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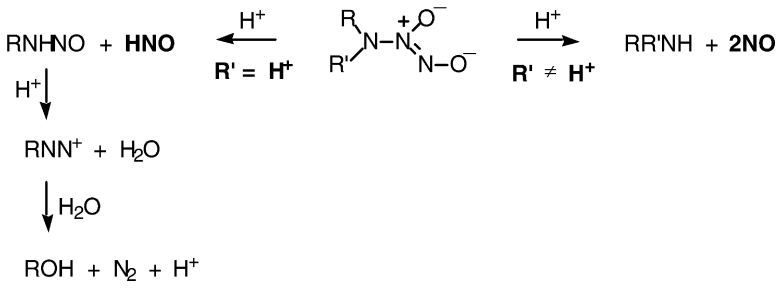

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Comparison of the NO and HNO Donating Properties of Diazeniumdiolates: Primary Amine Adducts Release HNO in Vivo

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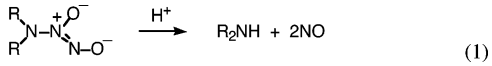
Diazeniumdiolates, more commonly referred to as NONOates, have been extremely useful in the investigation of the biological effects of nitric oxide (NO) and related nitrogen oxides. The NONOate Angeli's salt ($Na_2N_2O_3$) releases nitroxyl (HNO) under physiological conditions and exhibits unique cardiovascular features (i.e., positive inotropy/lusitropy) that may have relevance for pharmacological treatment of heart failure. In the search for new, organic-based compounds that release HNO, we examined isopropylamine NONOate (IPA/NO; $Na[(CH_3)_2CHNH(N(O)NO])$, which is an adduct of NO and a primary amine. The chemical and pharmacological properties of IPA/NO were compared to those of Angeli's salt and a NO-producing NONOate, DEA/NO ($Na[Et_2NN(O)NO]$), which is a secondary amine adduct. Under physiological conditions IPA/NO exhibited all the markers of HNO production (e.g., reductive nitrosylation, thiol reactivity, positive inotropy). These data suggest that primary amine NONOates may be useful as HNO donors in complement to the existing series of secondary amine NONOates, which are well-characterized NO donors.

Introduction

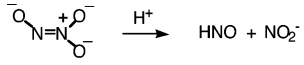
Exposure of amines to high pressures of nitric oxide (NO) results in production of anions[1−5] termed diazeniumdiolates or, more commonly, NONOates, after the $[N(O)NO]^-$ moiety. Many such adducts between NO and secondary amines are stable as solids and in alkaline solution but fragment back to the parent and two equivalents of NO with increased acidity (eq 1).



(1)

These compounds have proven to be highly useful for examining the effects of NO in chemical, biochemical, cellular, and even in vivo experiments (reviewed in refs 6−9). The rate of NO delivery is a function of the secondary amine component, and NONOates have been prepared with half-lives ranging from seconds to days at physiological pH and temperature.[4,5] NONOate decomposition is well-behaved, with little influence from media composition or external factors such as room light. These properties allow experimental simulation of biological fluxes of NO derived from NO synthase without the inconvenience of enzymatic preparations or procedures involving dissolved gases.[10] Consequently, secondary amine NONOates have contributed substantially to the current understanding of the redox biology of NO.

NONOates are essentially adducts of the NO dimer and a nucleophile, and stable complexes between the $[N(O)NO]^-$ functional group and O-, S-, and N-based nucleophiles have been formed by varieous synthetic routes (reviewed in refs 11 and 12). The NONOate with oxide as the nucleophilic component, $^-O[N(O)NO]^-$, commonly known as Angeli's salt with sodium counterions,[13] exhibits similar stability to amine-based NONOates. However, decomposition of Angeli's salt at physiological pH produces nitroxyl (HNO) (eq 2) rather than NO.[14,15]



(2)

Experimental elucidation of the chemical biology of NO has primarily focused on the oxidized products of NO such as peroxynitrite ($ONOO^-$) and dinitrogen trioxide (N_2O_3), while reduced species such as HNO have been of relatively minor interest. However, the similarity in the structures and rates of decomposition[4] of Angeli's salt and the secondary amine NONOate DEA/NO ($Na[Et_2NN(O)NO]$) has allowed convenient comparison of the pharmacological properties or HNO

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and NO donors. Such analyses have revealed that HNO repeatedly induces distinct effects from NO (reviewed in refs 16 and 17). For instance, infusion of Angeli's salt into conscious healthy dogs resulted in increased myocardial contractility and improved myocardial relaxation in a load-independent manner.[18] These hemodynamic modifications were accompanied by elevated plasma levels of calcitonin gene-related peptide (CGRP),[18] which is a small noncGMP vasoactive neuropeptide released from nonadrenergic/noncholinergic (NANC) fibers[19] that abundantly innervate some regions of the heart. In contrast, DEA/NO affected canine myocardial contractility in a load-sensitive (i.e. baro-reflex-mediated) fashion and augmented cGMP levels in plasma.[18]

The cardiovascular effects of Angeli's salt observed in both normal[18] and failing hearts[20] have initiated interest in HNO donors as attractive alternatives to organic nitrates in the treatment of congestive heart failure (see commentary by Feelisch[21]). Although Angeli's salt has proven to be a highly effective tool in the investigation of the chemical biology of HNO, continued progress in the pharmacological exploitation of HNO requires the availability of a variety of organic-based yet water-soluble donors, such as presently exist for NO production.

