ANTIOXIDANTS & REDOX SIGNALING
Volume 14, Number 9, 2011
© Mary Ann Liebert, Inc.
DOI: 10.1089/ars.2010.3269

Chronic Administration of the HNO Donor Angeli's Salt Does Not Lead to Tolerance, Cross-Tolerance, or Endothelial Dysfunction: Comparison with GTN and DEA/NO

Jennifer C. Irvine,[1,2] Barbara K. Kemp-Harper,[1] and Robert E. Widdop[1]

Abstract

Nitroxyl (HNO) displays distinct pharmacology to its redox congener nitric oxide ($NO^{\bullet}$) with therapeutic potential in the treatment of heart failure. It remains unknown if HNO donors are resistant to tolerance development following chronic *in vivo* administration. Wistar–Kyoto rats received a 3-day subcutaneous infusion of one of the $NO^{\bullet}$ donors, glyceryl trinitrate (GTN) or diethylamine/NONOate (DEA/NO), or the HNO donor Angeli's salt (AS). GTN infusion (10 μg/kg/min) resulted in significantly blunted depressor responses to intravenous bolus doses of GTN, demonstrating tolerance development. By contrast, infusion with AS (20 μg/kg/min) or DEA/NO (2 μg/kg/min) did not alter their subsequent depressor responses. Similarly, *ex vivo* vasorelaxation responses in isolated aortae revealed that GTN infusion elicited a significant 6-fold decrease in the sensitivity to GTN and reduction in the maximum response to acetylcholine (ACh). Chronic infusion of AS or DEA/NO had no effect on subsequent vasorelaxation responses to themselves or to ACh. No functional cross-tolerance between nitrovasodilators was evident, either *in vivo* or *ex vivo*, although an impaired ability of a nitrovasodilator to increase tissue cGMP content was not necessarily indicative of a reduced functional response. In conclusion, HNO donors may represent novel therapies for cardiovascular disease with therapeutic potential over clinically used organic nitrates. *Antioxid. Redox Signal.* 14, 1615–1624.

Introduction

NITROXYL (HNO), the one electron reduced and protonated congener of nitric oxide ($NO^{\bullet}$), is rapidly emerging as a novel nitrogen oxide with distinct biological actions as compared with $NO^{\bullet}$ (18, 36). Thus, unlike $NO^{\bullet}$, HNO is able to increase myocardial contractility (via direct thiol interaction) (37, 38), increase plasma levels of calcitonin gene-related peptide (CGRP) (38), activate vascular voltage-dependent (K_v) (1, 13, 16) and ATP-sensitive (K_{ATP}) (12) K^+ channels, and is resistant to scavenging by superoxide (28). Such unique properties of HNO offer considerable advantages in the treatment of cardiovascular disease (18, 36).

Thus, the concomitant ability of HNO to serve as a positive cardiac inotrope (37, 38) and cause vasodilation (unload the heart) (11, 14, 17, 45) confers protection in the setting of acute experimental heart failure (36, 37, 40), where $NO^{\bullet}$ donors have minimal impact. Such beneficial effects of HNO arise as a consequence of its ability to directly target thiol residues on cardiac sarcoplasmic ryanodine receptors (RyR2) (42), sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) (21) and myofilaments (5) to enhance Ca^{2+} cycling and sensitize the contractile apparatus, respectively. HNO also serves as a vasodilator (1, 11–14, 16, 17, 45) and inhibits platelet aggregation (2), mediating such effects predominantly via activation of soluble guanylyl cyclase (sGC) (11, 16, 17, 45) and a subsequent increase in cGMP (14, 17). Together, these findings suggest that HNO donors may represent an alternative to traditional $NO^{\bullet}$ donors such as the organic nitrate, glyceryl trinitrate (GTN), in the treatment of congestive heart failure (36, 37, 40) and vascular disorders such as angina.

Importantly, in addition to the beneficial hemodynamic effects of HNO donors, these compounds may also be resistant to tolerance development (17), a major limitation of clinically used organic nitrates such as GTN (6). Nitrate tolerance is defined as a rapid attenuation of the hemodynamic and vasodilatory effects of nitrovasodilators following continuous exposure. It is a multifactorial process that may arise as a consequence of neurohumoral counter-regulation, reduced biotransformation of organic nitrates, impaired sGC

[1]Department of Pharmacology, Monash University, Clayton, Victoria, Australia.
[2]Heart Failure Pharmacology, Baker IDI Heart and Diabetes Institute, Melbourne, Victoria, Australia.

function, increased activity of cGMP degrading phosphodiesterases (PDEs), and increased production of the reactive oxygen species (ROS), superoxide (for review, see Refs. 6 and 34). Nitrate tolerance is not only associated with a loss in vasodilator potency but is also accompanied by endothelial dysfunction due, at least in part, to a ROS-mediated decrease in endogenous $NO^{\bullet}$ bioavailability (6, 34).

Given that HNO donors such as Angeli's salt (AS) do not require bioactivation to release HNO, and HNO is resistant to scavenging by superoxide (28), it may be anticipated that HNO donors do not develop tolerance. Certainly we have shown that short-term (1 h) treatment of rat isolated aortae with high concentrations of AS (10–100 μM) does not induce tachyphylaxis to the vasodilator actions of this compound (17). However, from a clinical perspective it is imperative to determine if chronic *in vivo* administration of a low, therapeutically relevant dose of an HNO donor leads to vascular tolerance both *in vivo* and *ex vivo*. Such information can not be extrapolated from isolated vessel studies, given the mechanisms underlying nitrate tolerance induced *in vivo* and *in vitro* have been shown to be distinct (32). Moreover, given the reported ability of AS to inhibit mitochondrial aldehyde dehydrogenase (ALDH-2) (8), an enzyme believed to be critically involved in the biotransformation of GTN to $NO^{\bullet}$ (3, 4), it is important to determine whether chronic *in vivo* administration of AS may induce cross-tolerance to GTN. Similarly, investigation of vasodilatory responses to AS in GTN-tolerant animals will provide insight into the potential use of HNO donors in patients displaying tolerance to organic nitrates.

This study sought to determine if chronic *in vivo* administration of the HNO donor, AS, leads to tolerance to its vasodilatory actions both *in vivo* and *ex vivo*, endothelial dysfunction, and cross-tolerance to the $NO^{\bullet}$ donor, GTN. Findings were compared to those obtained following chronic administration of GTN and the spontaneous $NO^{\bullet}$ donor DEA/NO, which releases $NO^{\bullet}$ with similar kinetics as the release of HNO from AS, was investigated in order to allow us to determine if tolerance development was specific for the redox form of NO released (*e.g.*, HNO *vs.* $NO^{\bullet}$) or the mode of generation (*e.g.*, biotransformation versus spontaneous release). Our results indicate that neither tolerance to the vasodilatory effects of AS nor endothelial dysfunction occurs following chronic *in vivo* exposure. These findings further strengthen the therapeutic potential of HNO as a novel class of nitrovasodilator for the treatment of disorders such as heart failure and angina.

Materials and Methods

Animals

This study was approved by the Pharmacology Animal Ethics Committee, Monash University, Australia, and conforms to the US National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1996). Male Wistar–Kyoto rats (16–17 weeks of age, 300–350 g, $n = 73$) were housed in standard rat cages at $20 \pm 3^{\circ}C$, with a 12-hour day/night cycle. Food and water was available *ad libitum*.

Rats were anesthetized with pentobarbitone, 60 mg/kg i.p., supplemented as required, and catheters inserted into the right carotid artery and right jugular vein for direct blood pressure measurement and drug administration, respectively. Each catheter was externalized through the back, in the neck region, and secured by a custom-made harness. Rats were thereafter housed individually. Approximately 24 h after surgery, the carotid artery was connected to a pressure transducer (Gould Inc.) attached to a MacLab-8 data acquisition system (ADInstruments). Mean arterial pressure (MAP) and heart rate (HR) were derived from the phasic blood pressure signal. All experiments were performed in conscious unrestrained rats.

