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RESEARCH PAPER

The concomitant coronary vasodilator and positive inotropic actions of the nitroxyl donor Angeli's salt in the intact rat heart: contribution of soluble guanylyl cyclase-dependent and -independent mechanisms

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BACKGROUND AND PURPOSE

The NO redox sibling nitroxyl (HNO) elicits soluble guanylyl cyclase (sGC)-dependent vasodilatation. HNO has high reactivity with thiols, which is attributed with HNO-enhanced left ventricular (LV) function. Here, we tested the hypothesis that the concomitant vasodilatation and inotropic actions induced by a HNO donor, Angeli's salt (sodium trioxodinitrate), were sGC-dependent and sGC-independent respectively.

EXPERIMENTAL APPROACH

Haemodynamic responses to Angeli's salt (10 pmol–10 μmol), alone and in the presence of scavengers of HNO (L-cysteine, 4 mM) or of NO [hydroxocobalamin (HXC), 100 μM] or a selective inhibitor of sGC [1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), 10 μM], a CGRP receptor antagonist ($CGRP_{8–37}$, 0.1 μM) or a blocker of voltage-dependent potassium channels [4-aminopyridine (4-AP), 1 mM] were determined in isolated hearts from male rats.

KEY RESULTS

Angeli's salt elicited concomitant, dose-dependent increases in coronary flow and LV systolic and diastolic function. Both L-cysteine and ODQ shifted (but did not abolish) the dose–response curve of each of these effects to the right, implying contributions from HNO and sGC in both the vasodilator and inotropic actions. In contrast, neither HXC, $CGRP_{8–37}$ nor 4-AP affected these actions.

CONCLUSIONS AND IMPLICATIONS

Both vasodilator and inotropic actions of the HNO donor Angeli's salt were mediated in part by sGC-dependent mechanisms, representing the first evidence that sGC contributes to the inotropic and lusitropic action of HNO in the intact heart. Thus, HNO acutely enhances LV contraction and relaxation, while concomitantly unloading the heart, potentially beneficial actions in failing hearts.

Abbreviations

4-AP, 4-aminopyridine; CGRP, calcitonin gene-related peptide; HNO, nitroxyl; HXC, hydroxocobalamin; LV ± dP/dt, first derivatives of LV pressure; LV, left ventricle; LVDP, LV developed pressure; LVEDP, LV end-diastolic pressure; LVSP, LV systolic pressure; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; RyR, ryanodine receptor; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; sGC, soluble guanylyl cyclase; U46619, 9,11-dideoxy-9α,11α-methanoepoxy PG $F_{2\alpha}$

Introduction

Nitroxyl (HNO) is the one-electron reduced and protonated redox sibling of NO. Its therapeutic potential was first suggested when the effects of the anti-alcoholism drug, cyanamide, were found to be attributable to the release of HNO (Nagasawa *et al.*, 1990). HNO is a transient species, readily undergoing dimerization to form hyponitrous acid with subsequent decomposition into nitrous acid and water (Dumond and King, 2011). Therefore, HNO donors are utilized in pharmacological studies, often with the prototypical HNO donor, sodium trioxodinitrate ($Na_2N_2O_3$) or Angeli's salt (Miranda *et al.*, 2005a). In recent years, HNO has emerged as a novel regulator of cardiovascular function, with vasoprotective (vasodilator, anti-aggregatory) and cardioprotective (i.e. positive inotrope, anti-hypertrophic) properties (Irvine *et al.*, 2008; Bullen *et al.*, 2011; Tocchetti *et al.*, 2011; Lin *et al.*, 2012). Interestingly, HNO serves as a positive cardiac inotrope and is protective in an experimental model of heart failure (Paolocci *et al.*, 2001; 2003), an action not shared by NO. HNO also exhibits antihypertrophic actions in the myocardium, an effect mediated via inhibition of NADPH oxidase-derived superoxide generation (Lin *et al.*, 2012) and attenuation of the activity of a pro-hypertrophic signalling pathway, p38 MAPK (Wanstall *et al.*, 2001; Favaloro and Kemp-Harper, 2009; Lin *et al.*, 2012). As such, recent interest in the therapeutic potential of HNO has focused on cardiovascular disorders, such as vascular dysfunction, cardiac dysfunction, cardiac remodelling and heart failure (Irvine *et al.*, 2007; 2008; Ritchie *et al.*, 2009; El-Armouche *et al.*, 2010; Bullen *et al.*, 2011; Ding *et al.*, 2011; Yuill *et al.*, 2011; Lin *et al.*, 2012).

In contrast to NO, HNO possesses several unique pharmacological properties. Firstly, HNO is resistant to scavenging by the reactive oxygen species (ROS), superoxide (levels of which are commonly elevated in cardiovascular pathologies), whereas NO is highly reactive with superoxide, forming a second ROS, peroxynitrite (Miranda *et al.*, 2002). In addition, tolerance does not develop to vasodilator actions of HNO , a favourable difference from traditional clinically used nitrovasodilators (Irvine *et al.*, 2007; 2011). HNO reacts readily with metal centres of proteins such as iron-containing haem in oxymyoglobin and soluble guanylyl cyclase (sGC; nomenclature follows Alexander *et al.*, 2013a, and in contrast to NO, preferentially targets ferric (Fe^{3+}) rather than ferrous (Fe^{2+}) haem groups and thus may activate these proteins when their iron is in the oxidized state (Miranda *et al.*, 2003). Furthermore, HNO (but not NO) is highly thiolphilic, directly targeting thiol-containing proteins. Such an action of HNO underlies many of its unique properties in the CVS (Fukuto and Carrington, 2011). Indeed, the interaction of HNO with cysteine residues on Ca^{2+}-cycling proteins, that is ryanodine receptors (RyR) and the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) on the sarcoplasmic reticulum of cardiomyocytes leads to enhanced cardiac contractility (Fukuto and Carrington, 2011; Tocchetti *et al.*, 2011). The therapeutic advantages of HNO over NO are likely to be more obvious in settings where NO are exposed to significant levels of ROS which would limit the bioavailability of NO but not of HNO (Irvine *et al.*, 2008; Ritchie *et al.*, 2009; Bullen *et al.*, 2011), and/or where specific HNO interactions with key cysteine residues confers protection, as with SERCA, a property not shared by NO (Fukuto and Carrington, 2011; Tocchetti *et al.*, 2011). It is anticipated that HNO donors would thus be comparable with NO donors in other settings such as via inhalation for pulmonary hypertension (De Witt *et al.*, 2001). However, the distinct pharmacological profile of HNO suggests that it offers favourable therapeutic advantages over its free radical sibling, NO, in vascular dysfunction, cardiac dysfunction, cardiac remodelling and heart failure.

NO predominantly utilizes sGC/cGMP to mediate vasodilatation and suppression of cardiomyocyte hypertrophy. In contrast, HNO has been shown to signal via both sGC-dependent and -independent pathways in the vasculature and myocardium. The mechanism of vasodilator actions of the HNO donor, Angeli's salt are largely sGC-dependent (Fukuto *et al.*, 1992; Ellis *et al.*, 2000; Irvine *et al.*, 2003; 2007; Favaloro and Kemp-Harper, 2007; 2009), with a smaller contribution from K^+ channels (K_v and K_{ATP}; nomenclature follows Alexander *et al.*, 2013b) and calcitonin gene-related peptide (CGRP) evident in the resistance (Irvine *et al.*, 2003; Favaloro and Kemp-Harper, 2007) and coronary vasculature (Favaloro and Kemp-Harper, 2007) respectively. These vasodilator properties are evident in both large (e.g. aorta) as well as smaller vessels such as in rodent-isolated thoracic aorta, rodent-isolated mesenteric arteries or isolated hearts *in vitro* (Ellis *et al.*, 2000; Wanstall *et al.*, 2001; Irvine *et al.*, 2003; Favaloro and Kemp-Harper, 2007). The antihypertrophic actions of HNO donors in isolated cardiomyocytes are similarly cGMP dependent (Lin *et al.*, 2012), whereas the superoxide-suppressing actions have been variably reported as cGMP dependent (Lin *et al.*, 2012) or cGMP independent

(Bullen *et al.*, 2011), in cardiomyocytes and arteries respectively. In contrast, the acute enhancement of cardiac contractility elicited by HNO donors in the intact heart have been regarded as cGMP-independent, as no detectable changes in plasma cGMP content were observed *in vivo* (Paolocci *et al.*, 2003). These studies in the intact heart have not however investigated HNO actions on cardiac contractility in the presence of cGMP inhibition. Of note, cardiac contractility is acutely enhanced by HNO donors in failing and normal hearts to an equivalent extent (Paolocci *et al.*, 2001; 2003).