In the initial investigation of the vasodilatory effects of NONOates,[4] examples of different classes of NONOate, i.e., DEA/NO (secondary amine), Angeli's salt (oxide), and IPA/NO (primary amine, $Na[(CH_3)_2CHNH-(N(O)NO]$), were compared. The product yields and vasoactivity of IPA/NO and Angeli's salt were similar and substantially lower than those of DEA/NO. These results, in conjunction with the observation that decomposition of IPA/NO in phosphate buffer (100 mM) produced a significant yield of N_2O (unpublished data; see also Wink and Feelisch[22]), indicated that IPA/NO may function as an HNO donor and thus may serve as an organic alternative to Angeli's salt. Consequently, we investigated the solution chemistry and cardiovascular hemodynamic profile in vivo of IPA/NO in comparison to those of the HNO donor Angeli's salt and the NO donor DEA/NO.

Results and Discussion

In contrast to its free radical redox cousin NO, the dimerization of HNO is a favorable, rapid (8×10^6 M^{-1} s^{-1})[23] process that produces nitrous oxide via dehydration of the transient hyponitrous acid (eq 3).[24,25]

$$2HNO \rightarrow [HONNOH] \rightarrow N_2O + H_2O \qquad (3)$$

Although this reaction provides N_2O as an indirect marker of HNO formation, it also renders direct detection of HNO to be inherently challenging and requires that HNO be produced in situ, generally by reductive techniques or via a donor system. Specialized spectroscopic techniques can be utilized to detect HNO (see, for example, refs 26−30); however, at present, all conventional detection methods are indirect (reviewed in refs 17 and 22).

Currently, the method used most commonly to suggest the presence of HNO is observation of reductive nitrosylation (eq 4).[31,32]

$$M^{n+} + HNO \rightarrow M^{(n-1)+}NO + H^+ \qquad (4)$$

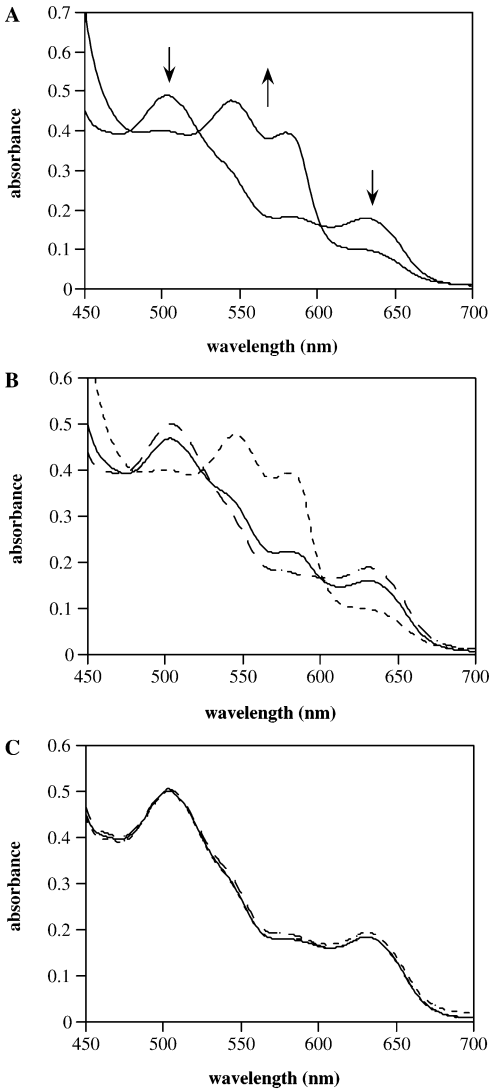

Figure 1. (A) Reductive nitrosylation of metMb (50 μM; 502 and 630 nm) to MbNO (543 and 582 nm) by Angeli's salt (100 μM); (B) comparative efficiency of MbNO formation by Angeli's salt (100 μM, --), IPA/NO (100 μM, −), or DEA/NO (50 μM, − −); and (C) quenching of reductive nitrosylation by 250 μM GSH. The reactions were performed at 37 °C in PBS (pH 7.4, 50 μM DTPA).

Heme proteins such as myoglobin (Mb) or synthetic analogues are particularly useful in this assay due to the dissimilar spectral signatures of the oxidized reactant and nitrosylated product. Reductive nitrosylation can be conveniently coupled with quenching by excess thiol, such as glutathione (GSH), which reacts rapidly (2×10^6 M^{-1} s^{-1})[33] with HNO (eqs 5 and 6).[34−36]

$$RSH + HNO \rightarrow RSNHOH \qquad (5)$$

$$RSNHOH + RSH \rightarrow RSSR + NH_2OH \qquad (6)$$

The spectral changes upon complete reductive nitrosylation of metMb (50 μM) to MbNO by Angeli's salt (100 μM) are shown in Figure 1A. An excess of Angeli's salt is required for complete conversion of metMb, presumably due to competitive HNO consumption by dimerization (eq 3). Consequently, this assay is not quantitiative for HNO release. The relative yield of MbNO from Angeli's salt, IPA/NO (100 μM), or DEA/NO (50 μM to provide stoichiometric reactive

nitrogen oxide; see eqs 1 and 2) is compared in Figure 1B, while the sensitivity to GSH (250 μM) of the spectral changes induced by Angeli's salt or IPA/NO is demonstrated in Figure 1C. This comparison strongly substantiates the formation of HNO during IPA/NO decomposition, although at a lower yield than that of Angeli's salt.

IPA/NO induces vasorelaxation in isolated aortic rings[4] although HNO has been shown to not stimulate soluble guanylyl cyclase,[37] which is the first enzyme in the vasodilatory cascade. The observation of a vasodilatory response, as well as the lower yields compared to other NONOates in the aortic[4] and metMb (Figure 1) assays, therefore indicates that IPA/NO decomposition is releasing both NO and HNO, presumably by competing pathways. At low pH the nitrogen-containing end product from Angeli's salt decomposition is exclusively NO,[38,39] indicating that NONOates that release HNO can in fact be converted to NO donors.

