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# Angeli's Salt, a nitroxyl anion donor, reverses endothelin-1 mediated vascular dysfunction in murine aorta

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## Abstract

Nitroglycerin (Gtn) is a treatment for cardiovascular patients due to its vasodilatory actions, but induces tolerance when given chronically. A proposed mechanism is the superoxide ($O_2^-$)oxidative stress hypothesis, which suggests that Gtn increases $O_2^-$ production. Nitric oxide (NO) exists in three different redox states; the protonated, reduced state, nitroxyl anion (HNO) is an emerging candidate in vascular regulation. HNO is resistant to scavenging and of particular interest in conditions where high levels of reactive oxygen species (ROS) exist. We hypothesize that treatment with Gtn will exacerbate endothelin 1 ( ET-1) induced vascular dysfunction via an increase in ROS, while treatment with Angeli's Salt (AS), an HNO donor, will not. Aorta from mice were isolated and divided into four groups: vehicle, ET-1 [0.1μM, 1μM], ET-1+Gtn [Gtn 1μM] and ET-1+AS [AS 1μM]. Concentration response curves (CRCs) to acetylcholine (ACh) and phenylephrine (Phe) were performed. Aorta incubated with ET-1 (for 20–22hrs) exhibited a decreased relaxation response to ACh and an increase in Phe-mediated contraction. Aorta incubated with AS exhibited a reversal in ET-1 induced vascular and endothelial dysfunction. ET-1 increased ROS in aortic vascular smooth muscle cells (VSMCs), visualized by dihydroethidium

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(DHE) staining. AS incubated reduced this ROS generation, yet maintained with Gtn treatment. These data suggest that aorta incubated with the HNO donor, AS, can reverse ET-1 mediated vascular dysfunction, which may be through a decrease or prevention of ROS generation. We propose that HNO may be vasoprotective and that HNO donors studied as a therapeutic option where other organic nitrates are contraindicative.

**Keywords**

HNO; Angeli's Salt; Nitroglycerin; Endothelin-1; Vascular Dysfunction

## 1 Introduction

Nitroglycerin (glyceryl nitrate, Gtn), was proposed by Murrell in 1879 as a treatment for angina (Murrell, 1879; Frame *et al.*, 2002). Gtn continues to be used for various conditions, including angina, heart failure and other acute cardiovascular crises. While this and other organic nitrovasodilators improve cardiac output by decreasing preload and afterload on the heart, and inducing vasodilation, these compounds are also of limited clinical efficacy. They induce tolerance when given over time, which has been demonstrated clinically and in various animal models (Munzel *et al.*, 2014). Additionally, when conditions of increased reactive oxygen species ( ROS) are present, they also have a diminished vasoprotective effect, and even induce vascular wall damage.

There has been considerable research regarding the one electron reduced congener of nitric oxide (NO), nitroxyl anion ($NO^-$) (Irvine *et al.*, 2003; Favaloro & Kemp-Harper, 2007; Irvine *et al.*, 2007; Irvine *et al.*, 2008; Andrews *et al.*, 2009; Chin *et al.*, 2016; Miao & King, 2016; Shoman & Aly, 2016). It was previously thought that $NO^-$ exists as an anion (pKa 4.7) at a physiological pH; however, this assumption was corrected in 2002, when investigators determined the actual pKa to be around 11.4. At physiological pH, $NO^-$ exists as HNO (Gratzel, 1970; Bartberger, 2001; Shafirovich & Lymar, 2002; Fukuto *et al.*, 2005a). It was also determined that this conjugated weak acid, HNO, can cross cellular membranes, leading investigators to divert research to this understudied molecule (Bartberger, 2001). Although the comparative mechanisms between HNO and NO are still being investigated, it is widely accepted that their physiology, pharmacology and biochemistry are vastly different (Fukuto *et al.*, 2005a; Fukuto *et al.*, 2005b; Andrews *et al.*, 2009; Favaloro & Kemp-Harper, 2009; Wynne *et al.*, 2012).

This phenomenon can be seen when using the HNO/$NO^-$ donor, Angeli's Salt (AS). Various studies have used AS to study HNO-mediated vasorelaxation. One of the most attractive effects of AS is the lack of tolerance induced (Irvine *et al.*, 2007; Irvine *et al.*, 2008; Irvine *et al.*, 2010). In functional studies performed using rat aorta, Irvine and colleagues demonstrated that the use of the HNO-donor AS, did not induce tolerance as compared to Gtn (Irvine *et al.*, 2007; Irvine *et al.*, 2010). Additionally, they also revealed that AS did not exhibit a cross tolerance to Gtn.(Irvine *et al.*, 2010) A recent study has demonstrated a similar phenomenon *in vivo;* chronic infusion of AS did not lead to tolerance or endothelial dysfunction (Irvine *et al.*, 2007; Irvine *et al.*, 2010). The exact mechanisms of Gtn-induced

tolerance have not been fully elucidated; however, several proposed models have been recommended (Munzel T, 1995; Szocs *et al.*, 2007; Daiber *et al.*, 2009). These mechanisms may overlap, but are suggested to include: reduced biotransformation of the organic nitrates to NO, neurohormonal activation, desensitization of soluble guanylate cyclase (sGC), increased phosphodiesterase 1A1 activity and increased production of ROS (Frame *et al.*, 2002; Fukatsu *et al.*, 2007; Irvine *et al.*, 2007; Szocs *et al.*, 2007; Daiber *et al.*, 2009; Kosmicki, 2009). In fact, there is a growing body of literature showing that administration of organic nitrates not only induces tolerance, but promotes vascular wall damage (Sage *et al.*, 2000; Bartberger, 2001; Schulz *et al.*, 2002; Munzel *et al.*, 2005; Irvine *et al.*, 2008).