The vasodilator and cardiac inotropic effects of HNO donors have been commonly attributed to cGMP-dependent and -independent mechanisms respectively. The concomitant effects of an HNO donor on vascular and cardiac function, and the net mechanism(s) of these actions, however, remain unresolved. The objective of the present study was to thus test the hypothesis that the concomitant vasodilator and inotropic actions induced by the HNO donor, Angeli's salt, are sGC-dependent and sGC-independent, respectively, in the rat isolated heart.

Methods

This investigation complies with the National Health and Medical Research Council of Australia code of practice for the care and use of animals for scientific purposes. All the procedures involved in this project were approved by The Alfred Medical Research Educational Precinct (AMREP) Animal Ethics Committee. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 53 animals were used in the experiments described here.

Hearts isolated from male Sprague-Dawley rats (350–450 g) n = 53 under ketamine-xylazine anaesthesia (100 and 12 mg·kg^{-1} i.p., respectively) were Langendorff perfused with Krebs buffer (pH 7.4, composition in mM: NaCl 118, KCl 4.7, $MgSO_4.7H_2O$ 1.18, KH_2PO_4 1.2, EDTA 0.5, $CaCl_2$ 1.75, $NaHCO_3$ 25.0 and D-glucose 11, bubbled with 95% O_2 and 5% CO_2 at 37°C) under constant pressure, using the ADInstruments Langendorff System® (ADInstruments Pty, Ltd., Bella Vista, Australia). The STH Pump Controller (ADInstruments Pty, Ltd.) continuously detected coronary flow, in addition to maintaining a constant perfusion pressure (set to achieve coronary flow at baseline of 10 mL·min^{-1}). A fluid-filled balloon was positioned in the left ventricle (LV) for continuous monitoring of LV systolic pressure (LVSP), LV end-diastolic pressure (LVEDP), LV developed pressure (LVDP) and the first derivatives of LV pressure (LV ± dP/dt). The ADInstruments PowerLab data acquisition system acquired these variables, as well as coronary perfusion pressure, coronary flow and heart rate, throughout the protocol.

After 30 min equilibration, the thromboxane A_2 mimetic U46619 (9,11-dideoxy-9α,11α-methanoepoxy PG $F_{2\alpha}$, 3 μM) was continuously infused into the aorta via a syringe infusion pump (0.1–2.5 mL·min^{-1}), via a port just above the aortic cannula, to preconstrict the coronary vasculature to give a ~50% reduction in baseline coronary flow-rate (i.e. from ~10 to ~5 mL·min^{-1}). A single bolus dose of NaOH (10 mM, vehicle for Angeli's salt) was then administered to the heart via an injection port just above the aortic cannula, followed by a serial dose–response curve to Angeli's salt (10 pmol–10 μmol), constructed by administering bolus doses of the HNO donor to the heart via a second injection port just above the aortic cannula, in increasing doses 1 min apart. All parameters of contractile function had returned to baseline levels achieved with U46619 preconstriction. For coronary flow, this had either returned to baseline levels or had stabilized to a plateau, prior to the addition of the next bolus dose of Angeli's salt. In a parallel series of experiments, hearts were administered serial bolus doses of the equivalent volume of 10 mM NaOH, as a vehicle control.

Subsequent experiments were performed to examine the mechanism of the haemodynamic effects of Angel's salt in the intact heart, in which dose–response curves to Angeli's salt were performed in the presence of various selective pharmacological inhibitors, added to the reservoir of Krebs perfusion buffer. The relative contribution of HNO and NO to the actions of Angeli's salt was investigated in the presence of the HNO scavenger L-cysteine (4 mM), the NO scavenger hydroxocobalamin (HXC, 0.1 mM) or the thiol DTT (100 μM). Parallel experiments utilized the sGC inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 μM), the CGRP receptor antagonist $CGRP_{8-37}$ (0.1 μM), or the K_v channel blocker 4-aminopyridine (4-AP, 1 mM) to further examine the mechanisms of Angeli's salt actions. For comparison, dose–response curves to the pure NO donor diethylamine-NONOate (DEA-NO) were also performed.

Data analysis

Changes in all haemodynamic variables induced by each vasodilator dose were measured as the change (Δ) in each response relative to that elicited by the vehicle control (10 mM NaOH for Angeli's salt). All results were expressed as group mean ± SEM, with the number of independent experiments denoted as 'n'. Data analysis was performed using Graphpad Prism® (version 5.0, La Jolla, CA, USA). Vasorelaxant responses were fitted to a sigmoidal logistic equation, to derive the pEC_{50} (vasodilator dose eliciting 50% maximal response, expressed as –log mol) and R_{max} (maximal vasodilator response). The coefficient of variation, R^2, for vasodilator responses was consistently >0.8 in all hearts studied. Dose–response curves to Angeli's salt in the absence and presence of each pharmacological inhibitor were compared on two-way ANOVA, with the Bonferroni *post hoc* test. Baseline haemodynamic variables and the pEC_{50} and R_{max} for Angeli's salt in the absence and presence of various inhibitors, were analysed using one-way ANOVA with Dunnett's *post hoc* test for multiple comparisons. In all cases, $P < 0.05$ was considered statistically significant.

Materials

Angeli's salt, U46619, ODQ and DEA-NO were obtained from Cayman Chemical Company (Ann Arbor, MI, USA). All other reagents were purchased from Sigma Aldrich (St. Louis, MO, USA).

All stock and working solutions of Angeli's salt or DEA-NO were prepared fresh daily in 10 mM NaOH, and kept on ice until required. Aliquots of U46619 (1 mM in 100% ethanol) were stored at –20°C, and were further diluted on

the day of use in Krebs buffer. Stock solutions of ODQ were prepared fresh daily (1 mM in 100% ethanol) with further dilution in Krebs buffer. Aliquots of $CGRP_{8–37}$ (0.1 mM in distilled water) were stored at –20°C, with subsequent dilution in Krebs buffer on the day of use. L-cysteine, HXC, 4-AP and isoprenaline solutions were all prepared in Krebs buffer.

Results

Angeli's salt elicits HNO/sGC-dependent vasodilator actions in the whole heart

The baseline characteristics of all buffer-perfused rat hearts used in this study, at the end of equilibration, prior to commencement of any interventions, are shown in Table 1. Haemodynamic variables after the commencement of infusion with pharmacological inhibitors and U46619 preconstriction are also shown. Baseline coronary flow prior to commencement of any interventions, as well as that immediately following U46619 preconstriction, was generally comparable across all experimental groups. A representative recording of all haemodynamic parameters on construction of a dose–response curve to Angeli's salt is shown in Figure 1. In the presence of U46619 preconstriction, the HNO donor, Angeli's salt (10 pmol–10 μmol) elicited a dose-dependent vasodilatation, with pEC_{50} (–log mol) of 8.55 ± 0.24 and R_{max} ($mL{\cdot}min^{-1}$) of 5.14 ± 0.69 (Table 2, Figure 2A). Significant increases in coronary flow were evident with doses of Angeli's salt ≥ 10 nmol. The selective HNO scavenger L-cysteine (4 mM, n = 6) caused a rightward shift in the dose–response curve of the vasodilator actions of Angeli's salt, with significant reductions in both the pEC_{50} and R_{max}. In contrast, the selective NO scavenger HXC (100 μM, n = 5) not only failed to blunt the vasodilator effect of Angeli's salt, but actually tended to enhance the vasorelaxant effect of Angeli's salt (Figure 2A). The thiol DTT (100 μM, n = 5) did not affect the dose–response curve for Angeli's salt.

