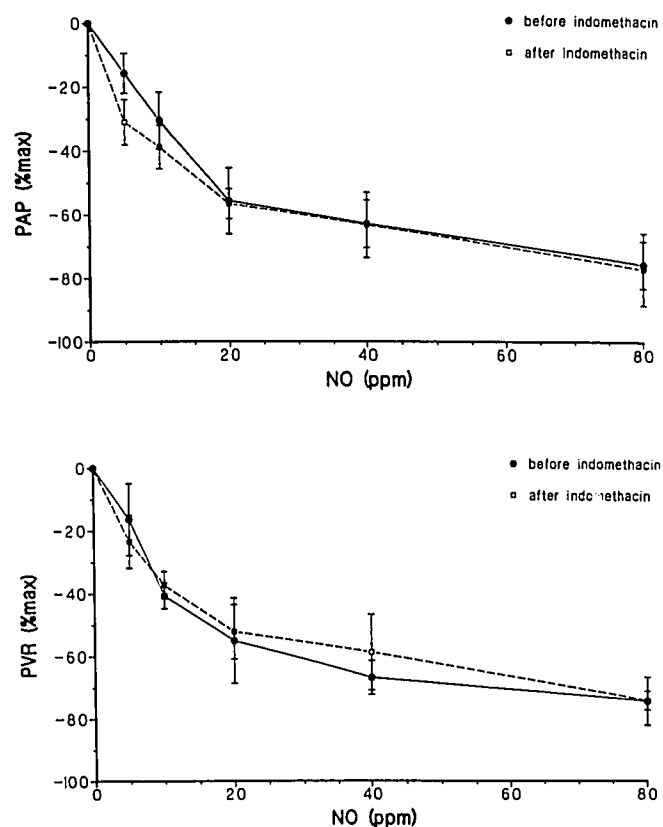


FIG. 3. Dose-response curves at varying inhaled NO concentration during the continuous infusion of U46619, for the 6-min percent maximal change of PAP and PVR in five lambs, before and after pretreatment by indomethacin. See Materials and Methods for details of computation of maximal change ($n = 5$, mean \pm SE).

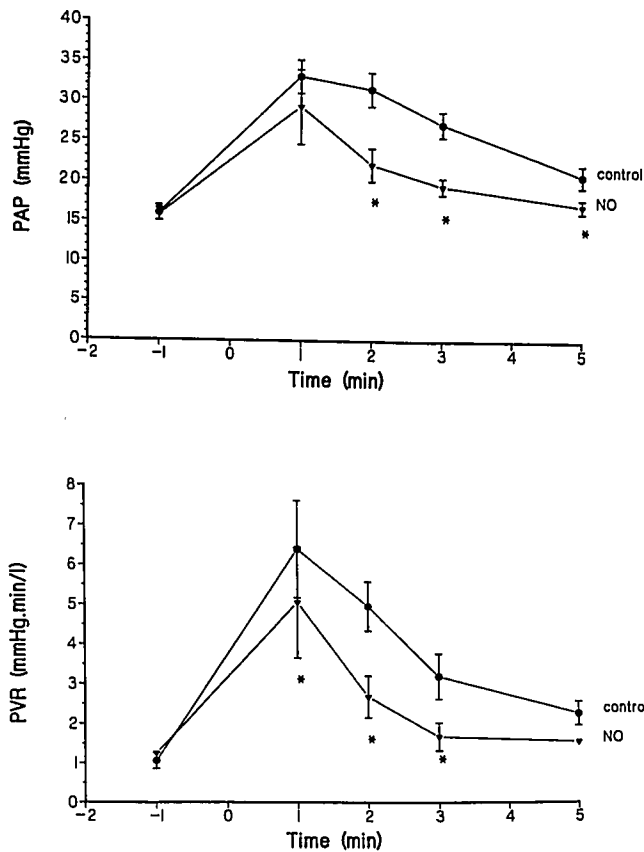


FIG. 4. Effects of a bolus injection of protamine given 5 min after heparin injection on mean pulmonary arterial pressure (PAP) and pulmonary vascular resistance (PVR) in 7 lambs without (control) or during inhalation of 40–80 ppm nitric oxide (NO). * $P < 0.05$ value differs from control at the same time.

considerably reduced the baseline MAP and SVR while significantly increasing CO and heart rate (table 2). Methemoglobin levels never exceeded 2% after inhalation of 180 ppm NO for 10 min ($0.82 \pm 0.07\%$, $n = 5$). Another lamb (data not reported) breathed 600 ppm NO for 10 min, and the methemoglobin level increased to 3.4%. Mean plasma cyanide concentration measured 5 min after protamine injection during the infusion of SNP were $741 \pm 251 \mu\text{g/l}$.