It has also been demonstrated that HNO is less reactive as compared to NO, which may lend HNO a superior stability against reactions with ROS or during conditions of high oxidative stress, such as hypertension (Savoia & Schiffrin, 2006; 2007; Schiffrin, 2007; Irvine *et al.*, 2008; Paolocci & Wink, 2009; Switzer *et al.*, 2009). Common mediators of hypertension-induced ROS generation, promotion of oxidative stress and vascular wall damage are angiotensin II (AngII) and endothelin-1 (ET-1) (Wilcox, 2002; Palm *et al.*, 2010; Wilcox, 2010; Pollock & Pollock, 2011; Nguyen Dinh C at *et al.*, 2013; Gonzalez *et al.*, 2014; Brito *et al.*, 2015; Montezano *et al.*, 2015 ). In this manuscript, we sought to determine whether the HNO/$NO^-$ donor (AS) would alleviate ET-1-induced vascular dysfunction. We hypothesized that treatment with Gtn will exacerbate ET-1 vascular dysfunction via an increase in ROS, while AS will not.

To our knowledge, this is the first study to compare the effects of Gtn *vs.* HNO and investigate vascular responses *ex vivo*. The importance of these experiments will further demonstrate how the use of organic nitrates during pathophysiological conditions such as hypertension, where there are significantly increased levels of ROS, may not always be beneficial. Additionally, we will also reveal that using donors for HNO, which has proven to be a more stable molecule and is resistant to scavenging from ROS, may be a better therapeutic option.

## 2 Materials and Methods

Male C57bl/6 mice, weighing between 25–30 grams were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were maintained on a 12-hour light dark cycle, housed five per cage and allowed access to chow and water *ad libitum*. Isoflurane (10%) in oxygen was used for surgeries with carbon dioxide (CO2) for euthanasia. All procedures were performed in accordance with the Guiding Principles in the Care and Use of Animals, approved by the Medical College of Georgia at Augusta University Committee on the Use of Animals in Research and Education.

### 2.1 Primary Cell Culture

Aorta from mice were carefully excised and cleaned in sterile Dubelco's Modified Eagle's Medium (high glucose, DMEM), supplemented with 1% penicillin/streptomycin and 30% fetal bovine serum (FBS). Per previous protocols and experimental procedures used in this laboratory, the endothelium was removed and a primary vascular smooth muscle cell (VSMC) culture obtained via explant technique (Carrillo-Sepulveda *et al.*, 2010). The

VSMCs were maintained in DMEM (low glucose), supplemented with 1% penicillin/streptomycin and 10% FBS under normal cell culture conditions (37°C, 5% $CO_2$). Cells were grown to confluency, passed using trypsin and used within 4–6 passages.

### 2.2 Aortic Ring Incubation

After euthanasia with $CO_2$, the aorta was rapidly excised and bathed in ice-cold physiological salt solution (PSS) (NaCl 120 mM, KCl 4.7 mM, $KH_2PO_4$ 1.18 mM, $NaHCO_3$ 14.9 mM, dextrose 5.6 mM, $CaCl_2 \cdot H_2O$, 0.06 mM EDTA). Increased concentrations of EDTA were used in PSS buffer to aid in preventing the extracellular conversion of HNO to NO. Aorta were carefully isolated and incubated in DMEM (low glucose) supplemented with 1% penicillin/streptomycin. Vessels were incubated overnight in incubator per standard cell culture technique and divided into the following groups: vehicle, endothelin-1 (ET-1, 0.1μM or 1 μM, Pheonix Pharmaceuticals, Burlingame CA), ET-1 plus the HNO donor, Angeli's Salt (ET-1 + AS, 1 μM, Cayman Chemical, Ann Arbor MI) or ET-1 plus Gtn (ET-1 + Gtn, 1 μM, American Regent, Shirley NY). Vessels were incubated overnight in either vehicle or ET-1 for 20–22 hrs. During the last hour of incubation, vessels were given two treatments of either AS or Gtn (one treatment at one hour prior and second 30 minutes prior to functional experiment).

### 2.3 Functional Studies

Aortas were mounted as ring preparations on two stainless steel pins in a myograph (Danish MyoTech, Aarhus, Denmark). Vessels were maintained at 37°C and continuously aerated with 95% $O_2$, 5% $CO_2$ and allowed to stabilize for at least 45 mins, at an optimal passive force of 5.0 mN. After stabilization, tissues were contracted with KCl (120 mM) solution to determine the reactivity of the vascular smooth muscle cells. To determine endothelium viability, contraction was stimulated via phenylephrine (Phe; 1 μM) followed by relaxation with acetylcholine (ACh; 10 μM). Vessels were then washed before performing concentration response curves (CRC) and after each CRC. CRCs to ACh in Phe (10 μM)-contracted vessels were performed in the presence of vehicle or the following: tempol ($O_2^-$ scavenger), Iron (III) $^{5,10,15,20}$-Tetrakis (4-sulfonatophenyl) porphyrinato, chloride (Fe-TPPS, $ONOO^-$ degradation catalyst). All other chemicals and drugs were purchased from Sigma Aldrich, St. Louis, MO. Force measurements were collected using Chart™ Software (ADI Instruments, Colorado Springs, CO) for PowerLab data acquisition systems (ADI Instruments).