As shown in Figure 2B, the selective sGC inhibitor, ODQ (10 μM, n = 6) also caused a rightward shift in the dose–response curve of the vasodilator actions of Angeli's salt, with significant reduction in the pEC_{50} (Figure 2B). The R_{max} to Angeli's salt was not significantly affected by ODQ (Table 2). Both the selective CGRP receptor antagonist $CGRP_{8–37}$ (0.1 μM, n = 5) and the K_v channel inhibitor 4-AP (1 mM, n = 5) failed to affect the vasodilator actions of Angeli's salt (Figure 2B). Furthermore, serial bolus doses of 10 mM NaOH vehicle failed to elicit significant haemodynamic response (Figure 2B). As shown in Table 1, neither L-cysteine, HXC alone nor other pharmacological inhibitors had any significant effect on basal vascular function, although DTT tended to enhance coronary flow and heart rate. For comparison, the NO donor DEA-NO (10 pmol–10 μmol) elicited a dose-dependent vasodilatation which was also shifted rightwards by HXC (both n = 5, Figure 2C and Table 2).

Relative contribution of HNO/sGC (but not NO) to the inotropic effects of Angeli's salt

The vasorelaxant effect of Angeli's salt was accompanied by concomitant dose-dependent enhancement of myocardial inotropic function. Significant increases in LVSP (Figure 3A), LVDP (Figure 4A) and LV+dP/dt (Figure 5A), parameters of cardiac contractile function, were evident from ≥10 nmol Angeli's salt. Both L-cysteine and DTT (but not HXC) markedly blunted the effects of Angeli's salt on each of LVSP (Figure 3A), LVDP (Figure 4A) and LV+dP/dt (Figure 5A). Maximal increases in parameters of cardiac contractility induced by Angeli's salt were suppressed by ~60% in the presence of L-cysteine. Angeli's salt also tended to increase heart rate at the highest dose studied (by 59 ± 7 beats per min), this was unaffected by either L-cysteine or HXC. Further, there was no evidence of arrhythmic events observed at any time. Inhibition of sGC with ODQ also markedly blunted (but did not abolish) the positive inotropic effect of Angeli's salt, on each of LVSP (Figure 3B), LVDP (Figure 4B) and LV+dP/dt (Figure 5B), by ~50%. In contrast, inhibition of CGRP receptors or K_v channels failed to suppress the positive inotropic actions of Angeli's salt. Interestingly, the LV+dP/dt response tended to be exaggerated by 4-AP. For comparison, the NO donor DEA-NO elicited comparatively modest increases in LVSP (Figure 3C), LVDP (Figure 4C) and LV+dP/dt (Figure 5C), evident at higher doses of DEA-NO, which were insensitive to HXC (both n = 5). None of these inhibitors alone (L-cysteine, DTT, HXC, ODQ, $CGRP_{8–37}$ and 4-AP) affected these parameters of contractile function prior to the construction of the dose–response curve to Angeli's salt, as shown in Table 1).

Contribution of HNO/sGC to the effects of Angeli's salt on cardiac relaxation

Angeli's salt elicited dose-dependent enhancement of myocardial lusitropic function, with progressive reduction in LVEDP (Figure 6) and potentiation of LV-dP/dt (Figure 7). These actions were blunted by L-cysteine, DTT and ODQ (Angeli's salt enhancement of LV-dP/dt was particularly sensitive to these inhibitors), but not by HXC or 4-AP (both of which tended to enhance the Angeli's salt effect). $CGRP_{8–37}$ was without effect on the cardiac relaxation response to Angeli's salt (Figures 6 and 7). None of these inhibitors alone (L-cysteine, DTT, HXC, ODQ, $CGRP_{8–37}$ and 4-AP) affected these parameters of cardiac relaxation alone, prior to the construction of the dose–response curve to Angeli's salt (Table 1).

Discussion

The key findings of the present study are that the HNO donor, Angeli's salt, elicits concomitant coronary vasodilator, inotropic and lusitropic actions in the intact rat heart, all of which are mediated by L-cysteine-sensitive, HNO-dependent mechanisms, with a significant contribution mediated via sGC. There appeared to be no role for extracellular oxidation of HNO to NO, or for CGRP receptors or K_v channels in the haemodynamic responses to Angeli's salt. These results are the first evidence that sGC may contribute, at least in part, to the inotropic and/or lusitropic action of HNO in the intact heart.

Our observations here that Angeli's salt induces HNO/sGC-mediated, dose-dependent vasodilatation in the intact rat heart are consistent with previous reports in isolated large

Table 1

Characteristics of all hearts in each experimental group, at each of baseline (at the end of the equilibration period), after pretreatment with each pharmacological inhibitor alone, and then the subsequent commencement of U46619 infusion (prior to the addition of AS or DEA-NO, shown as mean ± SEM)

