Exhaled Nitric Oxide as a Marker for Organic Nitrate Tolerance

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**Background** This study was designed to demonstrate the development of biochemical tolerance to organic nitrates by measuring levels of exhaled gaseous nitric oxide (NO) in lambs given intravenous (IV) nitroglycerin or sodium nitroprusside.

**Methods and Results** IV injections of nitroglycerin or sodium nitroprusside produced dose-dependent and sustained increases in the exhaled levels of nitric oxide measured by chemiluminescence in awake lambs with tracheostomies. After a 6-hour IV infusion of 25 μg·kg⁻¹·min⁻¹ nitroglycerin, peak exhaled NO levels were significantly reduced (−53.6±4.9%, mean±SEM, P<.001) and systemic hypotensive responses were attenuated (−52.6±5.9%, P<.001) after an IV challenge of nitroglycerin but not sodium nitroprusside. After a subsequent 12-hour nitroglycerin-free period, there was complete recovery of NO excretion in exhaled breath and a return to baseline of systemic hypotensive changes on administration of IV nitroglycerin boluses. For IV sodium nitroprusside challenges, pulmonary NO excretion and systemic hypotensive responses remained constant throughout the study. Challenges with IV nitroglycerin but not sodium nitroprusside during a 12-hour nitroglycerin-free period resulted in delayed biochemical recovery with various exhaled NO levels and systemic hypotensive responses to challenges with IV nitroglycerin.

**Conclusions** Measurements of exhaled NO provide in vivo, noninvasive evidence for the development of biochemical tolerance to nitroglycerin. There was reduced NO release into exhaled gas from the pulmonary vasculature concomitant with evidence of tolerance to nitroglycerin vaso-dilation in the systemic circulation. (*Circulation. 1994;89:2498-2502.*)

**Key Words** • nitroglycerin • sodium nitroprusside

The mechanism of tolerance to organic nitrates remains controversial despite more than a century of clinical application and investigation. Continuing debate regarding the development of tolerance is due in part to relatively small patient cohort studies in which hemodynamic nitrate tolerance was observed in some but not all of the patients.¹ At least four mechanisms have been proposed to explain the development of tolerance to organic nitrates: (1) diminished biotransformation to nitric oxide (NO), (2) counterregulatory neurohormonal activation, (3) desensitization of soluble guanylate cyclase, and (4) plasma volume shifts.¹² The lack of a suitable in vivo model has further limited definitive analysis.

Recently, low levels of endogenous NO were measured in the exhaled breath of animals and humans.⁴ We hypothesized that quantification of NO excretion in exhaled breath might provide a sensitive in vivo model for studying nitrate pharmacology and tolerance.

Specifically, we hypothesized that (1) bolus intravenous injections of sodium nitroprusside, which produces vaso-dilation via the nonenzymatic release of NO, or nitroglycerin, which requires enzymatic conversion to release NO, would produce proportionate and dose-dependent increases of NO concentration in expired gas and concomitant reductions of arterial blood pressure; (2) bolus intravenous injections of nitroglycerin but not sodium nitroprusside would produce decreased levels of exhaled NO and an attenuated systemic vasodilator response when tolerance to nitroglycerin is produced by a prolonged continuous nitroglycerin infusion, and (3) if a sufficient nitroglycerin-free period were allowed for recovery, enzymatic function and hemodynamic vasodilator reactivity to nitroglycerin injection would be restored.

**Methods**

**Animal Preparation**

This study was approved by the Subcommittee on Research Animal Care at the Massachusetts General Hospital. Sixteen Suffolk lambs weighing 29 to 31 kg were anesthetized by inhalation induction with halothane in oxygen. Their tracheas were intubated and their lungs mechanically ventilated at 15 breaths per minute and 15 mL/kg tidal volume with a large-animal ventilator (Harvard Apparatus). A 7F thermocatheter pulmonary artery catheter (Edwards Laboratories) was placed via the right external jugular vein through an 8F introducer (Cordis). The femoral artery was cannulated with a polyvinyl chloride catheter (2-mm internal diameter) advanced 20 cm into the aorta for continuous arterial pressure monitoring. A tracheostomy was performed, and an 8.0-mm cuffed tracheostomy tube (Portex) was inserted to allow for spontaneous ventilation. Studies began 2 hours after emergence from the anesthetic. The lambs were housed in a Babraham cage with access to food and water.

**Hemodynamic Measurements**

Systemic arterial pressure was measured by a calibrated pressure transducer (Cobe Laboratories) zeroed at the mid-chest level and continuously recorded on a thermal chart recorder (Mark 10-1, Western Graphitec, Inc).

**NO Measurements and Calculations**

The chemiluminescence analyzer (model 14A, Thermo Environmental) was calibrated with certified NO (360 ppb, Airco) mixed with 100% oxygen (0 ppb NO)³ by precision

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flowmeters (Air Products and Chemicals). The detection limit and resolution for NO gas were <2 ppb (vol/vol).