### 2.4 Dihydroethidium Staining

VSMC primary cells were used in determining ROS and $O2^-$ generation with dihydroethidium (DHE, Invitrogen, California), which oxidizes in the presence of ROS, generating fluorescence (Bruder-Nascimento *et al.*, 2014; Silva *et al.*, 2015). VSMCs were grown to confluence, and then seeded to 6-well plates. Once VSMCs reached 80% confluency, experiments were performed using: vehicle, ET-1 (0.1 μM), ET-1 plus AS (10 μM) and ET-1 plus Gtn (10 μM). VSMCs were treated with ET-1 for 2 minutes, in the presence of vehicle, AS or Gtn administered simultaneously. After washing 3 times in phosphate buffered saline (PBS) VSMCs were incubated with DHE (2 μM) for 20 minutes.

VSMCs were washed with PBS and then fluorescence visualized using confocal imaging (Zeiss, Carl Zeiss MicroImaging, Thornwood, NY).

### 2.5 Statistical Analysis

Agonist concentration-response curves (CRCs) were fitted using a nonlinear interactive fitting program (GraphPad Prism, Graph Pad Software Inc., San Diego CA), and values expressed as percent of maximal relaxation graphed against increasing molar concentrations of agonist. Agonist potencies and maximum response are expressed as negative logarithm of the molar concentration of agonist producing 50% of the maximum response ($EC_{50}$) and maximum effect elicited by the agonist ($R_{max}$), *respectively*. Non-linear regression analysis was used to determine EC50 values, where *Rmax* was normalized to 100 percent for calculations. Data are expressed as mean ± S.E.M. (n), where n is the number of experiments performed. Statistical analysis of the CRCs was performed by using the *F test* for comparisons of best-fit data between groups ($EC_{50}$ and $R_{max}$).

### 2.6 Drugs

Angeli's Salt was prepared using a 0.01M NaOH solution. All other stock solutions were prepared by using water. Stock solutions originally diluted in DMSO or ethanol was used with a final concentration of less than 0.003% v/v in the muscle bath; this concentration has been demonstrated to have no effect on vascular reactivity. Additionally, solutions containing vehicle levels of ethanol, 0.01M NaOH or DMSO were also used throughout the experimental protocol.

## 3 Results

### 3.1 Aortic rings incubated with ET-1 exhibit vascular dysfunction

To establish this model of vascular dysfunction, aortas were treated with ET-1 (0.1 μM and 1 μM) overnight, and vascular reactivity assessed by performing CRCs to Phe and ACh. Aortic rings treated with ET-1 [0.1 μM], did not exhibit an increased contractility to Phe ($R_{max}$ 105.1% ± 2.06 *vs.* 110.0% ± 2.56) (Fig. 1A), or decreased endothelium-mediated relaxation response to ACh ($EC_{50}$ −6.84 ± 0.11 *vs.* −6.95 ± 0.12) (Fig. 1B). When the concentration of ET-1 was increased to [1 μM], vascular dysfunction was observed. ET-1 treated aortic rings exhibited a significantly enhanced Phe-mediated contraction, as compared to vehicle alone ($R_{max}$ 128.3% ± 3.5 *vs.* 115.5% ± 3.26, P<0.01) (Fig. 1C). CRCs were then performed to ACh, and ET-1 treated aortic rings exhibited a significantly decreased ACh-mediated relaxation response, indicative of endothelial dysfunction ($R_{max}$ 58.33% ± 3.2 *vs.* 81.68% ± 2.6, P<0.001) (Fig. 1D). For the remainder of the experiments, the higher ET-1 treatment (1 μM) dose was used.

### 3.2 Incubation with Angeli's Salt, the HNO donor, reversed the increase in Phe-mediated contraction induced by ET-1

To determine if Gtn would exacerbate ET-1 mediated vascular dysfunction, CRCs were performed in aortic rings incubated with ET-1 overnight, and co-treated with Gtn (1 μM, twice). As shown in Fig. 2A, vessels incubated with ET-1 exhibited increased Phe-mediated contraction, which was not altered with Gtn co-treatment ($R_{max}$ 121.7% ± 2.37 *vs.* 128.3%

± 3.46). In contrast, when ET-1 vessels were treated with AS, there was a significant decrease in Phe-mediated contraction, as compared to those incubated with ET-1 alone ($R_{max}$ 117.2% ± 3.58 *vs.* 128.3% ± 3.46, P<0.05), which was not different from vehicle (Fig. 2B). These data reveal that AS may be able to reverse ET-1-mediated vascular dysfunction.

### 3.3 Incubation with the HNO donor, Angeli's Salt, reverses ET-1-mediated endothelial dysfunction

To determine if Gtn treatment would further exacerbate ET-1-mediated endothelial dysfunction, CRCs were performed to ACh. Vessels were treated with ET-1 or vehicle overnight, and then given Gtn (1 μM, twice). Aortic rings incubated with ET-1 exhibited a marked endothelial dysfunction ($R_{max}$ 58.33% ± 3.2 *vs.* 81.68% ± 2.6, P<0.001) (Fig. 3). When these vessels were co-incubated with Gtn, there was an improvement in endothelial dysfunction ($R_{max}$ 71.3% ± 2.6 *vs.* 58.3% ± 3.2, P<0.05) (Fig. 3), yet relaxation responses remained significantly decreased as compared to vehicle. When these vessels were treated with AS, a complete reversal of endothelial dysfunction was observed ($R_{max}$ 83.2% ± 2.9 *vs.* 58.3% ± 3.2, P<0.0001) (Fig. 3), suggesting AS may also be capable of improving ET-1-induced endothelial dysfunction.

