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EXPERIMENTAL

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Inhibition of lung phosphodiesterase improves responsiveness to inhaled nitric oxide in isolated-perfused lungs from rats challenged with endotoxin

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Abstract *Objectives:* To investigate the ability of phosphodiesterase (PDE) selective inhibitors to improve responsiveness to inhaled nitric oxide (NO) in isolated-perfused lungs of rats pretreated with endotoxin/lipopolysaccharide (LPS).
Design and setting: Prospective, controlled animal study in the animal research facility of a university hospital.
Interventions: Sixteen hours after adult Sprague-Dawley rats were injected intraperitoneally with 0.4 mg/kg *E. coli* 0111:B4 LPS administration, lungs were isolated and perfused, and the thromboxane mimetic U46619 was employed to increase the mean pulmonary artery pressure by 5–7 mmHg. The lungs were then ventilated with or without 0.4 ppm NO, and erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA; PDE type 2 inhibitor), milrinone (PDE type 3 inhibitor), or zaprinast (inhibitor of PDE types 5 and 9) were added to the perfusate.
Measurements and results: In the presence of EHNA (12.5, 25, 50 μM) the vasodilator response to inhaled NO was not greater than in its absence (0.25 ± 0.25, 0.5 ± 0.25, 0.75 ± 0.25 mmHg vs. 0.25 ± 0.25, 0.5 ± 0.25, 0.75 ± 0.25 mmHg, respectively). In the presence of milrinone (125, 250, 500 nM), the vasodilator response to inhaled NO was also not improved. In contrast, zaprinast (3.7, 7.4, 14.8 μM) augmented the pulmonary vasodilatory effect of inhaled NO in lungs from LPS-pretreated rats from 0.25 ± 0.25, 0.5 ± 0.25, 0.75 ± 0.25 mmHg to 0.75 ± 0.25, 1.5 ± 0.5, 1.75 ± 0.75 mmHg, respectively ($p < 0.05$).
Conclusions: Our results demonstrate that inhibition of pulmonary PDE enzyme activity with zaprinast increases vasodilator responsiveness to inhaled NO in lungs obtained from rats 16 h after LPS challenge.

Key words Nitric oxide · Hyporesponsiveness · Phosphodiesterase · Isolated perfused lung

Introduction

Inhalation of nitric oxide (NO) selectively dilates the pulmonary circulation and improves arterial oxygenation in patients with adult respiratory distress syndrome (ARDS). In many ARDS patients minimal or no pulmonary vasodilatory response to inhaled NO is measured [1, 2, 3]. The precise mechanisms responsible for hyporesponsiveness to inhaled NO are unknown, but clinical evidence suggests that Gram-negative bacteremia and endotoxemia, the most common causes of ARDS, are associated with NO hyporesponsiveness. In septic patients with ARDS, 60 % had little or no pulmonary artery pressure reduction upon inhaling 18 or 36 ppm NO [2].

NO is a lipid-soluble free radical molecule with a short half-life in biological fluids. In the presence of oxygen, nitric oxide synthase (NOS) enzymes produce NO via the conversion of the amino acid L-arginine to L-citrulline. In pulmonary vascular smooth muscle cells, NO stimulates soluble guanylate cyclase (sGC), an enzyme that promotes the conversion of GTP to 3′, 5′-cyclic guanosine monophosphate (cGMP). cGMP activates cGMP-dependent protein kinases leading to decreased vascular smooth muscle tone. cGMP is metabolized to guanosine monophosphate (GMP) by a family of enzymes called phosphodiesterases (PDEs).

We previously observed that in isolated, perfused, and ventilated lungs obtained from rats challenged with endotoxin (lipopolysaccharide, LPS) the ability of inhaled NO to reduce an elevated pulmonary vascular tone is impaired [4]. Hyporesponsiveness to inhaled NO is associated with a lower NO-induced cGMP release rate into the perfusate than in lungs from unchallenged control rats. NO hyporesponsiveness is also associated with an increased lung PDE activity. Moreover, perfusion with a PDE-resistant cGMP analog dilates preconstricted lungs obtained from LPS-treated rats to the same extent as the lungs from control rats.