Discussion

This study demonstrates in awake lambs that 1) during an infusion of the pulmonary vasoconstrictor U46619 the selective pulmonary vasodilation caused by inhaling NO at 5–80 ppm is independent of cyclooxygenase products; 2) pretreatment by continuously breathing 180 ppm NO can markedly reduce the peak pulmonary vasoconstriction and hypertension produced by the heparin-protamine reaction; and 3) NO breathing did not significantly reduce

endogenous thromboxane release during the heparin-protamine reaction.

In a recent study¹¹ Frostell *et al.* demonstrated that NO inhalation by lambs could reverse pulmonary vasoconstriction and hypertension produced by either the continuous infusion of U46619 (a stable thromboxane analog) or by the inhalation of a hypoxic gas mixture. Furthermore, the pulmonary vasodilator effect of breathing NO occurred selectively in the lung since systemic arterial pressure and vascular resistance remained unchanged. The first portion of the present study reproduced the dose-response curve to breathing 5–80 ppm NO during U46619 infusion. We then examined the effects of pretreating these lambs with a cyclooxygenase inhibitor to learn if eicosanoid mediators played a role in NO vasodilation. Neither cyclooxygenase nor thromboxane synthetase blockade has been demonstrated to affect the degree of U46619-induced pulmonary vasoconstriction.¹⁶ In our study, pretreating the lambs with indomethacin did not significantly alter the subsequently ob-

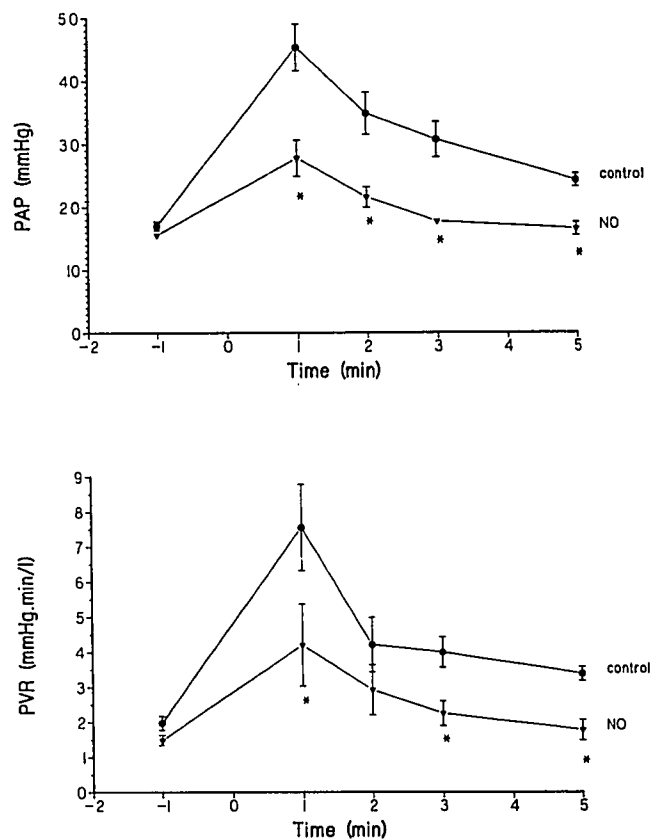


FIG. 5. Effects of a bolus injection of protamine given 5 min after heparin injection on mean pulmonary arterial pressure (PAP) and pulmonary vascular resistances (PVR) in nine lambs without (control) or during inhalation of 180 ppm nitric oxide (NO). * $P < 0.05$ value differs from control at the same time.