Infusion with NAC

After a 24 h recovery period, a subset of rats was used to test the hypotensive effects of bolus intravenous administration of each of glyceryl trinitrate (GTN, 5-100 μg/kg), the HNO donor Angeli's salt (AS, 10–200 μg/kg), or the $NO^{\bullet}$ donor diethylamine NONOate (DEA/NO, 1–50 μg/kg) over the following three experimental days (Fig. 1a). From this preliminary dose-ranging, three doses were chosen for each nitrovasodilator that induced depressor responses of similar magnitude and that did not exceed a decrease in MAP of more than 50 mmHg. These doses were then used in an additional subset of instrumented rats in which a 3-point dose-response curve was constructed using one of these nitrovasodilators on any given day, prior to the rats receiving a 60 min infusion of the HNO scavenger *N*-acetyl-L-cysteine (NAC; 6.7 μmol/kg/min, i.v. (38)). The 3-point dose-response curve was then repeated in the presence of this infusion (Figs. 1b–1d).

Tolerance protocol

In vivo tolerance. In different groups of animals, a mini-osmotic Alzet pump (2ML1; 10 μl/h pump rate) was implanted into the flank of the animal during the catheterization procedure. GTN tolerance was induced via continuous infusion of GTN (10 μg/kg/min) over a 3-day period as previously described (19, 31). Chronic infusion rates of AS (20 μg/kg/min) and DEA/NO (2 μg/kg/min) were chosen in accordance with their depressor potency following acute intravenous administration relative to that of GTN (Fig. 1a). Specifically, 2 μg/kg DEA/NO and 20 μg/kg AS decreased mean arterial blood pressure (MAP) to a similar magnitude as 10 μg/kg GTN and hence chronic infusion rates were based on these potency differences. Rats received either GTN, AS, or DEA/NO, or their respective vehicles (GTN vehicle = propylene glycol, DEA/NO & AS vehicle = 0.1 mol/l NaOH) over the 3-day period. To investigate the possible development of tolerance to GTN, AS, or DEA/NO (and cross-tolerance between these drugs) at the end of this period, changes in MAP in response to intravenous bolus doses of GTN (5, 20, and 50 μg/kg), AS (10, 50, and 200 μg/kg), and DEA/NO (1, 5, and 20 μg/kg) were examined in each of these animals.

To verify that the solutions of AS and DEA/NO had retained their biological activity throughout their time in the osmotic minipumps, the 4-day-old solutions were removed from the osmotic minipumps of rats euthanized at the end of the treatment period and their activity tested in additional naïve rats, with comparisons made to the depressor activity of freshly constituted solutions. Moreover, to ensure that AS had retained its identity as an HNO donor throughout its time in the minipump, the depressor activity of the AS minipump solution was further assessed following a 60 min infusion of NAC.

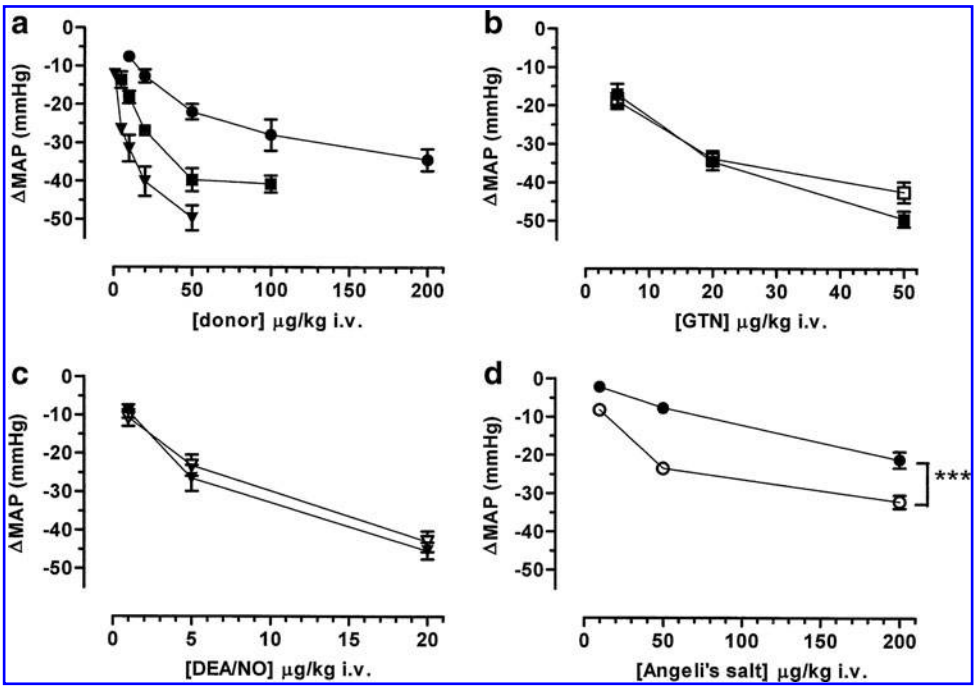

FIG. 1. NAC inhibits the dose-dependent vasodepressor effects of HNO. Dose-dependent depressor responses induced in conscious rats by the bolus intravenous (i.v.) administration of **(a)** GTN ($NO^{\bullet}$, ■), DEA/NO ($NO^{\bullet}$, ▼), or Angeli's salt (HNO, ●); **(b–d)** responses measured before (*open symbols*) or after (*closed symbols*) a 60 min infusion of NAC (6.7 μmol/kg/min). Values are expressed as the maximum change in mean arterial pressure (MAP) from baseline and are given as mean ± SEM, where $n = 6$–10 for all groups. $^{***}P < 0.0001$ for response in the presence of NAC *vs.* untreated control (2-way repeated measures ANOVA).

Tissue preparation and ex vivo *tolerance*

After 4 days of continuous infusions, the animals were killed via CO_2 and exsanguination. The entire aorta was excised and cleaned of connective tissue. 5 mm ring preparations were cut from the thoracic aorta, leaving the endothelium intact. The vessels were then mounted in 20 ml organ baths and isometric tension measured as described (17). Data was captured using the CVMS data acquisition system (World Precision Instruments, Sarasota, FL). Vessels were maintained in physiological Krebs' solution (composition in mmol/l: NaCl 119, KCl 4.7, $MgSO_4$ 1.17, $NaHCO_3$ 25, KH_2PO_4 1.18, $CaCl_2$ 2.5, glucose 11.1, and EDTA 0.026) at 37°C and bubbled continuously with carbogen (95% O_2, 5% CO_2). After a 30-min equilibrium period, vessels were stretched to an optimal passive tension of 2 g.

Functional experiments

Vessels were maximally contracted with a K^+-depolarizing solution (composition of KPSS in mmol/l: KCL 123, $MgSO_4$ 1.17, KH_2PO_4 2.37, $CaCl_2$ 2.5, glucose 11.1, and EDTA 0.026). Subsequent responses to vasorelaxants were examined in vessels precontracted to ~50% KPSS following a priming dose of U46619 (1 nmol/lL) and titrated concentrations of cirazoline (0.005–0.5 μmol/l) and cumulative concentration-response curves to either GTN (1 nmol/l to 10 μmol/l), AS (1 nmol/l to 10 μmol/l), DEA/NO (1 nmol/l to 10 μmol/l), or acetylcholine (ACh; 1 nmol/l to 10 μmol/l) were constructed. Maximal relaxation was obtained with isoprenaline (1 μmol/l) and only one concentration-response curve to any vasodilator was obtained for each vessel segment.

cGMP assay

In addition, four thoracic aortic rings were immediately dissected from each animal as described previously and placed in Eppendorf vials in Krebs' solution (1 ml), maintained at 37°C and bubbled continuously with carbogen. After a 30-min equilibration period, vessels were incubated alone or with either 30 μmol/l GTN, AS, or DEA/NO for 1 min prior to being snap-frozen in liquid nitrogen and stored at −80°C until cGMP analysis. Frozen tissues were crushed in ice-cold 6% tricholoroacetic acid, sonicated and centrifuged at 6000 rpm for 15 min. The supernatant was extracted 4X with saturated diethylether and air dried. cGMP analysis was performed using a RIA kit (Perkin Elmer) according to manufacturer's instructions and results expressed as fmol per mg tissue (wet weight).

Data and statistical analysis

All data are expressed as mean ± SEM. Statistical analysis was performed using a Student unpaired *t*-test, or by 1-way, 2-way, or 2-way with repeated measures analysis of variance (ANOVA) with Bonferroni corrections where appropriate (GraphPad Prism 4.0, La Jolla, CA). Depressor responses are expressed as the maximum change in MAP from baseline readings. Vasorelaxation responses are expressed as a percentage reversal of cirazoline precontraction. Individual relaxation curves were fitted to a sigmoidal logistic equation and pEC_{50} values (concentration of agonist giving a 50% relaxation) calculated and expressed as −logmol/l. $P < 0.05$ was accepted as statistically significant.