		Haemodynamic Variable Prior to Vasodilator Dose–response Curve								
Experimental group	**Timepoint**	**Coronary flow ($mL \cdot min^{-1}$)**	**Perfusion pressure (mmHg)**	**Heart rate ($beats \cdot min^{-1}$)**	**LVSP (mmHg)**	**LVDP (mmHg)**	**LVEDP (mmHg)**	**LV+dP/dt ($mmHg \cdot s^{-1}$)**	**LV-dP/dt ($mmHg \cdot s^{-1}$)**	***n***
AS	Baseline	10.6 ± 0.4	44.8 ± 1.3	242 ± 21	75.6 ± 6.2	76.7 ± 7.0	–1.1 ± 1.8	1963 ± 94	–1855 ± 101	8
	U46619	5.7 ± 0.5	51.1 ± 2.2	217 ± 18	55.9 ± 6.9	56.4 ± 8.4	0.6 ± 2.0	1721 ± 165	–1617 ± 185	
AS + L-cysteine	Baseline	10.2 ± 1.0	43.0 ± 1.6	268 ± 19	57.8 ± 6.0	52.6 ± 6.0*	5.2 ± 1.3*	1687 ± 105	–1435 ± 83	6
	L-cysteine	11.7 ± 1.1	42.3 ± 3.5	201 ± 23	57.5 ± 7.3	52.1 ± 6.6	5.4 ± 1.2	1635 ± 149	–1366 ± 120	
	U46619	7.2 ± 0.8	49.2 ± 3.5	187 ± 18	51.8 ± 5.9	47.1 ± 6.1	4.7 ± 0.8	1533 ± 98	–1280 ± 82	
AS + HXC	Baseline	10.5 ± 0.4	45.2 ± 1.5	296 ± 21	53.3 ± 5.0*	55.2 ± 3.9*	–2.0 ± 1.4	1949 ± 153	–1232 ± 131**	5
	HXC	9.1 ± 0.5	46.4 ± 2.0	280 ± 14	56.9 ± 7.9	61.0 ± 6.2	–4.1 ± 2.0	2138 ± 211	–1267 ± 58	
	U46619	5.7 ± 0.5	48.7 ± 1.5	269 ± 11	41.7 ± 8.5	44.7 ± 7.9	–3.0 ± 1.8	1588 ± 242	–968 ± 96	
AS + DTT	Baseline	11.2 ± 0.4	50.5 ± 0.6*	291 ± 25	55.1 ± 1.3*	50.7 ± 1.4*	4.1 ± 1.3	1687 ± 218	–1150 ± 81**	5
	DTT	16.5 ± 1.2**	50.0 ± 2.1	299 ± 9	52.5 ± 4.1	52.3 ± 4.8	0.2 ± 2.0	1818 ± 193	–1211 ± 92	
	U46619	8.8 ± 0.5*	54.9 ± 1.7	318 ± 19**	42.4 ± 3.4	41.5 ± 3.6	0.9 ± 1.6	1530 ± 156	1123 ± 118	
AS + ODQ	Baseline	10.2 ± 0.7	41.6 ± 0.9	294 ± 18	54.1 ± 4.5*	51.0 ± 3.0*	3.2 ± 1.2	1832 ± 177	–1578 ± 180	6
	ODQ	10.4 ± 0.6	42.6 ± 0.9	244 ± 20	69.5 ± 6.3	67.3 ± 5.4*	2.2 ± 1.7	2047 ± 186	–1812 ± 211	
	U46619	5.5 ± 0.5	47.4 ± 1.8	208 ± 22	55.8 ± 9.5	52.6 ± 9.0	3.1 ± 1.1	1636 ± 192	–1372 ± 222	
AS + $CGRP_{8–37}$	Baseline	10.1 ± 0.3	41.4 ± 0.3	239 ± 16	64.8 ± 3.8	64.7 ± 2.9	0.1 ± 1.4	1674 ± 66	–1320 ± 21	5
	$CGRP_{8–37}$	10.1 ± 0.2	43.2 ± 1.6	255 ± 16	69.6 ± 3.4	71.6 ± 3.0	–2.0 ± 1.5	1758 ± 116	–1505 ± 99	
	U46619	5.3 ± 0.2	49.9 ± 1.9	233 ± 14	53.7 ± 1.3	54.6 ± 1.5	–0.8 ± 1.1	1481 ± 82	–1201 ± 71	
AS + 4-AP	Baseline	10.1 ± 0.8	46.1 ± 1.6	295 ± 19	53.5 ± 3.6*	53.7 ± 3.4*	–0.2 ± 1.5	1718 ± 43	–1500 ± 89	5
	4-AP	8.4 ± 1.5	52.0 ± 2.1	241 ± 9*	72.7 ± 13.9	76.1 ± 15.2	–3.4 ± 2.1	2324 ± 348	–2079 ± 314	
	U46619	4.5 ± 0.9	56.3 ± 2.2	226 ± 20	47.9 ± 11.5	47.9 ± 12.4	–0.0 ± 1.8	1549 ± 319	–1362 ± 245	
DEA-NO	Baseline	10.4 ± 0.4	51.9 ± 3.1	254 ± 12	63.9 ± 6.7	58.6 ± 8.1	5.3 ± 2.6	1956 ± 248	–1109 ± 57	5
	U46619	5.6 ± 0.3	57.9 ± 3.2	267 ± 10	51.5 ± 4.5	47.2 ± 6.2	4.3 ± 2.6	1637 ± 177	–978 ± 67	
DEA-NO + HXC	Baseline	10.6 ± 0.3	47.8 ± 2.2	271 ± 12	54.3 ± 2.4	55.5 ± 1.6	–1.2 ± 3.4	1834 ± 66	–1203 ± 92	5
	HXC	10.8 ± 1.4	46.8 ± 2.3	257 ± 10	48.0 ± 7.6	50.5 ± 7.1	–2.5 ± 3.2	1705 ± 193	–1079 ± 63	
	U46619	7.0 ± 1.2	50.5 ± 1.8	250 ± 11	44.7 ± 5.6	46.8 ± 3.9	–2.1 ± 2.7	1589 ± 117	–1080 ± 49	

*$P < 0.05$, **$P < 0.01$ versus the analogous time point in hearts allocated to treatment with AS alone ;one-way ANOVA (Dunnett's *post hoc* test).

AS, Angeli's salt; DEA-NO, diethylamine-NONOate; HXC, hydroxocobalamin; LVDP, left ventricle diastolic pressure; LVEDP, left ventricle end-diastolic pressure; LVSP, left ventricle systolic pressure; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one.

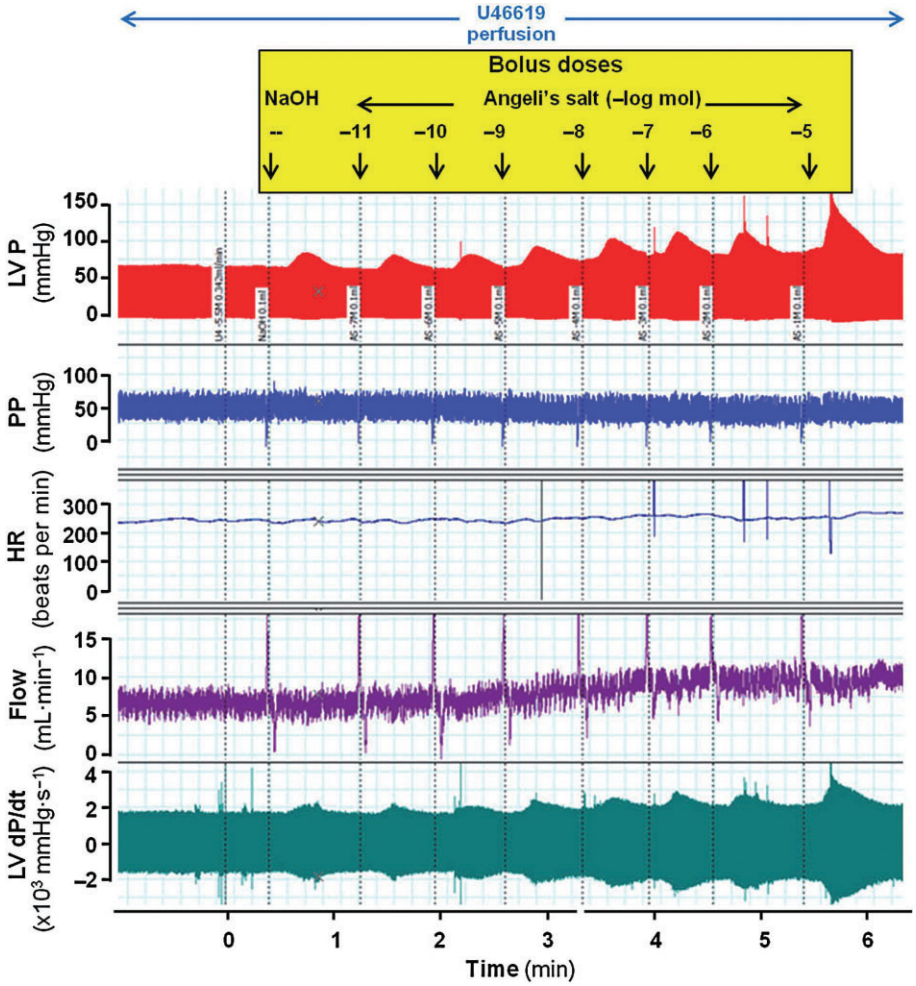

Figure 1

Representative dose–response curve to Angeli's salt, showing effects on LV pressure (LVP), perfusion pressure (PP), heart rate (HR), coronary flow and LV dP/dt.