In the mammalian lung at least six different isoforms of PDE metabolize cGMP, including PDE_1 (calcium/calmodulin-activated PDE), PDE_2 (cGMP-stimulated), PDE_3 (cGMP-inhibited), and PDE_5 (cGMP-specific), as well as the recently discovered isoforms PDE_9 and PDE_{10} [5, 6, 7, 8] (Table 1). All of these isoforms with the exception of PDE_5 and PDE_9 also promote the conversion of 3′, 5′-cyclic adenosine monophosphate (cAMP) to adenosine monophosphate (AMP) [7, 9]. We examined whether cGMP-specific inhibition of PDE enzyme activity can augment the decrease in pulmonary vasodilation in response to NO after LPS challenge. We studied the effects of specific PDE inhibitors on pulmonary vasoreactivity after LPS challenge in the absence and presence of inhaled NO. We report here that inhibition of pulmonary PDE enzyme activity by zaprinast markedly improves vasodilator responsiveness to inhaled NO after LPS exposure.

Materials and methods

These investigations were approved by the Subcommittee for Research Animal Studies of the Massachusetts General Hospital, Boston, Massachusetts.

Isolated, perfused rat lungs

Lungs obtained from rats were isolated, perfused, and ventilated as described previously [4]. Briefly, adult Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass., USA) weighing 450–550 g were injected intraperitoneally with 0.4 mg/kg *Escherichia coli* 0111:B4 lPS (Difco Laboratories Detroit, Mich., USA) and 16 h later were anesthetized by an intraperitoneal injection of sodium pentothal (100 mg/kg body wt.). Following a midline thoracotomy the rats were killed by blood withdrawal via puncture of the heart. Then pulmonary artery and left atrium were cannulated. Lungs were perfused with Hank's balanced salt solution containing 5 % dextran, 5 % bovine albumin, and 30 μM indomethacin, using a roller pump (Cardiovascular Instrument, Wakefield, Mass., USA) at a pulsatile flow of 0.03 ml/g body wt./min in a recirculat-

Table 1 Distribution of PDE isoforms that metabolize cGMP in the lung. At least six different cGMP-metabolizing PDE isoforms are expressed in lungs. Selective inhibitors for each of these isoforms are available

PDE isoform		Substrate	Location	Inhibited by
PDE_1	Ca/CaM-activated [23] (calcium- and calmodulin-dependent)	cGMP/cAMP [24]	Lung, heart, brain, kidney [24]	Vinpocetine, IBMX [25]
PDE_2	cGMP-stimulated [15, 26]	cGMP [26]	Lung, heart, brain, kidney [26]	EHNA [15]
PDE_3	cGMP-inhibited [27]	cAMP > cGMP [27]	Lung, heart, brain, liver, smooth muscle [28, 29]	cGMP, milrinone, amrinone [27, 29]
PDE_5	cGMP-specific [30, 31]	cGMP [30, 31]	Lung, heart, liver, skeletal muscle, smooth muscle, brain and other [32]	Zaprinast, dipyridamole, sildenafil [22, 33]
PDE_9	cGMP-specific [7, 14]	cGMP [7, 14]	Lung, kidney, spleen, brain, liver [7, 14]	Zaprinast [7]
PDE_{10}	cAMP-inhibited cGMP/cAMP PDE [9, 34]	cAMP > cGMP [9, 34]	Human fetal lung, thyroid, brain, testis [34]	Dipyridamole [34]

ing system at 37 °C. The perfusate was continuously equilibrated with 95 % O_2, 5 % CO_2 in the presence of $NaHCO_3$ to maintain pH at 7.35–7.40 as well as to maintain PCO_2 (35–40 mmHg) and PO_2 (150–250 mmHg). The pulmonary artery pressure (PAP) and left atrial pressure (set to 3–4 mmHg) were measured via a small catheter (PE-50) placed within the lumen of the inflow and outflow perfusion catheters, respectively. The time between death and perfusion of the lungs was about 3–5 min.

Dose-response curves of PDE inhibitors

The PAP in the isolated-perfused lungs was increased 5– 7 mmHg by addition to the perfusate of the stable thromboxane mimetic U46619. The amount of U46619 used to increase and maintain PAP was 2.6 ± 0.4 μg/min and 230 ± 140 ng/min, respectively. Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA; 12.5, 25, 50 μM; $n = 3$), milrinone (125, 250, 500 nM; $n = 3$), or zaprinast (3.7, 7.4, 14.8 μM; $n = 3$) was added to the perfusate, and values are expressed as final concentrations. First, for each PDE inhibitor dose-response curves were generated to assess the threshold doses at which pulmonary vasodilation occurred. After administration of each dose of the PDE inhibitor the PAP was allowed to stabilize for 5 min.