Drugs

Drugs and their sources were glyceryl trinitrate (50 mg/10ml, Mayne Pharma, Warwickshire, UK); Angeli's salt (sodium trioxodinitrate), U46619 [9,11-dideoxy-9α,11α-methanoepoxy-prosta-5Z,13E-dien-1-oic acid] (Sapphire Bioscience, Crows Nest, Australia); diethylamine NONOate [diethylammonium (Z)-1-(N,N-diethylamino)diazen-1-ium-1,2-diolate], cirazoline hydrochloride [2-[(2-cyclopropylphenoxy)methyl]-4,5-dihydro-1H-imidazole], *N*-acetyl-L-cysteine

Table 1. Basal Mean Arterial Pressures, Heart Rates, and Basal Aortic cGMP Content After 3–4 Day Continuous Infusion of Nitrovasodilator or Its Vehicle

Variable	*Vehicle (GTN)*	*GTN (10 μg/kg/min)*	*Vehicle (DEA/NO)*	*DEA/NO (2 μg/kg/min)*	*Vehicle (AS)*	*AS (20 μg/kg/min)*
MAP (mmHg)	127 ± 4	131 ± 5	132 ± 3	132 ± 4	130 ± 3	126 ± 1
HR (bpm)	311 ± 8	341 ± 11	312 ± 10	323 ± 13	309 ± 5	326 ± 7
cGMP (fmolmg^{-1} tissue)	12.6 ± 0.4	12.4 ± 2.3	16.5 ± 3.1	11.5 ± 1.4	18.1 ± 2.5	16.7 ± 4.4

Values are given as mean ± SEM. $n = 7$–9 per group.
AS, Angeli's salt; DEA/NO, diethylamine/NONOate; GTN, glyceryl trinitrate; HR, heart rate; MAP, mean arterial pressure.

(Sigma-Aldrich, St. Louis, MO). Stock solutions of AS and DEA/NO (10 mmol/l) were constituted in 0.01 mol/l NaOH, as were all subsequent dilutions. Stock solutions of U46619 (10 mmol/l) were made up in absolute ethanol (EtOH), and GTN (10 mmol/l) in 50% EtOH. All subsequent dilutions of stock solutions were in distilled water. All other drugs were made up in distilled water. All dilutions were prepared fresh daily. For infusion from osmotic pumps, glyceryl trinitrate (ADI Limited, Mulwala, NSW, Australia; 5% w/w solution in EtOH) was diluted in propylene glycol, and AS and DEA/NO were constituted in 0.1 mol/l NaOH.

Results

AS causes a dose-dependent depressor response in vivo

Both the NO$^{\bullet}$ donors GTN and DEA/NO and the HNO donor AS caused transient dose-dependent decreases in MAP in conscious rats (Fig. 1a). DEA/NO was the most potent depressor agent with a rank order of potency of DEA/NO > GTN > AS. In subsequent experiments, three doses of GTN (5, 20, and 50 μg/kg), AS (10, 50, and 200 μg/kg), and DEA/NO (1, 5, and 20 μg/kg) were used to produce equivalent decreases in MAP.

NAC discriminates between NO$^{\bullet}$ and HNO

NAC infusion (60 min) increased MAP on average by 15 ± 6 mmHg ($P < 0.001$; $n = 26$) with no significant change in HR (4 ± 9 bpm), as measured prior to the repeat administration of nitrovasodilator. Importantly, depressor responses to AS were significantly ($P < 0.0001$, Fig. 1d) attenuated following the infusion of the HNO scavenger NAC, yet the responses to GTN and DEA/NO were unchanged (Figs. 1b and 1c).

AS and DEA/NO retain their depressor effects following 4 days in minipumps

There was no change in the depressor response to a 5 μg/kg i.v. bolus dose of DEA/NO derived from the 4-day old minipump solutions (-24 ± 4 mmHg MAP; $n = 4$) compared to that obtained from freshly prepared DEA/NO (-21 ± 3 mmHg MAP; $n = 4$). Similarly, the depressor response to 50 μg/kg i.v. AS obtained from the minipump (-21 ± 3 mmHg MAP; $n = 4$) or freshly prepared AS (-21 ± 4 mmHg MAP; $n = 4$) did not differ. In additional experiments, minipump-derived solutions of AS (50 μg/kg i.v.; −25 ± 2 mmHg MAP; $n = 7$) were significantly attenuated following a 60 min infusion of NAC (-9 ± 2 mmHg MAP; $n = 7$; $P < 0.001$).

Tolerance development to GTN but not AS or DEA/NO in vivo

Following 3 days continuous infusion of either GTN, DEA/NO, or AS, baseline MAP and HR were unchanged as compared with vehicle-treated animals (Table 1). Pretreatment of animals with GTN (10 μg/kg/min, 3-day infusion via osmotic minipump) significantly ($P < 0.0001$) blunted the dose-dependent depressor response to GTN (Fig. 2a) such that a 50 μg/kg i.v. bolus dose of GTN decreased MAP by 9 ± 1 mmHg ($n = 9$) and 40 ± 2 mmHg ($n = 8$) in GTN- and vehicle-treated rats, respectively. In contrast, the depressor responses to AS in AS treated (3 days; 20 μg/kg/min) rats remained unchanged from those in vehicle treated rats (Fig. 2c). Likewise, responses to DEA/NO in DEA/NO treated (3 days; 2 μg/kg/min) rats were similar to those obtained in vehicle treated rats (Fig. 2b). In addition, there was no cross-tolerance evident between any of the nitrovasodilators (Figs. 2a–2c); for example, chronic GTN pretreatment did not alter the vasodepressor effect of AS.

Tolerance development to GTN but not AS or DEA/NO ex vivo

Ex vivo vasorelaxation responses to GTN in isolated aortae from GTN-treated rats showed a significant 6-fold ($P < 0.05$) decrease in sensitivity and 15% ($P < 0.01$) decrease in the response to 10 μmol/l GTN ($pEC_{50} = 6.72 \pm 0.22$; $R_{max} = 79.4$ 3.4%; Fig. 3a) as compared with vehicle-treated rats ($pEC_{50} = 7.51 \pm 0.14$; $R_{max} = 93.4 \pm 2.1\%$). This impaired vasorelaxation was associated with a reduced ability of GTN to elevate aortic cGMP content, such that GTN-induced cGMP accumulation was significantly ($P < 0.01$) attenuated in GTN-treated rats (86.5 ± 20.7 fmolmg^{-1} tissue) versus vehicle-treated (270.4 ± 53.1 fmolmg^{-1} tissue; Fig. 3a inset) rats. Chronic GTN infusion also resulted in a significant ($P < 0.01$) reduction in the maximum response to ACh (GTN treated: $R_{max} = 54.4 \pm 5.4\%$ *vs.* GTN vehicle: $R_{max} = 77.1 \pm 2.3\%$; Fig. 3b). In contrast, GTN treatment had no effect on vasorelaxations to either AS or DEA/NO (Figs. 3c and 3d). However, in the latter case, GTN treatment significantly reduced ($P < 0.01$) cGMP accumulation in response to an *in vitro* challenge with DEA/NO (781.0 ± 64.2 fmolmg^{-1} tissue) compared with vehicle-treated rats (1069.0 ± 63.1 fmolmg^{-1} tissue; Fig. 3d inset). However, AS-induced cGMP accumulation was not significantly reduced (GTN treated: 597.6 ± 74.0 *vs.* GTN vehicle: 624.3 ± 135.6 fmolmg^{-1} tissue; Fig. 3c inset).