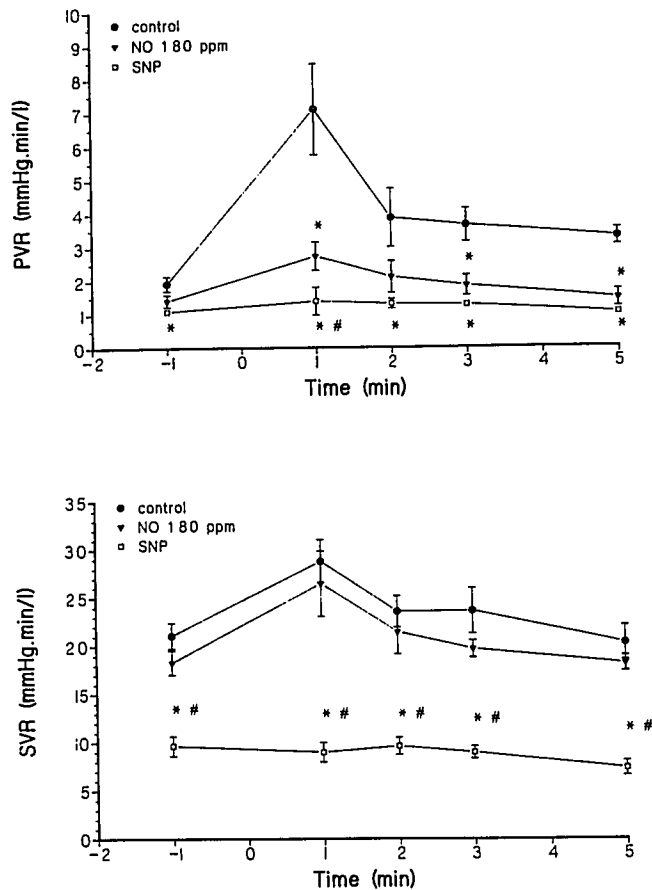


FIG. 6. Effects of a bolus injection of protamine given 5 min after heparin injection on pulmonary vascular resistance (PVR) and systemic vascular resistance (SVR) in six lambs before and after inhalation of 180 ppm nitric oxide (NO) and after infusion of sodium nitroprusside (SNP). * $P < 0.05$ value differs from control values at same time. # $P < 0.05$ value differs from 180 ppm NO breathing at the same time.

tained pulmonary vasodilator dose-response curves to inhaled NO during the infusion of U46619 (figs. 2 and 3). Although we did not measure the plasma levels of 6-keto-prostaglandin $F_{1\alpha}$, the metabolite of prostacyclin, our results support the widely held view that the vasodilator effect of NO is not mediated by cyclooxygenase products such as the potent pulmonary vasodilator prostacyclin. Thus, NO inhalation vasodilates the lung independently of cyclooxygenase products, as was previously demonstrated for systemic vessels treated with S-nitrosothiols, which are unstable and spontaneously decompose to yield NO.¹⁷ In these *in vitro* studies, NO vasodilator responses were not antagonized by propranolol, indomethacin, or antihistamines.

The proposed mechanism for the vasodilator effect of NO and nitro-vasodilators is *via* the increase of intracellular cyclic guanosine monophosphate (cGMP)¹⁸ (fig. 1). To test the hypothesis that cGMP mediates vascular smooth muscle relaxation, the actions of NO on bovine

coronary arteries were examined. NO was found to activate soluble guanylate cyclase and elevate cGMP levels, causing marked but transient relaxation responses that were antagonized by hemoproteins and methylene blue.^{19,20} The molecular mechanism by which NO stimulates intracellular cGMP accumulation in mammalian cells is well understood, but the precise mechanism by which cGMP causes smooth muscle relaxation is uncertain; it may do so by rapidly reducing intracellular calcium levels. The heme moieties of soluble guanylate cyclase have a high affinity for NO and react rapidly with NO to generate the NO-heme complex,¹⁸ which binds to the porphyrin site on soluble guanylate cyclase, presumably causing a conformational change and resulting in enzyme activation and stimulation of cGMP formation. NO is extremely lipophilic and can readily permeate biologic membranes to reach and bind to intracellular guanylate cyclase. Thus, NO exposure stimulates cGMP formation, relaxing vascular smooth muscle and inhibiting platelet aggregation.^{17,21} cGMP analogs inhibit the release and subsequent metabolism of AA in human platelets.²² It appears that a portion of the antiplatelet aggregation activity of NO is mediated *via* the elevation of cGMP that inhibits AA release.