### 3.4 Angeli's Salt reduces ET-1-induced $O_2^-$ generation

Using a primary VSMC culture, experiments were performed to further investigate the mechanism of AS-mediated vasoprotection. VSMCs obtained from murine thoracic aorta were maintained in cell culture for 4–6 passages before use, which has been demonstrated to be an acceptable time period during which VSMC maintain their phenotype in culture (Poliseno *et al.*, 2006). VSMCs were treated with: vehicle, ET-1 (0.1 μM, 2 min), ET-1 plus AS (10 μM, 2 min) or ET-1 plus Gtn (10 μM, 2 min). After treatment, DHE staining was performed to visualize $O_2^-$ generation. As shown in Fig. 4, ET-1 treatment increased $O_2^-$ generation, as compared to basal levels. With Gtn co-incubation (Fig. 4D), no changes in $O_2^-$ levels were apparent. However, when VSMCs were co-incubated with AS (Fig. 4C), a decrease in DHE staining was observed. These data suggest that AS may be preventing or reducing $O_2^-$ generation in VSMCs.

### 3.5 Superoxide scavenging improved vascular reactivity in ET-1 treated aortic rings

To determine if $O_2^-$ and NO were reacting to form $ONOO^-$, resulting in decreased vascular function, vessels were incubated with $ONOO^-$ decomposition catalyst FeTPPS (FT, 10 μM) for 30min prior to CRCs. Interestingly, ET-1 treated aorta incubated with FeTPPS exhibited a marked decrease in ACh sensitivity ($EC_{50}$ −6.19 ± 0.09) and maximal relaxation response ($R_{max}$ 52.0% ± 2.4) (Fig. 5A). In addition, incubation with FeTPPS in ET-1+Gtn (Fig. 5B) resulted in no significant changes while FeTPPS incubation in ET-1+AS (Fig. 5C) reduced ACh-mediated relaxation responses ($R_{max}$ 56.1% ± 3.85 *vs.* 83.2% ± 2.85, P<0.0001).

In order to determine if the prevention or reduction of $O_2^-$ would induce similar effects as AS, tempol (T, 1mM) was used. Vessels co-incubated with Gtn and tempol exhibited no changes in ACh-mediated relaxation responses, as compared to ET-1+Gtn (Fig. 6A) ($R_{max}$ 71.2% ± 3.6 *vs.* 71.3% ± 2.6). We also observed no additional effects of tempol whin ET-1 + AS co-incubated vessels (Fig. 6B) ($R_{max}$ 75.2% ± 2.4 *vs.* 83.2% ± 2.9). However, ET-1

treated vessels (Fig. 6C) incubated with tempol alone exhibited a significant improvement in the maximal ACh-mediated relaxation responses (*Rmax* 82.6% ± 2.6 *vs.* 58.3% ± 3.2, P<0.0001), with no changes in ACh sensitivity. Taken together, these data suggest that reduction of ROS/$O_2^-$ does enhance relaxation responses in ET-1-damaged aorta.

## 4 Discussion

The use of nitrosovasodilators, such as Gtn, for the treatment of cardiovascular conditions is well established and has been used for decades (Boden *et al.*, 2015; Investigators *et al.*, 2015; Woodhouse *et al.*, 2015; Krishnan *et al.*, 2016 ). Although nitrosovasodilators mediate rapid and pronounced vasodilation, they are limited clinically due to the development of tolerance and reported increases in ROS generation (Sage *et al.*, 2000; Schulz *et al.*, 2002; Munzel *et al.*, 2005). The redox variant of NO, HNO, has recently become the subject of increased investigation and produces vasodilatation in multiple vascular beds (Favaloro & Kemp -Harper, 2007; Irvine *et al.*, 2007; Martin, 2009 ). Data from our laboratory demonstrate that aorta from AngII-hypertensive mice exhibit a preserved vasodilatory response to AS, even with significant endothelial dysfunction (Wynne *et al.*, 2012). Moreover, researchers have clearly established that AS does not induce vascular tolerance, using *ex vivo* preparations, in *in vivo* chronic models and in human vessels (Irvine *et al.*, 2007; Irvine *et al.*, 2010; Andrews *et al.*, 2015). However, there are no data demonstrating whether the use of HNO, a more stable gas, would also decrease the vascular wall damage seen with NO donors. In this study we proposed that the NO donor, Gtn, would exacerbate ET-1-mediated vascular dysfunction while the HNO donor, AS would improve vascular function.

In this study, we used an ET-1-induced model of endothelial dysfunction, as studies have demonstrated that ET-1 induces hypertension, with endothelial dysfunction and increased ROS generation (Savoia & Schiffrin, 2006; 2007; Schiffrin, 2007; Wynne *et al.*, 2009; Pollock & Pollock, 2011; Rodrigo *et al.*, 2011; Idris-Khodja *et al.*, 2016; Leurgans *et al.*, 2016). Our data demonstrate that aorta incubated overnight with ET-1 induces increased Phe-mediated contraction and a decrease in ACh-mediated relaxation. The underlying ROS production known to be present with ET-1 was a perfect model for these studies. We found that Gtn improved vascular function, although relaxation and contraction responses remained significantly different from vehicle-treated aorta. Although there was some benefit with Gtn treatment, the HNO donor (AS), completely reversed the ET-1-induced vascular and endothelial dysfunction. We acknowledge the limitations in our current experimental model of overnight *ex vivo* incubations. Further experiments will be needed to determine how AS will affect vascular function in a chronic, *in vivo*, model.