Ex vivo vasorelaxation responses to GTN, ACh, AS, and DEA/NO in isolated aortae were unchanged following 4-day AS treatment (Fig. 4a–4d). Furthermore, AS-induced cGMP

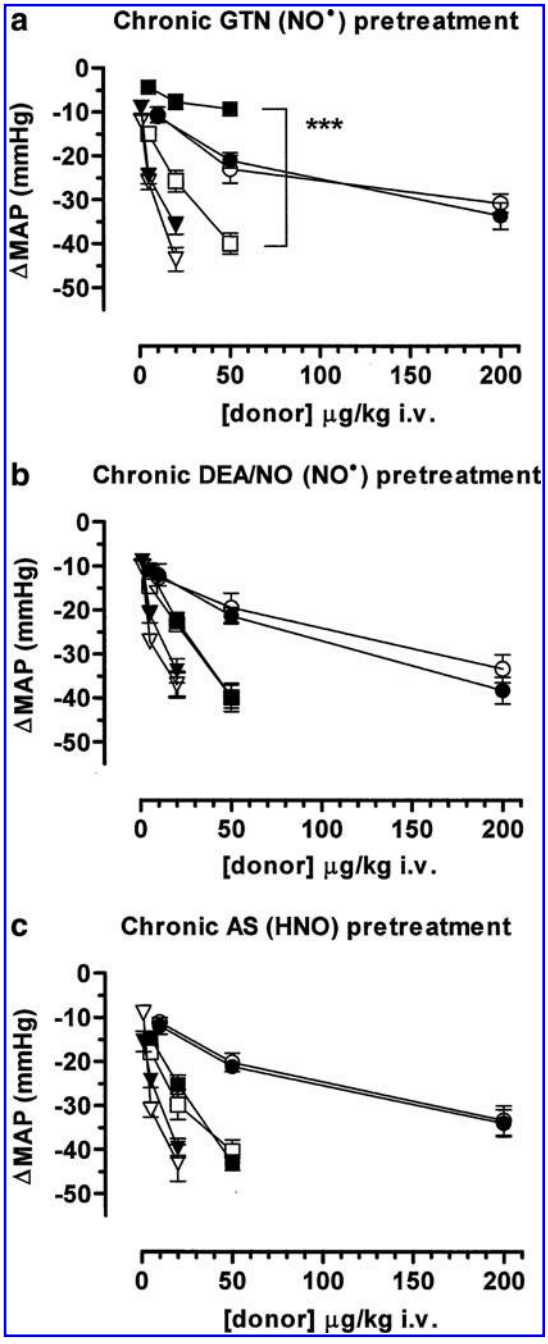

FIG. 2. Tolerance development to GTN but not Angeli's salt or DEA/NO *in vivo*. Depressor responses induced in conscious rats by the bolus intravenous administration of GTN (□,■), DEA/NO (▽,▼), or Angeli's salt (○,●) after a 3-day subcutaneous infusion via osmotic minipump of either **(a)** GTN vehicle (*open symbols*) or GTN (10 μg/kg/min; *closed symbols*), **(b)** DEA/NO vehicle (*open symbols*) or DEA/NO (2 μg/kg/min; *closed symbols*) or **(c)** Angeli's salt (AS) vehicle (*open symbols*) or AS (20 μg/kg/min; *closed symbols*). Values are expressed as the maximum change in mean arterial pressure (MAP) from baseline and are given as mean ± SEM, where $n = 7$–9 for all groups. ***$P < 0.0001$ for responses in GTN pretreated *vs.* vehicle treated control (2-way ANOVA).

accumulation was not significantly reduced following AS treatment (Fig. 4c inset). Interestingly however, cGMP accumulation in response to an *in vitro* challenge with GTN was significantly ($P < 0.05$; Fig. 4a inset) attenuated in the AS-treated animals, yet DEA/NO-induced cGMP accumulation remained unchanged (Fig. 4d inset).

Similarly, 4-day treatment with DEA/NO had no effect on subsequent vasorelaxations to GTN, ACh, AS, and DEA/NO in isolated aortae (Figs. 5a–5d), yet cGMP accumulation was significantly attenuated in response to *in vitro* challenges with DEA/NO ($P < 0.05$; Fig. 5d inset), GTN ($P < 0.01$; Fig. 5a inset), and AS ($P < 0.01$; Fig. 5c inset).

Basal levels of aortic cGMP content remained unchanged following 4-day treatment with either GTN, AS, or DEA/NO, compared to their respective vehicle controls (Table 1). It is interesting to note that the magnitude of cGMP accumulation differed substantially between the nitrovasodilators such that DEA/NO > AS > GTN.

Discussion

This study has demonstrated for the first time that chronic *in vivo* treatment with a low, therapeutically relevant dose of the HNO donor AS does not lead to the development of tolerance. Specifically, we found that chronic AS infusion had no effect either on subsequent *in vivo* vasodepressor responses to AS or to AS-induced vasorelaxations *ex vivo*. Furthermore, chronic administration of AS did not induce cross-tolerance to GTN or DEA/NO or cause endothelial dysfunction. Importantly, with regards to its clinical usefulness, the vasodilator capacity of AS both *in vivo* and *ex vivo* was preserved in animals tolerant to GTN.

AS caused transient dose-dependent decreases in blood pressure, akin to those induced by the NO• donors GTN and DEA/NO. We found the rank order of potency of these donors *in vivo* to be DEA/NO > GTN > AS, which is comparable to the study of Miranda and co-workers who found 2 μg/kg/min DEA/NO decreased left ventricular end-systolic pressure in dogs to a similar level as 10 μg/kg/min AS (26). Of note, such potency differences between the nitrovasodilators studied are reflective of the parent compounds rather than the species donated (*i.e.,* NO• or HNO) as DEA/NO decomposes to generate two equivalents of NO•, whilst AS generates one equivalent of HNO. Our results are the first demonstration of a blood pressure-lowering effect of HNO in conscious rats and are consistent with studies in anesthetized rabbits (23) and cats (7), and conscious dogs (37, 38).

HNO and NO• donors can be distinguished both *in vivo* and *in vitro* via their sensitivity to thiols. Specifically, thiols attenuate the actions of HNO, yet enhance and prolong the activity of NO• (11, 16, 17, 22, 38, 42, 45, 48). Accordingly, we found the depressor response to AS to be attenuated in the presence of the thiol NAC, yet hemodynamic responses to GTN and DEA/NO remained unchanged. These findings are in agreement with previous studies in which NAC has been shown to selectively inhibit AS-mediated vasodilatory responses in the isolated perfused heart (12), inotropic effects in conscious dogs (38), and protective preconditioning actions during myocardial ischaemia/reperfusion (35), and suggest that the *in vivo* vasodepressor response to AS was mediated by HNO. Interestingly, the observation that NAC infusion caused a significant increase in baseline MAP may suggest

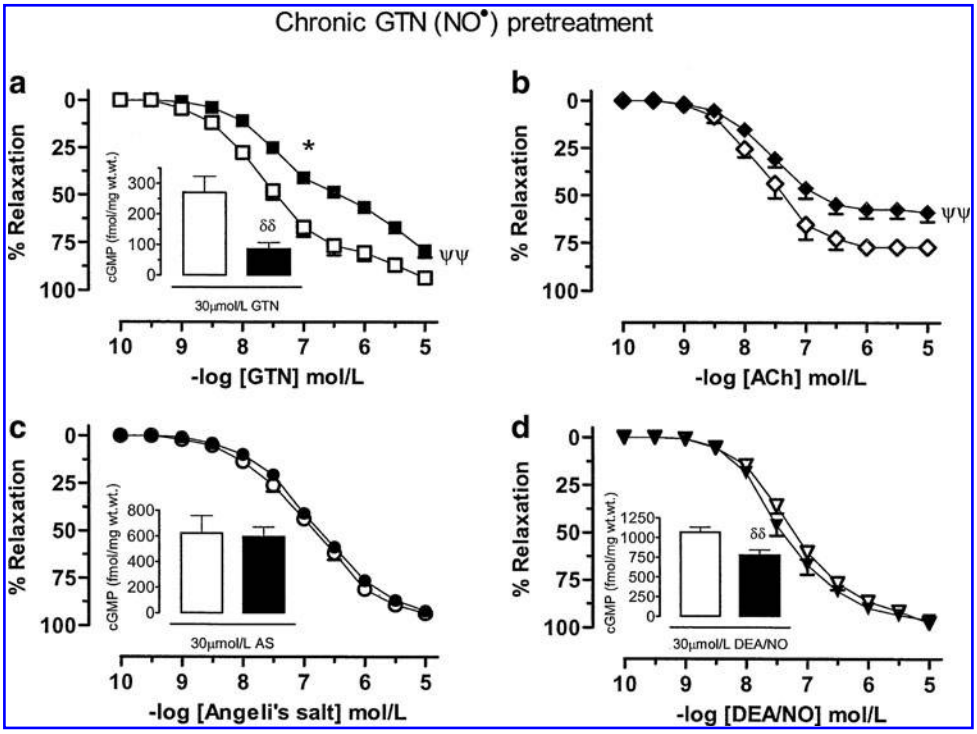

FIG. 3. Tolerance development to GTN *ex vivo*. Concentration-dependent vasorelaxation responses to **(a)** GTN, **(b)** ACh, **(c)** Angeli's salt, and **(d)** DEA/NO in isolated aortae from rats having received a 4-day subcutaneous infusion via osmotic minipump of either GTN vehicle (*open symbols*; n = 5-7) or GTN (10 μg/kg/min; *closed symbols*; $n = 6$–8). Values are expressed as percentage reversal of pre-contraction and given as mean ± SEM, where $n =$ number of vessel segments. **Insets**: cGMP accumulation in response to 30 μmol/l (1 min) **(a)** GTN, **(c)** Angeli's salt (AS), and **(d)** DEA/NO in aortae isolated from rats treated for 4 days with either GTN vehicle (*open bars*; $n = 7$–9) or GTN (10 μg/kg/min; *closed bars*; $n = 7$–9). * $P < 0.05$ for pEC_{50} value *vs.* vehicle control (unpaired *t*-test). $^{\psi\psi}$ $P < 0.01$ for response at 10 μmol/l *vs.* vehicle control (unpaired *t*-test). $^{\delta\delta}P < 0.01$ *vs.* vehicle (unpaired *t*-test).

