Expedited Articles

Localizing Antithrombotic and Vasodilatory Activity with a Novel, Ultrafast Nitric Oxide Donor

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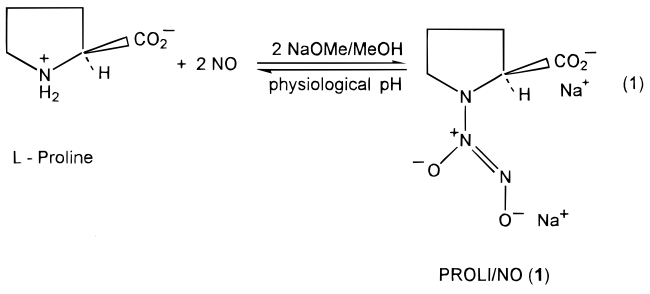
Reaction of nitric oxide (NO) with L-proline in methanolic sodium methoxide yields a diazeniumdiolate product, $C_5H_7N_3O_4Na_2 \cdot CH_3OH$ (PROLI/NO), that can be stabilized in basic solution but that dissociates to proline (1 mol) and NO (2 mol) with a half-life of only 1.8 s at pH 7.4 and 37 °C. This kinetic behavior has allowed the generation of highly localized antiplatelet and vasodilatory effects. By infusing solutions containing 4 μM PROLI/NO in 0.1 M sodium hydroxide at the rate of 1 nmol·min^{-1} immediately upstream from a polyester vascular graft in the unheparinized baboon circulatory system, for example, platelet deposition at the normally thrombogenic graft surface was substantially reduced relative to controls receiving only 0.1 M sodium hydroxide. In a second study, infusion of PROLI/NO into the right atrium of sheep with induced pulmonary hypertension selectively dilated the lung vasculature, dose-dependently reducing the pulmonary arterial pressure by as much as 9 mmHg with no observable effect on the systemic arterial pressure at an infusion rate of up to 24 nmol·kg^{-1}·min^{-1}. PROLI/NO could also be formulated as an insoluble polymer blend that released NO smoothly for prolonged periods. The results suggest that localized delivery of diazeniumdiolates such as PROLI/NO which generate NO with extreme rapidity on entering the blood stream may hold considerable promise for inhibition of thrombus formation, selective dilation of the vasculature, and other research and clinical applications.

Introduction

Nitric oxide (NO) has been discovered to play so many crucial signaling roles (e.g., in the cardiovascular, nervous, genitourinary, immune, and gastrointestinal systems) during the past decade that development of means for delivering this critical bioregulatory molecule to sites of deficiency without adverse effect on other NO-sensitive parts of the body has become an essential goal of the search for improved "nitrovasodilator" therapies.[1−4] We have developed a novel approach to achieving such targeted NO delivery that relies on the extreme rapidity with which certain newly discovered drugs of the diazeniumdiolate class generate NO under physiological conditions.

In the present report, we describe the facile, inexpensive synthesis of one such agent and show how local infusion thereof can be used both to inhibit adhesion of platelets to a normally thrombogenic vascular graft and to dilate the pulmonary vasculature without detectable systemic effects. We also show insoluble polymer blends thereof to be capable of emitting NO for prolonged periods, suggesting their use for localization of NO exposures to cell types with which they are in intimate physical contact.

Scheme 1. Synthesis of PROLI/NO (Right Arrow) and Its Dissociation in Aqueous Media to Proline and NO (Left Arrow)



Chemistry

Despite repeated failures in our earliest attempts to prepare diazeniumdiolate derivatives of amino acids, the target compound, PROLI/NO (**1**), could be conveniently isolated in good yield by exposing methanolic solutions of L-proline and sodium methoxide to gaseous NO, as in Scheme 1, and filtering the white, powdery product. Solutions of **1** in aqueous alkali showed the spectral characteristics expected of a diazeniumdiolate ion, including a strong ultraviolet maximum at 252 nm.[5]

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While previously characterized NO donor compounds of the diazeniumdiolate class generate NO in pH 7.4 phosphate buffer at 37 °C with half-lives ranging from 1 min to 1 day,[5] depending on their structure, PROLI/NO was found to dissociate with noteworthy speed. The course of decomposition as followed by loss of the chromophore at 252 nm was first order, with a rate constant of 0.39 s^{-1} (half-life 1.8 s). Chemiluminescence measurements showed that PROLI/NO released the full theoretical amount of NO during the dissociation (Scheme 1).