Inhibition of AA release by NO with reduced thromboxane A_2 (TXA₂) formation might provide another route moderating the heparin-protamine reaction. If this hypothesis were true, administration of NO should reduce the release of endogenous thromboxane. We examined this hypothesis in the lambs during neutralization of heparin anticoagulation with protamine sulfate. NO inhalation at high concentrations (180 ppm) did not significantly reduce the mean peak circulating TXB₂ levels during the heparin-protamine reaction. We believe that this provides indirect evidence that NO is not reaching sufficiently high levels in the cells that release thromboxane. These cells are likely to be the recently described ovine pulmonary intravascular macrophages²³ or possibly other cells. It is probable that circulating hemoglobin markedly and rapidly reduces the intravascular levels of NO, thereby preventing NO from reaching intravascular cells. During NO inhalation, the rapid intravascular neutralization of NO is also reflected by the absence of systemic vasodilation and an unchanged bleeding time (data not shown). Intravenous SNP infusion during the heparin-protamine reaction markedly reduced plasma thromboxane release and blocked the increase of PVR; however, we do not know if SNP exerted its effect by inhibiting AA metabolism or cyanide toxicity, since the plasma cyanide doses we administered and levels we measured are considered toxic in humans.²⁴

Severe acute pulmonary vasoconstriction, bronchoconstriction, and systemic hypotension occur occasionally when the anticoagulant effect of heparin is neutralized

TABLE 1.

	Thromboxane B ₂ (ng/ml)			White Blood Cells (cells/mm ³)		
	-1'	+1'	+3'	-1'	+1'	+3'
Group 1 (n = 7)						
C	0.23 ± 0.02	3.76 ± 0.68*	2.73 ± 0.46*	7608 ± 471	2974 ± 479*	3537 ± 404*
NO 40-80 ppm	0.22 ± 0.03	2.92 ± 0.69*	1.77 ± 0.39*	7521 ± 469	3808 ± 445*	4239 ± 689*
Group 2 (n = 9)						
C	0.25 ± 0.03	6.41 ± 1.68*	3.58 ± 0.37*	10380 ± 1045	4436 ± 884*	5298 ± 918*
NO 180 ppm	0.24 ± 0.04	4.53 ± 1.77*	1.92 ± 0.76	9899 ± 828	5470 ± 1396	6426 ± 1286
Group 3 (n = 6)						
C	0.25 ± 0.04	3.24 ± 1.05*	2.2 ± 0.51*	9434 ± 1282	5171 ± 1210	6137 ± 1250
NO 180 ppm	0.19 ± 0.05	3.44 ± 2.32	2.19 ± 1.44	7056 ± 810	5064 ± 673*	5384 ± 994*
SNP	0.22 ± 0.08	0.43 ± 0.06†	0.37 ± 0.06†	9921 ± 1266	5344 ± 2128*	6248 ± 1964*

Means ± SEM.
C = control; NO = nitric oxide; SNP = sodium nitroprusside 40 μg · kg⁻¹ · min⁻¹.

* P < 0.05 value differs from -1 min in same treatment group.
† P < 0.05 value differs from control measurements at same time.

by protamine sulfate in humans.²⁵ Explosive release of TXA₂ has been identified as the key event in this life-threatening complication.^{15,26} TXA₂ is an unstable and potent vasoconstrictor synthesized from AA and rapidly hydrolyzed to its inactive stable metabolite TXB₂.²⁷ Inhibition of TXA₂ production by pretreating the lambs with either a cyclooxygenase inhibitor or a thromboxane synthetase inhibitor, or reducing the effects of TXA₂ by blocking thromboxane receptors, can reduce or prevent pulmonary hypertension induced by heparin-protamine neutralization in lambs.^{14,26}

Although breathing 180 ppm NO lowered the peak PAP and PVR at 1 min during the heparin-protamine reaction as compared with control heparin-protamine reaction, lower inhaled levels of NO (40-80 ppm) did not reduce the mean peak PAP at 1 min (fig. 5). High inhaled levels of NO are necessary to prevent pulmonary vasoconstriction during the heparin-protamine reaction, probably because of the markedly elevated pulmonary

vascular concentrations of TXA₂. This contrasts with the marked vasodilation of the constricted pulmonary circulation achieved by inhaling ≥ 40 ppm NO during an infusion of U46619¹¹ or during the breathing of a hypoxic gas mixture.

NO did not modify the transient leukopenia that occurs during the heparin-protamine reaction. It is believed that circulating white blood cells decrease because of the production of anaphylatoxins such as C5a,²⁸⁻³⁰ a potent leukoaggregating substance. NO breathing should not modify intravascular leukoaggregation.

