Nitroxyl Anion Donor, Angeli’s Salt, Does Not Develop Tolerance in Rat Isolated Aortae

Jennifer C. Irvine, Joanne L. Favaloro, Robert E. Widdop, Barbara K. Kemp-Harper

Abstract—The nitroxyl anion (HNO) is emerging as a novel regulator of cardiovascular function with therapeutic potential in the treatment of diseases such as heart failure. It remains unknown whether tolerance develops to HNO donors, a limitation of currently used nitrovasodilators. The susceptibility of the HNO donor, Angeli’s salt (AS), to the development of vascular tolerance was compared with the NO donors, glyceryl trinitrate (GTN) and diethylamine/NONOate (DEA/NO) in rat isolated aortae. Vasorelaxation to AS was attenuated \( (P<0.01) \) by the HNO scavenger L-cysteine, whereas the sensitivity to GTN and DEA/NO was decreased \( (P<0.01) \) by the NO\(^\cdot \) scavenger carboxy-[2-(4-carboxyphenyl]-4,4,5,5-tetramethyl-imidazole-1-oxo-3-oxide]. The soluble guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one impaired responses to GTN\( \geq \)AS\( \geq \)DEA/NO. Pretreatment with 10, 30, and 100 \( \mu \)mol/L of GTN for 60 minutes induced a 4- \( (P<0.05) \), 13- \( (P<0.01) \), and 48-fold \( (P<0.01) \) decrease in sensitivity to GTN, demonstrating tolerance development. In contrast, pretreatment with AS or DEA/NO (10, 30, and 100 \( \mu \)mol/L) did not alter their subsequent vasorelaxation. All of the nitrovasodilators (30 \( \mu \)mol/L) displayed a similar time course of vasorelaxation and cGMP accumulation over a 60-minute period. Unlike vasorelaxation, the magnitude of peak cGMP accumulation differed substantially: DEA/NO\( \geq \)AS\( \geq \)GTN. GTN did not induce cross-tolerance to either AS or DEA/NO. In contrast, pre-exposure to DEA/NO, but not AS, caused a concentration-dependent attenuation \( (P<0.01) \) of GTN-mediated relaxation, which was negated by the protein kinase G inhibitor guanosine 3',5'-cyclic monophosphorothioate, 8-(4-chlorophenylthio)-Rp-isomer, triethylammonium salt. In conclusion, vascular tolerance does not develop to HNO, nor does cross-tolerance between HNO and GTN occur. Thus, HNO donors may have therapeutic advantages over traditional nitrovasodilators. (Hypertension. 2007;49:885-892.)

Key Words: NO ■ nitroxyl anion ■ nitrate tolerance ■ vasorelaxation ■ vasculature ■ Angeli’s salt ■ glyceryl trinitrate

Nitric oxide is an important biological signaling molecule that plays an integral role in the control of vascular tone and blood pressure.\(^1\) NO can exist in 3 different redox states: as the uncharged form (NO\(^\cdot \)), in the reduced state as the nitroxyl anion (NO\(^{-} \)), and in the oxidized state as the nitrosonium cation (NO\(^{+} \)). Recently, interest has been renewed in NO\(^{-} \), which exists almost entirely at physiological pH in its protonated form (HNO)\(^2\) and is emerging as a novel regulator of cardiovascular function.\(^3\)

Using HNO donors such as Angeli’s salt (AS; sodium trioxodinitrate), HNO has been shown to be a potent vasodilator of both large conduit\(^4-6\) and small resistance\(^7\) arteries, mediating its response, like NO\(^\cdot \), via stimulation of soluble guanylate cyclase (sGC)\(^5,7\) and a subsequent rise in cGMP.\(^4\) However, here the similarities between HNO and NO\(^\cdot \) cease. Thus, HNO activates voltage-dependent K\(^+\) channels in small resistance-like arteries,\(^7\) yet NO\(^\cdot \) activates calcium-activated K\(^+\) channels in the same preparation.\(^8\) Furthermore, unlike NO\(^\cdot \), HNO targets ferric rather than ferrous heme proteins,\(^9\) is resistant to scavenging by superoxide,\(^10\) has positive inotropic effects in vivo,\(^11,12\) and elevates plasma levels of calcitonin gene-related peptide.\(^11\) Excitingly, the distinct pharmacology of HNO versus NO\(^\cdot \) offers considerable therapeutic advantages, particularly in the setting of heart failure.\(^12,13\)