Antithrombotic Activity of Infused PROLI/NO

The combination of ultrafast dissociation kinetics with the production of 2 mol of NO and 1 mol of an innocuous natural product, proline, per mol of **1** that decomposes suggested that PROLI/NO could be an especially useful vehicle for localizing the effects of infused solutions thereof. Dissociation is acid-catalyzed and thus sufficiently slow in alkaline solution to permit infusion of essentially pure NO donor, but sufficiently rapid at physiological pH to be effectively complete within a few seconds after entering the blood stream. The resulting NO is known to react at near diffusion-controlled rates with oxyhemoglobin, curtailing its lifetime in the blood,[1] and systemic effects should be further limited by infusing the short-lived PROLI/NO slowly at the boundary layer where the blood velocity is substantially smaller than at the center of the mainstream.[6,7]

As an initial test of the hypothesis that PROLI/NO infusions might produce highly localized NO donor effects in the vasculature, we compared its ability to inhibit adhesion of platelets to a thrombogenic vascular graft in the baboon circulatory system ex vivo[6,7] to that of MAHMA/NO (**2**), the fastest NO generator among previously described diazeniumdiolates[5] with a half-life of 67 s at 37 °C and pH 7.4. Both compounds were infused 2 cm upstream from a polyester vascular graft inserted in a plastic shunt connecting the femoral artery and vein as previously described[4] (except that the PROLI/NO was introduced as the un-neutralized alkaline solution via a single infusion line rather than with the co-infusion system used for MAHMA/NO). Since the animals were not heparinized or otherwise given systemic anticoagulants, control infusions of vehicle (0.1 M sodium hydroxide) alone led to complete blockage of flow in the graft within 1 h of its insertion into the circulation. Both diazeniumdiolates strongly inhibited this platelet deposition, but PROLI/NO proved 1 order of magnitude more potent. As shown in Figure 1, PROLI/NO inhibited thrombus formation by 58 ± 19% at an infusion rate of 0.2 nmol·min^{-1} while MAHMA/NO had to be administered at more than 10 times this rate to achieve a similar antiplatelet effect. Very importantly, PROLI/NO induced no detectable change in mean arterial pressure at 1 nmol/min, confirming the localization of its pharmacological effects; dose-dependent depression of iliac blood pressure was seen at 3 nmol/min and higher, however.

Selective Dilation of the Pulmonary Vasculature

To determine whether intravenous infusion of an NO donor with a 1.8-s half-life could be used for selective relaxation of an entire vascular bed, we chose an ovine model of pulmonary hypertension for further study. Animals were initially given the lung-selective vasoconstrictor, U46619, to increase pulmonary arterial pressure (PAP), and then PROLI/NO was infused into the right atrial blood to determine its effects on lung function. PROLI/NO decreased PAP dose-dependently by up to 9 mmHg without any observable impact on systemic arterial pressure (SAP) at dose rates as high as 24 nmol·kg^{-1}·min^{-1}, while sodium nitroprusside lowered both SAP and PAP at all infusion rates studied (0.8−13 nmol·kg^{-1}·min^{-1}). PROLI/NO lowered SAP only at the highest infusion rate, 48 nmol·kg^{-1}·min^{-1}. The results are summarized in Figure 2. The data indicate that intravenous infusion of alkaline PROLI/NO at the rate of 24 nmol·kg^{-1}·min^{-1} relieves pulmonary hypertension in U46619-treated sheep as effectively and lung-specifically as inhalation of 40 ppm NO, the concentration metered into inhaled gas to treat certain types of respiratory distress in modern clinical