Previously, we determined that decomposition of Angeli's salt (10 μM) and DEA/NO (5 μM) produced oxidants of significantly different potentials, as assessed by the extent of two-electron oxidation of dihydrorhodamine (DHR) to the fluorescent dye rhodamine (RH).[40] Fluorophore production was O_2-dependent, indicating that the reactive oxidants were the autoxidation products of the initial species released by each donor.[40] Formation of RH was shown to increase in a donor concentration-dependent manner; thus, the relative reactivity toward DHR (2−100 μM) can be conveniently visualized with double-reciprocal plots, where a steeper slope indicates a lower reaction rate.[41] The similarity of the double-reciprocal plots of IPA/NO (10 μM) and Angeli's salt (Figure 2) supports the metMb result (Figure 1) that IPA/NO decomposition produces HNO.

The similarity of the extent of DHR oxidation by IPA/NO and Angeli's salt suggests that the yields of oxidant are comparable. Dimerization of HNO (eq 3) competes with autoxidation such that the DHR assay is not quantitative for HNO release from Angeli's salt.[33] Consumption of HNO by NO (eqs 7−9)[23] has a similar initial rate constant to HNO dimerization (8×10^6 M^{-1} s^{-1}).

$$HNO + NO \rightarrow HN_2O_2 \quad (6 \times 10^6\ M^{-1}\ s^{-1}) \qquad (7)$$

$$HN_2O_2 + NO \rightarrow HN_3O_3 \quad (8 \times 10^6\ M^{-1}\ s^{-1}) \qquad (8)$$

$$HN_3O_3 \rightarrow N_2O + HNO_2 \quad (2 \times 10^4\ M^{-1}\ s^{-1}) \qquad (9)$$

Although the simultaneous production of NO and HNO by IPA/NO would limit HNO dimerization, the overall rate of HNO consumption by pathways other than autoxidation would not be altered significantly. NO produces a nearly negligible signal compared to HNO in this assay. Thus, since the rates of decomposition of Angeli's salt and IPA/NO are comparable,[4] the trapping efficiency of HNO by O_2 is expected to be similar as long as HNO is the major product from IPA/NO.

Whereas GSH reacts directly with HNO (eq 5), urate quenches the chemistry of the HNO/O_2 product.[40] Thus, the scavenging ability of urate toward the reactive species produced by IPA/NO was compared by the DHR assay to that of Angeli's salt and DEA/NO. The profile of quenching for IPA/NO is similar to that of Angeli's salt (Figure 3A), supporting the production of a comparable amount of oxidant from both donors.

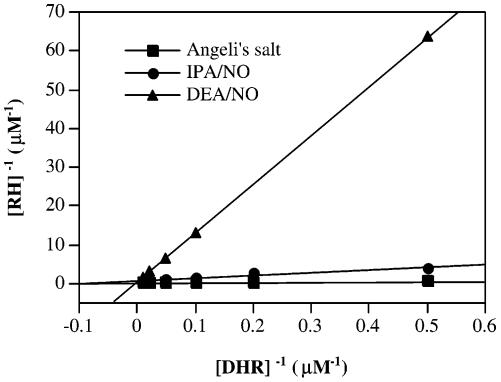

Figure 2. Oxidation of DHR by Angeli's salt (squares; 10 μM), IPA/NO (circles; 10 μM), or DEA/NO (triangles; 5 μM). The experiment was performed as described in the Experimental Section.

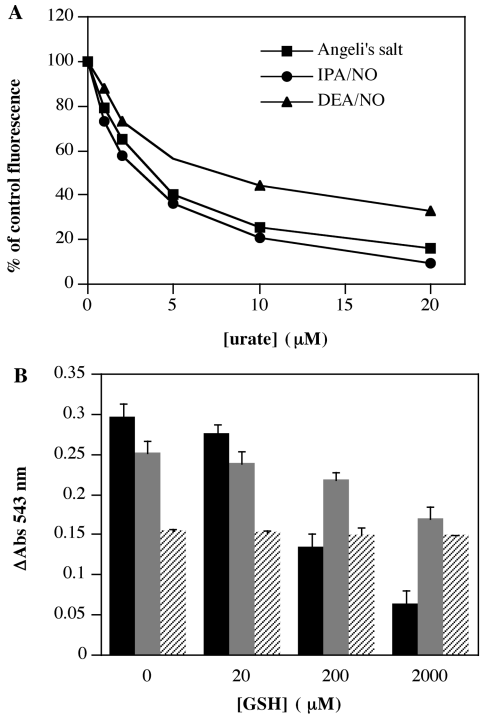

Figure 3. (A) Quenching of donor-mediated DHR oxidation by urate. Angeli's salt (squares, 10 μM), IPA/NO (circles, 10 μM) or DEA/NO (triangles, 5 μM) was added to 1 mL of PBS containing DTPA (50 μM), DHR (50 μM) and urate (1−20 μM), and fluorescence was determined as for Figure 2. The data were normalized to fluorescence for each donor in assay buffer alone. The data points are the means from triplicate sets; SEM error bars were of similar size to the symbols and were thus omitted for clarity. (B) Quenching of MbO_2 oxidation to metMb by GSH. Angeli's salt (black bars, 20 μM), IPA/NO (gray bars, 20 μM) or DEA/NO (hatched bars, 10 μm) were added to 1 mL of PBS containing DTPA (50 μM), MbO_2 (40 μM), and GSH (20, 200 or 2000 μM) and treated as described in the Experimental Section.