Table 2

Sensitivity (pEC_{50}) and maximal relaxation response (R_{max}) for the dose–response curves to AS and DEA-NO on coronary flow, in the absence and presence of selective inhibitors

Experimental group	pEC_{50} (-log mol)	R_{max} (mL·min^{-1})	*n*
AS	8.55 ± 0.24	5.14 ± 0.69	8
AS + L-cysteine	7.53 ± 0.18**	2.62 ± 0.44*	6
AS + HXC	9.12 ± 0.12	6.85 ± 0.47	5
AS + DTT	7.85 ± 0.40	5.65 ± 0.93	5
AS + ODQ	7.36 ± 0.29**	3.88 ± 0.52	6
AS + $CGRP_{8-37}$	8.49 ± 0.26	4.76 ± 0.52	5
AS + 4-AP	8.40 ± 0.30	5.36 ± 0.85	5
DEA-NO	9.60 ± 0.18	8.82 ± 0.61	5
DEA-NO + HXC	8.56 ± 0.19$^{\#\#}$	4.77 ± 1.01$^{\#\#}$	5

*P < 0.05, **P < 0.01 versus AS alone and $^{\#\#}P$ < 0.01 versus DEA-NO alone.
AS, Angeli's salt; DEA-NO, diethylamine-NONOate; HXC, hydroxocobalamin; LVDP, left ventricle diastolic pressure; LVEDP, left ventricle end-diastolic pressure; LVSP, left ventricle systolic pressure; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one

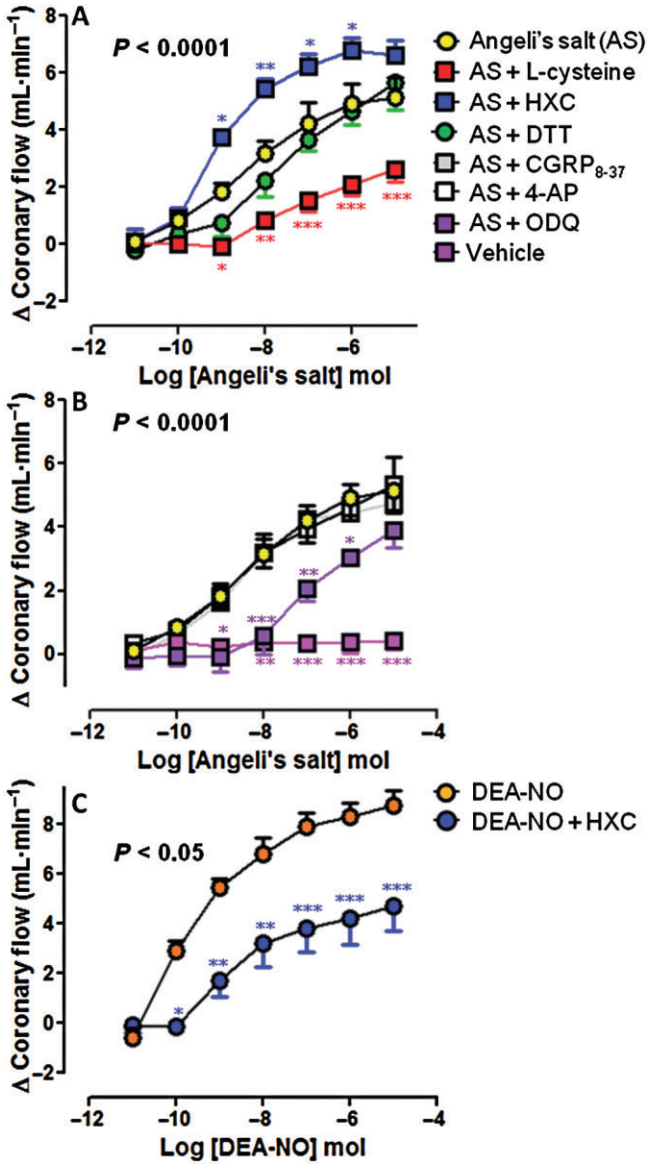

Figure 2

Dose–response curves to Angeli's salt (AS) (n = 8) on coronary flow in the absence and presence of (A) the HNO scavenger L-cysteine (4 mM, n = 6), the NO scavenger HXC (100 μM, n = 5) or the reducing agent DTT (100 μM, n = 5); and (B) the sGC inhibitor ODQ (10 μM, n = 6), the CGRP receptor antagonist $CGRP_{8-37}$ (0.1 μM, n = 5) and the K_v channel inhibitor 4-AP (1 mM, n = 5). Serial bolus doses of 10 mM NaOH vehicle are shown for comparison (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 versus AS; two-way ANOVA with Bonferroni *post hoc* test for multiple comparisons. (C) The dose–response curves to DEA-NO (n = 5) on coronary flow in the absence and presence of HXC (100 μM, n = 5) are shown for comparison.

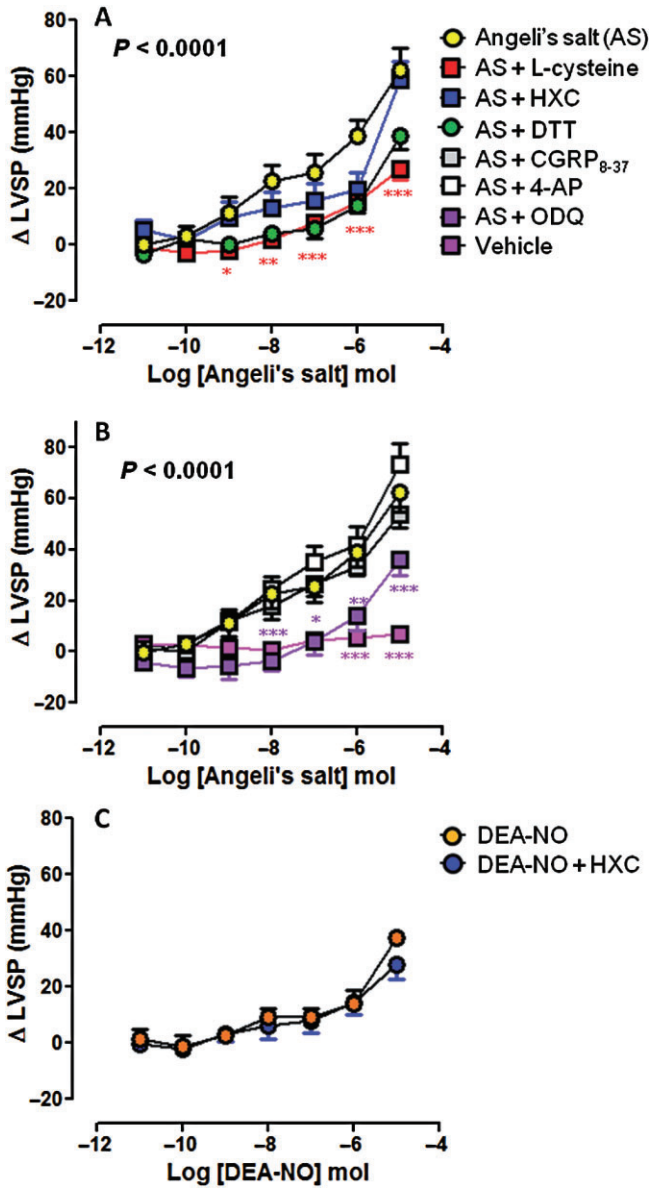

Figure 3

Dose–response curves to Angeli's salt (AS) (n = 8) on LVSP in the absence and presence of (A) L-cysteine (n = 6), HXC (n = 5) or DTT (n = 5); and (B) ODQ (n = 6), $CGRP_{8-37}$ (n = 5) and 4-AP (n = 5). Serial bolus doses of 10 mM NaOH vehicle are shown for comparison (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 versus AS; two-way ANOVA with Bonferroni *post hoc* test for multiple comparisons. (C) The dose–response curves to DEA-NO (n = 5) on LVSP in the absence and presence of HXC (100 μM, n = 5) are shown for comparison.

conduit and smaller resistance-like vessels *in vitro* (Irvine *et al.*, 2003; Favaloro and Kemp-Harper, 2009), as well as in the intact heart studied under conditions of constant flow *ex vivo* (Favaloro and Kemp-Harper, 2007). Although coronary vascular tone under basal, physiological conditions is largely regulated by K_v channels (Leblanc *et al.*, 1994; Shimizu *et al.*, 2000), we observed no role for K_v signalling in the vasodilator response to Angeli's salt in the rat coronary vasculature, consistent with previous observations (Irvine *et al.*, 2003; Favaloro and Kemp-Harper, 2007). In contrast, the vasorelaxant actions of Angeli's salt are mediated, in part, via K_v channels in the mesenteric circulation (Irvine *et al.*, 2003; Favaloro and Kemp-Harper, 2009), perhaps because of regional differences in K^+ channel subtype distribution. Although K_{ATP} channels may also play a role in coronary vasodilatation in