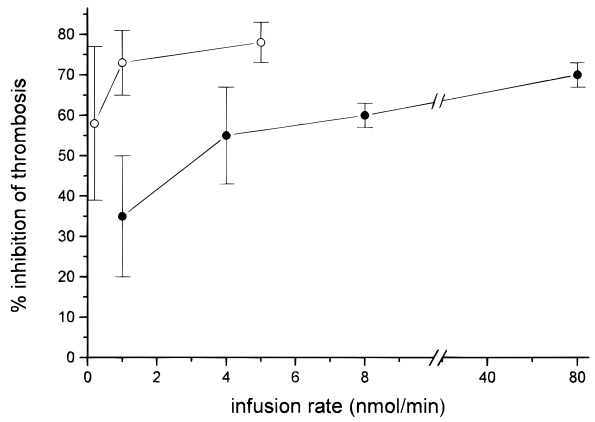


Figure 1. Inhibition of thrombus formation in polyester vascular grafts placed 2 cm downstream from the point at which PROLI/NO (○) and MAHMA/NO (●) were infused for 1 h into an arteriovenous shunt placed in a baboon's blood stream. Points are means ± SD, $N = 4$.

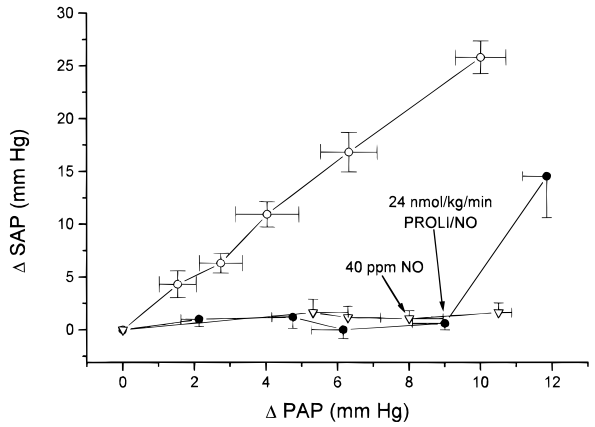
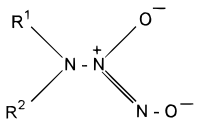


Figure 2. Degree of lung selectivity in reduction of arterial pressure when PROLI/NO (●) or sodium nitroprusside (○) were infused intravenously, or when NO gas was administered via inhalation (▽), at various rates in sheep with U46619-induced pulmonary hypertension. Initial dose rates (those with the smallest effects) were 3.0 and 0.8 nmol·kg^{-1}·min^{-1} for PROLI/NO and sodium nitroprusside, respectively, and 10 ppm for NO; these rates were doubled successively to give the increasing effects shown. Points represent the decrease in mean systemic arterial pressure (ΔSAP) ± SE and the decrease in mean pulmonary arterial pressure (ΔPAP) ± SE observed at each dose rate ($N = 8$).

practice.[8] The inhalation of 40 ppm NO at a typical minute ventilation of 0.2 $L \cdot kg^{-1} \cdot min^{-1}$ in our sheep[9] would deliver approximately 0.33 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ to the lung. The extent of pulmonary uptake of NO gas is, however, quite variable, making a molar potency comparison between inhaled NO and infused PROLI/NO difficult to interpret with confidence.

Fabrication of PROLI/NO Polymer Blends

The data described above documenting the localized action of infused PROLI/NO suggest that this compound may allow the preparation of improved coatings for stents, pump components, vascular grafts, and other materials that are kept in more than transient contact with blood. A previously described blend of DETA/NO, a low-molecular-weight diazeniumdiolate of structure **3**, in insoluble polycaprolactone was shown to release



2: R^1 = Me; R^2 = $(CH_2)_6NH_2^+$ Me

3: R^1 = $(CH_2)_2NH_2$; R^2 = $(CH)_2$ NH_3^+

detectable NO for a week on immersion in pH 7.4 buffer,[10] but this duration of action was not significantly longer than that of solutions of DETA/NO itself, a long-lived NO donor with a half-life of 20 h.[5] In addition, the possibility that some unchanged DETA/NO could leach out of the polycaprolactone to generate NO at sites remote from the insoluble solid could not be excluded.