The reactivity of HNO is substantially more extensive than that of NO, despite the fact that NO is a free radical (reviewed in ref 17). We thus compared the susceptibilities of the reactive products from Angeli's salt and IPA/NO to scavenging by common biomolecules such as GSH, ascorbate, *N*-acetyl-L-cysteine and the SOD mimic tempol (4-hydroxy-2,2,6,6-tetramethyl-

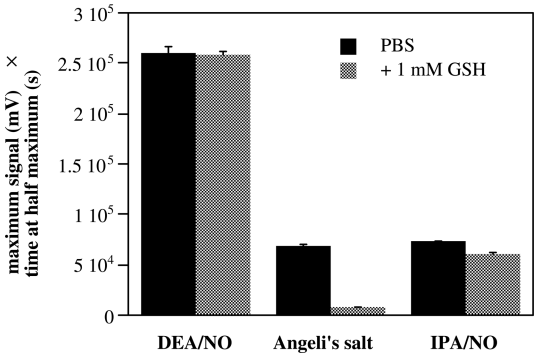

Figure 4. Signal area estimation (maximal chemiluminescent signal × peak width at half-maximum height, mV s) during the decomposition at 37 °C of DEA/NO (2.5 nmol), Angeli's salt (5 nmol), or IPA/NO (5 nmol) in PBS (∼ 6 mL, pH 7.4, containing 50 μM DTPA) ± 1 mM GSH. Triplicate data are presented as the mean ± SEM.

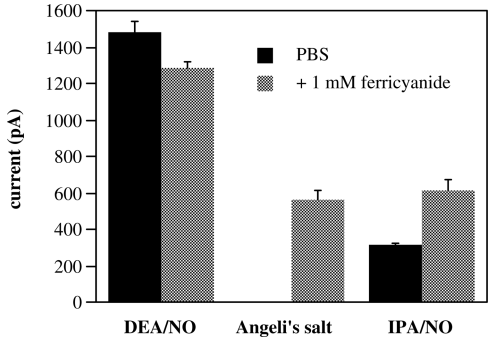

Figure 5. Maximal electrochemical signal by NO-specific electrode during the decomposition at room temperature of DEA/NO (2.5 μM), Angeli's salt (5 μM), or IPA/NO (5 μM) in PBS (20 mL, pH 7.4, containing 50 μM DTPA) ± 1 mM ferricyanide. Duplicate data are presented as the mean ± SEM.

piperidine-*N*-oxyl). Although reductive nitrosylation of metMb is an excellent assay for detection of HNO, the nitrosylated product is not air stable. Consequently, this assay is not suitable for large sample sizes or end product analyses. HNO also reacts with MbO_2 to form metMb,[31,34] and this reaction has the advantage of both large spectral changes and an air stable product.

The scavenging profile of GSH (Figure 3B) is representative, in which oxidation of MbO_2 (40 μM) was affected to a lower extent by the presence of scavenger during decomposition of IPA/NO (20 μM) compared to Angeli's salt (20 μM). This lower susceptibility to species that bind HNO can be explained by the simultaneous production of NO, which also readily oxidizes MbO_2.

$$MbO_2 + NO \rightarrow metMb + NO_3^- \qquad (10)$$

The relative yield of metMb from DEA/NO (10 μM) was lower than that of both Angeli's salt and IPA/NO. Moreover, product formation was insensitive to GSH, as expected by the lack of direct reactivity between NO and thiols.[42]

These results warranted direct assessment of NO production from IPA/NO. Detection of NO often involves chemiluminescence and electrochemical methodologies due to the relative sensitivity and specificity compared to other assays.[43,44] Similar methods are not currently available for HNO, but these techniques can be applied to HNO donors in the presence of excess ferricyanide, which converts HNO to NO.[45,46] As demonstrated previously,[4,47] decomposition of DEA/NO (2.5 nmol) resulted in a substantially larger chemiluminescent signal than Angeli's salt (5 nmol) (Figure 4), while a current increase was only apparent by NO-specific electrode for DEA/NO (2.5 μM) (Figure 5). Addition of ferricyanide (1 mM) did not significantly affect the current maximum or the temporal decay of the DEA/NO signal, indicating that ferricyanide does not react with either NO or the parent NONOate. Ferricyanide substantially enhanced the current during Angeli's salt (5 μM) decomposition. The temporal response was similar to that for DEA/NO, suggesting that the rate of decomposition of Angeli's salt is rate-limiting and that ferricyanide reacts with HNO rather than the parent. Given that the concentration of released nitrogen oxides should have been of similar magnitude, the lower signal for Angeli's salt (5 μM, ferricyanide) compared to DEA/NO (2.5 μM) is likely a function of incomplete conversion of HNO to NO or of rapid consumption (eqs 3 and 7). Thus, despite the large excess of ferricyanide, measurement of HNO is not quantitative by this method.

Maintenance of the baseline signal of the NO-specific electrode during Angeli's salt decomposition (Figure 5) indicated undetectable formation of NO at pH 7.4. However, a signal, although of much reduced intensity compared to that of DEA/NO, was apparent under similar conditions in the chemiluminescence analyzer (Figure 4), which has a substantially higher sensitivity than the electrode. GSH was added to the analyzer vessel in order to discern whether the observed signal was a result of NO production below the detection limit of the electrode or to detection of HNO itself. That GSH essentially eliminated the signal (Figure 4) suggests that the analyzer is not specific for NO.

Decomposition of IPA/NO (5 μM) in assay buffer produced a maximum current nearly 5-fold lower than that of DEA/NO (2.5 μM) but significant compared to Angeli's salt (5 μM) (Figure 5). Interestingly, the current from IPA/NO in the presence of ferricyanide was comparable with that of Angeli's salt, indicating a similar yield of total nitrogen oxides measured. The chemiluminescent signals of IPA/NO and Angeli's salt were essentially identical and again approximately 5-fold lower than that of DEA/NO (Figure 4). However, the attenuation by GSH of the chemiluminescent signal from IPA/NO was much lower than for Angeli's salt. This can be explained by an increased yield of NO at the detector due to reduced consumption by HNO (eq 7).