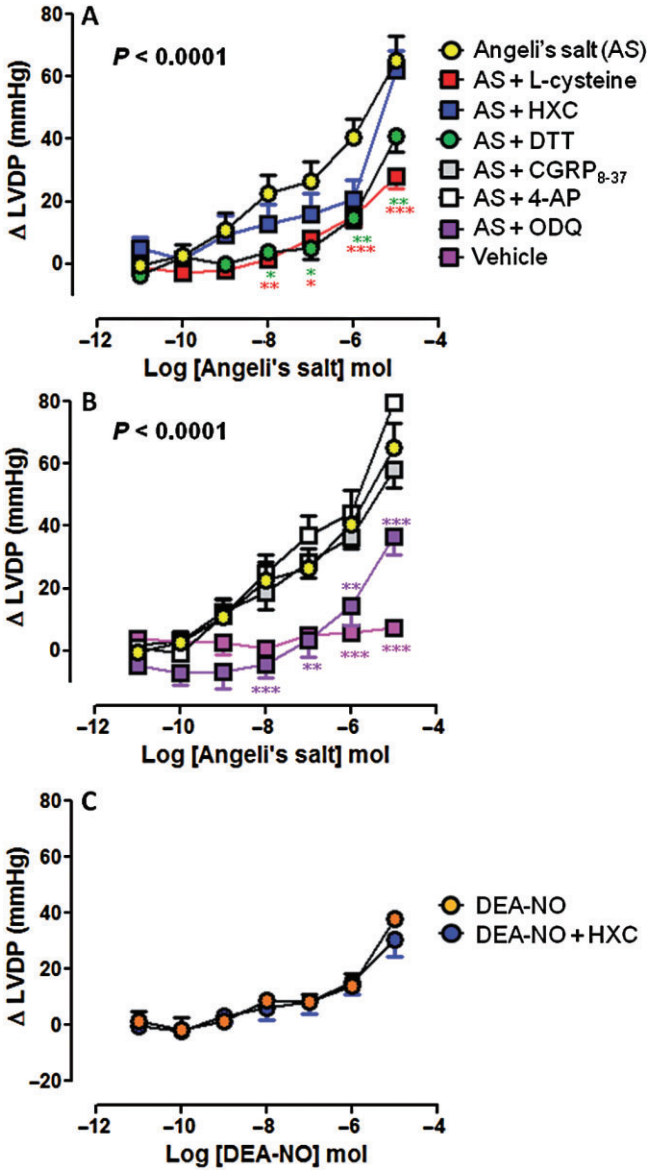

Figure 4

Dose–response curves to Angeli's salt (AS) (n = 8) on LVDP in the absence and presence of (A) L-cysteine (n = 6), HXC (n = 5) or DTT (n = 5); and (B) ODQ (n = 6), $CGRP_{8–37}$ (n = 5) and 4-AP (n = 5). Serial bolus doses of 10 mM NaOH vehicle are shown for comparison (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 versus AS two-way ANOVA with Bonferroni *post-hoc* test for multiple comparisons. (C) The dose–response curves to DEA-NO (n = 5) on LVDP in the absence and presence of HXC (100 μM, n = 5) are shown for comparison.

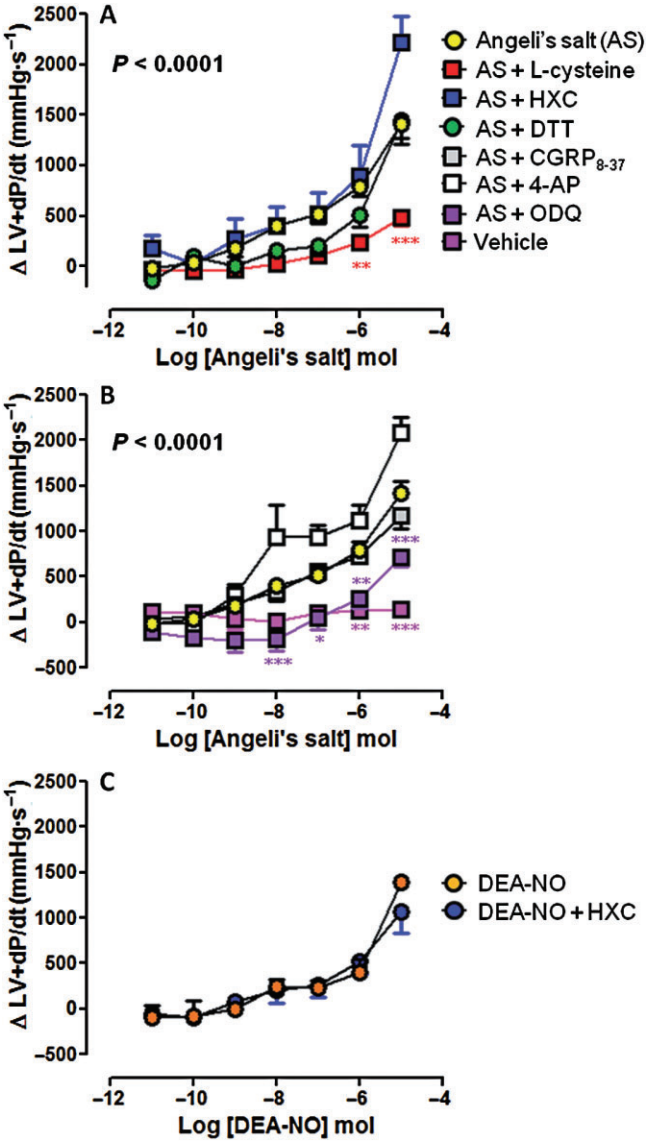

Figure 5

Dose–response curves to Angeli's salt (AS) (n = 8) on LV+dP/dt in the absence and presence of (A) L-cysteine (n = 6), HXC (n = 5) or DTT (n = 5); and (B) ODQ (n = 6), $CGRP_{8–37}$ (n = 5) and 4-AP (n = 5). Serial bolus doses of 10 mM NaOH vehicle are shown for comparison (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 versus AS; two-way ANOVA with Bonferroni *post hoc* test for multiple comparisons. (C) The dose–response curves to DEA-NO (n = 5) on LV+dP/dt in the absence and presence of HXC (100 μM, n = 5) are shown for comparison.

response to Angeli's salt (Favaloro and Kemp-Harper, 2007), this was not investigated in the present study.

Previous studies have suggested a potential contribution of CGRP to the coronary vasodilator response to Angeli's salt, as described in the isolated rat heart studied under constant flow conditions *ex vivo* (Favaloro and Kemp-Harper, 2007), but not to the peripheral arterial or venous vasorelaxation, as reported in a canine model *in vivo* (Paolocci *et al.*, 2001). Although we detected no contribution of CGRP-dependent signalling to the vasodilator actions of Angeli's salt in the isolated rat heart studied under constant pressure conditions *ex vivo*, the reason for this discrepancy remains unresolved. Angeli's salt co-releases both HNO and nitrite at physiological pH (Miranda *et al.*, 2005a), HNO rather than nitrite is likely to mediate the vasodilator responses observed here. Firstly, the HNO-selective scavenger, L-cysteine, markedly impaired these responses, and secondly, nitrite has almost negligible dilator activity in the rat coronary vasculature, with 15 000-fold less potency than Angeli's salt (Irvine *et al.*, 2003;