We reasoned that replacing DETA/NO with the short-lived PROLI/NO should guarantee localization of NO release as was the case in the infusion experiments, but it was not clear a priori whether polymer−PROLI/NO blends would allow prolonged generation of NO at the polymer surface. Initial attempts to fabricate such a blend led to complete decomposition of the diazeniumdiolate, but means were ultimately devised to prepare composites from which the compound with its 252-nm chromophore could be recovered in reasonable yield. One such material was prepared by dissolving polyurethane in tetrahydrofuran and mixing the resulting solution with powdered PROLI/NO. Thorough removal of solvent under high vacuum gave a solid residue that generated NO fairly steadily during the 7 weeks it was soaked in pH 7.4 buffer at 37 °C. The quantitative NO release profile, shown in Figure 3, revealed a curious 5-fold surge of unknown origin at one point, but otherwise the rate remained at 0.2−0.7 $pmol \cdot min^{-1} \cdot mg^{-1}$ during the entire observation period. The results confirm that PROLI/NO can be incorporated into an insoluble, solid matrix that exudes molecular NO and/or a locally-acting NO donor for many days on exposure to aqueous media at physiological pH.

To demonstrate that prolonged release of NO from a PROLI/NO-containing coating could be achieved, glass fiber filter paper was soaked in a similarly constituted tetrahydrofuran solution, and solvent was removed in vacuo. The resulting coated paper released NO steadily for 3 days when immersed in pH 7.4 buffer at 37 °C.

Significance

The results suggest that localized delivery of PROLI/NO may provide an ideal basis for NO donor drug therapy in many applications. We have shown that stable alkaline solutions thereof markedly retard platelet deposition in vascular grafts placed in the baboon circulatory system when infused at the rate of a few picomoles per kilogram per minute during a 1-h observation period. If planned experiments show that a 3-day infusion reduces thrombus formation over several weeks thereafter, then significant clinical benefit might be forthcoming.

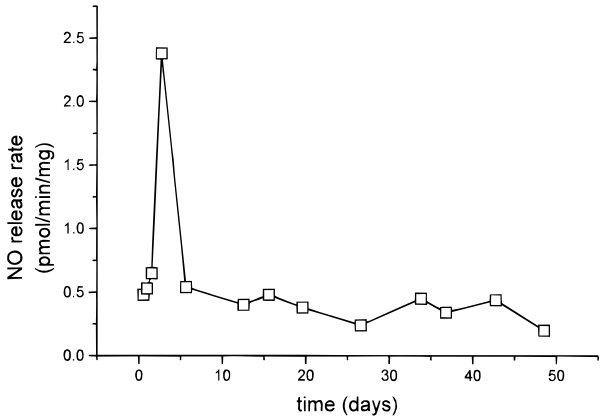


Figure 3. Time course of NO release from a polyurethane blend containing 70 nmol of PROLI/NO per milligram of polymer. Rates of NO generation are plotted as a function of time after immersion in pH 7.4 phosphate buffer at 37 °C.

Our experiments with the sheep show that infusion at higher rates can increase the size of the vascular bed blanketed with NO while nevertheless highly localizing the effect. In particular, infusions at the 3−24 $nmol \cdot kg^{-1} \cdot min^{-1}$ rate (2−3 orders of magnitude higher than those given to the baboons) into the venous blood just prior to its entry into the lung produced substantial lowering of the pulmonary arterial pressure without detectable effect on systemic hemodynamics in sheep with drug-induced pulmonary hypertension. Indeed, the lung-selective effects of intravenous PROLI/NO compared favorably with those of NO gas concentrations currently administered via inhalation to treat certain types of respiratory distress.

Finally, we have shown that PROLI/NO can be incorporated into solid polymer matrices that should provide prolonged NO release into tissues and cell types with which they are in intimate contact without affecting NO-sensitive sites elsewhere in the system.

Very importantly, PROLI/NO is among the most advantageous of the known NO donor drugs from the toxicological point of view. Its dissociation has been shown to lead cleanly to just two products, NO and the natural amino acid, proline. Even if some of the released NO were oxidatively converted to a nitrosating agent that recombined with the amino nitrogen[11] to form *N*-nitrosoproline, the ultimate product would not pose the carcinogenic threat that most secondary amine/NO combinations do because *N*-nitrosoproline has never proven carcinogenic in any of the long-term toxicity studies to which it has been subjected.[12−18]

Future work will aim to exploit these and other important advantages that localized delivery of this ultrafast NO donor may offer the medicinal chemist as well as the clinician.