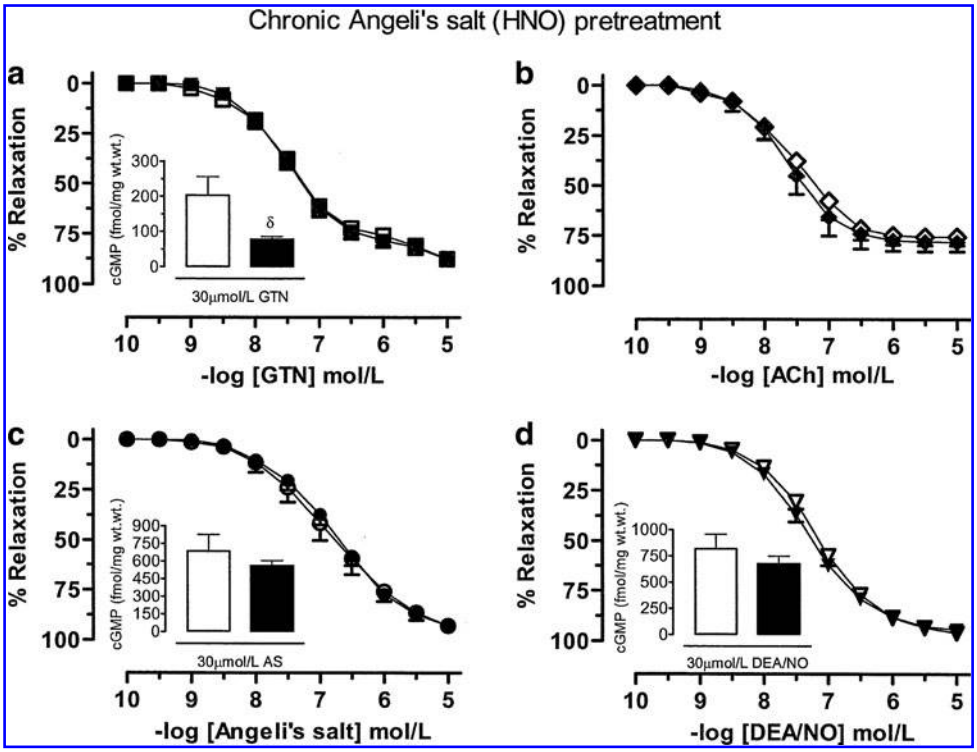

FIG. 4. Angeli's salt does not develop tolerance *ex vivo*. Concentration-dependent vasorelaxation responses to **(a)** GTN, **(b)** ACh, **(c)** Angeli's salt, and **(d)** DEA/NO in isolated aortae from rats having received a 4-day subcutaneous infusion via osmotic minipump of either Angeli's salt vehicle (op*en symbols*; n = 5–6) or Angeli's salt (20 μg/kg/min; *closed symbols*; $n = 6$–8). Values are expressed as percentage reversal of pre-contraction and given as mean ± SEM, where $n =$ number of vessel segments. **Insets**: cGMP accumulation in response to 30 μmol/l (1 min) **(a)** GTN, **(c)** Angeli's salt (AS), and **(d)** DEA/NO in aortae isolated from rats treated for 4 days with either Angeli's salt vehicle (*open bars*; n = 7–9) or Angeli's salt (20 μg/kg/min; *closed bars*; $n = 7$–9). $^{\delta}P < 0.05$ *vs.* vehicle (unpaired *t*-test).

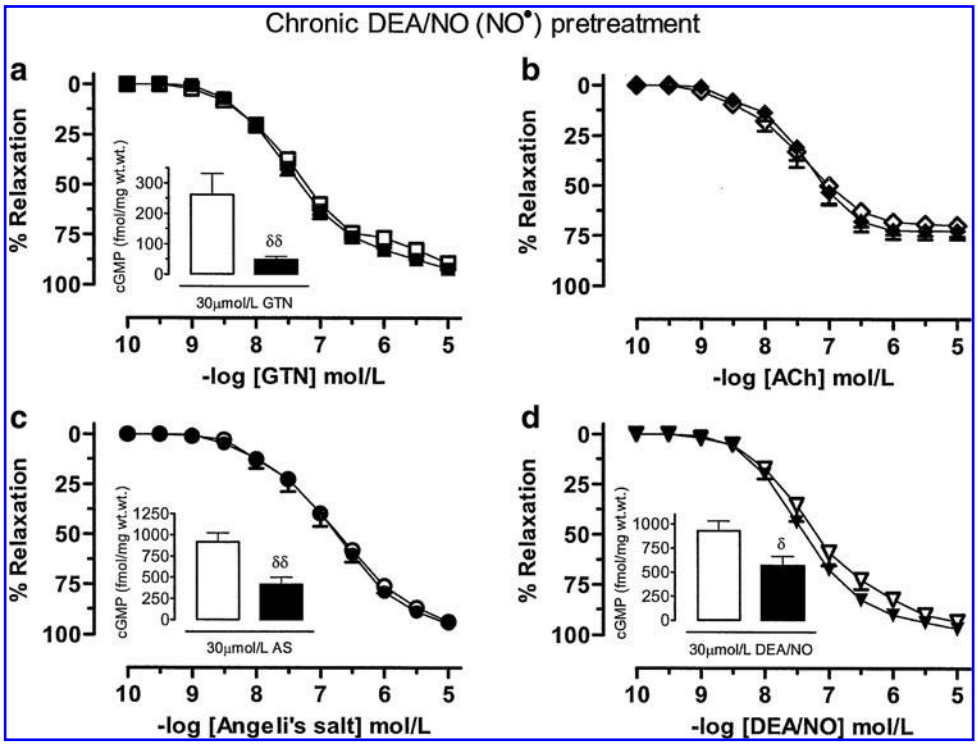

FIG. 5. DEA/NO does not develop tolerance *ex vivo*. Concentration-dependent vasorelaxation responses to **(a)** GTN, **(b)** ACh, **(c)** Angeli's salt, and **(d)** DEA/NO in isolated aortae from rats having received a 4-day subcutaneous infusion via osmotic minipump of either DEA/NO vehicle (*open symbols*; n = 5–6) or DEA/NO (2 μg/kg/min; *closed symbols*; n = 7–9). Values are expressed as percentage reversal of precontraction and given as mean ± SEM, where n = number of vessel segments. **Insets**: cGMP accumulation in response to 30 μmol/l (1 min) **(a)** GTN, **(c)** Angeli's salt (AS), and **(d)** DEA/NO in aortae isolated from rats treated for 4 days with either DEA/NO vehicle (*open bars*; n = 7–9) or DEA/NO (20 μg/kg/min; *closed bars*; n = 7–9). $^{\delta}P < 0.05$, $^{\delta\delta}P < 0.01$ *vs.* vehicle (unpaired *t*-test).

that putative endogenous HNO (1) contributes to blood pressure regulation. Moreover, whilst AS decomposes to produce both HNO and nitrite, it is unlikely that the vasodepressor response is attributable to nitrite since the latter is 15,000-fold less potent than AS (16). Similarly, nitrite was reported to lower blood pressure in rats but only at very high concentrations (0.3–1.0 g/kg body weight) (43).