Before the therapeutic potential of HNO donors can be realized, their susceptibility to the development of tolerance, a phenomenon that limits the effectiveness of clinically used organic nitrates, such as glyceryl trinitrate (GTN), must be determined.\(^13\) Currently, the mechanisms underlying the phenomenon of nitrate tolerance remain unclear and are likely to be multifactorial. Specifically, they may involve reduced biotransformation of organic nitrates to NO\(^\cdot \), physiological counterregulatory mechanisms (eg, neurohormonal activation), desensitization of sGC, increased activity of phosphodiesterase 1A,\(^11\) increased production of reactive oxygen species leading to scavenging of NO\(^\cdot \) and a decreased bioavailability.\(^14,15\)

Given that HNO donors, such as AS, spontaneously donate HNO via a process that does not require biotransformation and are not susceptible to scavenging by superoxide, we hypothesize that tolerance will not develop to this class of nitrovasodilator. Conversely we predict that HNO donors may induce cross-tolerance to organic nitrates, given the ability of
HNO to inhibit aldehyde dehydrogenase (ALDH),\textsuperscript{16,17} an enzyme that has been implicated recently in the biotransformation of GTN in the vasculature.\textsuperscript{18,19}

To test these hypotheses, this study compared the susceptibility of the HNO donor, AS, with the NO\textsuperscript{-} donors, GTN and diethylamine/NONOate (DEA/NO), to the development of tolerance and cross-tolerance in vitro. Given that GTN undergoes intracellular biotransformation to generate NO\textsuperscript{-} and DEA/NO releases NO\textsuperscript{-} spontaneously with similar kinetics to the release of HNO from AS, such a comparison will allow us to determine whether tolerance depends on either the way in which NO is generated (eg, biotransformation versus spontaneous release) or the redox form of NO produced (NO\textsuperscript{-} versus HNO).

**Methods**

This study was approved by the Pharmacology Animal Ethics Committee, Monash University, Australia, and conforms to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Tissue Preparation**

Male Wistar–Kyoto rats (16 to 17 weeks of age) were killed by stunning and cervical dislocation. The thoracic aorta was isolated, cut into 5-mm ring preparations (endothelium intact), and mounted in organ baths for the measurement of isometric tension. Data were captured using the CVMS data acquisition system (World Precision Instruments). Vessels were maintained in physiological Krebs’ solution at 37°C and bubbled continuously with carbogen (95\% O\textsubscript{2} and 5\% CO\textsubscript{2}). After a 30-minute equilibration period, vessels were stretched to an optimal passive tension of 2 g.

**Functional Experiments**

Vessels were maximally contracted with a K\textsuperscript{+}-depolarizing solution. Responses to vasorelaxants were examined in vessels precontracted to \( \sim 50\% \) K\textsuperscript{+}-depolarizing solution with U46619 (0.3 mmol/L) and titrated concentrations of cirazoline (0.005 to 0.5 mmol/L).

Cumulative concentration–response curves to GTN (0.1 mmol/L to 10 mmol/L), the HNO donor AS (0.1 mmol/L to 10 mmol/L), and the NO\textsuperscript{-} donor DEA/NO (0.1 mmol/L to 10 mmol/L) were constructed in the absence or presence of one of the following: (1) the HNO scavenger L-cysteine (3 mmol/L; 3 minutes); (2) the NO\textsuperscript{-} scavenger carboxy-[2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxo-3-oxide] (200 \( \mu \)mol/L; 15 minutes); and (3) ODQ (0.1, 1, 3 or 10 \( \mu \)mol/L, 30 minutes). Only 1 concentration–response curve to any vasodilator was obtained for each vessel.