Experimental Section

MAHMA/NO (**2**) and DETA/NO (**3**) were prepared as previously described.[19] NO was purchased from Matheson Gas

Products (Montgomeryville, PA). Ultraviolet (UV) spectra were recorded on a Hewlett-Packard Model 8451A diode array spectrophotometer. Nuclear magnetic resonance spectra were collected with a 300-MHz Varian Unity Plus or a Varian XL-200 NMR spectrometer. Elemental analyses were performed by Atlantic Microlab, Inc. (Norcross, GA).

Disodium 1-[2-(Carboxylato)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate (1). A solution of 10 g (87 mmol) of L-proline (Aldrich Chemical Co., Milwaukee, WI) in 39 mL (0.18 mol) of 25% sodium methoxide in methanol (Aldrich) and 20 mL of methanol was degassed and exposed to 3 atm of NO for 20 h. The pressure was released, and the precipitate was collected by filtration, washed with ether, and dried under vacuum to give 17 g of white product. NMR and elemental analysis indicated that the product of this preparative method consists of **1** and methanol in 1:1 molar ratio (see below). Since the methanol could not be removed by holding the sample at 1−2 mmHg overnight, we take the molecular weight of **1** to be 251: UV (10 mM sodium hydroxide) λ_{max} (ϵ) 252 nm (8.4 mM^{-1} cm^{-1}); ^1H NMR (D_2O) δ 1.71 (m, 1 H), 1.91 (m, 2 H), 2.27 (m, 1 H), 3.27−3.43 (m, 2 H), 3.34 (s, 3 H [methanol CH_3]), 4.04 (m, 1 H); ^{13}C NMR δ 24.45, 30.97, 48.73 (methanol), 54.95, 67.70, 182.75. The amount of NO generated on dissolution in pH 7.4 buffer was found using a previously described chemiluminescence method[20] to be 2.0 (theoretical: 2.0) mol/mol of PROLI/NO consumed. The rate of NO release was determined by rapidly mixing a small aliquot of alkaline PROLI/NO with a large excess of pH 7.4 buffer at 37 °C in a cuvette and measuring the absorbance at 252 nm every 0.6 s through several half-lives; semilog plots of [absorbance ($A - A_\infty$)] versus time yielded a first-order plot ($r^2 = 0.995$) with a half-life of 1.8 s. The half-life in this buffer at 22 °C was measured as 6.4 s, while that in 0.1 M sodium hydroxide solution was found by periodic scanning of the ultraviolet spectrum to be about 3 weeks; the half-life at 37 °C in 0.1 M sodium hydroxide was estimated to be 2 days. Anal. Calcd for $C_5H_7N_3O_4Na_2 \cdot CH_3OH$: C, 28.69; H, 4.41; N, 16.73; Na, 18.30. Found: C, 28.65; H, 3.99; N, 16.74; Na, 18.04.

The methanol could be removed from this preparation by triturating the solid with 1:10 (v:v) acetonitrile:diethyl ether followed by filtration and drying under vacuum, but the material used for the following experiments was the methanol solvate (molecular weight 251).

Antiplatelet Activity in the Baboon Circulatory System. Adult male baboons (*Papio cynocephalus*) weighing approximately 12−20 kg each were employed for these studies in accord with federal guidelines (Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23) using protocols approved by the Yerkes Primate Research Center's Institutional Animal Care and Use Committee. Each baboon had an exteriorized silicone rubber shunt placed between its common femoral artery and vein. Each arteriovenous shunt was equipped with a specially designed local infusion device.[7,21] A 1-cm dacron vascular graft was placed 2 cm downstream from each infusion device. Blood flow through the circuit was measured with a transit time Transonic T106 flowmeter (Transonic Inc., Ithaca, NY) placed downstream from the graft.[7] Blood flow was kept constant at 100 mL/min by adjusting a clamp placed downstream from the flow probe. The experiments were conducted on awake animals.