Using a well-established model of *in vivo* nitrate tolerance (19, 31, 33, 46), we clearly demonstrated tolerance to GTN, such that its depressor response in the intact animal, as well as *ex vivo* vasorelaxation and cGMP accumulation in isolated aortae, were markedly impaired. In agreement with previous studies (33, 41), chronic GTN treatment also induced endothelial dysfunction as evidenced by slightly impaired vasorelaxation to the endothelium-dependent vasodilator, ACh. Such dysfunction has previously been attributed to reduced endogenous NO• bioavailability as a consequence of increased oxidative stress (41) and/or nitric oxide synthase (NOS) uncoupling (15).

In contrast, the HNO donor AS did not develop tolerance either *in vivo* or *ex vivo*. Specifically, following chronic administration, AS maintained its ability to induce dose-dependent depressor responses in the intact animal, elicit vasorelaxation, and elevate cGMP in isolated aortae. Moreover AS treatment did not lead to endothelial dysfunction, with ACh-mediated vasorelaxation preserved. These findings support and extend our previous observations that AS does not develop tachyphylaxis in the isolated vasculature (17). Further, the vasodilatory efficacy of AS, both *in vivo* and *ex vivo*, was preserved in GTN-tolerant animals. Thus, the lack of tolerance of AS and cross-tolerance with GTN suggests that HNO donors may represent an alternative to traditional nitrovasodilators and be of use in patients tolerant to organic nitrates.

The susceptibility of the spontaneous NO• donor, DEA/NO, to tolerance development was also studied in order to determine if tolerance was dependent upon the redox form of NO donated (*i.e.,* NO• *vs.* HNO) or the mode of release (*i.e.,* spontaneous release *vs.* biotransformation). Like AS, DEA/NO was resistant to the development of self-tolerance both *in vivo* and *ex vivo* following chronic administration. The lack of tolerance development to AS and DEA/NO did not reflect inadequate drug administration nor was there a time-dependent decrease in the biological activity of each nitrovasodilator whilst in the minipump, since the residual solutions of AS and DEA/NO obtained from minipumps after 4 days of continuous use elicited vasodepressor responses in naïve rats of similar magnitude to freshly constituted solutions. Furthermore, it is unlikely that over the 4-day infusion period AS decomposed to generate nitrite as depressor responses to AS removed from the minipump retained their sensitivity to NAC. Last, chronic infusions of both AS and DEA/NO influenced the ability of nitrovasodilators to increase acutely aortic cGMP content (see later) providing further evidence for their sustained bioactivity during chronic infusion.

Taken together, our findings suggest that tolerance development is not dependent upon the redox form of NO donated by a nitrovasodilator, given both the HNO (AS) and NO• (DEA/NO) donors were resistant, yet the NO• donor GTN was susceptible to tolerance development. Rather, the resistance of AS and DEA/NO to tolerance development may be due to their ability to spontaneously donate HNO and NO• respectively, versus GTN which requires biotransformation to generate NO• (3, 41). Certainly there is an increasing body of evidence that impaired biotransformation of GTN by mitochondrial ALDH-2 (4) contributes to nitrate tolerance (3, 41). However, tolerance is a complex process and multiple

mechanisms may underlie the phenomenon, including neurohumoral counter-regulation, impaired sGC function, increased activity of PDEs, and increased production of superoxide (6, 34). Previous findings that *in vivo* GTN treatment does not cause cross-tolerance to nitrovasodilators such as DEA/NO *ex vivo* (24, 47), coupled with our observation that both AS and DEA/NO retained their vasodilatory efficacy and, for the most part, ability to elevate vascular cGMP in GTN-tolerant rats indicates that neither impaired sGC/cGMP/cGMP-dependent protein kinase (cGK-I) signaling nor increased PDE activity alone accounts for organic nitrate tolerance.

Although chronic infusion of AS and DEA/NO did not lead to self-tolerance, we assessed their ability to induce cross-tolerance to GTN given they have been shown to inhibit ALDH-2 with IC_{50}s in the micromolar range (8, 9). Importantly, chronic infusion with either AS or DEA/NO did not translate to a functional change in the response to GTN, such that its vasodepressor and vasorelaxant effects were preserved. Such findings lend support to the idea that HNO donors may be co-administered with GTN, thereby facilitating the use of lower doses of organic nitrates and minimizing the potential for tolerance development.

Another novel aspect of the present study relates to the changes in cGMP production when assessing tolerance development. We clearly showed that aortae from GTN-tolerant rats had a significantly reduced ability to increase cGMP content in response to an acute *in vitro* challenge with GTN, which is consistent with previous *in vivo* studies in mice (44) and rats (19). This finding appears to correlate with the attenuated depressor response and reduced vasorelaxant effect of GTN in GTN-tolerant rats and upon initial consideration, concurs with the conclusion of Dikalov and colleagues (10) that nitrate-induced effects on cGMP content can be used as a marker of tolerance development. However, in the present study, we report several instances of mismatch between tolerance and cGMP production that show an impaired ability of a nitrovasodilator to increase cGMP may not be necessarily indicative of an impaired functional response.

Thus, chronic infusion of either GTN, AS, or DEA/NO attenuated the subsequent ability of one or more of the nitrovasodilators to elevate cGMP acutely in isolated aortae, despite their sustained *in vivo* depressor and *ex vivo* vasorelaxation responses. The preservation of functional responses in the face of cGMP reduction is perhaps not so surprising given the recent evidence that only very low amounts of cGMP are needed for a full biological response to sGC activators (25). Certainly in the present study, DEA/NO, AS, and GTN displayed similar vasodilator potencies, yet DEA/NO elevated cGMP content to a level 2- and 4-fold higher than that for AS and GTN, respectively. Furthermore, a recent study showed that maximum relaxation to GTN occurred at cGMP levels of only 3.4% of the maximal levels obtained with 100 μmol/l DEA/NO (20). Alternatively, such findings may be indicative of an ability of $NO^{\bullet}$ and HNO to target sGC/cGMP-independent signaling pathways. Indeed we have provided evidence that the sensory neuropeptide, CGRP, contributes to HNO-mediated vasorelaxation of the coronary vasculature (12). Moreover, HNO has been shown to target both K_v (1, 13, 16) and K_{ATP} (12) channels in the resistance vasculature, albeit via an apparent cGMP-dependent mechanism (13). $NO^{\bullet}$ can also directly activate K_{Ca} channels in resistance arteries (39). Thus, cGMP-independent vasodilatory mechanisms may serve to compensate for the reduced capacity of $NO^{\bullet}$ and HNO to elevate cGMP.

The mechanisms underlying the observed mismatch between cGMP accumulation and functional response remain to be elucidated but may involve changes at the level of ALDH-2 (8), sGC expression (46), and/or downstream targets of cGMP, such as cGK-I (31, 46) and the cGMP metabolizing enzyme phosphodiesterase 5 (PDE5) (30), all of which may be differentially affected by nitrovasodilators. Future studies in our laboratory will address these salient points. Importantly, however, our findings clearly indicate that measurement of cGMP alone is not a sensitive indicator of vascular tolerance.

From a clinical perspective, the findings of this study have significant impact. First and foremost, the unique hemodynamic actions of HNO (*i.e.*, positive cardiac inotrope) coupled with its resistance to tolerance development, indicate that HNO donors may offer a superior alternative to currently used organic nitrates in the treatment of heart failure and possibly other vascular disorders such as angina. In addition, we hypothesize that the cardio- and vasoprotective actions of HNO may be preserved under disease conditions where those to $NO^{\bullet}$ are compromised (*i.e.,* during oxidative stress). Thus, unlike $NO^{\bullet}$, HNO is (i) resistant to scavenging by superoxide (29), (ii) can target distinct signaling pathways in the myocardium (RyR2, SERCA2a) (5, 42) and vasculature (K_v, K_{ATP}, CGRP) (12, 16), in part via its ability to modify critical cysteine residues, (iii) preferentially activates oxidized, ferric hemeproteins (27), and (iv) its bioavailability may be augmented in the face of disease-associated thiol depletion (18). Whilst this concept remains to be fully explored, it appears that the vasodepressor actions of HNO are preserved in an experimental model of heart failure (37). Importantly we also have evidence to suggest that the lack of tolerance to HNO donors persists in the setting of hypercholesterolemia, a disease state associated with elevated vascular superoxide and impaired endogenous $NO^{\bullet}$ bioavailability (Bullen, Kemp–Harper *et al.,* unpublished). Taken together these findings suggest that the clinical efficacy of HNO donors may be maintained under disease conditions associated with oxidative stress.