Pulmonary vasoreactivity to inhaled NO in combination with PDE inhibitors

NO was added to the inspired gas mixture at a low concentration of 0.4 ppm which decreased the PAP of preconstricted lungs from LPS-challenged rats by 10–15 %. NO gas was obtained from Airco (Hingham, Mass., USA) as a mixture of 800 ppm NO in pure N_2. NO was mixed with O_2 and N_2 just before the ventilator, and levels were measured by chemiluminescence (Eco Physics CLD 700AL, Dürnten, Switzerland). Lungs were ventilated continuously with 0.4 ppm NO, and PAP was measured before and after the PDE inhibitor was added to the perfusate in escalating doses. After each PDE inhibitor dose the PAP was allowed to stabilize for 5 min.

Statistics

All data are expressed as means ± SE. A multivariate analysis of variance with repeated-measures techniques was used to examine the effects of NO (with vs. without PDE inhibitor treatment) with all active doses combined. Statistical significance level was reached when $p < 0.05$. Additionally, the change in PAP after a given dose of PDE inhibitor in the absence of NO was compared with the corresponding dose when NO was inhaled using a two-sample t test. To guard against the inflation of the type due to multiple comparisons, a p value less than 0.017 (0.05/3) was considered as statistically significant for this part of analysis.

Results

Selective inhibition of PDE_2 does not alter inhaled NO responsiveness

To investigate whether selective inhibition of PDE_2 can augment pulmonary vasodilator responsiveness to inhaled NO, EHNA was added to the perfusate of preconstricted isolated lungs obtained from LPS-treated rats in the absence ($n = 3$) and presence ($n = 6$) of 0.4 ppm NO. In the absence of inhaled NO increasing doses of EHNA (12.5, 25, 50 μM final concentration) decreased the PAP by 0.25 ± 0.25, 0.5 ± 0.25, and 0.75 ± 0.25 mmHg, respectively, in a dose-dependent matter ($p < 0.05$; Fig. 1). During NO inhalation increasing doses of EHNA caused a further decrease in PAP (0.25 ± 0.25, 0.75 ± 0.25, and 1.0 ± 0.25 mmHg, respectively, $p < 0.05$, Fig. 1). However, the magnitude of the vasodilator response to inhaled NO did not differ in the presence or absence of EHNA ($p = 0.26$), suggesting that the vasodilatory effect of the combination of inhaled NO and EHNA is additive rather than synergistic.

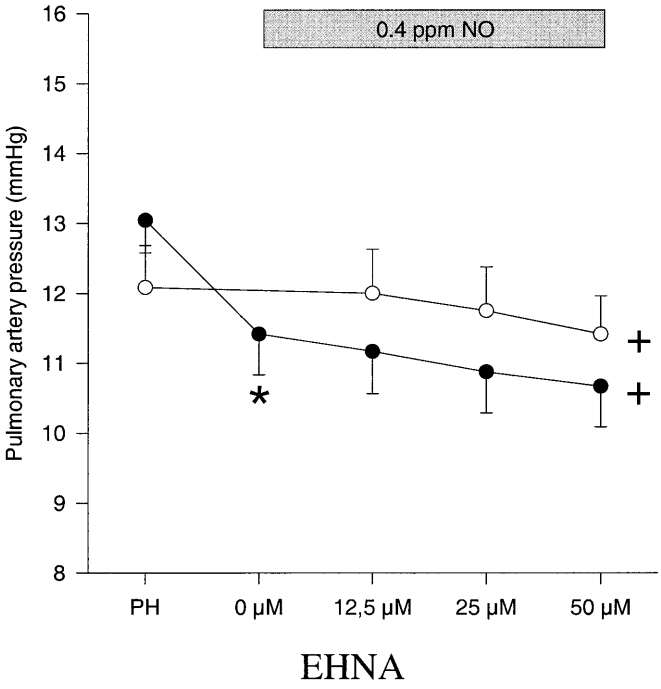

Fig. 1 EHNA, a PDE_2 inhibitor, decreased the PAP of lungs obtained from LPS-treated rats alone and with inhaled NO. Isolated-perfused lungs from LPS-treated rats were preconstricted with U46619 *(PH)* and EHNA was added to the perfusate in increasing doses in the absence *(open circles)* and presence *(filled circles)* of 0.4 ppm NO. Inhalation of 0.4 ppm NO decreased PAP at constant flow of perfusate (*$p < 0.05$ vs. PH). EHNA dilated the pulmonary circulation in a dose-dependent manner with and without inhaled NO ($^{+}p < 0.05$ vs. 0.4 ppm NO and PH, respectively). The magnitude of the EHNA-induced decrease in PAP did not differ in lungs ventilated with ($n = 6$) and without ($n = 3$) 0.4 ppm NO. Data are means ± SE

