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A Novel Inhaled Organic Nitrate That Affects Pulmonary Vascular Tone in a Piglet Model of Hypoxia-Induced Pulmonary Hypertension

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ABSTRACT

Persistent pulmonary hypertension of the newborn is characterized by elevated pulmonary vascular resistance after birth leading to right-to-left shunting and systemic arterial hypoxemia. Inhaled nitric oxide (NO) is effective in reducing the need for extracorporeal membrane oxygenation, but it has potential toxicities, especially in an oxygen-rich environment. A number of other NO-based molecules have been given by inhalation, but their structure–function relationships have not been established. Recent studies have raised the idea that toxic and beneficial properties can be separated. We synthesized a novel organic nitrate [ethyl nitrate (ENO_2)], tested it *in vitro*, and administered it to hypoxic piglets. ENO_2 lowered pulmonary artery pressure and raised the Po_2 in arterial blood but did not alter systemic vascular resistance or methemoglobin levels. In addition, we tested the effect of ENO_2 in the presence of the thiol glutathione, both *in vivo* and *in vitro*, and found its action to be enhanced. Although ENO_2 is less potent than inhaled NO on a dose-equivalency basis, pretreatment of hypoxic animals with glutathione, which may be depleted in injured lungs, led to a markedly enhanced effect (largely mitigating the difference in potency). These results suggest that ENO_2 may hold promise as a safe alternative to NO, particularly in hypoxemic conditions characterized by thiol depletion. (***Pediatr Res* 58: 531–536, 2005**)

Abbreviations

CO, cardiac output
ENO, ethyl nitrite
ENO_2, ethyl nitrate
GSH, glutathione
FIO_2, fraction of inspired oxygen
NMR, nuclear magnetic resonance
NO, nitric oxide
NO_x, oxides of nitrogen
PaO_2, arterial partial pressure of oxygen
PAP, pulmonary artery pressure
PPHN, persistent pulmonary hypertension of the newborn
PVR, pulmonary vascular resistance
SNO, S-nitrosothiol
SVR, systemic vascular resistance

The disease entity persistent pulmonary hypertension of the newborn (PPHN) is characterized by elevated pulmonary vascular resistance (PVR) after birth leading to extrapulmonary right-to-left shunting through the patent foramen ovale and patent ductus arteriosus with resulting systemic arterial hypoxemia. These patients are frequently treated with inhaled nitric oxide (NO) and may require extracorporeal membrane oxygenation; those with severe respiratory failure are at increased risk for neurodevelopmental impairment (1,2). NO's clinical effectiveness has been well documented (3–6), although some reports suggest that it may exacerbate lung inflammation (7), raising the concern that it may have toxic properties in some clinical settings. Infants who receive inhaled NO for PPHN are often treated with high inspired oxygen, and in the O_2-rich environment of the lung, higher oxides of nitrogen (NO_x) including peroxynitrite may form (8), which can cause hemorrhage, inflammation, and edema (9,10). Airways of animal and human subjects who have received inhaled NO showed the same patterns of oxidative injury found in animals and humans who were exposed to NO_x or hyperoxia (11,12).

Inhaled, exogenous NO is delivered to well-ventilated parts of the lung and will stimulate guanylate cyclase activity, leading to vascular smooth muscle relaxation. Thus, NO has been effective in lowering pulmonary artery pressure (PAP)

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and PVR without compromising left ventricular function or systemic arterial pressure. As a result, it has been effective in preventing progression to severe PPHN (3), reducing ventilatory support (3), and lessening the need for extracorporeal membrane oxygenation (5). However, in addition to questions about its toxicity, its clinical use has potential drawbacks, which may include methemoglobinemia (13), impairment of left ventricular function (14), impairment of platelet aggregation (15), and "rebound" pulmonary hypertension (16). NO is frequently difficult to administer, and the cost of its use may be staggering (17).

We previously tested a novel inhaled drug, ethyl nitrite (ENO; O-nitrosoethanol), which mimicked NO synthase activity by forming S-nitrosothiols (SNOs) *in vitro* (18). This drug was effective in mitigating hypoxia-induced pulmonary hypertension and showed promise in improving oxygenation in pulmonary hypertensive neonates (19). ENO did cause, however, modest increases in methemoglobin concentration and a rise in blood ethanol level (in a single infant with liver injury). To better understand the structure–function relationship of this class of compounds, we synthesized a closely related derivative, ethyl nitrate (ENO_2; O-nitroethanol). We hypothesized that as a volatile organic nitrate, it might have an advantage over other vasodilators in that it could be converted easily into an inhaled form and thus selectively dilate the pulmonary vascular bed; in addition, although it would not fortify the body's SNO reservoir as markedly as ENO, its action might be enhanced by thiols such as glutathione, which may be depleted in lung injury (20). We tested our synthetic compound in a vascular bioassay *in vitro* and in a hypoxic model of PPHN.