In conclusion, this study has clearly demonstrated that neither self-tolerance, cross-tolerance to other nitrovasodilators, nor endothelial dysfunction develops following chronic *in vivo* treatment with an HNO donor. As such, HNO donors with their distinct pharmacology and lack of tolerance development may offer considerable therapeutic advantages over traditional nitrovasodilators in the treatment of cardiovascular disorders.

Acknowledgments

JCI was supported by an Australian Postgraduate Award and a Monash University Postgraduate Publication Award. BKK-H was the recipient of a Foundation for High Blood Pressure Research Postdoctoral Fellowship (Australia). BKK-H and REW are supported by grants from the National Health and Medical Research Council (Australia) and Heart Foundation (Australia).

We gratefully acknowledge the excellent technical assistance of Ms Iresha Welungoda.

Author Disclosure Statement

No competing financial interests exist.

References

1. Andrews KL, Irvine JC, Tare M, Apostolopoulos J, Favaloro JL, Triggle CR, and Kemp–Harper BK. A role for nitroxyl (HNO) as an endothelium-derived relaxing and hyperpolarizing factor in resistance arteries. *Br J Pharmacol* 157: 540–550, 2009.
2. Bermejo E, Saenz DA, Alberto F, Rosenstein RE, Bari SE, and Lazzari MA. Effect of nitroxyl on human platelets function. *Thromb Haemost* 94: 578–584, 2005.
3. Chen Z, Foster MW, Zhang J, Mao L, Rockman HA, Kawamoto T, Kitagawa K, Nakayama KI, Hess DT, and Stamler JS. An essential role for mitochondrial aldehyde dehydrogenase in nitroglycerin bioactivation. *Proc Natl Acad Sci USA* 102: 12159–12164, 2005.
4. Chen Z, Zhang J, and Stamler JS. Identification of the enzymatic mechanism of nitroglycerin bioactivation. *Proc Natl Acad Sci USA* 99: 8306–8311, 2002.
5. Dai T, Tian Y, Tocchetti CG, Katori T, Murphy AM, Kass DA, Paolocci N, and Gao WD. Nitroxyl increases force development in rat cardiac muscle. *J Physiol* 580: 951–960, 2007.
6. Daiber A, Wenzel P, Oelze M, and Münzel T. New insights into bioactivation of organic nitrates, nitrate tolerance and cross-tolerance. *Clin Res Cardiol* 97: 12–20, 2008.
7. De Witt BJ, Marrone JR, Kaye AD, Keefer LK, and Kadowitz PJ. Comparison of responses to novel nitric oxide donors in the feline pulmonary vascular bed. *Eur J Pharmacol* 430: 311–315, 2001.
8. DeMaster EG, Redfern B, and Nagasawa HT. Mechanisms of inhibition of aldehyde dehydrogenase by nitroxyl, the active metabolite of the alcohol deterrent agent cyanamide. *Biochem Pharmacol* 55: 2007–2015, 1998.
9. DeMaster EG, Refern B, Quast BJ, Dahlseid T, and Nagasawa HT. Mechanism for the inhibition of aldehyde dehydrogenase by nitric oxide. *Alcohol* 14: 181–189, 1997.
10. Dikalov S, Fink B, Skatchkov M, Stalleicken D, and Bassenge E. Formation of reactive oxygen species by pentaerithrityltetranitrate and glyceryl trinitrate *in vivo* and development of nitrate tolerance. *J Pharmacol Exp Ther* 286: 938–944, 1998.
11. Ellis A, Li CG, and Rand MJ. Differential actions of L-cysteine on responses to nitric oxide, nitroxyl anions and EDRF in the rat aorta. *Br J Pharmacol* 129: 315–322, 2000.
12. Favaloro JL and Kemp–Harper BK. The nitroxyl anion (HNO) is a potent dilator of rat coronary vasculature. *Cardiovasc Res* 73: 587–596, 2007.
13. Favaloro JL and Kemp–Harper BK. Redox variants of NO (NO and HNO) elicit vasorelaxation of resistance arteries via distinct mechanisms. *Am J Physiol Heart Circ Physiol* 296: H1274–1280, 2009.
14. Fukuto JM, Chiang K, Hszieh R, Wong PSY, and Chaudhurri G. The pharmacological activity of nitroxyl: A potent vasodilator with activity similar to nitric oxide and/or endothelium-derived relaxing factor. *J Pharmacol Exp Ther* 263: 546–551, 1992.
15. Gori T, Mak SS, Kelly S, and Parker JD. Evidence supporting abnormalities in nitric oxide synthase function induced by nitroglycerin in humans. *J Am Coll Cardiol* 38: 1096–1101, 2001.
16. Irvine JC, Favaloro JL, and Kemp–Harper BK. NO^- activates soluble guanylate cyclase and K_v channels to vasodilate resistance arteries. *Hypertension* 41: 1301–1307, 2003.
17. Irvine JC, Favaloro JL, Widdop RE, and Kemp–Harper BK. Nitroxyl anion donor, Angeli's salt, does not develop tolerance in rat isolated aortae. *Hypertension* 49: 885–892, 2007.
18. Irvine JC, Ritchie RH, Favaloro JL, Andrews KL, Widdop RE, and Kemp–Harper BK. Nitroxyl (HNO): The Cinderella of the nitric oxide story. *Trends Pharmacol Sci* 29: 601–608, 2008.
19. Kim D, Rybalkin SD, Pi X, Wang Y, Zhang C, Munzel T, Beavo JA, Berk BC, and Yan C. Upregulation of phosphodiesterase 1A1 expression is associated with the development of nitrate tolerance. *Circulation* 104: 2338–2343, 2001.
20. Kollau A, Hofer A, Russwurm M, Koesling D, Keung WM, Schmidt K, Brunner F, and Mayer B. Contribution of aldehyde dehydrogenase to mitochondrial bioactivation of nitroglycerin: Evidence for the activation of purified soluble guanylate cyclase through direct formation of nitric oxide. *Biochem J* 385: 769–777, 2005.
21. Lancel S, Zhang J, Evangelista A, Trucillo MP, Tong X, Siwik DA, Cohen RA, and Colucci WS. Nitroxyl activates SERCA in cardiac myocytes via glutathiolation of cysteine 674. *Circ Res* 104: 720–723, 2009.
22. Laursen JB, Boesgaard S, Poulsen HE, and Aldershvile J. Nitrate tolerance impairs nitric oxide-mediated vasodilation *in vivo*. *Cardiovasc Res* 31: 814–819, 1996.
23. Ma XL, Gao F, Liu G–L, Lopez BL, Christopher TA, Fukuto JM, Wink DA, and Feelisch M. Opposite effects of nitric oxide and nitroxyl on postischemic myocardial injury. *Proc Natl Acad Sci USA* 96: 14617–14622, 1999.
24. MacPherson JD, Gillespie TD, Dunkerley HA, Maurice DH, and Bennett BM. Inhibition of phosphodiesterase 5 selectively reverses nitrate tolerance in the venous circulation. *J Pharmacol Exp Ther* 317: 188–195, 2006.
25. Mergia E, Friebe A, Dangel O, Russwurm M, and Koesling D. Spare guanylyl cyclase NO receptors ensure high NO sensitivity in the vascular system. *J Clin Invest* 116: 1731–1737, 2006.
26. Miranda KM, Katori T, Torres de Holding CL, Thomas L, Ridnour LA, McLendon WJ, Cologna SM, Dutton AS, Champion HC, Mancardi D, Tocchetti CG, Saavedra JE, Keefer LK, Houk KN, Fukuto JM, Kass DA, Paolocci N, and Wink DA. Comparison of the NO and HNO donating properties of diazeniumdiolates: Primary amine adducts release HNO *in vivo*. *J Med Chem* 48: 8220–8228, 2005.
27. Miranda KM, Nims RW, Thomas DD, Espey MG, Citrin D, Bartberger MD, Paolocci N, Fukuto JM, Feelisch M, and Wink DA. Comparison of the reactivity of nitric oxide and nitroxyl with heme proteins. A chemical discussion of the differential biological effects of these redox related products of NOS. *J Inorg Biochem* 93: 52–60, 2003.
28. Miranda KM, Paolocci N, Katori T, Thomas DD, Ford E, Bartberger MD, Espey MG, Kass DA, Feelisch M, Fukuto JM, and Wink DA. A biochemical rationale for the discrete behavior of nitroxyl and nitric oxide in the cardiovascular system. *Proc Natl Acad Sci USA* 100: 9196–9201, 2003.
29. Miranda KM, Yamada K, Espey MG, Thomas DD, DeGraff WG, Mitchell JB, Krishna MC, Colton CA, and Wink DA. Further evidence for distinct reactive intermediates from nitroxyl and peroxynitrite: Effects of buffer composition on the chemistry of Angeli's salt and synthetic peroxynitrite. *Arch Biochem Biophys* 401: 134–144, 2002.
30. Mullershausen F, Lange A, Mergia E, Friebe A, and Koesling D. Desensitization of NO/cGMP signaling in smooth muscle: Blood vessels versus airways. *Mol Pharmacol* 69: 1969–1974, 2006.

