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cGMP-independent inotropic effects of nitric oxide and peroxynitrite donors: potential role for nitrosylation

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Paolocci, Nazareno, Ulf E. G. Ekelund, Takayoshi Isoda, Michitaka Ozaki, Koenraad Vandegaer, Dimitrios Georgakopoulos, Robert W. Harrison, David A. Kass, and Joshua M. Hare. cGMP-independent inotropic effects of nitric oxide and peroxynitrite donors: potential role for nitrosylation. *Am J Physiol Heart Circ Physiol* 279: H1982–H1988, 2000.—Nitric oxide (NO) has concentration-dependent biphasic myocardial contractile effects. We tested the hypothesis, in isolated rat hearts, that NO cardiostimulation is primarily non-cGMP dependent. Infusion of 3-morpholinosydnonimine (SIN-1, 10^{-5} M), which may participate in S-nitrosylation (S-NO) via peroxynitrite formation, increased the rate of left ventricular pressure rise (+dP/d*t*; 19 ± 4%, $P < 0.001$, $n = 11$) without increasing effluent cGMP or cAMP. Superoxide dismutase (SOD; 150 U/ml) blocked SIN-1 cardiostimulation and led to cGMP elaboration. Sodium nitroprusside (10^{-10}–10^{-7} M), an iron nitrosyl compound, did not augment +dP/d*t* but increased cGMP approximately eightfold ($P < 0.001$), whereas diethylamine/NO (DEA/NO; 10^{-7} M), a spontaneous NO· donor, increased +dP/d*t* (5 ± 2%, $P < 0.05$, $n = 6$) without augmenting cGMP. SIN-1 and DEA/NO +dP/d*t* increase persisted despite guanylyl cyclase inhibition with 1*H*-(1,2,4)oxadiazolo-(4,3,-*a*)quinoxalin-1-one (10^{-5} M, $P < 0.05$ for both donors), suggesting a cGMP-independent mechanism. Glutathione (5×10^{-4} M, $n = 15$) prevented SIN-1 cardiostimulation, suggesting S-NO formation. SIN-1 also produced SOD-inhibitable cardiostimulation in vivo in mice. Thus peroxynitrite and NO donors can stimulate myocardial contractility independently of guanylyl cyclase activation, suggesting a role for S-NO reactions in NO/peroxynitrite-positive inotropic effects in intact hearts.

myocardial contractility; 3-morpholinosydnonimine; cyclic nucleotides; superoxide dismutase; glutathione; 1*H*-(1,2,4) oxadiazolo-(4,3,-*a*)quinoxalin-1-one; guanosine 3′,5′-cyclic monophosphate

THE MECHANISM(S) by which nitric oxide (NO) influences myocardial contractility remains controversial (17). At least two biochemical mechanisms may be relevant to NO signaling in the heart: activation of heme-containing proteins and nitrosylation (35). First, NO activates soluble guanylyl cyclase by binding to its heme moiety, leading to the production of cGMP (13,25). Second, NO, a free radical, may also react with sulfhydryl moieties on either low molecular compounds or proteins (34, 37, 44, 45). Protein nitrosylation has been shown in vitro to activate various proteins involved in the regulation of myocardial contractility. Most prominently, NO may activate the L-type calcium channel (3) and the ryanodine receptor (46).

NO influences contractility in a concentration-dependent biphasic manner. In vitro studies (5, 19, 23, 41) indicate that lower concentrations of NO may be cardiostimulatory, whereas higher concentrations become cardiodepressant. In vivo, organic nitrate NO donors have no effect or a weak stimulatory effect (29). With regard to the chemical signaling pathways responsible, cGMP has been shown in some studies to mediate biphasic effects (19, 41). On the other hand, direct protein nitrosylation that is cGMP independent could potentially augment contractility via increases in Ca^{2+} cycling (3, 46).

The purpose of this study was to test the hypothesis that NO donors well characterized to participate in nitrosylation reactions (24) would have a positive inotropic effect independent of cGMP activation in both isolated hearts and in vivo. To further clarify the determinants of this reaction, we tested the effect of agents that inhibit guanylyl cyclase activity [e.g., 1-*H*-(1,2,4)oxadiazolo-(4,3,-*a*)quinoxalin-one (ODQ)] or modify nitrosylation [e.g., superoxide dismutase (SOD) and the reduced form of glutathione (GSH)] on this inotropic response.