To investigate the possible development of tolerance to GTN, AS, or DEA/NO (and cross-tolerance between these drugs), vessels were incubated in either Krebs’ solution alone or in the presence of 10, 30, or 100 \( \mu \)mol/L of GTN, AS, or DEA/NO for a period of 60 minutes. Vessels were washed thoroughly every 15 minutes for 1 hour after incubation. After precontraction to \( \sim 50\% \) K\textsuperscript{+}-depolarizing solution with cirazoline, concentration–response curves were obtained to GTN, AS, or DEA/NO. To investigate the mechanism by which DEA/NO pretreatment caused cross-tolerance to GTN, arteries were treated with the protein kinase G (PKG) inhibitor guanosine 3’,5’-cyclic monophosphorothioate, 8-(4-chlorophenylthio)-Rp-isomer, triethylammonium salt (Rp-8-pCPT-cGMPS; 10 \( \mu \)mol/L) alone or in combination with 30 \( \mu \)mol/L of DEA/NO for 60 minutes, followed by a 1-hour washout period.

**Time Course Studies**

The time course of vasorelaxation and cGMP production in response to GTN, DEA/NO, and AS was compared to elucidate potential differences in the functional half-lives of these nitrovasodilators. A subset of aortae was precontracted to \( \sim 50\% \) K\textsuperscript{+}-depolarizing solution with cirazoline before the single addition of 30 \( \mu \)mol/L of GTN, AS, or DEA/NO. Vasorelaxation responses were then measured over the ensuing 60 minutes.

To measure cGMP concentration, aortic rings were placed in Eppendorf vials in Krebs’ solution (1 mL), maintained at 37°C, and bubbled continuously with carbogen. After a 30-minute equilibration period, vessels were incubated with 30 \( \mu \)mol/L of GTN, AS, or DEA/NO for 0, 10, 15, 30, 45, or 60 minutes, after which they were snap frozen in liquid nitrogen and stored at \(-80\)°C until cGMP analysis. Frozen tissues were crushed in ice-cold 6\% trichloroacetic acid, sonicated, and centrifuged at 6000 rpm for 15 minutes. The supernatant was extracted \( \times 4 \) with saturated diethyl ether and air dried. cGMP analysis was performed using a radioimmunoassay kit (Perkin Elmer) according to manufacturer’s instructions and results expressed as finomol\textsuperscript{-1} of tissue (wet weight).

**Data and Statistical Analysis**

Relaxation responses are expressed as a percentage reversal of cirazoline precontraction. Individual relaxation curves were fitted to a sigmoidal logistic equation (Graphpad Prism 4.0), and EC\textsubscript{50} values (concentration of agonist giving a 50\% relaxation) were calculated and expressed as \(-\log\) mol per liter (pEC\textsubscript{50}). Differences between mean pEC\textsubscript{50} and maximum relaxation values were tested using either a Student’s unpaired t test or 1-way ANOVA. Concentration–response curves were compared by means of a 2-way ANOVA (Sigma Stat 3.1).

After a single addition of 30 \( \mu \)mol/L of GTN, AS, or DEA/NO, a 1-way ANOVA was used to compare the time course of vasorelaxation and cGMP accumulation against peak (10 minutes) and basal levels (0 minutes), respectively. Data are expressed as mean±SEM, and \( P<0.05 \) was accepted as statistically significant.

An expanded Methods section can be found in an online data supplement available at http://hyper.ahajournals.org.

**Results**

**Carboxy-PTIO and L-Cysteine Discriminate Between NO\textsuperscript{-} and HNO**

Vasorelaxation to both the NO\textsuperscript{-} donors GTN and DEA/NO was unchanged in the presence of the HNO scavenger, l-cysteine (3 mmol/L), yet their potencies were decreased up to 4-fold (\( P<0.01 \)) by the NO\textsuperscript{-} scavenger carboxy-PTIO (200 \( \mu \)mol/L; Figure 1a and 1c). In contrast, carboxy-PTIO failed to alter the response to the HNO donor AS (Figure 1b), yet l-cysteine caused a significant rightward shift in the concentration–response curve to AS (\( P<0.01 \); Table S1).

**Concentration-Dependent Inhibition of Responses to GTN, AS, and DEA/NO by ODQ**

The sGC inhibitor ODQ caused a marked and concentration-dependent (\( P<0.05 \)) attenuation of the relaxation responses to GTN and AS (Figure 2a and 2b), such that 3 \( \mu \)mol/L of ODQ virtually abolished vasorelaxation. In contrast, relaxation responses to DEA/NO were more resistant to the inhibitory effects of ODQ, with 10 \( \mu \)mol/L of ODQ causing an \( \sim 200\)-fold (\( P<0.05 \)) decrease in sensitivity yet only reducing the response to 10 \( \mu \)mol/L DEA/NO by 17.6±3.1\% (\( P<0.01 \); Table S2).