Platelets were labeled by diluting 60 mL of whole baboon blood with 6 mL of 3.8% citrate.[22] The mixture was divided into two conical centrifuge tubes. After separation from plasma by centrifugation, the platelets were mixed with 1 mCi of $^{111}InCl_3$; a labeling efficiency of >90% was routinely achieved. Labeled platelets were reinfused back into the animal the day before establishing the arteriovenous shunt.

Infusions were continued for 1 h each with a Harvard infusion pump (Harvard Apparatus Inc., South Natick, MA) at a constant flow rate of 3 mL/h. The infusate was either 0.1 M sodium hydroxide (as a control), PROLI/NO dissolved in 0.1 M sodium hydroxide, or MAHMA/NO. The latter compound was infused such that the alkaline stock solution was neutralized with an equimolar stream of acid immediately before entering the infusion device as previously described.[4] Platelet accumulation in the Dacron graft was measured with a General Electric or a Searle gamma counter with a fixed region of interest at 172 keV with a 15% window.[7,10] Measurements were made in 5-min periods for 1 h. At the end of the experiments, the animals were returned to the animal pool for future experiments.

Relief of Pulmonary Hypertension in U46619-Treated Sheep. Eight Suffolk lambs each weighing 25−30 kg were employed in protocols approved by the Subcommittee for Research Animal Studies at Massachusetts General Hospital, as separately described.[23] Systemic arterial pressure was monitored via a catheter advanced through the femoral artery 20 cm into the aorta, while pulmonary arterial pressure was measured with a three-port thermodilution pulmonary artery catheter placed via the right external jugular vein. A tracheotomy was performed, and a cuffed tracheostomy tube was inserted. All animals were then allowed to recover from anesthesia for 2 h and were studied awake and spontaneously ventilating via the tracheotomy tube. All animals had free access to food and water during the study.

Mean pulmonary artery pressure was increased to 30 mmHg by infusing U46619 (9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$, Cayman Chemical Co., Ann Arbor, MI) at the rate of 0.4−0.8 $\mu g \cdot kg^{-1} \cdot min^{-1}$. Stock solutions of either PROLI/NO dissolved in saline made 0.1 M in sodium hydroxide or sodium nitroprusside (Elkins-Sinn, Inc., Cherry Hill, NJ) in lactated Ringer's solution were infused via the right atrial port of the Swan-Ganz catheter for 15 min, whereupon the decreases in pressure relative to preadministration baseline values were recorded and infusion of the NO donor was stopped. Complete recovery to baseline hemodynamic parameters occurred within 15 min. Alternatively, NO (Airco, Murray Hill, NJ) was introduced into the inhaled gas (100% oxygen) for 6 min, systemic and pulmonary arterial pressures were recorded, and NO administration was halted for 6 min to allow complete return to baseline hemodynamic readings. Both the order of administering the PROLI/NO, sodium nitroprusside, and NO and the doses given were randomized in treating each of the eight sheep with four different concentrations of NO gas plus five different infusion rates for each of the drugs given intravenously.

Preparation of Nitric Oxide-Releasing PROLI/NO−Polymer Blends. Polyurethane (Tecoflex, 393 mg; obtained from Thermedics, Inc., Woburn, MA) was dissolved in 6 mL of tetrahydrofuran. The resulting lacquer was mixed with 7 mg (0.028 mmol) of finely powdered PROLI/NO and homogenized thoroughly via a vortex mixer. The solvent was evaporated under a stream of nitrogen, and the residue was placed under high vacuum to give 400 mg of composite containing 70 nmol of PROLI/NO per milligram. Rates of NO release were measured as a function of time after immersing a 10.7-mg aliquot in 50 mM phosphate buffer, pH 7.4, with a chemiluminescence detector as previously described.[10]

A 4.25-cm sheet of glass fiber filter paper (GF/A, Whatman International Ltd., Maidstone, U.K.) was submerged in a similar mixture of 253 mg of polyurethane in tetrahydrofuran with 0.1 mmol of PROLI/NO and then dried under vacuum. Extraction of an aliquot with 10 mL of 0.1 M sodium hydroxide led to recovery of PROLI/NO in 89% yield, based on the absorbance of the solution at 252 nm. NO release rates were determined as above.

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