MATERIALS AND METHODS

Reagents. 3-Morpholinosydnonimine-HCl (SIN-1), diethylenetiamine pentaacetic acid (DTPA), Cu/Zn SOD, and GSH were purchased from Sigma Chemicals (St. Louis, MO). SIN-1 was prepared immediately before use as a 10 mM stock solution. ODQ was purchased from Tocris Cookson (Ballwin, MO) and was dissolved in 10% ethanol (0.1% final concentration in the heart), which did not alter contractility per se (data not shown). Sodium nitroprusside (SNP) was from

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Elkins-Sinn (Cherry Hill, NJ). Diethylamine/NO (DEA/NO) complex was a generous gift from Dr. D. A. Wink (Radiation Biology Branch, National Institutes of Health, Bethesda, MD). DEA/NO was dissolved and diluted in ice-cold 10 mM NaOH to prevent NO release until addition to the heart perfusate. NaOH alone did not alter contractility (data not shown).

Isolated heart preparation. Hearts were rapidly excised from male Wistar rats ($n = 77$) premedicated with 1,000 U im heparin and retrogradely perfused with oxygenated perfusion buffer at 37°C (15). A polyvinyl chloride balloon attached to a polyethylene-190 tubing balloon (Clay Adams-Becton-Dickinson, Parsipanny, NJ) was placed through the left atrium and mitral valve into the left ventricle. The balloon was filled with saline to achieve a maximum isovolumic developed pressure, which typically occurred at an end-diastolic pressure of 10–15 mmHg. Hearts were perfused at a constant flow by a peristaltic pump, initially titrated to achieve a coronary perfusion pressure of 80 mmHg. Constant flow was used to avoid confounding alterations in contractility due to the Gregg effect (11). The perfusate contained (in mmol/l) 144 sodium, 5 potassium, 1.5 calcium, 17.5 bicarbonate, 1.2 magnesium, and 134 chloride, along with 5 μg/ml lidocaine. This was equilibrated with a gas mixture of 95% O_2-5% CO_2, resulting in a perfusate pH of 7.4. Finally, the metal-chelating agent DTPA (100 μM) was added (made from a stock solution of 1 mM in water) to prevent lipid oxidation (43). The hearts were placed in a heated bath at 37°C and paced at 300 beats/min. Left ventricular (LV) pressure, the rate of change of LV pressure (dP/d*t*), and the mean coronary perfusion pressure were measured continuously (Gould, Cleveland, OH) and digitized at 1,000 Hz. The animal protocol was approved by the Johns Hopkins University School of Medicine Animal Care and Use Committee.

Drug protocol. In preliminary experiments, SIN-1 was observed to have long-lasting effects on myocardial contraction. Accordingly, the effects of 1, 10, and 100 μM SIN-1 (final concentrations within the coronary circulation) were tested in separate experiments. In all experiments, steady-state conditions were established over a 15-min period. The SIN-1 solution was then infused (Harvard Instruments) through a sidearm of the aortic cannula at 1% of the coronary flow rate. The effect of SIN-1 (10 μM for 15 min) was also evaluated in a subset of experiments omitting DTPA in the perfusate ($n = 5$).

Potential mechanisms for the effects of SIN-1 were probed by the following experiments: *1*) To prevent SIN-1 decomposition to $ONOO^-$, Cu/Zn SOD (150 IU/ml) was infused 5 min before and continued during SIN-1 administration (for 15 min). *2*) To test whether a cGMP-dependent mechanism contributed to inotropic effects of SIN-1, ODQ (10^{-5} M) was infused for 15 min and continued during SIN-1 (10^{-5} M) coinfusion. *3*) In additional hearts, experiments were conducted with coinfusion of GSH (5×10^{-4} M), which served as a "competing" thiol to block myocardial nitrosylation-based reactions. *4*) To contrast NO donors with nitrosylating effects versus those without nitrosylating effects (20), SNP (an iron nitrosyl) was infused (10^{-10}-10^{-7} M) to test for effects on myocardial contractility and cyclic nucleotide levels (cGMP and cAMP) in the coronary sinus drainage. SNP (10^{-8}–10^{-7} M) was also coinfused with ODQ (10^{-5} M) to confirm the activity of this agent to block cGMP elaboration. *5*) Finally, to verify whether other NO donors can enhance myocardial contractility, we infused DEA/NO (10^{-7} M), a spontaneous NO· donor in aqueous solutions, alone and after ODQ.

Cyclic nucleotide assays. In additional experiments, effluent from perfused hearts was collected to determine the concentration of cAMP and cGMP in response to SIN-1 (10^{-5} M) alone, SNP (10^{-10}–10^{-7} M) alone, SIN-1 in the presence of SOD (150 U, $n = 6$) and ODQ (10^{-5} M, $n = 9$), SNP in presence of ODQ ($n = 6$), and DEA/NO (10^{-7} M, $n = 6$) alone. Samples (10 mL) were immediately frozen in liquid nitrogen. Assays were performed using an enzyme immunoassay (Biotrak, Amersham Pharmacia Biotech, Piscataway, NJ) using lyopholized samples. Cyclic nucleotide concentrations are expressed in picomolars per minute per gram of heart tissue. The assay has a sensitivity of detection of 2 fM.