**Tolerance Development to GTN But Not AS or DEA/NO**

Pretreatment of aortae with 10, 30, and 100 \( \mu \)mol/L of GTN for 60 minutes caused a significant 4– (\( P<0.05 \)), 13– (\( P<0.01 \)), and 48-fold (\( P<0.01 \)) decrease in sensitivity to subsequent responses to GTN, respectively (Figure 3a). Pretreatment with 100 \( \mu \)mol/L of GTN also resulted in a significant reduction in the response to 10 \( \mu \)mol/L of GTN (\( P<0.05 \), Table S3). In contrast, treatment of aortae with AS (10, 30, and 100 \( \mu \)mol/L) or DEA/NO (10 and
30 μmol/L) had no effect on their subsequent responses (Figure 3b and 3c). A total of 100 μmol/L of DEA/NO caused a significant 3-fold (P<0.01) decrease in sensitivity to DEA/NO (Table S3).

GTN, AS, and DEA/NO Have a Similar Time Course of Response
A single addition of 30 μmol/L of GTN, AS, or DEA/NO induced maximal vasorelaxation in isolated aortae that peaked at 10 minutes (Figure 4a through 4c). Although GTN and AS maintained vasorelaxation over the 60-minute period, the vasorelaxation response to DEA/NO was reduced 45 (P<0.01) and 60 (P<0.01) minutes after addition.

Like vasorelaxation, the time course of cGMP accumulation after the single addition of 30 μmol/L GTN, AS, or DEA/NO was similar (Figure 4), with cGMP levels peaking at 10 minutes (P<0.01). However, the magnitude of peak cGMP accumulation differed substantially between the nitrovasodilators such that DEA/NO (543.7±167.7 fmol/mg¹) > AS (84.7±53.1 fmol/mg⁻¹) > GTN (21.9±9.0 fmol/mg⁻¹). Despite vasorelaxation to GTN and AS and, to a lesser extent, DEA/NO, being well sustained over the 60-minute period, cGMP levels for GTN, AS, and DEA/NO returned to basal levels by 30 minutes.

Lack of Cross-Tolerance to AS or DEA/NO After GTN Pretreatment
Pretreatment of vessels with 10, 30, or 100 μmol/L of GTN had no effect on the sensitivity to AS (Figure 5a), although pretreatment with 100 μmol/L of GTN did cause a small, but
significant, decrease in the response to 10 μmol/L of AS (P<0.05) compared with control (Table S3). Similarly, relaxation responses to DEA/NO were unaffected by pre-exposure to GTN (Figure 5b).

**DEA/NO, But Not AS, Induces Cross-Tolerance to GTN**

Relaxation responses to GTN were unchanged after pre-exposure to either 10 or 30 μmol/L of AS, yet 100 μmol/L of AS caused a small, but significant, decrease in both sensitivity and maximum response to GTN (P<0.05; Figure 6a).

In contrast, pretreatment of aortae with DEA/NO at concentrations of 10, 30, and 100 μmol/L induced cross-tolerance to GTN, as evidenced by significant 3-, 5- (P<0.01), and 14-fold (P<0.01) rightward shifts in the relaxation response to GTN, respectively (Figure 6b), and reductions (P<0.01) in the response to 10 μmol/L of GTN (Table S3).

The decomposed DEA/NO (10 μmol/L) solution contained negligible amounts of NO (81±22 nmol; n=4) compared with 10 μmol/L of freshly prepared DEA/NO (233±170 nmol; n=4), as determined using an NO electrode, and was subsequently used as a control for the effects of the diethylamine component of this compound. Pretreatment with
100 μmol/L of diethylamine had no significant effect on subsequent responses to GTN (Figure 6b). Interestingly, the cross-tolerance to GTN after pretreatment with DEA/NO (100 μmol/L) was not apparent with AS (Figure 6c).