METHODS

Preparation of ethyl nitrate. ENO_2 was prepared by sulfuric acid–induced nitration of ethanol. Nitric acid (25 mL) was mixed slowly with sulfuric acid (25 mL). Urea (160 mg) was added. The solution was cooled in an ice water bath. Ethanol (15 mL) was added dropwise in portions over a period of 1 h. After complete addition, the solution was stirred for 10 min more. The mixture was added to a solution of cold (4°C) brine in a separation funnel. The bottom (aqueous) layer was discarded, and the top layer was washed twice with cold, saturated sodium bicarbonate (15 mL), resulting in the evolution of gas. The top layer was washed with cold brine (15 mL), dried with powdered magnesium sulfate, and filtered to give ENO_2 as a clear, colorless liquid.

The ^1H nuclear magnetic resonance (NMR) spectrum showed a single organic product with no residual ethanol or other impurities. Observed ^1H NMR peaks ($CDCl_3$) were as follows: 1.38 ppm, 3H, triplet; 4.52 ppm, 2H, quartet. Literature ^1H NMR values (21) were as follows: 1.40 ppm, 3H, triplet; 4.52 ppm, 2H, quartet. Infrared (IR) nitrate peak was observed at 1664 cm^{-1}. Gas chromatography–mas spectrometry analysis (J&W Scientific GS-Q column, Folson, CA) of the head space of a vial of prepared ENO_2 showed the compound to be free of higher NO_x: two peaks were observed, one of room air, the other with a mass spectrum consistent with ENO_2. ENO_2 did not measurably decompose on exposure to air, water, propylene glycol, DMSO, methanol, or ethanol.

An ENO_2 gas admixture was generated by passing either 14 or 21% oxygen, at 0.6 L/min, through a small glass mixing column (URG, Chapel Hill, NC) filled with rasching rings and containing liquid ENO_2 in a propylene glycol solution. Concentrations of ENO_2 delivered by this system were changed by varying solution concentrations (0.0025, 0.025, 0.125, 1.25, and 2.5%). Experiments done with "control" gas used propylene glycol in the mixing column, without the addition of ENO_2.

Pulmonary artery ring preparation. The ring procurement protocol was approved by the Duke University animal care and use committee. New Zealand White rabbits (2.5–3 kg) were killed by carbon dioxide inhalation. As vessels would be chemically constricted to mimic hypoxic constriction, vessels from mature rabbits were deemed sufficient. The pulmonary artery was removed and cleared of fat and connective tissue, and second- and third-order branches were cut into 3-mm rings. The rings were prepared and hung as previously described (22). A total of eight pulmonary artery rings from two rabbits were used. Contractions were initiated with phenylephrine, and endothelial function was assessed by the response to 10^{-7} mol/L acetylcholine; relaxation of >50% was considered adequate. Liquid ENO_2 was added to the baths at an initial concentration of 10^{-9} mol/L and then increased 10-fold at a time to a final concentration of 10^{-4} mol/L. Glutathione (GSH) was tested at up to 10^{-3} mol/L final concentration.

Surgical model of pulmonary hypertension. The protocol was approved by the Duke University animal care and use committee. A total of 21 neonatal swine (1–2 wk of age; 2.6–4.0 kg) were anesthetized and instrumented as previously described (23). Pressure monitors directly measured the pressure in the main pulmonary artery, right ventricle, and left atrium. A flow probe around the pulmonary artery continuously measured cardiac output (CO). In each of a subgroup of these animals ($n = 7$), the ductus arteriosus was found to be patent by placement of a pressure catheter in the ductus during hypoxia, as well as by postmortem examination**.** Mechanical ventilation was initiated with a V.I.P. Bird ventilator (SensorMedics, Yorba Linda, CA) in volume control mode. Ketamine (22 mg/kg) and acepromazine (1.1 mg/kg) were given intramuscularly as preanesthetics. Anesthesia was maintained during the study with a continuous infusion of fentanyl (20 $\mu g \cdot kg^{-1} \cdot h^{-1}$), abating some of the inherent instability of the open-chest preparation, and intermittent doses of pancuronium (0.1 mg/kg) were given for neuromuscular blockade. Blood pressure and heart rate were monitored continuously. Before data acquisition, animals were allowed to stabilize for 20 min.