Published studies show the limited efficacy of organic nitrates, including nitrate tolerance and cross-tolerance, yet the mechanism still not clearly defined (Daiber *et al.*, 2009; Munzel *et al.*, 2014). Literature suggests that diminished bioconversion of Gtn, neurohumoral adaptations, and vascular oxidative stress may be a cause (Frame *et al.*, 2002; Fukatsu *et al.*, 2007; Irvine *et al.*, 2007; Szocs *et al.*, 2007; Daiber *et al.*, 2009; Kosmicki, 2009). Although the process is likely multifactorial, oxidative stress has emerged as a principal factor (Munzel T, 1995; Szocs *et al.*, 2007). Gtn treatment increases ROS generation in platelets,

endothelial and smooth muscle cells, as well as in experimental animal models (Dikalov *et al.*, 1998; Szocs *et al.*, 2007). Other studies demonstrated that patients treated with Gtn for 24 hours exhibited endothelial dysfunction and increased ROS production, in addition to nitrate tolerance (Sage *et al.*, 2000; Schulz *et al.*, 2002). Here we report that Gtn does not exacerbate ET-1 mediate vascular dysfunction, but slightly improves Phe- mediated contractile and ACh-mediated relaxation responses. However, there are questions as to whether more doses or increased incubation times would alter the results we observed.

There are limited studies demonstrating whether HNO treatment can reduce ROS generation in VSMCs. Using DHE staining for ROS generation, we observed an increase in ET - 1 - mediated ROS production in primary VSMCs. ROS is a known contributor to vascular dysfunction, and is present during hypertension and other cardiovascular disease (Gonzalez *et al.*, 2014; Brito *et al.*, 2015). We hypothesized that Gtn, or NO as a free radical gas, may interact with the ET-1-induced ROS present, further increasing the oxidant environment. However, we did not observe an increase following Gtn treatment. Importantly, DHE staining revealed a reduction in the ET-1-induced increases in ROS generation with AS co-incubation.

To investigate a possible mechanism for the improvement following AS treatment, functional studies were performed in the presence of the tempol. ET-1 incubated vessels treated with tempol did exhibit an increase in maximal relaxation responses. Interestingly, relaxation was not improved in vessels co-incubated Gtn and tempol. *In vitro*, AS incubation also reduced ROS generation in ET-1-treated VSMCs. Together, these data suggest that AS may be exerting vasoprotective effects through a decrease ROS generation. However, ROS generation alone does not seem entirely responsible for the effect of AS treatment.

To determine if the increase in $O_2^-$ production may be reacting with Gtn to produce increased levels of peroxynitrite ($ONOO^-$), CRCs were performed in vessels incubated with the $ONOO^-$ degradation catalyst, FeTPPS. ET-1 treated aorta with FeTPPS worsened the endothelial dysfunction. These data were not completely surprising. In general, ROS are beneficial if within physiological parameters, yet damaging if excessive (Szewczyk *et al.*, 2015). There are also data suggesting a regulatory role for basal $ONOO^-$ levels; thus the quantity of $ONOO^-$ necessary to maintain proper vascular function may have been compromised in this study (Villa *et al.*, 1994; Li *et al.*, 2004; Maneen *et al.*, 2006; Maneen & Cipolla, 2007).

We also observed no further improvements in relaxation responses when ET-1 and Gtn co-incubated vessels were treated with FeTPPS. This suggests that in our model, addition of Gtn does not seem to induce increase in $ONOO^-$ levels. Furthermore, we can only conclude that ET-1 induced $ONOO^-$ production may not be a primary cause for the vascular dysfunction present in this model. 5 Conclusions

Overall, our data demonstrate that AS, the HNO donor, can confer vasoprotection in aorta with ET-1 mediated vascular and endothelial dysfunction. Our data suggest that the mechanism may be via a reduction in VSMC ROS and/or $O2^-$ production; however, other

mechanisms are also likely involved. The use of HNO donors may prove to be a beneficial therapeutic alternative to organic nitrates.