**PKG Mediates DEA/NO-Induced Cross-Tolerance to GTN**

In the presence of the PKG inhibitor Rp-8-pCPT-cGMPS (10 μmol/L), pretreatment with 30 μmol/L of DEA/NO did not induce cross-tolerance to GTN (Figure 7). Rp-8-pCPT-cGMPS alone had no effect on vasorelaxation to GTN (Table S4).

**Discussion**

This study has demonstrated for the first time that the vasorelaxant effects of the HNO donor AS are resistant to the development of tolerance. Furthermore, the vasodilator capacity of HNO is sustained in vessels tolerant to GTN, and unlike the NO donor DEA/NO, AS does not induce cross-tolerance to GTN.

Using the NO donor DEA/NO scavengers carboxy-PTIO and L-cysteine, respectively, we confirmed that vasorelaxation responses to AS were mediated by HNO, and those to GTN and DEA/NO by NO. Importantly, the lack of effect of carboxy-PTIO on the response to AS, in the presence of the Cu螯合剂 EDTA, suggests that extracellular oxidation of HNO to NO does not occur.

In agreement with previous findings, ODQ virtually abolished vasorelaxation to both GTN and AS, suggesting that their responses are mediated almost entirely through the sGC/cGMP pathway. Given that it has been suggested that only NO can directly activate sGC, the sensitivity of AS to ODQ may be indicative of intracellular oxidation of HNO to NO. Conversely, the observation that AS is more susceptible to ODQ inhibition than authentic NO gas may indicate direct activation of sGC by HNO, yet this remains to be verified. Unlike GTN and AS, DEA/NO was more resistant to the inhibitory effects of ODQ, suggesting that it may mediate
vascular smooth muscle relaxation in rat isolated aorta through a cGMP-independent mechanism.23–25

Using a well established in vitro model of nitrate tolerance in the rat isolated aorta,26,27 we clearly demonstrated tolerance to GTN. Furthermore, GTN did not induce cross-tolerance to DEA/NO or AS. Similarly, GTN does not cause cross-tolerance to NO itself or NO donors such as sodium nitroprusside, spermine NONOate, and S-nitroso-N-acetylpenicillamine in rat26,28 and bovine29 arteries. Such findings suggest that in vitro vascular tolerance may arise because of impairment at the level of GTN biotransformation rather than dysfunction at the level of sGC. In support of this concept, De la Lande et al29 showed that GTN tolerance in bovine coronary arteries is largely independent of sGC activity.

Importantly, using the same experimental protocol that clearly demonstrated tolerance to GTN, no tolerance to AS was observed. Specifically, vasorelaxation responses to AS were unchanged after a 60-minute incubation with AS up to a concentration of 100 μmol/L. Similarly, DEA/NO was relatively resistant to self-tolerance development, consistent with a previous study in rat pulmonary arteries in which no tolerance to another NONOate, spermine NONOate, was observed.28

Despite the half-lives of DEA/NO (2.1 minutes30) and AS (2.3 minutes30) being shorter than that of the GTN metabolites 1,2-glyceryl dinitride and 1,3-glyceryl dinitride (≈40 minutes31,32), all 3 of the nitrovasodilators displayed a similar onset, magnitude, and duration of vasorelaxation over a 60-minute period, thereby mimicking the conditions used on inducing tolerance. Moreover, the time course of cGMP accumulation, albeit more transient when compared with vasorelaxation, did not differ between these 3 vasodilators. Furthermore, despite displaying similar vasodilator potencies in the rat isolated aorta, the elevation in cGMP content in response to DEA/NO was 6- and 25-fold higher than that for AS and GTN, respectively. Similar high levels of cGMP accumulation by DEA/NO have been reported previously in rat small mesenteric arteries23 and the human internal mammary artery.33 Thus, it appears that DEA/NO and AS elevate vascular cGMP to levels in excess of that required for vasorelaxation. Indeed, it has been shown that very low amounts of cGMP are sufficient for a full biological response to sGC activators.34 Taken together, our findings suggest that a shorter duration of action of these nitrovasodilators does not underlie their lack of tolerance development.