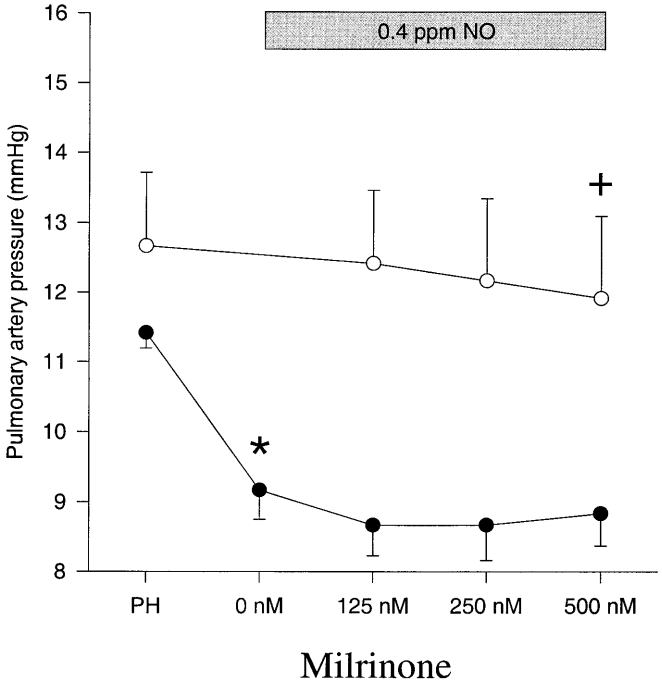

Fig. 2 Milrinone, a PDE_3 inhibitor, does not induce vasodilation in lungs from LPS-treated rats. Isolated-perfused lungs from LPS-treated rats were preconstricted with U46619 *(PH)* and milrinone was added to the perfusate in increasing doses in the absence *(open circles)* and presence *(filled circles)* of 0.4 ppm NO). In lungs from rats treated with milrinone in the absence of NO the highest dose of milrinone (500 nM final concentration) decreased the PAP at a constant flow of perfusate ($^{+}p < 0.05$ vs. PH). Inhalation of 0.4 ppm NO decreased PAP ($^{*}p < 0.05$ vs. PH). Addition of milrinone did not augment the pulmonary vasodilator response to inhaled NO ($n = 3$). Data are means ± SE