A decreased PAP and PVR during the heparin-protamine reaction was also achieved by infusing SNP (whose vasodilator action is due to NO release) at a dose producing a marked reduction of MAP and SVR. Furthermore, plasma cyanide levels obtained at the end of the reaction were above the toxic level. During SNP infusion, the plasma TXB₂ levels at 1 min were significantly reduced to below heparin-protamine control challenge val-

TABLE 2. Systemic Hemodynamics for Group 3 (n = 6)

		Time (min)				
		-1	1	2	3	5
Cardiac output (l/min)	C	4.88 ± 0.33	4.13 ± 0.29	4.48 ± 0.29	4.78 ± 0.39	5.30 ± 0.37
	NO	5.56 ± 0.38	4.43 ± 0.48	4.94 ± 0.43	5.18 ± 0.26	5.50 ± 0.21
	SNP	7.44 ± 0.77*	7.85 ± 0.89*	7.62 ± 0.65*	8.24 ± 0.55*	9.57 ± 0.89*
Heart rate (beats per min)	C	112 ± 17	110 ± 13	108 ± 17	119 ± 22	117 ± 25
	NO	134 ± 6	141 ± 10	130 ± 7	132 ± 5	143 ± 7
	SNP	173 ± 15*	188 ± 11*	192 ± 7*	200 ± 11*	217 ± 12*
Mean arterial pressure (mmHg)	C	104.9 ± 3.4	120.4 ± 5.6	108.2 ± 2.4	113.4 ± 3.1	106.6 ± 2.0
	NO	101.9 ± 6.1	109.6 ± 7.5	104.8 ± 6.1	105.3 ± 6.5	103.2 ± 6.4
	SNP	73.5 ± 3.7*	71.9 ± 4.3*	77.7 ± 3.5*	80.6 ± 3.9*	77.3 ± 5.7*
Systemic vascular resistance (mmHg · l ⁻¹ · min ⁻¹)	C	21.1 ± 1.3	28.8 ± 2.2	23.5 ± 1.6	23.6 ± 2.3	20.3 ± 1.8
	NO	18.3 ± 1.2	26.5 ± 3.4	21.4 ± 2.3	19.7 ± 0.9	18.2 ± 0.8
	SNP	9.7 ± 1.0*	9.0 ± 1.0*	9.6 ± 0.9*	9.0 ± 0.6*	7.3 ± 0.8*

Mean ± SEM.
C = control; NO = nitric oxide 180 ppm; SNP = sodium nitroprusside 40 μg · kg⁻¹ · min⁻¹.

ues, although TXB₂ values were elevated when compared to baseline values before protamine injection. Thus, a significant but incomplete inhibition of the increase of TXB₂ was produced by SNP infusion.

NO can be converted by oxidation to NO₂ and other toxic oxides of nitrogen. Therefore we also measured inhaled NO and NO₂ levels, by chemiluminescence. In the lambs breathing 180 ppm NO in 30–40% O₂, we measured inhaled NO at 180 ppm and NO₂ at less than 5 ppm. Mixed exhaled gas contained 120 ppm NO and less than 5 ppm NO₂. We consider these NO and NO₂ levels to be safe for brief periods of inhalation because they are near the levels permitted for occupational NO₂ excursions.³¹ Rabbits have breathed 43 ppm NO and 3.6 ppm NO₂ for 6 days without change of lung morphology by electron microscopy or increased lung water.³² Mice have breathed 10 ppm NO for 6 months without lung injury.³³ Inhalations of cigarette smoke contain up to 1,000 ppm NO,³⁴ and cigarette smoke dilates the hypoxic porcine pulmonary circulation.³⁵ Methemoglobin levels are increased by breathing high levels of NO³⁶; however, methemoglobin levels in five lambs measured after breathing 180 ppm NO for 10 min never exceeded 2%. However, studies of pulmonary metabolism and lung mechanics have not been obtained at 180 ppm NO. A detailed discussion of NO inhalation toxicity has recently been published.¹¹

Since pretreatment by NO inhalation attenuates heparin–protamine–induced pulmonary hypertension and vasoconstriction in awake lambs, NO inhalation represents an alternative approach to treating pulmonary hypertension associated with the heparin–protamine reaction as well as other acute and reversible causes of pulmonary hypertension. The advantages of such an inhalation therapy are its speed and ease of administration, its pulmonary selectivity, and the absence of systemic vasodilation. Since breathing NO at high levels for long periods can produce toxic side effects (methemoglobinemia, NO₂ contamination, or lung injury), future trials of NO breathing in patients with heparin–protamine reactions should be brief and carefully conducted, measuring the levels of these toxic products while searching for therapeutic efficacy by reversing life-threatening pulmonary hypertension.

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