A two-way nonrebreathing valve (Hans Rudolph, Inc) was attached to the tracheostomy. The lambs breathed 100% oxygen administered through a 50-L rubber reservoir bag. Exhaled gas was sampled from a tetrafluoroethylene reservoir collecting bag (Cole-Parmer Instrument Co) that was connected to the exhalation port of the two-way valve. Before analysis, the exhaled gas was passed through a solid carbon dioxide–cooled (−70°C) glass vapor trap (Thomas Scientific) to remove any moisture. Minute ventilation was measured continuously with a pneumotachometer (Medi-mold Enterprises, Inc) calibrated with precision flowmeters. Fractional excretion of NO was determined by integrating the exhaled NO curve from the graphic output. Since minute ventilation was known, the number of molecules of NO in the total exhaled gas volume was calculated assuming that 1 mol of gas occupies 22.4 L at standard temperature and pressure. The molecular weights of nitroglycerin and sodium nitroprusside are 227.09 and 297.95, respectively. By calculation of the total number of molecules of either nitroglycerin or sodium nitroprusside injected, fractional molar excretion of NO into the exhaled gas was calculated. We assumed that 1 mol nitroglycerin or sodium nitroprusside releases 1 mol of NO.

**Experimental Protocol**

Exhaled NO levels and systemic arterial pressure were measured and recorded at baseline and then continuously throughout the entire experiment. Incremental bolus injections of nitroglycerin (ICI Americas; 0.5, 1.0, 2.0, and 3.0 mg) were administered via the right atrial port of the pulmonary artery catheter at 5-minute intervals. All parameters returned to baseline within 5 minutes. After a 15-minute recovery period, incremental bolus injections of sodium nitroprusside (Elkins-Sinn, Inc; 0.1, 0.2, 0.4, and 0.6 mg) were administered at 5-minute intervals. All parameters returned to baseline within 5 minutes. After these bolus injections, intravenous nitroglycerin was infused continuously (Harvard Apparatus) at a dose of 25 µg·kg⁻¹·min⁻¹ for 6 hours. Fifteen minutes after discontinuation of the nitroglycerin infusion, bolus injections of nitroglycerin and sodium nitroprusside were repeated as outlined above. The nitroglycerin and sodium nitroprusside bolus administration experiments were again repeated in 6 of the lambs after a 12-hour nitroglycerin-free period. Three of 11 lambs were similarly studied with additional intravenous bolus injections of nitroglycerin and sodium nitroprusside at hours 2, 4, 8, and 12 of the nitroglycerin-free recovery period.

To compare exhaled NO levels when nitroglycerin was administered via different routes, two additional lambs were studied; intravenous bolus injections of nitroglycerin were administered via the femoral and external jugular vein, in addition to the right atrial port of the pulmonary artery catheter. In these additional lambs, the venous admixture (Qa/Va) was calculated from a standard formula at baseline and after an intravenous nitroglycerin bolus injection. These measurements were obtained both before and after a 6-hour continuous infusion of nitroglycerin to determine whether changes of exhaled NO levels might be related to changes of arteriovenous shunting induced by nitroglycerin.

**Statistical Analysis**

The data are presented as mean±SEM. Statistical significance of changes measured before and after nitroglycerin infusion and after the 12-hour nitroglycerin-free recovery period was calculated by repeated-measures ANOVA. When a significant difference was found between two groups, an adjusted paired t test for each level of the drug, using a Bonferroni correction for multiple comparisons, was performed. A value of P<.05 was considered significant.

**Results**

The baseline level of exhaled NO in the absence of intravenous nitroglycerin or sodium nitroprusside was 3±1 ppb. These levels remained stable. Bolus injections of either nitroglycerin or sodium nitroprusside produced dose-dependent increases of the peak exhaled NO concentration and concomitant decreases of systemic arterial blood pressure (Figs 1 and 2). After a 6-hour infusion of nitroglycerin, the baseline level of NO in the exhaled gas was unchanged, but the peak NO levels after challenge with boluses of nitroglycerin were significantly decreased (−53.6±4.9%, P<.001, see Fig 2B). The systemic hypotensive response after an intravenous nitroglycerin challenge was also significantly attenuated (−52.6±5.9%, P<.001). The dose-response profile of exhaled NO levels and systemic hemodynamic responses to a bolus of nitroglycerin returned to baseline (P>.05) after the 12-hour nitroglycerin-free recovery period.

The peak exhaled levels of NO and the systemic vasodilator responses after bolus injections of sodium nitroprusside were not affected by the nitroglycerin infusion (Fig 2A). Interestingly, serial intravenous nitroglycerin bolus injections in the three lambs studied at hours 2, 4, 8, and 12 during recovery showed a marked variability of exhaled NO excretion. The fractional molar excretions of the injected NO dose of nitroglycerin and sodium nitroprusside calculated by the method described above are 0.0002% and 0.0001%, respectively.