Murine in vivo experiments. Male C57BL/6 mice ($n = 25$, 12–18 wk old, 20–35 g body wt; Jackson Laboratories) were used. Animals were housed under diurnal lighting conditions and allowed food and tap water ad libitum. Animal treatment and care was provided in accordance with institutional guidelines, and the protocol was approved by the Animal Care and Use Committee of the Johns Hopkins University.

Mice were anesthetized and ventilated as described (8). A combined micromanometer-conductance catheter (9) (model SPR-719, Millar Instruments, Houston, TX) was placed retrograde into the left ventricle in open-chest animals. Infusions were administered via the right jugular vein cannulated with a 30-gauge needle. The recorded volume signal from the conductance catheter requires calibration for absolute volume (offset) and stroke volume (gain). Absolute volume was derived using a saline wash in technique (1). Stroke volume calibration was derived from cardiac output obtained from direct measurements of aortic flow, obtained using a flow probe (AT01RB, Transonic Systems, Ithaca, NY) placed around the aorta, and the flow per minute was recorded (AT106, Transonic Systems) via a lateral thoracotomy. Pressure, volume, and flow signals were digitized at 1,000 Hz, stored to disk, and analyzed with custom software.

Statistical analysis. Data are presented as means ± SE. Concentration-effect responses were analyzed by two-way ANOVA tests using an identification term for each individual experiment and the Student-Newman-Keuls post hoc test (42). For experiments comparing single dose effects, paired *t*-tests were applied. A P value <0.05 was considered significant.

RESULTS

Effects of SIN-1 on myocardial contractility and cyclic nucleotide concentrations. The baseline conditions of isolated hearts are shown in Table 1. Figure 1 depicts the concentration-dependent effects of SIN-1 on myocardial contractility. The peak rate of LV pressure rise (peak +dP/d*t*) rose by 9 ± 4% ($P < 0.05$, $n = 5$) at

Table 1. *Baseline conditions in isolated rat hearts*

Conditions Measured	Baseline
HR, beats/min	320 ± 0.8
LVSP, mmHg	98 ± 4
LVEDP, mmHg	14 ± 2
Peak + dP/d*t*, mmHg/s	2,669 ± 205
τ, ms	27.6 ± 2.4
CPP, mmHg	80.5 ± 0.9
cGMP, pmol·ml^{-1}·g^{-1}	1.04 ± 0.22
cAMP, pmol·ml^{-1}·g^{-1}	0.51 ± 0.1

All values presented as means ± SE; $n = 11$ hearts. HR, heart rate; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; peak +dP/d*t*, peak rate of left ventricular pressure rise; τ, time constant of relaxation; CPP, coronary perfusion pressure.

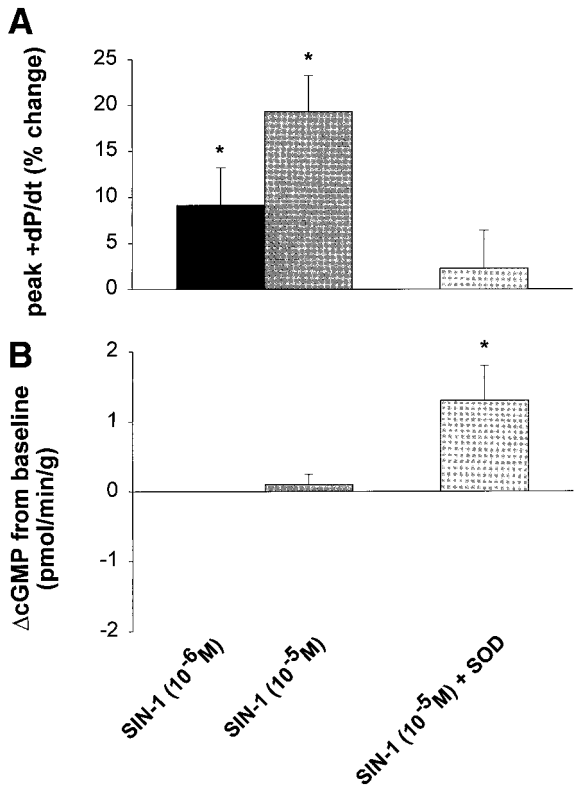

Fig. 1. Effect of 3-morpholinosydnonimine (SIN-1), with and without superoxide dismutase (SOD), on myocardial contractility and cGMP production. Retrograde perfused rat hearts were administered SIN-1 alone or SIN-1 after pretreatment with SOD at the indicated molar concentrations. The percentage of increase in peak rate of left ventricular pressure rise (peak +dP/d*t*) from baseline (*A*) and the increase in cGMP concentration in coronary sinus drainage (*B*) is depicted. Data represent means ± SE. *$P < 0.05$ vs. baseline.