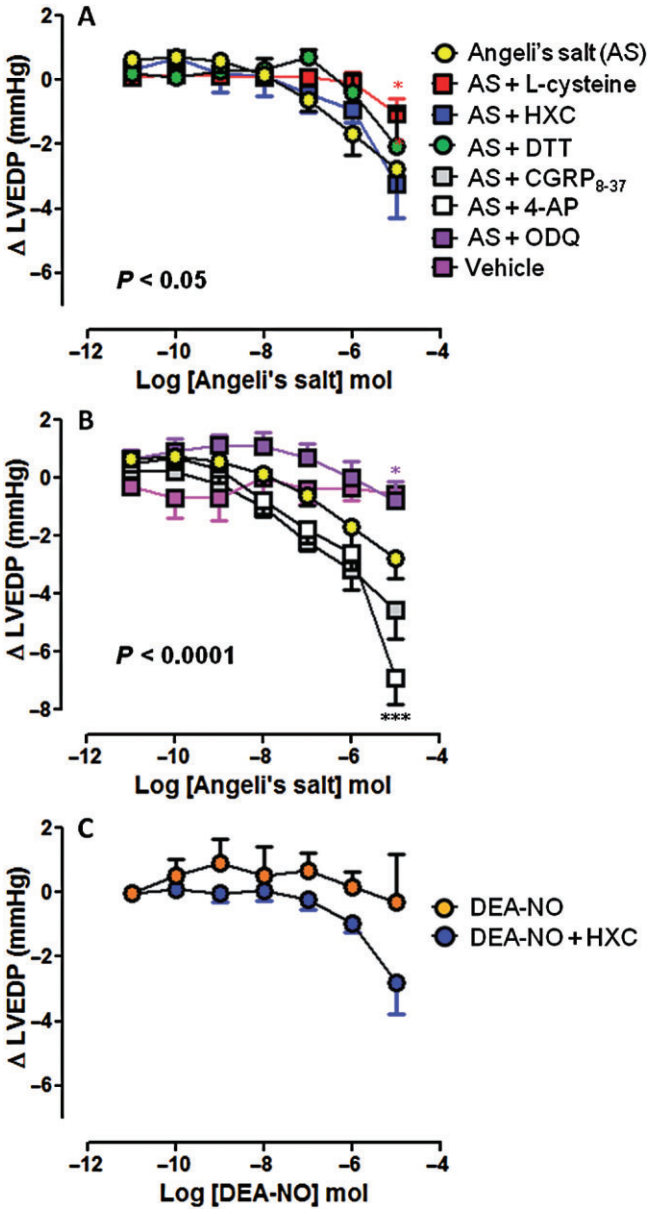

Figure 6

Dose–response curves to Angeli's salt (AS) (n = 8) on LVEDP in the absence and presence of (A) L-cysteine (n = 6), HXC (n = 5) or DTT (n = 5); and (B) ODQ (n = 6), $CGRP_{8-37}$ (n = 5) and 4-AP (n = 5). Serial bolus doses of 10 mM NaOH vehicle are shown for comparison (n = 3). *P < 0.05, ***P < 0.001 versus AS on two-way ANOVA with Bonferroni *post hoc* test for multiple comparisons. (C) The dose–response curves to DEA-NO (n = 5) on LVEDP in the absence and presence of HXC (100 μM, n = 5) are shown for comparison.

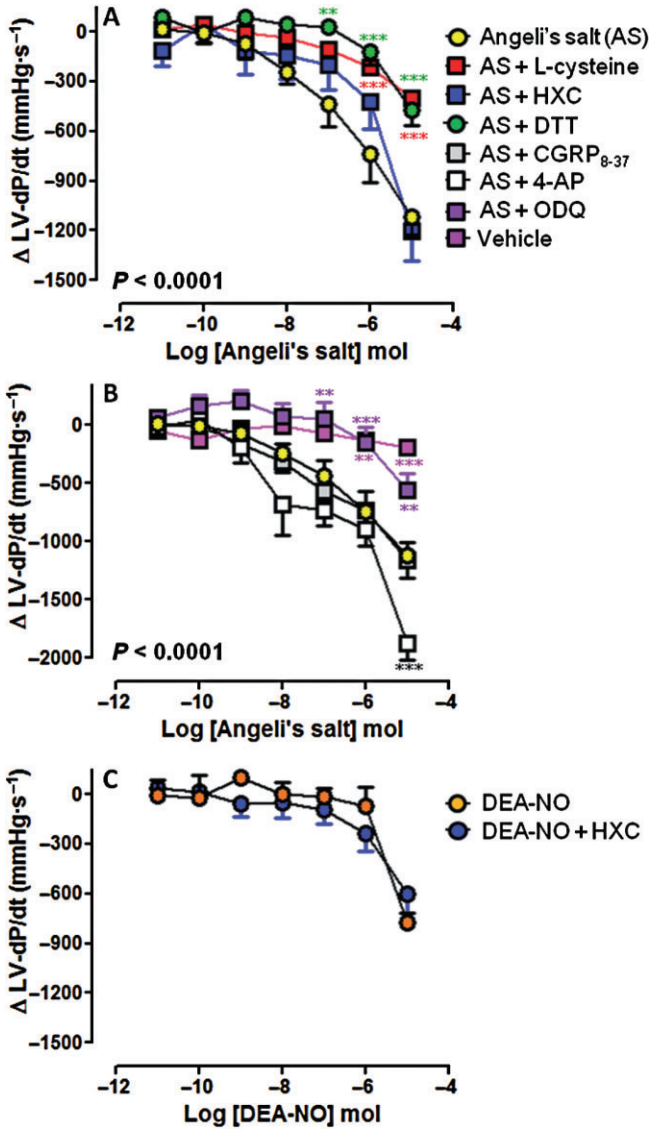

Figure 7

Dose–response curves to Angeli's salt (AS) (n = 8) on LV)-dP/dt in the absence and presence of (A) L-cysteine (n = 6), HXC) (n = 5) or DTT (n = 5); and (B) ODQ) (n = 6), $CGRP_{8-37}$ (n = 5) and 4-AP) (n = 5). Serial bolus doses of 10 mM NaOH vehicle are shown for comparison (n = 3). **P < 0.01, ***P < 0.001 versus AS on two-way ANOVA with Bonferroni *post hoc* test for multiple comparisons. (C) The dose–response curves to DEA-NO) (n = 5) on LV-dP/dt in the absence and presence of HXC (100 μM, n = 5) are shown for comparison.

Favaloro and Kemp-Harper, 2007). Given that a residual, modest Angeli's salt-induced vasodilatation remains in the presence of L-cysteine, we cannot rule out the possibility of oxidation of HNO to NO under our experimental conditions. However, the inability of the NO-selective scavenger HXC to blunt the vasodilator response to Angeli's salt suggests this is unlikely, at least in the extracellular milieu. Intriguingly, this vasodilator response was actually augmented in the presence of HXC; whether this reflects a loss of endogenous NO and thus an increased responsiveness of sGC to stimulation by HNO was however not determined.

The positive cardiac inotropic and lusitropic actions of HNO donors are well established, both in the intact heart *in vivo*, as well as in isolated cardiomyocytes and trabeculae *in vitro* (Paolocci *et al.*, 2001; Tocchetti *et al.*, 2007; Kohr *et al.*, 2010). We now confirm that the prototypical HNO donor, Angeli's salt, potently enhances both cardiac contraction and relaxation in the intact rat heart *ex vivo*. These actions were

markedly attenuated by both L-cysteine and DTT, specifically implicating HNO. The positive inotropic and dilator effects of Angeli's salt are not likely to be mediated by co-release of nitrite, as this has no appreciable effect on cardiomyocyte contractility (Kohr *et al.*, 2010). Early reports describing the positive inotropic actions implicated the neuropeptide CGRP at least in part in this mechanism of action, based on sensitivity to the CGRP receptor antagonist, $CGRP_{8–37}$ (Paolocci *et al.*, 2001; receptor nomenclature follows Alexander *et al.*, 2013c). CGRP itself elicits positive inotropic and lusitropic effects via activation of cAMP/PKA/L-type Ca^{2+} channel signalling (Huang *et al.*, 1999). These actions are however dependent on β-adrenoceptor signalling (Katori *et al.*, 2005), in contrast to those of HNO, which are β-adrenoceptor-independent (Paolocci *et al.*, 2003). Our results here are consistent with the absence of a role for CGRP in the inotropic and lusitropic actions of Angeli's salt.