The exhaled NO levels and hemodynamic responses resulting from peripheral (femoral and external jugular vein) administration of nitroglycerin boluses were not significantly different from central administration (right atrium) (Fig 3). The level of venous admixture (Qa/Va) was not different from baseline levels during intravenous nitroglycerin bolus injections and was unaffected by a 6-hour continuous infusion of nitroglycerin (Fig 4).

**Discussion**

In an attempt to monitor the development of nitrate tolerance in a large-animal model, we quantitatively measured gaseous NO excretion in the exhaled breath of sheep. We demonstrated that bolus injections of nitroglycerin or sodium nitroprusside produce dose-related increases of exhaled NO levels and decreases of systemic blood pressure. After a 6-hour continuous infusion of intravenous nitroglycerin, subsequent bolus injections of nitroglycerin but not sodium nitroprusside produced significantly decreased levels of exhaled NO and less systemic hypotension. These findings suggest that the prolonged nitroglycerin infusion produced tolerance to nitroglycerin but not sodium nitroprusside. Exhaled NO levels and systemic vasodilator reactivity were restored to baseline after a 12-hour nitroglycerin-free period. Shorter recovery periods produced highly variable responses.

That exhaled NO levels in response to bolus nitroglycerin but not sodium nitroprusside injections were reduced after a prolonged continuous infusion of nitroglycerin suggests that biochemical mechanisms are at least in part responsible for the development of tolerance to nitroglycerin. Sodium nitroprusside relaxes vascular smooth muscle and produces vasodilation via the nonenzymatic release of NO and subsequent activation of soluble guanylate cyclase. Nitroglycerin, on the other
hand, is first enzymatically converted to S-nitrosothiol intermediaries.6–8 Biochemical tolerance to nitroglycerin could occur by local substrate depletion, by enzymatic alteration, or conceivably, by metabolism of nitroglycerin to pharmacologically inactive degradation products that do not liberate NO. The variability of NO excretion and hemodynamic responses with nitroglycerin but not sodium nitroprusside bolus administration in the three lambs studied at several time points during the nitroglycerin-free recovery periods suggests that the enzyme system responsible for nitroglycerin metabolism is sensitive to further challenges to intravenous nitro-
The clinical relevance of nitrate tolerance remains controversial. Evaluation of the effect is difficult, particularly when small clinical trials are conducted. Despite evidence for nitrate tolerance, nitrate treatment appears to be effective.\textsuperscript{9,10} The variability in our study in biochemical response by the three lambs subsequently exposed to nitroglycerin after the development of tolerance may explain some of the heterogeneous responses observed in humans. Some patients develop nitrate tolerance very rapidly, while others appear resistant to tolerance. Furthermore, nitroglycerin tolerance may be partial.\textsuperscript{1}

Our estimation that the fractional gaseous excretion of a nitroglycerin or sodium nitroprusside dose is only 0.0002\% or 0.0001\%, respectively, is consistent with the fact that hemoglobin is an avid scavenger of NO.\textsuperscript{11} The NO level that we measured in the exhaled breath of the lambs is probably a sum of the normal levels (3±1 ppb) and that released from nitroglycerin or sodium nitroprusside. While relatively large bolus concentrations were injected to maximize the ratios of signal to background noise for exhaled NO detection, these doses are not outside the range of what is occasionally administered clinically. Recent advances in accurate gaseous NO detection by chemiluminescence in the parts per trillion range may allow precise quantification of exhaled NO after injection of far smaller doses of sodium nitroprusside or nitroglycerin. The exhaled NO levels and the systemic vasodilation response did not depend on the site of drug injection in our sheep. This suggests that the effects we observed were not due to the direct injection of very high concentrations of drug into pulmonary arterial blood.

Because the fractional exhaled excretion of NO was very small, increased intrapulmonary or extrapulmonary shunting of blood might significantly reduce the concentration of NO in exhaled gas. Nitrate infusions have been reported to release hypoxic pulmonary vasoconstriction and increase the venous admixture.\textsuperscript{12} In our study, the use of 100\% oxygen as the inhaled gas probably minimized the effect of hypoxic pulmonary vasoconstriction. We found no significant changes of venous admixture in response to boluses or an infusion of nitroglycerin or sodium nitroprusside in our model.

In summary, we have demonstrated a novel, noninvasive, in vivo method of studying tolerance to organic nitrates. We have shown that both nitroglycerin and sodium nitroprusside when injected intravenously release NO, which can be detected in the exhaled breath of lambs. Our results support the concept that nitroglycerin metabolism occurs via enzymatic mechanisms.

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