After instrumentation, lowering the fraction of inspired oxygen (F_{IO_2}) to 0.14 induced pulmonary hypertension. By 7 min, the PAP had plateaued and the other parameters had reached a constant level. At this point, baseline measurements were taken; measurements were retaken after 5 min of breathing ENO_2. The inhaled ENO_2 then was stopped, and the animals continued on the low F_{IO_2} for an additional 5 min, at which point hemodynamic measurements were obtained and the F_{IO_2} returned to 0.21. The procedure was repeated three times using different doses of ENO_2 in random order. For the experiments involving thiol supplementation, seven of the animals were pretreated with GSH (50 mg/kg, by i.v. infusion over 20 min) before induction of pulmonary hypertension (hypoxia); the procedure described above then was followed.

Ventilation and gas administration. Mechanical ventilation was used with an initial rate of 35 breaths/min and a tidal volume sufficient to return 7–9 mL/kg. Positive end expiratory pressure was set at 5 cm H_2O, and flow was 6 L/min. F_{IO_2} was 0.21 before the start of all experiments. Blended air from the ventilator flow meter was directed into the glass mixing column at a flow rate of 0.6 L/min. The gas that came out of the mixing column (containing gaseous ENO2) was added *via* t-connector to the inspiratory tubing of the ventilator.

Inspired gas (400 μL) was sampled by gastight syringe (Hamilton Co., Reno, NV) through a rubber septum on a port 100 mm proximal to the endotracheal tube. The minute quantities of ENO_2 gas to be analyzed were hydrolyzed with potassium hydroxide (KOH) to potassium nitrite (KNO_3), which were quantified, after vanadium chloride (VCL_3) reduction to NO, with a commercial NO (NOA) analyzer (Model 280 NO analyzer; Sievers Instruments, Boulder, CO), which was configured and run according to the manufacturer's instructions. ENO_2 was unexpectedly resistant to base-induced hydrolysis; solutions of authentic ENO_2 required 72 h at 95°C in 100 mM KOH in a 9:1 mixture of propylene glycol:water to yield 100% nitrate (KOH concentration was minimized to reduce background nitrate). The gas was injected into a 200-μL solution of 100 mM KOH in 9:1 propylene glycol:water in a 2-mL screw-cap vial. The vial was immediately covered with a screw cap that contained an intact PTFE-faced septum and put on a heat block at 95°C for at least 72 h. After cooling, aliquots were removed and analyzed for nitrate content by the NOA. Molar quantities of ENO_2 were converted to parts per million (ppm) by application of the ideal gas law.

Data collection and analysis. *In vivo* data were collected *via* pressure catheters connected to a data acquisition system customized in our laboratory. Least squares means for the different interventional states were calculated using multiple linear regression, and tests of statistical significance were carried out using repeated measures ANOVA followed by paired *t* tests with Bonferroni correction. All data were expressed as mean ± SE. *In vitro* data were analyzed using ANOVA with Tukey-Kramer honestly significant difference (JMP statistical software, Cary, NC)**.** A $p < 0.05$ was considered statistically significant. Analyses were performed using SAS statistical software (SAS Institute, Inc., Cary, NC).

RESULTS

***Dose-dependent response to ENO_2* in vitro.** Pulmonary artery rings that were exposed to ENO_2 showed no vasodilation

at concentrations below 10^{-7} mol/L. Dose-dependent relaxation was seen between 10^{-7} and 10^{-4} mol/L with $IC_{50} = 10^{-4.5}$ mol/L (Fig. 1). Addition of GSH (1 mM) resulted in a small increase in ENO_2 potency ($p < 0.05$ by multivariate analysis). Univariate analysis also showed a significant potentiation of ENO_2 relaxations in the presence of 10^{-4} mol/L GSH ($p < 0.05$). An equivalent volume of ethanol, with and without GSH, produced no relaxation.