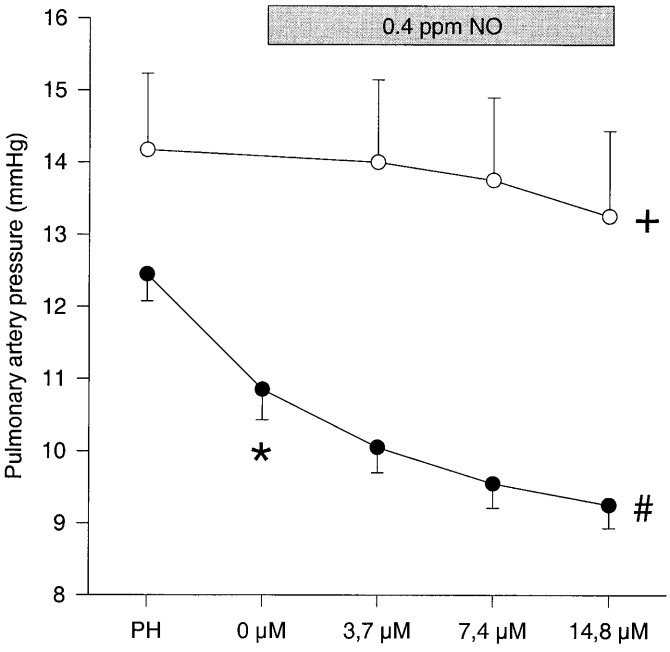

Fig. 3 Zaprinast, a PDE_5 and PDE_9 inhibitor, augments inhaled NO responsiveness in lungs of LPS-treated rats. Isolated-perfused lungs from LPS-treated rats were preconstricted with U46619 *(PH)* and increasing doses of zaprinast were added to the perfusate in the absence *(open circles)* and presence *(filled circles)* of inhaled NO. In the absence of inhaled NO ($n = 3$), zaprinast decreased PAP in a dose-dependent manner ($n = 5$, $^{+}p < 0.05$ vs. PH). Inhalation of 0.4 ppm NO decreased PAP ($^{*}p < 0.05$ vs. PH). In the presence of NO the vasodilation attributable to increasing doses of zaprinast was greater than in the absence of NO ($p = 0.09$, 0.05, and 0.003, respectively). The pulmonary vasodilator response to inhaled NO was greater in lungs perfused with zaprinast than in lungs perfused without zaprinast ($^{\#}p < 0.017$). Data are means ± SE

Selective inhibition of PDE_3 does not improve hyporesponsiveness to inhaled NO

In isolated-perfused lungs from LPS-treated rats, milrinone decreased PAP by 0.5 ± 0.25 mmHg at the highest dose studied in the absence of inhaled NO ($n = 3$, $p < 0.05$; Fig. 2). In the presence of NO ($n = 3$), milrinone (125, 250, 500 nM) did not further vasodilate the lungs of LPS-treated rats [0.5 ± 0.1, 0.5 ± 0.25, and 0.25 ± 0.1 mmHg, respectively, vs. milrinone with NO (0.25 ± 0.25, 0.5 ± 0.25, and 0.5 ± 0.25 mmHg, respectively, without NO; NS)].

Zaprinast augments vasodilator responsiveness to inhaled NO

Isolated-perfused lungs from LPS-treated rats were preconstricted with U46619, and zaprinast was added to the perfusate in increasing doses (3.7, 7.4, 14.7 µM) in the absence ($n = 3$) and presence ($n = 5$) of ventilation with 0.4 ppm NO. In the absence of inhaled NO, zaprinast decreased the PAP in a dose-dependent manner (0.25 ± 0.25, 0.5 ± 0.5, and 0.75 ± 0.75 mmHg, respectively; $p < 0.05$; Fig. 3). In the presence of inhaled NO the magnitude of the vasodilator dose-response curve attributable to zaprinast was greater than in the absence of NO (0.75 ± 0.1, 1.25 ± 0.25, and 1.75 ± 0.25 mmHg, respectively; $p < 0.05$). In the lungs of LPS-treated rats the magnitude of the pulmonary vasodilator response to inhaled NO was much greater in the presence of zaprinast than in its absence, suggesting that inhaled NO and zaprinast acted synergistically to augment pulmonary vasodilation ($p < 0.017$).

Discussion

In patients with ARDS the inhalation of NO selectively dilates the pulmonary circulation and improves arterial oxygenation. However, in up to 60% of septic ARDS patients minimal or no pulmonary vasodilatory response to inhaled NO is measured [1, 2, 3]. Variable responses to inhaled NO are also reported in clinical trials examining the effectiveness of inhaled NO in newborns with persistent pulmonary hypertension (PPHN) [10, 11, 12]. We previously reported that in isolated-perfused lungs obtained from rats challenged 16 h previously with LPS the ability of inhaled NO to induce pulmonary vasodilation was impaired [4]. Because hyporesponsiveness to inhaled NO in rats treated with LPS is associated with increased pulmonary PDE enzyme activity, we studied the effects of selective inhibition of various PDE isoforms on the pulmonary vascular response to inhaled NO.

Nine different isoforms of PDE are known to be present in the lungs [13, 14]. Six of them may participate in pulmonary cGMP metabolism or are regulated by cGMP, three are responsible for cAMP metabolism or show less affinity for cGMP than cAMP (see Table 1). cGMP-stimulated PDE (PDE_2) and cGMP-inhibited PDE (PDE_3) metabolize both cGMP and cAMP, whereas a cGMP-binding PDE (PDE_5) is specific for cGMP (reviewed in [5]). Recently PDE_9 was identified and was found to be abundantly expressed in human lungs [7, 8]. Various selective inhibitors of PDE enzyme activity are currently available including EHNA, milrinone, and zaprinast.