Together, the data in Figures 1−5 demonstrate conclusively that IPA/NO is functioning as both an HNO and NO donor at pH 7.4. Unfortunately, the lack of a method to quantitate HNO and the rapid consumption pathways (eqs 3 and 7) limit accurate assessment of the ratio of the two product pathways. However, HNO is suggested to be the major product.

In acidic medium, below pH 3, Angeli's salt converts to a NO donor,[14,38,39] but the mechanism has not been fully elucidated. NO has been proposed to be a secondary product arising through a free radical chain mechanism between nitrous acid (HNO_2; pK_a of 3.5) and the fully protonated conjugate acid of Angeli's dianion, $H_2N_2O_3$.[15] A theoretical analysis[48] has instead predicted

that NO results through the intermediacy of a diprotic species with a water:NO dimer structure (eq 11).

$$[ON(O)=NOH]^- + H^+ \rightleftharpoons H_2ON(O)=NO \rightarrow H_2O + [NO]_2^* \rightarrow 2NO \quad (11)$$

Regardless of the mechanism, the transition from production of HNO/NO_2^- to NO is quite sharp, which apparently is not the case for IPA/NO.

All three donor compounds utilized here are NONOates with varied functional groups. Recent quantum mechanical calculations[48,49] have predicted that upon dissolution of NONOates that release HNO, equilibria are established between stable and unstable isomers of varied protonation states. For instance, the water:NO dimer adduct in eq 11 is predicted to be a minor component of the equilibrium, as it is of higher energy than the tautomer protonated at both atoms of the nitroso group, $O_2N-NHOH$. This lowest energy species was calculated to be stable to decomposition and thus, although dominating the equilibrium, does not contribute to product formation. For primary amine NONOates such as IPA/NO, formation of HNO or NO is similarly suggested to result from dissociation of higher energy tautomers (eqs 12−16).[49]

$$[RN(H)-N(O)=NO]^- \rightleftharpoons [RN=N(O)-NHO]^- \quad (12)$$

$$[RN=N(O)-NHO]^- \rightarrow HNO + RNNO^- \quad (13)$$

$$[RN(H)-N(O)=NO]^- + H^+ \rightleftharpoons [RN(H)-N(O)=NOH] \quad (14)$$

$$[RN(H)-N(O)=NOH] \rightleftharpoons [RN(H)_2-N(O)=NO] \quad (15)$$

$$[RN(H)_2-N(O)=NO] \rightarrow RNH_2 + [NO]_2^* \rightarrow 2NO \quad (16)$$

Dissociation of minor components of the equilibria satisfactorily explains the slow decomposition rates ($<10^{-3}$ s^{-1} at 37 °C).[4] Further, kinetic parameters for tautomerization and protonation of $[RN(H)-N(O)=NO]^-$ resulting in dual decomposition pathways would account for production of both NO and HNO from IPA/NO. This competition at pH 7.4 as well as the fast reaction between NO and HNO (eq 7) can account for the lower yield of reductive nitrosylation by IPA/NO compared to Angeli's salt (Figure 1B). *N*-Nitrosated aliphatic primary amines are expected to be unstable and thus to rapidly dehydrate to the respective diazonium ion, RN_2^+. Subsequent hydrolysis with release of N_2 would yield the alcohol, ROH, as the final product of the NONOate amine component.

Protonation of secondary amine NONOates such as DEA/NO at the amine nitrogen, producing a species analogous to the reactant in eq 16, results in dissociation to R_2NH and 2NO (eq 1). The tautomers protonated at the terminal oxygens, similar to the product of eq 14, are of highest abundance in the equilibrium due to relative basicity.[50] In fact, the tautomer protonated at the terminal nitrogen, similar to eq 13, was predicted to be of such low basicity as to be nonexistent in the equilibrium. These analyses[48−50] suggest that the relative basicity of the atom bound to the $[N(O)NO]^-$ functionality compared to the terminal $[N(O)NO]^-$ nitrogen determines whether a pathway exists for HNO formation.

Our previous investigations showed that DEA/NO and Angeli's salt exhibited distinct cardiovascular properties in conscious dogs,[18,20] most profoundly with regard to contractility (inotropy), peripheral vascular dilation, and plasma signaling agent concentrations. For instance, Angeli's salt increased myocardial contractility and improved relaxation in a load-independent manner, while DEA/NO affected contractility in a load-sensitive fashion. Additionally, Angeli's salt stimulated an increase of plasma levels of CGRP, while cGMP was augmented in plasma by DEA/NO. Last, co-infusion of Angeli's salt with β-agonists such as dobutamine additively enhanced myocardial contractility, while DEA/NO dramatically blunted dobutamine-induced contractile improvement. These findings suggest that HNO donors may represent a novel class of compounds for treatment of cardiovascular conditions characterized by poor cardiac performance and pressure overload.