Induction of pulmonary hypertension. Table 1 shows the effect of lowering the F_{IO_2} to 0.14 for 7 min. This degree of hypoxia resulted in a 59% rise in PAP and a 49% increase in PVR ($p < 0.05$ for both). The arterial P_{O_2} (Pa_{O_2}) decreased by 52% ($p < 0.01$). The only statistically significant effect on systemic hemodynamics was a drop in mean arterial blood pressure by 16% ($p < 0.05$), although the characteristic decline in systemic vascular resistance (SVR) and compensatory increase in CO was observed. These results are similar to those found in other hypoxic models of pulmonary hypertension (18,24).

Effect of control gas on pulmonary hypertension. To test the effect of delivering air into the inspiratory limb of the ventilatory circuit at 0.6 L/min, we performed control experiments that consisted of filling the glass chamber with only propylene glycol (0% ENO_2) and then passing gas with an F_{IO_2} of 0.14 through it. In this set-up, the inspiratory and expiratory tidal volumes increased by 5–8%, but the minute ventilation increased by no more than 4%. None of changes in measured parameters reached statistical significance ($p < 0.05$, data not shown).

Effect of ENO_2 on pulmonary hypertension. Figure 2 shows the changes in several physiologic parameters after ENO_2 administration during hypoxia. There was both a significant decrease in PAP and an increase in Pa_{O_2} at doses of 0.125, 1.25, and 2.5% ENO_2 ($p < 0.05$), with the last two doses also achieving significance *versus* the control gas. The two lowest doses, 0.0025 and 0.025%, had changes that were similar in magnitude and were not significantly improved *versus* control gas alone. PVR showed a response similar to that of PAP ($p < 0.05$). With the systemic parameters, there was no effect on SVR or heart rate. CO decreased after 1.25 and 2.5% ENO_2, both when compared with the hypoxic, pre-ENO_2 state and *versus* control gas. Right-sided stroke work, an indirect measure of the resistance against the right ventricle, decreased with

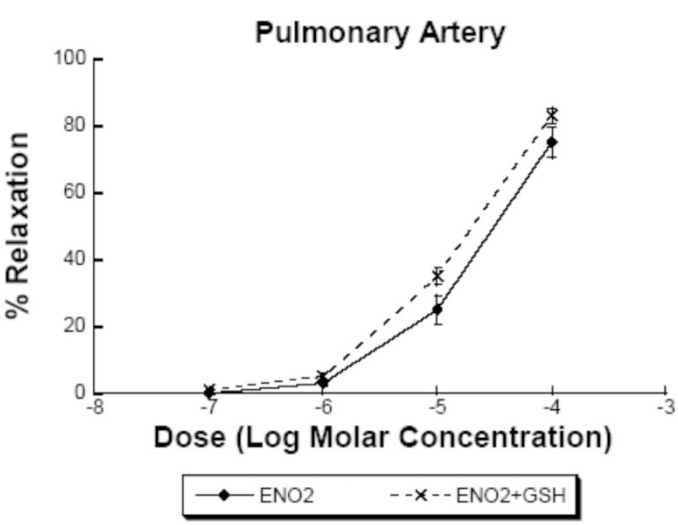

Figure 1. Dose-response curves in isolated pulmonary artery rings. In pulmonary artery rings that were preconstricted with phenylephrine, ENO_2 produces relaxation in a dose-dependent manner. The potency of ENO_2 is increased by GSH ($p < 0.05$, $n = 8$). Data shown are ± SEM.

Table 1. *Induction of pulmonary hypertension (14%O_2)*

	Normoxia	Hypoxia
PAP, mm Hg	13.9 (1.2)	22.1 (1.4)*
PaO_2, mm Hg	87.0 (4.2)	41.7 (2.1)*
PVR, dynes/s/cm^{-5}	2,947.1 (337.8)	4,384.5 (501.3)*
MnBP, mm Hg	54.0 (2.7)	45.3 (2.9)*
SVR, dynes/s/cm^{-5}	11,741.63 (1 332.3)	9,106.2 (1 111.1)
CO, mL/min	417.2 (36.0)	448.4 (36.2)

Values are shown as mean (SEM).
* $p < 0.05$.