1 μM and 19 ± 4% ($P < 0.001$, $n = 11$) at 10 μM but was unchanged ($n = 4$) at 100 μM. In separate experiments where DPTA was omitted from the perfusate, SIN-1 augmented +dP/d*t* to a similar degree (data not shown). Coronary perfusion pressure decreased by 12 ± 3% ($P = 0.002$) at 10 μM but not at 1 μM. Furthermore, SIN-1 (10 μM) decreased LV end-diastolic pressure (LVEDP) from 13 ± 2 to 8 ± 2 mmHg ($P = 0.004$ vs. baseline) and shortened the time constant of relaxation from 28 ± 2 to 20 ± 2 mmHg ($P =$ 0.005 vs. baseline). The positive inotropic effect of SIN-1 was not accompanied by changes in effluent concentrations of cGMP or cAMP (Fig. 1 and Table 2).

Table 2. *Cyclic nucleotide levels in control and SIN-1-treated hearts*

	cGMP	cAMP
Control hearts (n = 4)		
Saline (15 min)	1.03 ± 0.34	0.55 ± 0.14
Saline (30 min)	1.31 ± 0.32	0.44 ± 0.15
Saline (45 min)	0.98 ± 0.28	0.33 ± 0.07
SIN-1-treated hearts (n = 5)		
Saline (15 min)	1.04 ± 0.22	0.51 ± 0.1
Saline (30 min)	0.84 ± 0.19	0.35 ± 0.19
SIN-1 (15 min)	0.83 ± 0.14	0.34 ± 0.12

Data are expressed in picomolar per min per gram and shown as means ± SE. SIN-1, 3-morpholinosydnonimime; *n*, number of hearts.

Redox modulation of SIN-1-positive inotropic effect. To assess whether SIN-1 augmentation of contractility depended on a balance of superoxide and NO (i.e., peroxynitrite formation), SOD was coinfused with SIN-1. SOD, which had no effect on +dP/d*t* alone, fully prevented the SIN-1-positive inotropic effect (3,056 ± 164 and 3,100 ± 171 mmHg/s, SOD vs. SOD + SIN-1, respectively, $n = 17$). Moreover, in the presence of SOD, SIN-1 now modestly augmented effluent cGMP (Fig. 1).

Impact of GSH on SIN-1 inotropic effects. To test whether administration of a competing thiol could block the SIN-1-positive inotropic response, we coinfused the low-molecular-weight thiol GSH (5×10^{-4} M). GSH alone did not affect +dP/d*t* but fully prevented SIN-1 inotropy (2,511 ± 144 and 2,393 ± 126 mmHg/s, GSH vs. GSH + SIN-1, respectively, $n = 15$).

Impact of ODQ on SIN-1-positive inotropic effects and cGMP perfusate levels. To further assess the impact of guanylyl cyclase on SIN-1-positive inotropic responses, we performed an additional series of experiments in which SIN-1 (10^{-5} M) was administered after 15 min of ODQ (10^{-5} M). As shown in Fig. 2, ODQ alone significantly increased +dP/d*t* from 2,507 ± 152