The analysis of the solution chemistry of IPA/NO compellingly indicates that this NONOate is both an HNO and NO donor under physiological conditions. Thus, we examined whether IPA/NO functions as a source of HNO, NO or both in vivo by comparing donor effects on contractility and plasma levels of CGRP and cGMP. The IPA/NO dose (5 μg/kg/min) used in the present study was titrated to achieve a similar decrease in end-systolic left ventricular pressure (LVESP) as with other agents (see Table 1).[20] In healthy, conscious dogs IPA/NO resulted in significant positive inotropic action, as shown by the rise in end-systolic elastance (Ees) and the slope of the relation between dP/dt_{max} and end-diastolic dimension (D_{EDD}) (Figure 6 and Table 1), which are both load-independent indexes of contractile function.[51] Comparison of the contractility parameters revealed that the effect of IPA/NO on normal cardiac contractility in vivo was similar to that previously observed with Angeli's salt and dissimilar to that reported with DEA/NO (Table 1 and Paolocci et al.[18]). Additionally, infusion of IPA/NO resulted in a prominent decrease in cardiac preload, as indicated by the substantial reduction in left ventricular end-diastolic dimension (LVEDD in Table 1), which is an index of venodilation.

Although Katori and colleagues[51] recently demonstrated that CGRP release per se cannot account for HNO inotropy in vivo, plasma levels of CGRP do function as a biomarker of HNO activity.[33] Plasma CGRP was elevated by both IPA/NO and Angeli's salt (Table 2), while systemic cGMP levels where not affected (Table 2). These results are in stark contrast to the increases in cGMP levels previously reported[33] for DEA/NO and nitroglycerin. These observations indicate that the pharmacological behavior of IPA/NO is analogous to that of Angeli's salt. It, therefore, appears that IPA/NO functions predominantly as an HNO donor in vivo, despite the production of NO in solution. It is not presently clear if the lack of NO effects from IPA/NO in vivo results from inhibition of the NO-releasing pathway or if the low yield of NO is simply below the pharmacological threshold.

Conclusion. Angeli's salt has proven to be an effective tool in the investigation of the chemical biology of HNO. However, the half-life of HNO release from Angeli's salt (2.5 min under biological conditions)[4]

Table 1. Cardiovascular Effects in Normal Dogs[a]

	percent change (%) in parameters compared to baseline values					
volume restoration	DEA/NO (2 μg/kg/min; $n = 5$)		IPA/NO (5 μg/kg/min; $n = 8$)		Angeli's salt (10 μg/kg/min; $n = 7$)	
	−	+	−	+	−	+
Ees (mmHg/mm)	33.9 ± 12.7†	−10.4 ± 12.5	58 ± 8.1*	32.1 ± 13.4†	35.4 ± 7.9†	21.35 ± 7.6†
LVEDD (mm)	−7.9 ± 1.4**	0.28 ± 1.1	−8.6 ± 0.5*	−0.13 ± 0.2	−1.9 ± 0.4‡	−0.22 ± 0.6
LVESP (mmHg)	−29.6 ± 4.4†	−21.0 ± 3.3†	−24.3 ± 3.9*	−14.9 ± 2.7*	−16.4 ± 5.2*	−12.8 ± 3.0*
RT (mmHg/mm/s)	−19.2 ± 6.3†	−29.3 ± 4.9**	−16.7 ± 4.7‡	−28.2 ± 2.1*	−8.5 ± 5.7†	−25.9 ± 4.2*

[a] Ees, end-systolic elastance (contractility); LVEDD, left ventricular end-diastolic dimension; LVESP, left ventricular end-systolic pressure; RT, total resistance; †, $p < 0.05$; ‡, $p < 0.01$; **, $p < 0.005$; *, $p < 0.001$ vs baseline. The doses of each NONOate were chosen so as to produce similar decreases in LVESP.

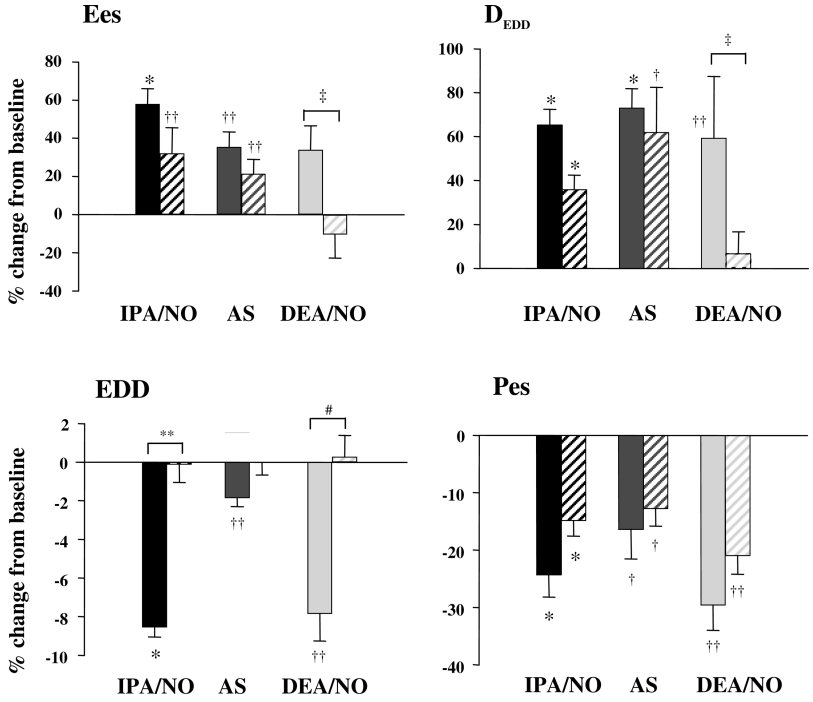

Figure 6. In vivo hemodynamic effects of IPA/NO, Angeli's salt, and DEA/NO in normal dogs. Studies were performed in the absence (solid bars) or presence (hatched bars) of volume repletion (10% Dextran 40 in 5% dextrose solution), as previously described.[20] The latter maneuver was performed to assess for reflex independence of HNO and NO effects by offsetting load decline with intravascular volume expansion. Data represent % changes from baseline and are shown as mean ± SEM. Angeli's salt, AS; Ees, end-systolic elastance; D_{EDD}, dP/dt, end-diastolic dimension relation; EDD, left ventricular end-diastolic dimension; Pes, left ventricular end-systolic pressure; ††, $p < 0.05$; †, $p < 0.01$; *, $p < 0.001$ vs baseline; ‡, $p < 0.05$; #, $p < 0.005$; **, $p < 0.001$ between before and after volume restoration.