As the myocardial effects of Angeli's salt are all evident even at relatively low doses (e.g. from 10 nmol), concomitant with doses required to elicit vasodilatation, this raises the possibility that these myocardial effects are a secondary effect to vasorelaxation, in accordance with the Gregg effect (Westerhof *et al.*, 2006). However, the vasodilator response plateaus at ~1 μmol, whereas the enhancement of LV contractility and relaxation induced by Angeli's salt progress further with increasing doses of the HNO donor, Angeli's salt. Given that previous reports suggest that the vasodilator actions of Angeli's salt are evident at markedly lower concentrations (e.g. 0.1 μM) than required for effects on cardiomyocyte function (e.g. 500 μM) (Favaloro and Kemp-Harper, 2007; Tocchetti *et al.*, 2007), it remains likely that Angeli's salt-mediated vasodilatation occurs at lower concentrations while the contractile effect of Angeli's salt occurs only at higher concentrations.

The cardiac inotropic and lusitropic effects of HNO donors have been traditionally attributed to cGMP-independent mechanisms, through a thiol-mediated interaction with the sarcoplasmic reticulum Ca^{2+}-handling proteins, RyR and SERCA (Tocchetti *et al.*, 2007; Kohr *et al.*, 2010). These previous reports concluded that the myocardial actions of HNO were cGMP-independent on the basis of an absence of detectable increases in plasma cGMP *in vivo* (Paolocci *et al.*, 2001), as well as a perceived lack of sensitivity to ODQ (Tocchetti *et al.*, 2007). Of note, the only previous investigation of the role for cGMP in the cardiac inotropic and lusitropic effects of HNO donors utilized isolated cardiomyocytes rather than the intact heart, and the concentration of HNO donor (1 mM) far exceeded that used for ODQ (10 μM) (Tocchetti *et al.*, 2007). ODQ is considered an oxidizer (rather than a competitive inhibitor) of sGC, which irreversibly inhibits the enzyme. There is however one report that supraphamacological concentrations of Angeli's salt (1 mM) may still be able to stimulate any residual sGC still in its reduced state (Zeller *et al.*, 2009). In the present study, the effects of HNO on LV contractility and relaxation were determined in the intact heart, concomitantly with its vasorelaxant effects. Administration of ODQ under these conditions significantly attenuated (but did not abolish) the LV inotropic and lusitropic effects of Angeli's salt, suggesting for the first time that HNO may mediate a part of these actions via sGC/cGMP-dependent signalling.

Although the effects of both NO and sGC on cardiac contractile function have been previously examined in a broad range of scenarios, no consensus has yet been reached, with negative inotropic (Balligand *et al.*, 1993; Brady *et al.*, 1993; Grocott-Mason *et al.*, 1994; Weyrich *et al.*, 1994; Mohan *et al.*, 1995; Kojda *et al.*, 1996; Sandirasegarane and Diamond, 1999; Muller-Strahl *et al.*, 2000; Gonzalez *et al.*, 2008; Cawley *et al.*, 2011; Derici *et al.*, 2012), positive inotropic (Klabunde and Ritger, 1991; Smith *et al.*, 1991; Kojda *et al.*, 1995; 1996; 1997; Sarkar *et al.*, 2000; Layland *et al.*, 2002; Langer *et al.*, 2003) or no change observed (Ritchie *et al.*, 2006; 2009). Indeed, the relationship between NO/sGC and myocardial force may be differentially modulated by concentration, whereby smaller increases in NO/sGC levels elicit positive inotropic effects either secondary to phosphodiesterase-3 inhibition (elevating cAMP), while high concentrations elicit a cGMP-mediated negative inotropic effect, perhaps secondary to formation of S-nitrosothiols on key cardiomyocyte Ca^{2+}-handling proteins such as RyR, SERCA and phospholamban (Smith *et al.*, 1991; Kojda *et al.*, 1996; 1997; Zahradnikova *et al.*, 1997; Paolocci *et al.*, 2000; Layland *et al.*, 2002; Langer *et al.*, 2003; Gonzalez *et al.*, 2007; 2008; Rastaldo *et al.*, 2007; Wang *et al.*, 2008; Ziolo, 2008). It is also likely that distinct cardiomyocyte pools of cGMP also contribute to this lack of consensus with respect to the nature of any possible effect of NO/sGC on inotropic mechanisms, as has been suggested for natriuretic peptide receptors (Qvigstad *et al.*, 2010). There is however consensus with respect to cardiac relaxation, which is enhanced by NO (Paulus *et al.*, 1994; Carnicer *et al.*, 2013). In our study DEA-NO (which releases two NO molecules per molecule of DEA-NO) did tend to enhance systolic function, but this was more modest than that achieved by the equivalent concentration of Angeli's salt (despite it only releasing a single HNO molecule per molecule of Angeli's salt). We have previously demonstrated that HNO donors such as Angeli's salt and IPA-NO do not increase cardiomyocyte cAMP or CGRP content (Lin *et al.*, 2012; Irvine *et al.*, 2013).

In our hands, the thiols L-cysteine and DTT were similarly effective at blunting the Angeli's salt enhancement of inotropic and lusitropic function at the concentrations used (4 vs. 0.1 mM). In contrast, only L-cysteine (and not DTT) blunted the vasodilatation response. L-cysteine is conventionally used as an HNO scavenger (Tocchetti *et al.*, 2011), blocking both Angeli's salt-induced coronary vasodilator and positive inotropic actions by removing available HNO. HNO is considered to enhance cardiac contractility and relaxation by inducing a reversible oxidation of key thiol residues on specific cardiomyocyte Ca^{2+} cycling/sensitization proteins (e.g. RyR and SERCA), without altering net thiol redox status (i.e. GSH:GSSG ratio; for review see Fukuto and Carrington, 2011; Tocchetti *et al.*, 2011). Our findings with both thiols are perhaps consistent then with the Angeli's salt-induced vasodilatation dependent on HNO and sGC (but not proteins implicated in Ca^{2+} cycling/sensitization), whereas its enhancement of cardiac contractility and relaxation may be mediated at least in part by both sGC-dependent and sGC-independent mechanisms (such as HNO-mediated oxidation of RyR and SERCA).

The thiol modification induced by HNO is quite distinct to that induced by NO. NO leads to S-nitrosation via an

indirect action, as it is initially oxidized to nitrous anhydride, which then reacts with protein thiol groups to form protein-SNO (Lima *et al.*, 2010; Heinrich *et al.*, 2013). In contrast, the interaction of HNO with thiols is direct and thus extremely rapid (Jackson *et al.*, 2009), first generating the intermediate, N-hydroxysulphenamide, which can then either be irreversibly arranged to form N-hydroxysulphenamide, or alternatively can reversibly interact with an additional thiol, to form a disulphide and hydroxylamine. The predominant thiol modification induced by HNO is thus considered formation of a sulphinamide or disulphide, rather than S-nitrosation (Fukuto and Carrington, 2011). As Angeli's salt only releases NO at a very acidic pH (Miranda *et al.*, 2005b), together with our finding that the coronary vasodilator action of Angeli's salt was not diminished in the presence of the NO scavenger HXC, it is highly unlikely that Angeli's salt will form S-NO in the presence of thiols such as L-cysteine. Thus, in contrast to NO donors, Angeli's salt dose-dependent enhancement of cardiac contractility and relaxation is unlikely to result from S-nitrosation of Ca^{2+}-handling proteins.

In conclusion, the HNO donor Angeli's salt elicits dose-dependent enhancement of LV systolic and diastolic function, concomitant with vasodilatation, in the intact rat heart. These effects are all L-cysteine-sensitive and mediated by HNO, with contributions from both sGC-dependent and s-GC-independent mechanisms. No role for CGRP, NO or K_v in Angeli's salt cardiac effects was evident. HNO thus acutely modulates both LV contractile function and LV relaxation, while concomitantly unloading the heart. These properties, in combination with the powerful antihypertrophic and superoxide-suppressing actions we have previously demonstrated, may favour HNO donors as a potential strategy for managing heart failure (alone or in addition to standard care).

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Conflict of interest

The authors have no conflicts of interest to declare.

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