31. Mulsch A, Oelze M, Kloss S, Mollnau H, Topfer A, Smolenski A, Walter U, Stasch JP, Warnholtz A, Hink U, Meinertz T, and Munzel T. Effects of *in vivo* nitroglycerin treatment on activity and expression of the guanylyl cyclase and cGMP-dependent protein kinase and their downstream target vasodilator-stimulated phosphoprotein in aorta. *Circulation* 103: 2188–2194, 2001.
32. Munzel T, Hink U, Yigit H, Macharzina R, Harrison DG, and Mulsch A. Role of superoxide dismutase in *in vivo* and *in vitro* nitrate tolerance. *Br J Pharmacol* 127: 1224–1230, 1999.
33. Munzel T, Li H, Mollnau H, Hink U, Matheis E, Hartmann M, Oelze M, Skatchkov M, Warnholtz A, Duncker L, Meinertz T and Forstermann U. Effects of long-term nitroglycerin treatment on endothelial nitric oxide synthase (NOS III) gene expression, NOS III-mediated superoxide production, and vascular NO bioavailability. *Circ Res* 86: e7–e12, 2000.
34. Münzel TT, Daiber AA and Mülsch AA. Explaining the phenomenon of nitrate tolerance. *Circ Res* 97: 618–628, 2005.
35. Pagliaro P, Mancardi D, Rastaldo R, Penna C, Gattullo D, Miranda KM, Feelisch M, Wink DA, Kass DA and Paolocci N. Nitroxyl affords thiol-sensitive myocardial protective effects akin to early preconditioning. *Free Radic Biol Med* 34: 33–43, 2003.
36. Paolocci N, Jackson MI, Lopez BE, Miranda K, Tocchetti CG, Wink DA, Hobbs AJ and Fukuto JM. The pharmacology of nitroxyl (HNO) and its therapeutic potential: not just the Janus face of NO. *Pharmacol Ther* 113: 442-458, 2007.
37. Paolocci N, Katori T, Champion HC, St. John ME, Miranda KM, Fukuto JM, Wink DA, and Kass DA. Positive inotropic and lusitropic effects of HNO/NO^- in failing hearts: Independence from ß-adrenergic signaling. *Proc Natl Acad Sci USA* 100: 5537–5542, 2003.
38. Paolocci N, Saavedra WF, Miranda KM, Martignani C, Isoda T, Hare JM, Espey MG, Fukuto JM, Feelisch M, Wink DA, and Kass DA. Nitroxyl anion exerts redox-sensitive positive cardiac inotropy *in vivo* by calcitonin gene-related peptide signaling. *Proc Natl Acad Sci USA* 98: 10463–10468, 2001.
39. Plane F, Sampson LJ, Smith JJ, and Garland CJ. Relaxation to authentic nitric oxide and SIN-1 in rat isolated mesenteric arteries: Variable role for smooth muscle hyperpolarization. *Br J Pharmacol* 133: 665–672, 2001.
40. Ritchie RH, Irvine JC, Rosenkranz AC, Patel R, Wendt IR, Horowitz JD, and Kemp–Harper BK. Exploiting cGMP-based therapies for the prevention of left ventricular hypertrophy: $NO^{\bullet}$ and beyond. *Pharmacol Ther* 124: 279–300, 2009.
41. Sydow K, Daiber A, Oelze M, Cheng Z, August M, Wendt M, Ullrich V, Mulsch A, Schulz E, Keaney JF, Stamler JS, and Munzel T. Central role of mitochondrial aldehyde dehydrogenase and reactive oxygen species in nitroglycerin tolerance and cross-tolerance. *J Clin Invest* 113: 482–489, 2004.
42. Tocchetti CG, Wang W, Froehlich JP, Huke S, Aon MA, Wilson GM, Di Benedetto G, O'Rourke B, Gao WD, Wink DA, Toscano JP, Zaccolo M, Bers DM, Valdivia HH, Cheng H, Kass DA, and Paolocci N. Nitroxyl improves cellular heart function by directly enhancing cardiac sarcoplasmic reticulum Ca2+ cycling. *Circ Res* 100: 96–104, 2007.
43. Vleeming W, van de Kuil A, te Biesebeek JD, Meulenbelt J, and Boink AB. Effect of nitrite on blood pressure in anaesthetized and free-moving rats. *Food Chem Toxicol* 35: 615–619, 1997.
44. Wang EQ, Lee WI, and Fung HL. Lack of critical involvement of endothelial nitric oxide synthase in vascular nitrate tolerance in mice. *Br J Pharmacol* 135: 299–302, 2002.
45. Wanstall JC, Jeffery TK, Gambino A, Lovren F, and Triggle CR. Vascular smooth muscle relaxation mediated by nitric oxide donors: A comparison with acetylcholine, nitric oxide and nitroxyl ion. *Br J Pharmacol* 134: 463–472, 2001.
46. Wenzel P, Oelze M, Coldewey M, Hortmann M, Seeling A, Hink U, Mollnau H, Stalleicken D, Weiner H, Lehmann J, Li H, Forstermann U, Munzel T, and Daiber A. Heme oxygenase-1: A novel key player in the development of tolerance in response to organic nitrates. *Arterioscler Thromb Vasc Biol* 27: 1729–1735, 2007.
47. Wiley KE and Davenport AP. Comparison of the effects of atherosclerosis and nitrate therapy on responses to nitric oxide and endothelin-1 in human arteries *in vitro. Clin Sci* 103: 124S–127S, 2002.
48. Zamora Pino R and Feelisch M. Bioassay discrimination between nitric oxide and nitroxyl using L-cysteine. *Biochem Biophys Res Commun* 201: 54–62, 1994.

Address correspondence to:
Robert Widdop
Department of Pharmacology
School of Biomedical Sciences
Monash University
Clayton VIC 3800
Australia

E-mail: Robert.Widdop@monash.edu

Date of first submission to ARS Central, April 27, 2010; date of final revised submission, August 19, 2010; date of acceptance, September 19, 2010.

Abbreviations Used

ACh = acetylcholine
ALDH-2 = mitochondrial aldehyde dehydrogenase
ANOVA = analysis of variance
AS = Angeli's salt
cGK = cGMP-dependent protein kinase
cGMP = cyclic guanosine 3′,5′-monophosphate
CGRP = calcitonin gene-related peptide
DEA/NO = diethylamine/NONOate
EtOH = ethanol
GTN = glyceryl trinitrate
HNO = nitroxyl
HR = heart rate
K_{ATP} = ATP-sensitive K^+ channel
K_{Ca} = calcium-sensitive K^+ channel
K_v = voltage-sensitive K^+ channel
KPSS = K^+-depolarizing solution
MAP = mean arterial pressure
NAC = *N*-acetyl-L-cysteine
NaOH = sodium hydroxide
$NO^{\bullet}$ = nitric oxide
PDE5 = phosphodiesterase 5
ROS = reactive oxygen species
RyR = ryanodine receptor
SEM = standard error of the mean
SERCA = sarcoplasmic reticulum Ca^{2+}-ATPase
sGC = soluble guanylyl cyclase

This article has been cited by:

1. Michelle L. Bullen , Alyson A. Miller , Karen L. Andrews , Jennifer C. Irvine , Rebecca H. Ritchie , Christopher G. Sobey , Barbara K. Kemp-Harper . 2011. Nitroxyl (HNO) as a Vasoprotective Signaling MoleculeNitroxyl (HNO) as a Vasoprotective Signaling Molecule. *Antioxidants & Redox Signaling* **14**:9, 1675-1686. [Abstract] [Full Text] [PDF] [PDF Plus]
2. Barbara K. Kemp-Harper . 2011. Nitroxyl (HNO): A Novel Redox Signaling MoleculeNitroxyl (HNO): A Novel Redox Signaling Molecule. *Antioxidants & Redox Signaling* **14**:9, 1609-1613. [Abstract] [Full Text] [PDF] [PDF Plus]