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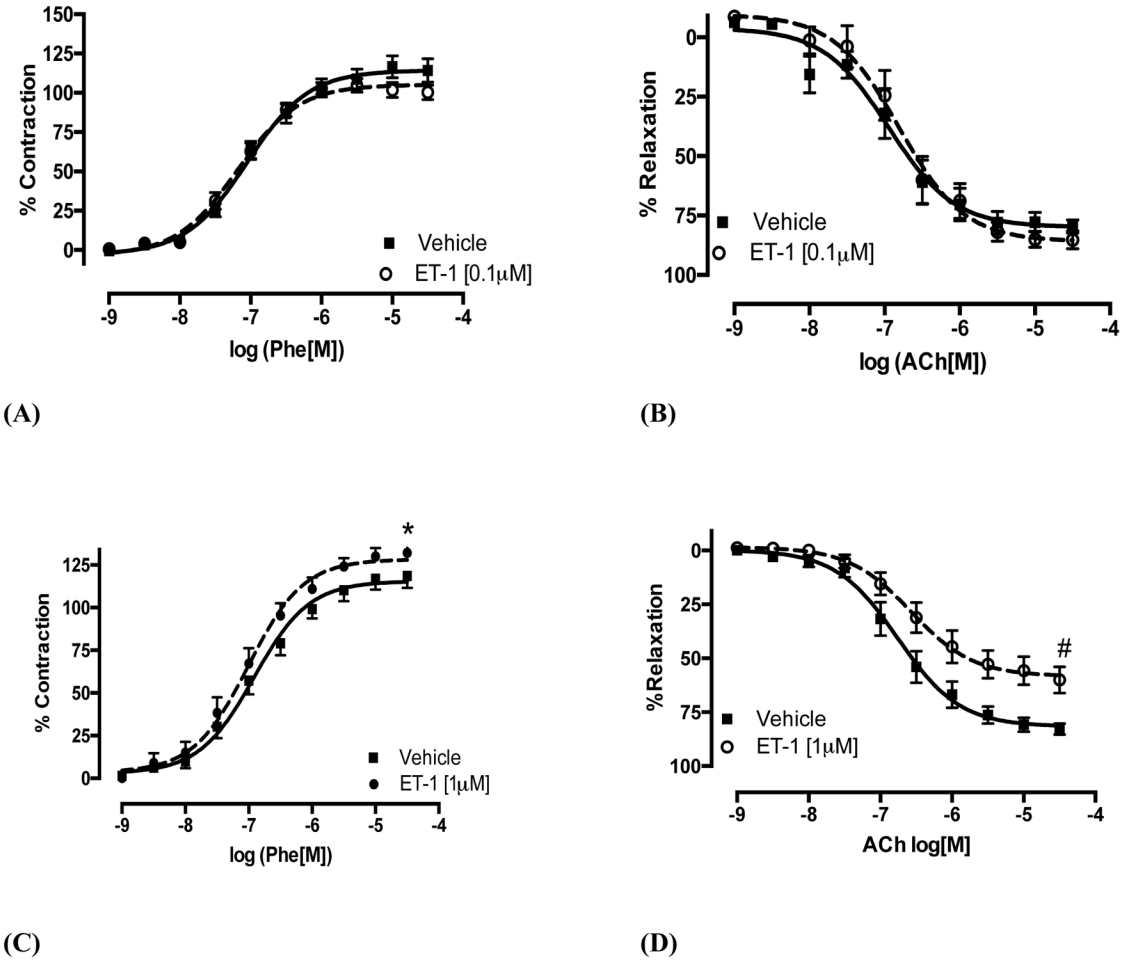
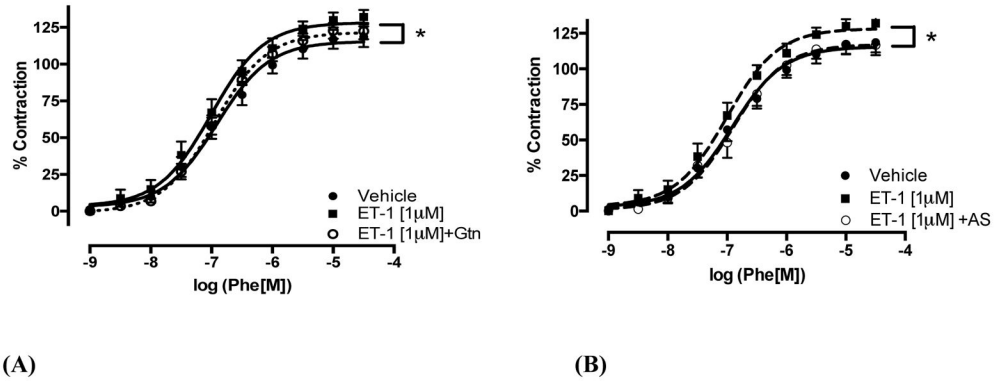



**Figure 1. Aorta incubated with endothelin-1 exhibit vascular dysfunction**

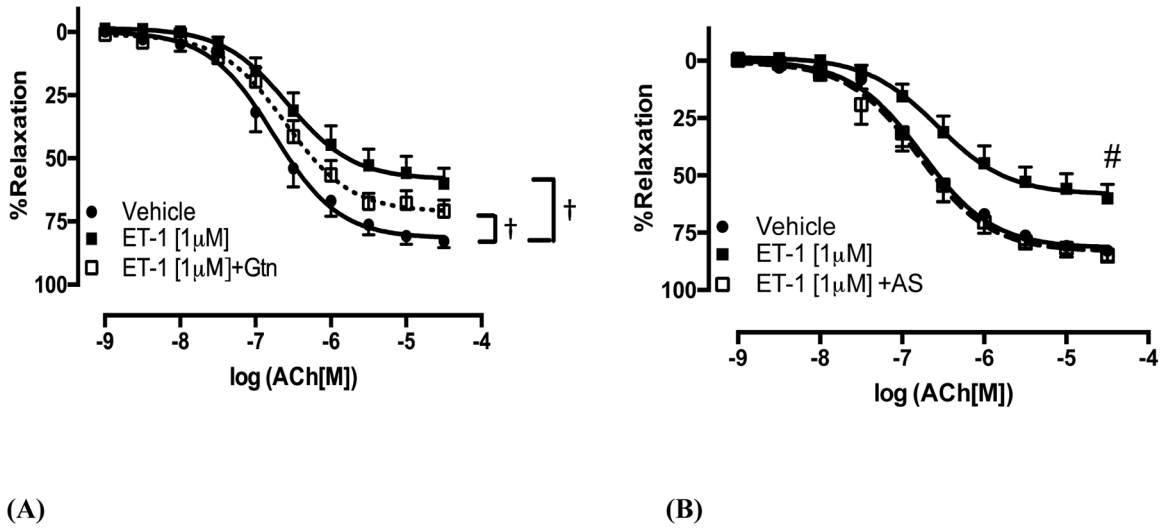
Concentration response curves to Phe performed in aorta incubated with [0.1 μM] (A) and [1 μM] (C) ET-1. Concentration response curves to ACh were performed in aorta incubated with [0.1μM] (B) and [1 μM] (D) ET-1 and pre-contracted with Phe (10 μM). Relaxation responses were calculated relative to the maximal contraction elicited by KCl. Data are represented as mean ± S.E.M.; n=4–9. *P<0.01, #P<0.0001, ET-1 *vs.* vehicle.