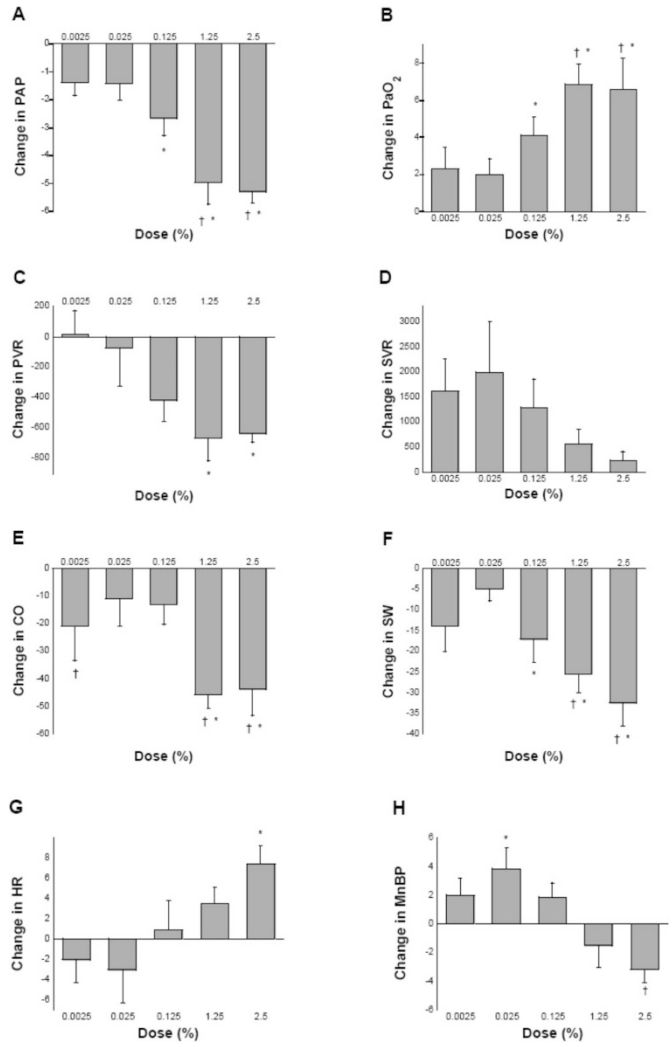

Figure 2. Effects of ENO_2 on physiologic parameters in hypoxic model of pulmonary hypertension. The graphs represent the change from hypoxic values after ENO_2 had been administered for 5 min ($n = 7$ for each dose). (*A*) PAP, mmHg. (*B*) Pa_{O_2}, mmHg. (*C*) PVR, dynes · s^{-1} · cm^{-5}. (*D*) SVR, dynes · s^{-1} · cm^{-5}. (*E*) CO, mL/min. (*F*) Right ventricular stroke work, erg*10^{-3}. (*G*) Heart rate, min^{-1}. (*H*) mean systemic blood pressure, mm Hg. *Significantly different from hypoxia ($p < 0.05$); †significantly different from the effect of the control dose ($p < 0.05$).

the higher doses ($p < 0.05$ for 0.125, 1.25, and 2.5%). [There was also a decrease in stroke work ($p < 0.05$) when compared with the control dose for 1.25 and 2.5 ENO_2]. Mean arterial blood pressure rose slightly in response to the three lower doses of ENO_2 and fell slightly during administration of 1.25 and 2.5% ENO_2. It is interesting that only the response to 0.025% reached significance ($p = 0.04$), whereas the two highest doses showed a trend toward significance ($p = 0.06$).

Rebound effect. We investigated the short-term effect of discontinuing ENO_2 infusion on various cardiovascular parameters, by recording for 5 min (of continued hypoxia) after the experimental period was complete. The post-ENO_2 value was statistically equivalent to the pre-ENO_2 value in each instance (data not shown).

***Potentiation of ENO_2 gas* in vivo.** To further investigate the effects of thiol supplementation on ENO_2, we gave glutathione (50 mg/kg) to the animals before the administration of inhaled drug. The effects of adding GSH are shown in Fig. 3. There was a marked potentiation of the decrease in PAP and the rise in Pao_2 in response to ENO_2 (Fig. 3*A* and *B*); PAP decreased by >2.5 mm Hg at the three lowest doses, whereas Pao_2 increased by 4–5 mm Hg. With regard to Pao_2, GSH effectively shifted the dose-effect of ENO_2 leftward, producing improvements from 0.0025 to 0.125% ENO_2 over baseline. GSH also potentiated the decrease in PVR at lower doses of ENO_2, whereas the effect of 0.125% ENO_2 was unchanged. GSH did not seem to affect mean systemic blood pressure. An analysis of the subgroup of animals that received GSH showed no attenuation of the hypoxic vasoconstriction after GSH administration.