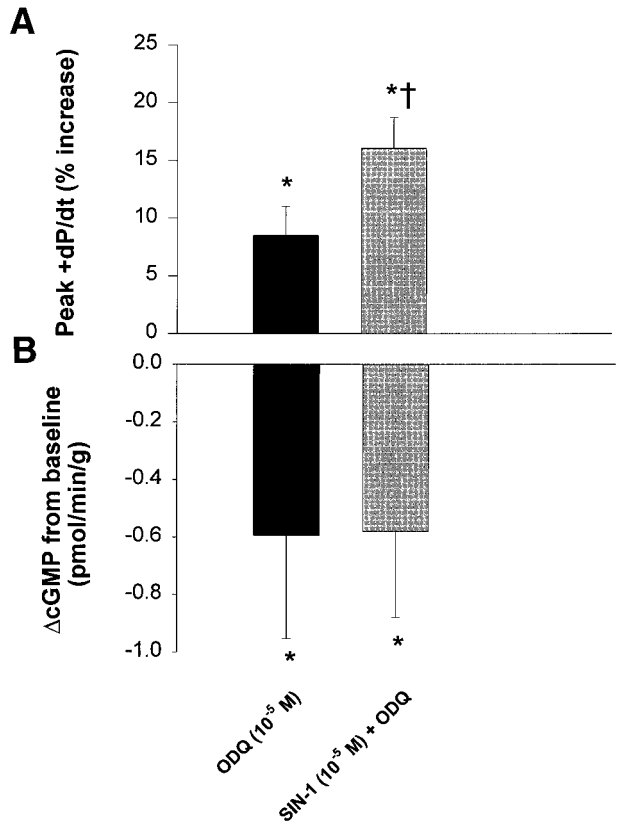

Fig. 2. Effect of 1-*H*-(1,2,4)oxadiazolo-(4,3,-*a*)quinoxalin-one (ODQ) on the inotropic and cGMP response to SIN-1. The percentage of increase in peak +dP/d*t* from baseline (*A*; $n = 9$) and the absolute change in cGMP perfusate level (*B*; $n = 5$) is depicted. Data represent means ± SE. *$P < 0.05$ vs. baseline; †$P < 0.05$ vs. ODQ alone.

to 2,731 ± 199 mmHg/s (9 ± 3%, $P < 0.05$ vs. baseline, $n = 9$), and SIN-1 further augmented +dP/dt to 2,898 ± 171 mmHg/s (16 ± 3%, $P < 0.05$ vs. baseline and ODQ alone). ODQ lowered cGMP levels in the perfusate by 40 ± 15% ($P < 0.05$ vs. control, $n = 5$), and SIN-1 did not change cGMP concentrations further ($P < 0.05$ vs. control; not significant vs. ODQ alone).

Effects of SNP on myocardial contractility and cyclic nucleotide concentrations. SNP infused over 10^{-10}–10^{-7} M did not alter peak +dP/dt ($n = 5$, Fig. 3). Similar to the effects of SIN-1, SNP decreased coronary perfusion pressure, with a maximal decrease of 15 ± 2% ($P < 0.0001$) at 10^{-7} M, and decreased LVEDP by 18 ± 3% ($P < 0.0005$). In marked contrast to SIN-1, SNP profoundly increased effluent cGMP concentrations (~8-fold, $P < 0.002$, $n = 3$; Fig. 3) but did not change cAMP levels (data not shown). In the presence of ODQ, the increase in cGMP was completely prevented (Fig. 3).

Effects of DEA/NO on myocardial contractility and cyclic nucleotide concentrations. To assess whether the SIN-1-positive inotropic effect was dependent on peroxynitrite, we infused another spontaneous NO donor, DEA/NO. As shown in Fig. 4, DEA/NO (10^{-7} M for 15 min) augmented +dP/dt by 5 ± 2% ($n = 6$, $P < 0.05$).

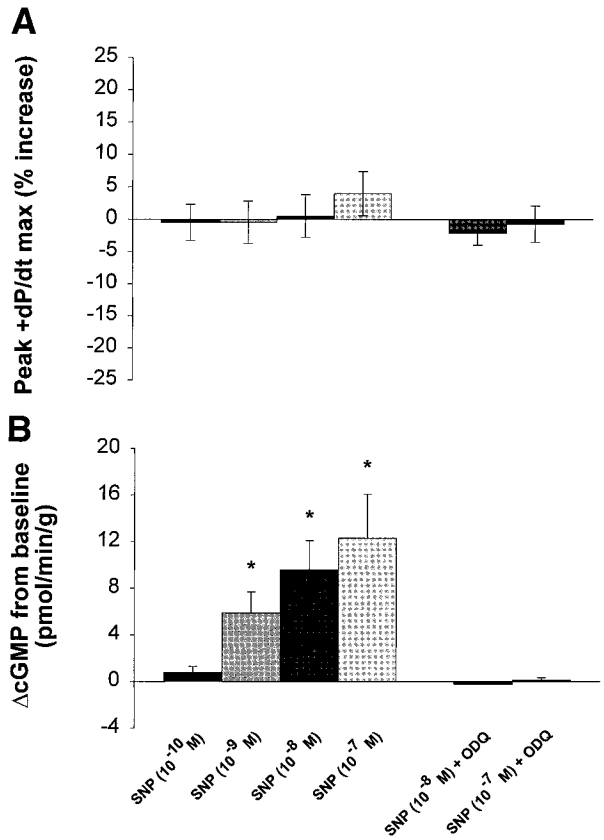

Fig. 3. Effect of sodium nitroprusside (SNP), with and without ODQ, on myocardial contractility and cGMP perfusate levels. *A*: dose-response curve to SNP alone ($n = 5$) and with ODQ ($n = 6$). *B*: increase in cGMP levels from baseline with SNP alone ($n = 3$) and SNP + ODQ ($n = 5$). Data represent means ± SE. *$P < 0.05$ vs. baseline.