Table 2. Circulating Plasma Levels of CGRP and cGMP in Normal Dogs[a]

plasma marker	donor	dose (μg/kg/min)	artery		vein	
			baseline	postinfusion	baseline	postinfusion
CGRP (pg/mL)	IPA/NO ($n = 7$)	5	30.3 ± 6.0	65.1 ± 2.8*	29.9 ± 6.7	52.0 ± 4.8**
	Angeli's salt ($n = 7$)	10	25.8 ± 1.5	52.3 ± 4.7*	27.8 ± 1.3	42.5 ± 1.3*
	DEA/NO ($n = 3$)	2	28.1 ± 3.2	30.6 ± 5.2	21.5 ± 1.5	22.0 ± 1.0
	nitroglycerin ($n = 4$)	10	29.4 ± 4.9	35.7 ± 7.0	26.3 ± 5.0	34.9 ± 12.3
cGMP (nM)	IPA/NO ($n = 4$)	5	26.1 ± 1.3	21.5 ± 2.3	30.7 ± 10.3	29.6 ± 5.0
	Angeli's salt ($n = 6$)	10	22.5 ± 8.5	20.2 ± 5.7	20.3 ± 8.1	23.2 ± 8.2

[a] * $p < 0.01$, ** $p < 0.05$ vs baseline. The doses of each NONOate were as in Table 1.

allows only acute properties of HNO to be examined. Additionally, the structure of this inorganic salt is not amenable to chemical modification without sacrificing the capacity to release HNO. Novel HNO donors will allow further elucidation of the biology of HNO and exploration of the therapeutic potential of HNO. The solution chemistry and cardiovascular properties of IPA/NO described here, including GSH-sensitive reductive nitrosylation, positive inotropy, and increased plasma levels of CGRP but not cGMP, suggest that this primary amine NONOate is both an HNO and NO donor under physiological conditions but effectively only an HNO donor in vivo. Whether the dual release of HNO and NO from IPA/NO is beneficial or even occurs in vivo remains to be determined. However, IPA/NO provides a novel lead compound in the design of organic-based donors of HNO for the treatment of heart failure as well as for other less studied applications. These include pharmacological preconditioning against reperfusion injury during myocardial infarct and stroke,[52,53] as well as inhibition of enzymes containing critical thiols, such as aldehyde dehydrogenase.[54] The decomposition mechanism proposed by Dutton et al.[49] suggests that any complicating factors of dual release of HNO and NO

from IPA/NO under physiological conditions can be surmounted by utilizing a primary amine of lower pK_b than isopropylamine.

Experimental Section

Abbreviations: Angeli's salt, sodium trioxodinitrate; cGMP, cyclic guanosine-3′,5′-monophosphate; CGRP, calcitonin gene-related peptide; DEA/NO, diethylamine NONOate; D_{EDD}, slope of the relation between dP/dt_{max} and end-diastolic dimension; DHR, dihydrorhodamine 123; DTPA, diethylenetriaminepentaacetic acid; Ees, end-systolic elastance; GSH, glutathione; HNO, nitroxyl; IPA/NO, isopropylamine NONOate; LVESP, end-systolic left ventricular pressure; MbNO, nitrosyl myoglobin; MbO_2, oxymyoglobin; metMb, ferric myoglobin; NANC, nonadrenergic/noncholinergic; NONOate, diazeniumdiolate; NO, nitric oxide; PBS, phosphate-buffered saline; RH, rhodamine 123; SOD, superoxide dismutase.

Chemicals. Angeli's salt ($Na_2N_2O_3$), DEA/NO [Na[Et_2NN(O)NO], sodium (*Z*)-1-(*N*,*N*-diethylamino)diazen-1-ium-1,2-diolate], and IPA/NO [Na[$(CH_3)_2$CHNH(N(O)NO], sodium 1-(*N*-isopropylamino)diazen-1-ium-1,2-diolate] were synthesized and utilized as previously described.[4,45] The purity of each batch of NONOate was confirmed spectrally from the extinction coefficients at 250 nm (ϵ = 8000 M^{-1} cm^{-1} for DEA/NO[4] or Angeli's salt[4] and 10 000 M^{-1} cm^{-1} for IPA/NO). The concentrations of stock solutions (>10 mM), prepared in 10 mM NaOH and stored at −20 °C, were also determined directly prior to use in 10 mM NaOH from the absorbance at 250 nm. The concentrations of DEA/NO utilized were half those for Angeli's salt, due to the release of 2 equiv of NO (eq 1).

Unless otherwise noted, chemicals were purchased from Sigma-Aldrich and were used without further purification. Stock solutions were prepared fresh daily at 100× in MilliQ or Barnstead Nanopure Diamond filtered H_2O unless specified. The assay buffer consisted of the metal chelator diethylenetriaminepentaacetic acid (DTPA, 50 μM) in calcium- and magnesium-free Dulbecco's phosphate-buffered saline (PBS, pH 7.4). Urate stock solutions were prepared at 10 mM in 50 mM NaOH and were prediluted in assay buffer, which was then brought to pH 7.4 with 6 M HCl. All reactions were performed at 37 °C except those measured with the NO-specific electrode, which were run at room temperature. Figures are representative data sets, each from $n \geq 2$ individual experiments.