Measurement of methemoglobin. Methemoglobin concentrations were measured after 5 min of ENO_2 exposure (Table 2). The average initial methemoglobin level was 0.7%, and there were no significant increases. The average methemoglobin after each concentration of ENO_2 was within the normal range.

Table 2. *Methemoglobin level by concentration*

Concentration	Methemoglobin (%)
Baseline	0.7 (0.04)
0.0025%	0.6 (0.08)
0.025%	0.6 (0.08)
0.125%	0.6 (0.08)
1.25%	0.8 (0.10)
2.5%	0.8 (0.10)

Values are shown as mean (standard error).

Quantification of ENO_2 gas. We devised a method of measuring the amount of ENO_2 in gas phase by obtaining samples of the gas just proximal to the endotracheal tube and then analyzing it for nitrate content. Liquid concentrations of 1.25 and 2.5% gave ~2000 and 4000 ppm, respectively (Fig. 4).

DISCUSSION

Inhaled NO has been shown to reduce pulmonary pressures and improve oxygenation indices (6,25,26) in patients with pulmonary hypertension, but there is remaining concern about its toxicity in certain populations (27–29) and its use may result in methemoglobinemia (13) and platelet inhibition (30). Inhaled alternatives to NO, including sodium nitroprusside (31,32), nebulized nitroglycerine (32,33), and various NONOates (34) have shown promise but are less well understood from the standpoint of structure–function relationships. We recently showed that the nitrosalcohol gas ENO has activity similar to that of NO but is resistant to reactions with O_2 that generate potentially toxic NO_x. In further contrast with NO, ENO preferentially reacted with cysteine thiols in GSH and hemoglobin to produce SNOs that mediate its effect (18). To better understand the structure–function relationship of these volatile drugs, we synthesized the nitrosalcohol ENO_2. That is, we effectively oxidized the NO moiety in ENO to an NO_2 group. We report that this modification attenuates the potency of the drug. We also report, however, that the potency was restored by the addition of a thiol. Thus, we have synthesized

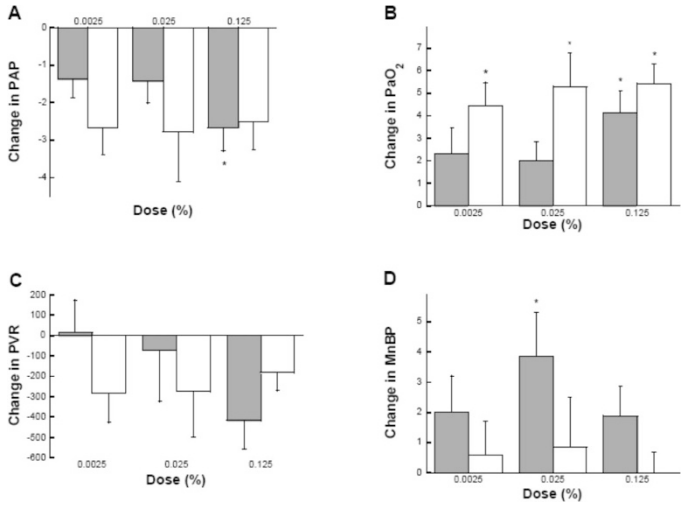

Figure 3. Effect of ENO_2 on animals that were pretreated with GSH (50 mg/kg), compared with ENO_2 alone. Pulmonary hypertension was induced by lowering the FIO_2 to 14%. Shown is the change in PAP (*A*), Pao_2 (*B*), PVR (*C*), and mean systemic blood pressure (*D*). Units as in Fig. 2. ▨, change with ENO_2 alone; □, ENO_2 + GSH. Mean systemic BP was not significantly affected ($n = 7$ for each dose). *Significantly different from hypoxia ($p < 0.05$).

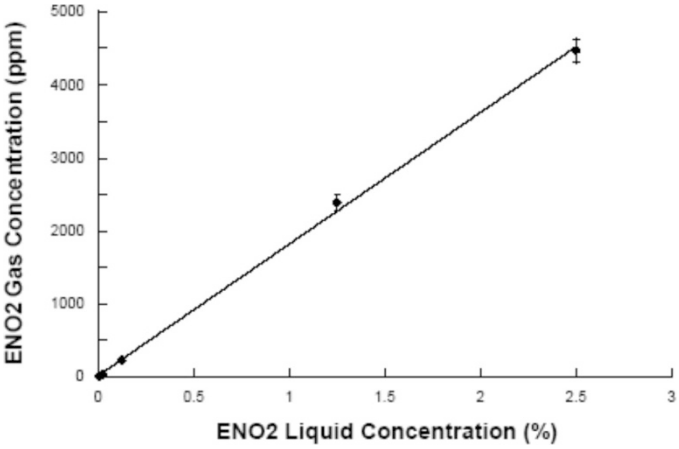

Figure 4. Measured dose of ENO_2 in gas phase as related to concentration in liquid phase.