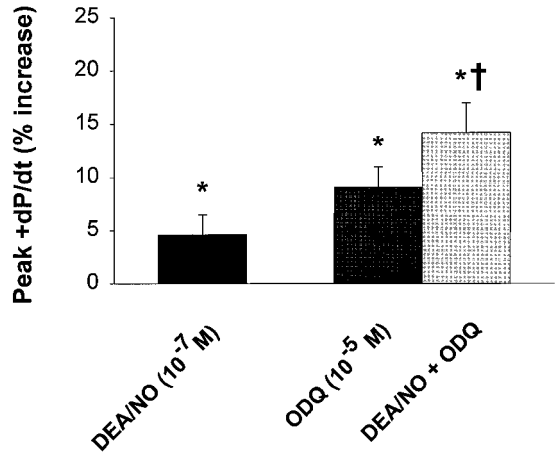

Fig. 4. Effect of diethylamine/nitric oxide (DEA/NO) alone ($n = 6$) and in combination with ODQ ($n = 10$) on myocardial contractility. Data represent means ± SE. *$P < 0.05$ vs. baseline; †$P < 0.05$ vs. ODQ alone.

This inotropic effect was not accompanied by any changes in cGMP in the heart perfusate (P = not significant, $n = 6$). Moreover, this augmentation also persisted in the presence of ODQ (10^{-5} M for 15 min). ODQ alone augmented +dP/dt by 9 ± 2% ($P < 0.05$ from baseline, $n = 10$), and the subsequent addition of DEA/NO further increased myocardial contractility (+14 ± 3% from baseline, $P < 0.005$, $n = 10$).

In vivo effects of SIN-1. To test the relevance of these findings in vivo, we infused SIN-1 with and without SOD pretreatment to anesthetized C57BL/6 mice instrumented with a combined pressure-volume catheter. Baseline conditions are depicted in Table 3. Graded infusion of SIN-1 (80, 160, and 320 $\mu g \cdot kg^{-1} \cdot min^{-1}$, ~9–36 μM, $n = 18$) caused a positive inotropic response. Ventricular elastance, the slope of the end-systolic pressure-volume relation, exhibited

Table 3. *Hemodynamic effects of SIN-1 and SIN-1 in combination with Cu/Zn SOD in mice*

	Baseline ($n = 24$)	SIN-1, % Change ($n = 18$)	SOD + SIN-1, % Change ($n = 6$)
HR, beats/min	700 ± 17	1.7 ± 1	−4.5 ± 4§
SBP, mmHg	97.6 ± 4.3	−2.5 ± 2.2	−8.5 ± 8.5
SV, μl	9.4 ± 1.9	17.5 ± 7*	−2.9 ± 6.4
CO, ml/min	5.7 ± 1.2	19.7 ± 7.7*	−5.4 ± 9.1
E_{es}, mmHg/μl	18.7 ± 5.9	149.6 ± 76.1*	5.1 ± 25.3
PRSW, mmHg	92.5 ± 4.3	31.9 ± 12†	−8.1 ± 7.3§
dP/dt-IP, s^{-1}	254.5 ± 13.6	10.0 ± 2.4‡	−13.4 ± 11.9§
+dP/dt, mmHg/s	13,475 ± 1,061	0.4 ± 7.6	−26.0 ± 9.2‡§
LVEDP, mmHg	7.6 ± 0.6	−0.9 ± 12.9	−7.2 ± 12
LVEDV, μl	18.6 ± 1.8	17.5 ± 7*	−2.9 ± −3.7
E_a, mmHg/μl	14.4 ± 6.4	−17 ± 5†	−24 ± 5‡

Baseline (control) values are expressed as means ± SE. SIN-1 was infused at a rate of 80 $\mu g \cdot kg^{-1} \cdot min^{-1}$, and Cu/Zn SOD was given at a dose of 30 IU. SBP, systemic blood pressure; SV, stroke volume; CO, cardiac output; E_{es}, ventricular elastance; PRSW, preload recruitable stroke work; dP/dt-IP, peak rate of LV pressure rise corrected for pressure; LVEDV, LV end-diastolic volume; E_a, arterial elastance; SOD, superoxide dismutase. *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$; §$P < 0.05$ vs. SIN-1 alone.