**(A)** **(B)**

**Figure 2. Incubation with AS reverses ET-1 induced vascular dysfunction in mouse aorta**

Concentration response curves to Phe performed in intact aorta incubated with either endothelin-1 (ET-1), ET-1 plus nitroglycerin (ET-1+Gtn [1 μM]) (A) or endothelin-1 plus Angeli's Salt (ET-1+AS [1 μM]) (B). Data represented as mean ± S.E.M., n=9–11, *P<0.01, ET-1 *vs.* vehicle, * P<0.01, ET-1+AS *vs.* ET-1.




**(A)** **(B)**

**Figure 3. Incubation with the HNO donor, Angeli's Salt, reverses ET-1 mediated endothelial dysfunction**

Concentration response curves to ACh were performed in Phe (10 μM) aorta. (A). Relaxation responses to ACh were assessed in intact aorta incubated with vehicle, ET-1 [1 μM] or ET- 1+Gtn [1 μM]. (B) Relaxation responses to ACh were assessed in intact aorta incubated with vehicle, ET-1 [1 μM] or ET-1+AS [1 μM]. Relaxation responses were calculated relative to the maximal contraction elicited by Phe. Data are represented as mean ± S.E.M.; n=5–10. †P<0.05, ET+Gtn *vs.* vehicle, #P<0.001, ET-1 *vs.* vehicle.

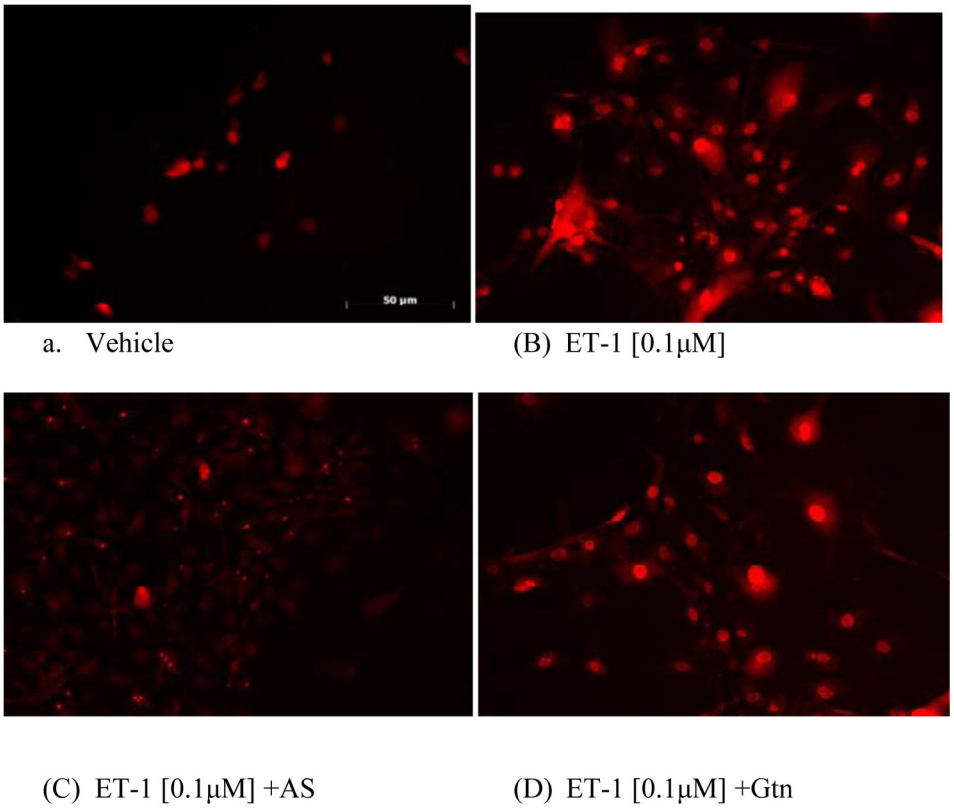
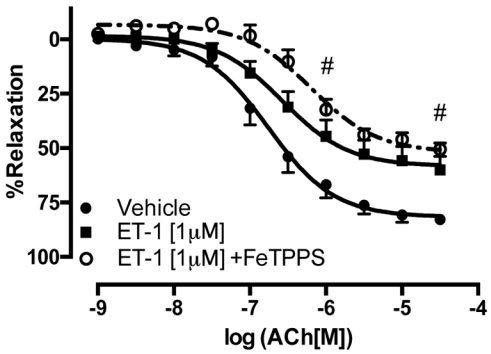
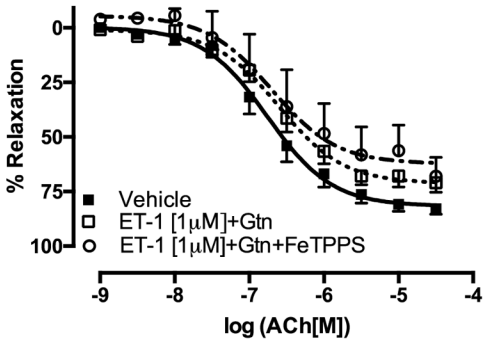



**Figure 4. Endothelin 1 treatment increases superoxide generation, which is decreased when co-incubated with Angeli's Salt**

Dihydroethidium (DHE) staining for superoxide production in a primary cell culture of aortic vascular smooth muscle cells (VSMC) Cells were treated with vehicle (A), endothelin-1 (ET-1 [0.1 µM]) (B), ET-1 plus Angeli's Salt (ET-1+AS [10 µM]) (C) or ET-1 plus nitroglycerin (ET-1+Gtn [10 µM]) (D) for 2 minutes, and then probed for superoxide production using DHE.