EHNA is a specific inhibitor of cGMP-stimulated PDE (PDE_2) [13, 15]. In isolated-perfused lungs obtained from rats, EHNA reversed the response to hypoxia in a dose-dependent manner [13]. In our study, the addition of EHNA to the perfusate of lungs obtained from LPS-treated rats that were ventilated with and without NO decreased the PAP (Fig. 1). However, this effect of EHNA was additive rather than synergistic with NO, because the magnitude of the pulmonary vasodilator response attributable to EHNA in lungs from rats perfused with EHNA alone did not differ from that in lungs of rats perfused with EHNA and ventilated with NO.

To investigate whether inhibition of PDE_3 enzyme activity augments vasodilation to inhaled NO in lungs obtained from LPS-treated rats, we added milrinone to the perfusate. Milrinone is a specific inhibitor of cGMP-inhibited PDE (PDE_3) enzyme activity. In dogs with monocrotaline-mediated chronic pulmonary hypertension, treatment with milrinone reduced both PAP and pulmonary vascular resistance [16]. In patients with pulmonary hypertension associated with congestive heart failure, a single intravenous bolus of milrinone decreased both PAP and pulmonary vascular resistance [17]. He et al. [18] reported that in human conduit arteries precontracted with either U46619 or potassium the combined treatment with milrinone and nitroglycerin resulted in greater relaxation than that with milrinone alone [18]. In our study, the addition of milrinone did not further vasodilate lungs obtained from LPS-treated rats ventilated with NO (Fig. 2). It is possible that in rats treated with LPS increased intracellular cGMP levels stimulated by inhaled NO maximally inhibit PDE_3, because the addition of milrinone to the perfusate vasodilates lungs obtained from LPS-treated rats in the absence of NO inhalation (Fig. 2).

To examine whether a selective inhibitor of cGMP metabolism enhances the pulmonary vascular response to inhaled NO in LPS-treated rats, zaprinast, an inhibitor of PDE_5, and the recently described PDE_9 [7], were added to the perfusate. A number of studies have demonstrated that zaprinast enhances NO-mediated pulmonary vasodilation [19, 20]. In awake lambs with U46619-induced pulmonary artery hypertension a continuous intravenous infusion of zaprinast prolongs the duration of pulmonary vasodilation produced by inhaled NO [20] and inhaled aerosolized zaprinast both potentiates and prolongs the pulmonary vasodilating effects of inhaled NO [21]. In our study, the addition of zaprinast to the perfusate of lungs obtained from LPS-treated rats potentiated the vasodilator effects of inhaled NO. Sildenafil, a highly selective inhibitor of PDE_5 [22], did not augment NO responsiveness in our model (data not shown). Dipyridamole, another PDE_5 inhibitor, did not enhance responsiveness to inhaled NO in isolated lungs obtained from LPS-challenged rats (data not shown). In contrast to zaprinast, sildenafil and dipyridamole do not inhibit PDE_9 [14]. These observations suggest that inhibition of pulmonary PDE_9 or another unidentified cGMP-metabolizing PDE can augment or prolong responsiveness to inhaled NO. It is unlikely that specific inhibition of PDE_1 by zaprinast improved vasodilatory responsiveness to inhaled NO in our study, because vinpocetine, a specific inhibitor of PDE_1 enzyme activity, did not augment pulmonary vasodilation to inhaled NO (data not shown).

Little is presently known about the regulation of pulmonary PDE enzyme activity in clinical sepsis or in animal models after LPS challenge. However, in lungs obtained from rats that were not challenged with LPS, EHNA, milrinone, and zaprinast all decreased PAP in a dose-dependent manner (data not shown). We have previously demonstrated that LPS challenge induced an increase in pulmonary PDE enzyme activity in rats [4]. In this study we tried to enhance the vasodilatory responsiveness to inhaled NO of rats previously challenged with LPS. Although it is possible that specific inhibition of PDE_9 enzyme activity can augment NO responsiveness, it remains uncertain whether increased PDE_9 enzyme activity and/or activity of other PDE iso-

forms contribute to the pulmonary vascular hyporesponsiveness to inhaled NO following exposure to LPS in the rat.

In summary, our results suggest that zaprinast, possibly by inhibiting PDE_9 or another PDE, can augment the pulmonary vasodilator response to inhaled NO in the lungs of rats 16 h after LPS challenge. If observations made in rodents after LPS challenge can be extrapolated to humans, an important clinical implication of our studies is that PDE inhibitors may be useful to augment the pulmonary vasodilator response to inhaled NO in patients with sepsis.

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