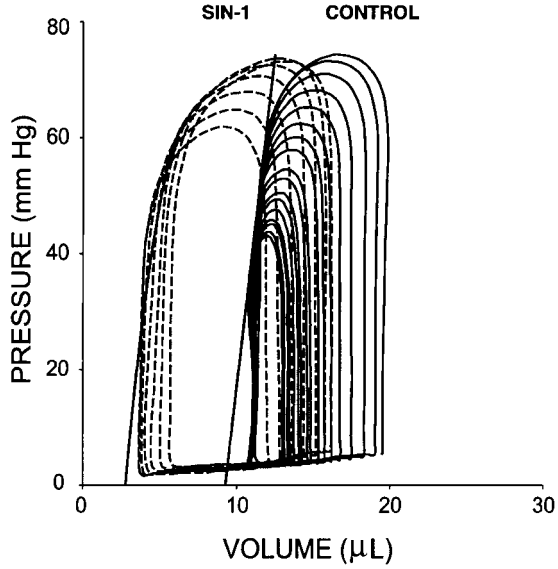

Fig. 5. Representative pressure-volume data generated with transient occlusion of the inferior vena cava. The pressure-volume loops before (solid loops) and after SIN-1 infusion (dashed loops, 160 $\mu g \cdot kg^{-1} \cdot min^{-1}$) are shown. The solid line indicates slope of the end-systolic pressure-volume relationship obtained by the nonlinear regression of the end-systolic pressure volume points. The pressure-volume relationship shifted to the left, indicating a positive inotropic effect.

marked increases in slope from 18.7 ± 4.2 to 29.7 ± 10.5 mmHg/μl, maximal at 320 $\mu g \cdot kg^{-1} \cdot min^{-1}$ ($P < 0.04$; Fig. 5). This positive inotropic effect was reflected in multiple indexes of myocardial contractility, namely the preload recruitable stroke work and the dP/dt corrected for pressure. In the presence of 30 IU Cu/Zn SOD ($n = 6$), the positive inotropic effect of SIN-1 was abolished or converted to a negative inotropic effect (Table 3).

SIN-1 in the presence or absence of SOD did not change preload, as measured by LVEDP (8 ± 1 vs. 8 ± 1 mmHg, respectively), LV end-diastolic volume (19 ± 2 vs. 21 ± 2 μl, respectively), or heart rate (Table 3). On the other hand, SIN-1 exerted a vasodilator action: arterial elastance (afterload) decreased 17 ± 5% from the control value of 14.4 ± 6.4 mmHg/μl ($P = 0.02$). This effect was not different in the presence of SOD. Thus SIN-1 had both a positive inotropic and vasodilator effect in mice.

DISCUSSION

In this study, we demonstrate in both isolated crystalloid perfused hearts and in vivo that the NO/peroxynitrite donor SIN-1 has positive inotropic effects independent of cGMP or cAMP formation. In contrast, SNP, which potently stimulated cGMP formation, did not augment myocardial contraction. The SIN-1-positive inotropic effect could be abrogated by SOD, which also led to increased cGMP. The SIN-1 response persisted in the presence of guanylyl cyclase inhibition but was blocked by GSH, supporting a role for a thiol-nitrosylation-dependent mechanism of action. Finally, a spontaneous donor of NO·, DEA/NO, also had a positive inotropic effect independent of cGMP release.

The conditions under which NO stimulates or inhibits its myocardial contractility remain controversial. Initial studies focused on the contractile impact of cGMP. NO has been reproducibly demonstrated in vivo to have a negative inotropic effect in the presence of β-adrenergic stimulation (14, 16, 18), likely mediated by cGMP formation (32, 33). Positive inotropic effects of NO donors in the absence of β-adrenergic stimulation have also been described (29). With regard to cGMP signaling, this nucleotide appears to have a biphasic effect on basal myocardial contractility, with low concentrations being cardiostimulatory (27). Whereas diverse metabolic (21, 39) and physiological (12) effects have been attributed to the NO synthase 3 isoform found in many cardiac cellular constituents, including myocytes, recent investigations (10, 40) have questioned the obligatory role of NO synthase 3 in myocardial contractile regulation.

Recently, the cardiac effects of NO that are cGMP independent are increasingly being appreciated in both amphibian (5) and mammalian (41) systems. One mechanism that could contribute to cardiac stimulation is nitrosylation of the L-type calcium channel (3) and the ryanodine receptor (46), which has been shown to activate these calcium-cycling proteins in vitro.