(A)

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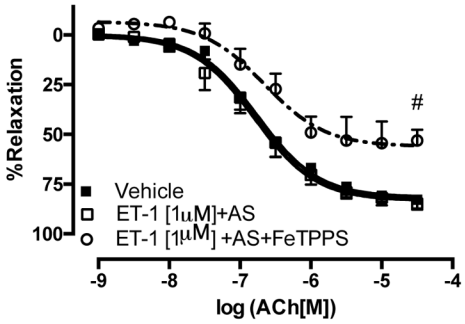


(C)

**Figure 5. Incubation with the ONOO⁻ degradation catalyst, FeTPPS, reduces ACh-mediated relaxation responses**

Concentration response curves to ACh were performed in Phe (10 μM) contracted aorta. (A). Relaxation responses to ACh were assessed in intact aorta incubated with vehicle, ET-1 [1 μM] or ET-1+FeTPPS [10 μM]. (B). Relaxation responses to ACh were assessed in intact aorta incubated with vehicle, ET-1 [1 μM], ET-1+Gtn [1 μM] or ET-1+Gtn+FeTPPS [10 μM]. (C). Relaxation responses to ACh were assessed in intact aorta incubated with vehicle, ET-1 [1 μM], ET-1+AS [1 μM] or ET-1+AS+FeTPPS [10 μM]. Relaxation responses were calculated relative to the maximal contraction elicited by Phe. Data are represented as mean ± S.E.M.; n=4–10. #P<0.0001, ET-1+FeTPPS *vs.* ET-1, #P<0.0001, ET-1+AS+FeTPPS *vs.* ET-1+AS.

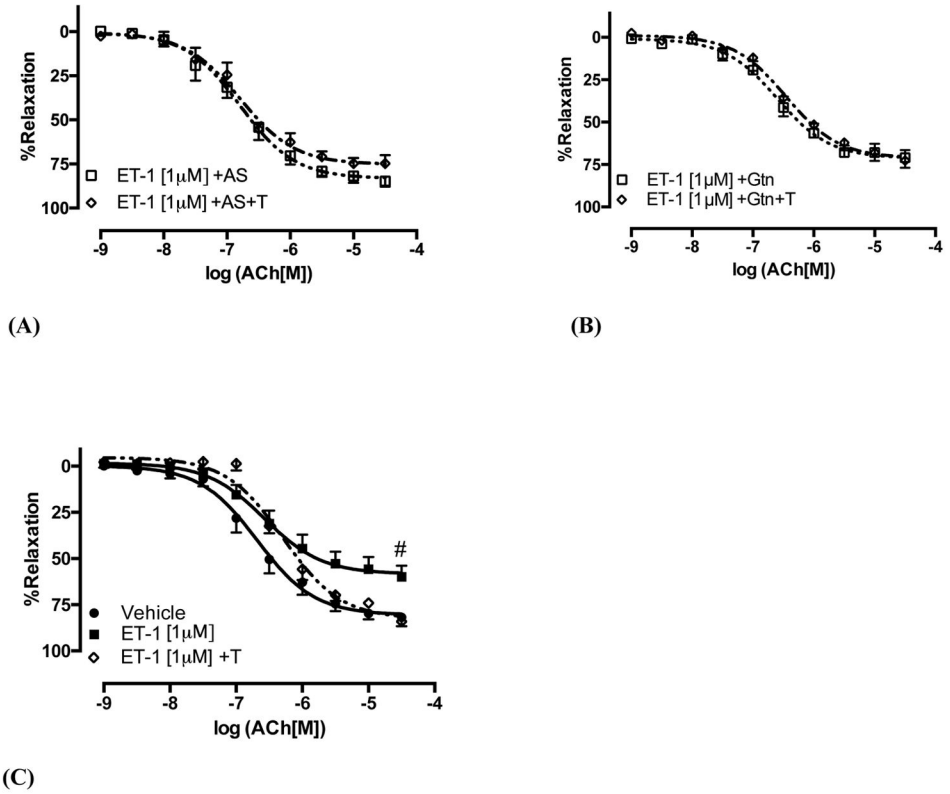


**Figure 6. Incubation with tempol does not improve ACh-mediated relaxation responses in Gtn and ET-1 treated aorta**

Concentration response curves to ACh were performed in Phe (10 μM) contracted aorta incubated overnight with endothelin-1. (A). Relaxation responses to ACh were assessed in intact aorta incubated with the HNO donor, AS [1 μM], and with or without tempol [1 mM] (A), with the NO donor, Gtn [1 μM], and with or without tempol (B) and with or without tempol (C). Relaxation responses were calculated relative to the maximal contraction elicited by Phe. Data are represented as mean ± S.E.M.; n=5–10. #P<0.0001, Et-1+T *vs.* vehicle.