a novel compound in our laboratory that selectively lowers PAP and raises P_{O_2} while also suppressing the tendency to produce methemoglobin and toxic NO_x (NO/O_2 byproducts). ENO_2's activity is further regulatable by thiols.

We based our experimental protocol on our previous experience with ENO (18), a potent vasodilator gas that is synthesized in ethanol. There are, however, some notable differences between ENO and ENO_2. Although volatile, ENO_2 is a liquid (it has a boiling point of 87.2°C). We synthesized ENO_2 using propylene glycol—an odorless, colorless, slightly viscous liquid that boils at 189°C—as a solvent. Propylene glycol has been "generally recognized as safe" by the U.S. Food and Drug Administration. Although heavy occupational exposure can lead to upper airway irritation (35), it has been recommended as an appropriate vehicle for routine administration of bronchodilator drugs (36,37). It was not anticipated that our procedure, performed at room temperature, would lead to significant exposure to inhaled propylene glycol.

We compared the *in vitro* activities of ENO and ENO_2 in organ chamber bioassays. Although both have vasodilatory properties, ENO_2 was almost 100-fold less potent. The potency of ENO_2 *in vivo* was also ~100 times less than that of NO and ENO. Notably, however, with the addition of GSH, the activity of ENO_2 *in vivo* mirrored that of ENO. Specifically, we found marked potentiation by GSH of the effects of ENO_2 on PAP, PVR, and Pa_{O_2} (Fig. 2). In addition, inhaled ENO_2 caused a dose-dependent decrease in right-sided stroke work ($p < 0.01$) and improved oxygenation, which suggests that the decrease in PVR led to improved V/Q matching. A fall in CO in these experiments is mainly attributable to a reversal of the rise in CO that accompanied induction of hypoxia. Systemically, there are minimal alterations, with a dose of 2.5% causing a drop in mean blood pressure comparable to the control gas and no difference in SVR.

The phenomenon of the rebound response is one of the major complications of stopping prolonged inhaled NO therapy (27,38,39). Our protocol allowed for monitoring the response of the animal for an interval of only 5 min after stopping ENO_2 inhalation. As the rebound effect was not the major focus of this investigation, we merely noted its absence in this protocol. Similarly, we recognize that our measurement of methemoglobin formation, after only 5 min of ENO_2 exposure, may not be representative of prolonged administration. However, it is important to note that in contrast to what is seen with NO at relatively high concentrations, the high doses of ENO (2000 ppm) did not cause an increase in methemoglobin.

The biotransformation of organic nitrates involves reactions with sulfhydryl groups (thiols), which may be used in converting NO_2 groups to NO and SNOs. Once in the vascular smooth muscle cell, NO or SNO activates soluble guanylate cyclase to increase intracellular cyclic GMP (40). This in turn leads to a decrease in intracellular calcium and relaxation of vascular smooth muscle. Previous studies have shown a potentiation of nitrate vasodilation by GSH (41,42), which constitutes >90% of the intracellular nonprotein thiol pool (40). In our studies, GSH increased the efficacy of ENO_2 (as measured by changes in PAP and Pa_{O_2}) at the two lowest doses but not at the higher doses (0.125% or higher), concentrations at which the effect of the drug had plateaued. That is, the blood vessels were evidently maximally dilated by the highest doses of ENO_2. In effect, pretreating animals with GSH shifted the dose-response curve to the left.

CONCLUSION

In summary, we have synthesized an organic nitrate that has not been previously studied in mammals. It relaxes constricted pulmonary artery segments *in vitro* and alleviates pulmonary hypertension and hypoxemia under clinically relevant conditions. Although its effective vaporized concentration is significantly higher than that recommended for NO, it does not seem after short-term administration to produce methemoglobinemia or systemic cardiovascular side effects. In addition, its activity is potentiated by the addition of thiols. This drug merits further studies for optimal dosing and long-term effects, and holds promise as a potential alternative to inhaled NO, particularly in inflammatory settings in which thiol depletion may occur.

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