In the present study, we infused different NO donors to isolated rat hearts and measured the contractile and cyclic nucleotide responses. The NO donor SIN-1 releases equimolar NO and O_2^-·, which react to form $ONOO^-$, in a reaction six times faster than SOD scavenging at physiological ionic strength (6, 24, 30, 36). $ONOO^-$ is an effective nitrosating species (24) and has been previously used in experiments regarding activation of the L-type calcium channel (3) and neutrophil-endothelium interactions in myocardial ischemia-reperfusion (26). SIN-1 stimulated myocardial contraction without altering the concentration of either cGMP or cAMP in the heart effluent. The iron nitrosyl NO donor SNP, which potently stimulated cGMP formation, did not increase contractility. Supporting this cGMP independence, SIN-1 inotropic responses were maintained in the presence of the soluble guanylyl cyclase inhibitor ODQ. In constrast, the response of SIN-1 could be converted to one resembling SNP (increasing cGMP without influencing contractility) by pretreatment with SOD. SOD would be anticipated to quench superoxide and prevent $ONOO^-$ formation. Finally, DEA/NO, which spontaneously releases NO· in aqueous solution (7), also stimulated myocardial contractility in the presence of ODQ, suggesting that other NO donors can similarly enhance contractility in a cGMP-independent manner. Thus different chemical signaling of NO/peroxynitrite donors produces different inotropic effects. The redox dependence of these reactions points out a factor that may not have been controlled for in previous studies.

In contrast to the divergence in inotropic effects, both SIN-1 and SNP had positive lusitropic effects, reducing LVEDP, the time constant of relaxation (τ), or

both. With regard to SNP, this response is most likely due to cGMP elevation, consistent with numerous in vitro (23, 33) and in vivo studies (28). The SIN-1-positive lusitropic effect, in the absence of cGMP elevation, is likely due to the same mechanism responsible for the positive inotropic effect (i.e., a Ca^{2+}-mediated action).

Several recent studies have examined NO-positive inotropic responses in vitro. Vila-Petroff et al. (41) demonstrated in rat myocytes that NO donors at low concentrations stimulated myocyte contractile amplitude in association with an increase in calcium transients as well as cAMP production. Higher concentrations of NO donors inhibited contractile amplitude in a cGMP-dependent manner. Chesnais and colleagues reported similar observations in frog myocytes and also described that positive inotropic responses to NO donors (5) or peroxynitrite (4) could be inhibited or converted to negative inotropic responses by SOD. An important regulatory link between NO synthase and superoxide has been suggested by the colocalization of NO synthase 3 and SOD in myocytes (2). Taken together, these studies support a redox-sensitive, non-cGMP dependent mechanism for NO-related positive inotropy. The present study extends these findings to a whole heart and in vivo preparations and raises the issue that these effects are due to nitrosylation-related mechanisms.

The physiological roles of nitrosylation-based reactions are being increasingly appreciated. Thiol-nitrosylation reactions have been shown to confer NO-like vasodilator activity to albumin (37) and other low-molecular-weight thiols (31), enhance tissue plasminogen activator function (34), participate in NO entry into cells (47), maintain balance between blood vessel tone and tissue oxygen requirements (38), and contribute to the regulation of caspases (22). The present findings extend in vitro observations that the L-type Ca^{2+} channel and the ryanodine receptor undergo thiol-nitrosylation leading to increased calcium cycling to the level of myocardial contractility.

Given that our infusions were performed in a crystalloid-perfused preparation, we sought to confirm the SIN-1 effects in vivo. Using mice, we confirmed that SIN-1 stimulates myocardial contraction and that this positive inotropic effect could be prevented by pretreatment with SOD. Thus these reactions are relevant in the intact cardiovascular system.

Two issues warrant mention. First, guanylyl cyclase inhibition with ODQ produced a positive inotropic effect, consistent with basal suppression of contractility by cGMP. Second, in the presence of ODQ, the SIN-1 inotropic effect was of less magnitude than that of SIN-1 alone (see Figs. 1 and 2). On the other hand, the NO donor DEA/NO produced similar responses (5% increases in $+dP/dt$) alone and after ODQ administration (see Fig. 4). These findings suggest that there may be important differences between the cross-talk between peroxynitrite versus NO· and cGMP signaling in the regulation of myocardial contractility.

This study is limited by the lack of direct biochemical measurement of protein nitrosylation. Endogenous nitrosylation of ryanodine receptors purified from dog myocytes has been demonstrated by Xu et al. (46). Future work is aimed at directly correlating NO effects on myocardial contractility with biochemical measures of protein nitrosylation. In addition, we did not measure intracellular cyclic nucleotide concentration changes in response to various stimuli. The cyclic nucleotide changes observed in the effluent of perfused hearts may not totally reflect intracellular events (e.g., SNP increases and ODQ decreases cGMP levels). Nevertheless, the changes observed are qualitatively those expected and are consistent with the intracellular observations previously reported (41).

In summary, the present data demonstrate positive inotropic effects of NO donors that are independent of cyclic nucleotide production. Conversely, NO donors that increase cGMP production do not increase myocardial contractility. Blockade of the response by GSH support the idea that this effect involves a nitrosylation reaction. The present findings may have broad implications for the regulation of a wide number of processes based on calcium cycling.

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