Importantly, the present study has not only demonstrated for the first time that tolerance does not develop to the HNO donor AS, at least in vitro, but it has also shed light on the possible mechanisms underlying nitrate tolerance development. Indeed, it does not appear, at least in our acute in vitro model, that tolerance development depends on the redox form of NO donated by a nitrovasodilator, given that both the HNO (AS) and NO (DEA/NO) donors were resistant; yet, the NO donor GTN was susceptible to tolerance development. Furthermore, given the similar dependence of GTN and AS on sGC activation for their vasorelaxation response, yet the lack of cross-tolerance to AS after GTN and the lack of self-tolerance to AS, it is unlikely that tolerance to GTN occurs as a result of dysfunction at the level of sGC itself. Although not directly tested here, previous studies have discounted a role for oxidative stress in in vitro tolerance.35,36 Rather, it is likely that tolerance development in our in vitro model is related to an impairment at the level of GTN biotransformation, because no tolerance was observed for DEA/NO and AS, which spontaneously liberate NO’ and HNO, respectively.

Recent evidence suggests that biotransformation of GTN to NO’ in the vasculature is mediated, at least in part, by mitochondrial ALDH-2.18,19 Despite an observed lack of self-tolerance to AS and DEA/NO, we explored the possibility that they may induce cross-tolerance to GTN, because both nitrovasodilators have been shown to inhibit yeast ALDH activity.16,37 with AS (IC50 1.3 μmol/L) the more potent inhibitor compared with DEA/NO (IC50 139 μmol/L), under aerobic conditions.17,37 Interestingly, pretreatment with AS at concentrations <100 μmol/L did not attenuate the vasodilator response to GTN. Presuming that AS inhibits ALDH-2 in the vasculature, the lack of cross-tolerance to GTN after AS pretreatment may suggest that GTN biotransformation is not mediated predominantly by ALDH-2 in the rat isolated aorta. This is in agreement with recent studies that have provided evidence to suggest that the inhibition of ALDH-2 may be only 1 of several causes of nitrate tolerance.38,39 Indeed, in an investigation into the role of ALDH-2 in the biotransformation of GTN in humans in vivo, complete ALDH-2 inhibition by disulfiram therapy only resulted in a 33% reduction in GTN responses.40 Recently, altered phosphodiesterase 5 activity was also implicated in the development of GTN tolerance in veins.51

In striking contrast to our findings with AS, pretreatment of vessels with DEA/NO caused marked cross-tolerance to GTN. To our knowledge, this is the first report of cross-tolerance to GTN, either in vitro or in vivo, after NONO-ate pretreatment. Furthermore, it is unlikely that DEA/NO affected the response to GTN at the level of sGC, because it did not develop self-tolerance or induce cross-tolerance to AS.
Rather, we hypothesized that the ability of DEA/NO to induce cross-tolerance may depend on its ability to substantially elevate cGMP and activate downstream mediators such as cGMP-dependent protein kinase (PKG-1)\(^{11,12,45}\) to a greater extent than AS and GTN. Indeed, the cross-tolerance to GTN by DEA/NO was abolished by the PKG-1 inhibitor Rp-8-pCPT-cGMPS,\(^{44}\) providing the first evidence that high levels of cGMP may impair vasorelaxation to GTN possibly via inhibition of biotransformation. Certainly in the pulmonary vasculature, tolerance to NO after prolonged exposure (20 hours) may result from PKG-mediated down-regulation of PKG itself.\(^{44}\) However, given that self-tolerance to DEA/NO was not observed in the present study, it is unlikely that PKG is exerting a negative feedback effect on itself in our experimental setting. Rather, PKG may interfere with GTN biotransformation, and this concept requires further investigation. Importantly, however, given that GTN is a much less efficacious stimulator of cGMP than DEA/NO, the mechanisms underlying GTN self-tolerance versus its cross-tolerance after DEA/NO preemt may differ.

**Perspectives**

In conclusion, this study has provided the first evidence that neither vascular tolerance nor cross-tolerance develops to an HNO donor and further highlights the distinct pharmacology of HNO and NO\(^{-}\). From a clinical perspective, HNO donors may offer considerable advantages over traditional nitrovasodilators,\(^{11,12,45}\) and their full therapeutic potential will be realized once their susceptibility to tolerance development is also explored in the venous vasculature and under disease conditions.

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**Disclosures**

None.

**References**


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