Instrumentation. UV−visible spectroscopy was performed with a Hewlett-Packard 8453 diode-array spectrophotometer. Fluorescence measurements were acquired on a Perkin-Elmer LS50B fluorometer or a Thermo Spectronic Aminco Bowman Series2 luminescence spectrometer. Electrochemical detection was accomplished with a World Precision Instruments Apollo 4000 system equipped with NO-, O_2-, and H_2O_2-sensitive electrodes. Chemiluminescent detection of NO was obtained with a Sievers 280i NO analyzer (Ionics, Boulder, CO).

Fluorescence and Colorimetric Assays. Two-electron oxidation was evaluated by formation of the fluorescent dye RH from DHR (Molecular Probes, Eugene, OR) as described previously.[40] Briefly, donor (10 μL of 5 or 10 mM stock) was added to 1 mL of PBS (pH 7.4, 50 μM DTPA) containing DHR (2−100 μM), and the solution was immediately vortexed and incubated for 1 h at 37 °C. Subsequently, 1 mL of H_2O was added, and the fluorescence was measured at 530 nm with excitation at 500 nm. The concentrations of RH produced were determined from a standard curve of fluorescence from authentic RH. Double-reciprocal plots of the concentration of total RH produced vs initial DHR can provide the relative reactivity toward DHR of each oxidative intermediate.[40,41] Here, such plots were used for qualitative assessment only to compare the intermediates of IPA/NO to those of known HNO and NO donors.

Reductive nitrosylation of metMb (50 μM) to MbNO by IPA/NO (100 μM), Angeli's salt (100 μM), or DEA/NO (50 μM) was monitored directly in a quartz cuvette in assay buffer at 37 °C. The formation of HNO was further examined by quenching with GSH (250 μM).

MbO_2 [542 nm (13.9×10^3 M^{-1} cm^{-1}) and 580 nm (14.4×10^3 M^{-1} cm^{-1})][55] was prepared by aerobic reduction of metMb [408 nm (188×10^3 M^{-1} cm^{-1}), 502 (10.2×10^3 M^{-1} cm^{-1}), and 630 nm (3.9×10^3 M^{-1} cm^{-1})][55] with excess dithionite ($Na_2S_2O_4$), which was removed by passage through a Sephadex G-25 M column (PD-10; Pharmacia Biotech). Typically, MbO_2 (40 μM) was added to 1 mL of assay buffer containing quenching agent (2, 20, 200, or 2000 μM; adjusted to pH 7.4) as appropriate. Following addition of IPA/NO (20 μM), Angeli's salt (20 μM), DEA/NO (10 μM), or NaOH (10 mM, control), the solutions were incubated for 1 h at 37 °C, after which the conversion to metMb was monitored spectrophotometrically. Data were plotted as the difference in the averages (n = 3) of the absorbance values at 543 nm in control and donor exposed solutions.

Chemiluminescence and Electrochemical Assays. The relative yields of NO and HNO from DEA/NO, Angeli's salt, or IPA/NO were examined via chemiluminescence detection and with a NO-specific electrode. In the chemiluminescence assay, IPA/NO (5 nmol), Angeli's salt (5 nmol), or DEA/NO (2.5 nmol) was injected by Hamilton syringe into the argon-purged, temperature-controlled reaction vessel, which contained ∼6 mL of assay buffer maintained at 37 °C. Triplicate runs were performed in series following signal return to baseline. To compensate for the severalfold slower decay rate of IPA/NO compared to DEA/NO and Angeli's salt, the signal area was estimated by multiplying the maximum peak height (mV) by the peak width at half-maximum peak height (s). The specificity of the NO analyzer for NO was examined by addition of a large excess of GSH (1 mM) to the assay buffer, which was returned to pH 7.4 with 1 M NaOH. Despite the rapid purging of the reaction vessel, the signal from Angeli's salt was reduced under these conditions, indicating that HNO is also being detected by the instrument.

For the electrochemical assay, IPA/NO (5 μM), Angeli's salt (5 μM), or DEA/NO (2.5 μM) was injected into the reaction vessel after a baseline for the NO-specific electrode was established in room-temperature assay buffer (20 mL). After the signal reached a maximum and began to decline, the assay buffer was replaced and the process repeated to obtain triplicate maximum signal values. The production of HNO during decomposition of each donor was estimated by addition of the oxidant ferricyanide (1 mM) to the assay buffer.

Cardiovascular Protocol and Plasma Analysis. Adult male mongrel dogs (22−25 kg) were chronically instrumented for pressure-dimension hemodynamic analysis as previously described.[51,56] Animals were anesthetized with 1−2% halothane after induction with sodium thiopental (10−20 mg/kg). The surgical and experimental animal protocol was approved by the Johns Hopkins University Animal Care and Use Committee. Cardiovascular parameters were obtained as previously described.[18,51,56] Arterial, venous, and coronary sinus plasma cGMP was detected by enzyme immunoassay (Biotrak; Amersham Pharmacia), and CGRP was assessed by radioimmunoassay (Peninsula Labs; San Carlos, CA) using CGRP antiserum (RAS 6012) as specified by the manufacturer.

Cytotoxicity Assay. Cell culture was performed as previously described for the cytotoxicity determinations of Angeli's salt.[45] Cell survival of Chinese hamster V79 lung fibroblasts was assessed by clonogenic survival. The LD_{50} dose of IPA/NO was determined to be 2 mM, with no appreciable necrosis observed at 1 mM (data not shown), in direct correlation to Angeli's salt.[45] The dose of IPA/NO used for the cardiovascular studies (5 μg/kg/min) is >1000-fold lower than the cytotoxic level toward V79 cells. The total dose of IPA/NO administered (typically a 30 min infusion) was also far below the maximum tolerated dose in mice of 106 mg/kg.[57]

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