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Nitrite exerts potent negative inotropy in the isolated heart via eNOS-independent nitric oxide generation and cGMP–PKG pathway activation

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ABSTRACT

The ubiquitous anion nitrite (NO_2^-) has recently emerged as an endocrine storage form of nitric oxide (NO) and a signalling molecule that mediates a number of biological responses. Although the role of NO in regulating cardiac function has been investigated in depth, the physiological signalling effects of nitrite on cardiac function have only recently been explored. We now show that remarkably low concentrations of nitrite (1 nM) significantly modulate cardiac contractility in isolated and perfused Langendorff rat heart. In particular, nitrite exhibits potent negative inotropic and lusitropic activities as evidenced by a decrease in left ventricular pressure and relaxation, respectively. Furthermore, we demonstrate that the nitrite-dependent effects are mediated by NO formation but independent of NO synthase (NOS) activity. Specifically, nitrite infusion in the Langendorff system produces NO and cGMP/PKG-dependent negative inotropism, as evidenced by the formation of cellular iron–nitrosyl complexes and inhibition of biological effect by NO scavengers and by PKG inhibitors. These data are consistent with the hypothesis that nitrite represents an eNOS-independent source of NO in the heart which modulates cardiac contractility through the NO–cGMP/PKG pathway. The observed high potency of nitrite supports a physiological function of nitrite as a source of cardiomyocyte NO and a fundamental signalling molecule in the heart.

1. Introduction

It has been proposed that the circulating anion nitrite (NO_2^-) may represent the largest physiological reservoir of nitric oxide (NO) in the body [1]. Nitrite, present in mammalian blood at high nanomolar concentrations, can be reduced to bioactive NO along a physiological oxygen and pH gradient non-enzymatically by acidic disproportionation [2,3] or by a number of enzymes including xanthine oxidoreductase [4], nitric oxide synthase [5], mitochondrial cytochromes [6] and deoxygenated haemoglobin and myoglobin [7–9,21,22]. A number of studies now show that nitrite mediates various biological responses including hypoxic vasodilation [1,10], inhibition of mitochondrial respiration [8,10], cytoprotection following ischemia/reperfusion [11–14], and regulation of protein and gene expression [15]. While most of these effects are thought to be dependent on the reduction of nitrite to NO, it has been suggested that nitrite may also mediate effects independent of NO generation [15].

NO is an important paracrine mediator of cardiovascular signalling, with low concentrations modulating a number of vascular responses. In regards to cardiac contractility, both positive and negative inotropic effects of NO have been observed, depending on preparation of the model system and the type and dose of NO donor being used [16–19]. Given these effects of NO on cardiac function and the hypoxic environment of the heart [20], we sought to determine whether nitrite could act as an endocrine store of NO that modulates cardiac mechanical performance. We recently reported that deoxygenated cardiac myoglobin can reduce nitrite to NO via heme-based nitrite reductase chemistry [21]. This reaction produces NO under hypoxic and anaerobic conditions and inhibits mitochondrial respiration by NO binding to cytochrome *c* oxidase of the mitochondrial electron transport system [21]. Such an effect would be expected to modulate oxygen consumption, possibly adjusting myocardial energetics and mechanical performance [22]. Consistent with such a mechanism, Rassaf and colleagues recently studied the myoglobin knock-out mouse and reported that nitrite-dependent NO formation in the heart, negative inotropic effects, and in vivo inhibition of mitochondrial ATP

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formation were critically dependent on the presence of myoglobin [22]. We have now carefully evaluated the potency of nitrite in the heart as a negative inotrope and find that in rat heart physiological concentrations of nitrite as low as 1 nM elicit negative inotropy. In particular, we show that the inotropic effect is exerted by a mechanism involving eNOS-independent formation of NO, the generation of iron-nitrosylated myoglobin and activation of cGMP–PKG-pathway. This study suggests that in the beating heart nitrite is a significant physiological source of NO that modulates cardiac performance. These studies compliment our growing understanding of the role of nitrite as a vasodilator.

2. Materials and methods

2.1. Chemicals

All chemicals/drugs were purchased from Sigma unless otherwise indicated and prepared immediately before each experiment.

2.2. Animals

Male Wistar rats (Morini, Bologna, Italy S.p.A.), weighing 180–250 g, were housed three per cage in a ventilated cage rack system and were fed ad libitum.

The animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2.3. Langendorff preparation

Rats were anaesthetized with ethyl carbamate (2 g/kg rat, i.p.), and the hearts rapidly excised and transferred in ice-cold buffered Krebs–Henseleit solution (KHs). The aorta was immediately cannulated with a glass cannula and connected with the Langendorff apparatus to start perfusion at a constant flow-rate of 12 ml/min as previously described [23]. To avoid fluid accumulation, the apex of the left ventricle (LV) was pierced. A water-filled latex balloon, connected to a BLPR gauge (WPI, Inc. USA), was inserted through the mitral valve into the LV to allow isovolemic contractions and to continuously record mechanical parameters. Coronary pressure was recorded using another pressure transducer placed just above the aorta. Hemodynamic parameters were assessed using a PowerLab data acquisition system and analyzed using chart software (ADInstruments, Basile, Italy).

2.4. Experimental protocol

The Langendorff-perfused paced rat heart performance was evaluated by analyzing the left ventricular pressure (LVP), which is an index of contractile activity, the rate-pressure product (RPP) (as an index of cardiac work) [24], the maximal values of the first derivative of LVP [+(LVdP/dt)max] which indicates the maximal rate of left ventricular contraction, the time to peak tension of isometric twitch (Ttp) for inotropic effect and the maximal rate of left ventricular pressure decline of LVP [−(LVdP/dt)max], the half time relaxation (HTR), and $T/-t$ ratio obtained by +(LVdP/dt)max/−(LVdP/dt)max for lusitropic effects [25]. The mean coronary pressure was calculated as the average of values obtained during several cardiac cycles [23].

The response of the hearts to pharmacological agents (L-arginine, SNP, SNAP, GSNO, SIN-1, SOD, L-NMMA, L-NIO, C-PTIO, PTIO, Hb, ODQ, KT5823, RT 8-Br-cGMPs and 8-Br-cGMP) was obtained by perfusing the cardiac preparations with the buffer containing the specified pharmacologic agent at the desired concentration in the presence or absence of increasing concentrations of sodium nitrite.

2.5. Nitrite-dependent modification of cardiac proteins

To measure nitrite-dependent protein modifications, perfused hearts were frozen and homogenized in a solution of N-ethylmaleimide (20 mM), Nonidet P-40, and diethylenetriaminepentaacetic acid (100 μM). Nitrite levels were determined by directly injecting these samples into a solution of acidified tri-iodide, purging with helium in-line with a gas-phase chemiluminescence NO analyzer (Sievers, Boulder, CO). To determine the levels of specific NO adducts (iron–nitrosyl–hemoglobin and S-nitrosohemoglobin) samples were reacted with acidified sulfanilamide (0.5% v/v) to eliminate nitrite, and mercuric chloride (5 mM), to eliminate S-nitrosothiols, before being subjected to reductive chemiluminescence.

2.6. Statistics

Data were expressed as the mean ± SEM. Since each heart represents its own control, the statistical significance of differences within-group was assessed using the paired Student's *t*-test ($P<0.05$). Comparison between groups was made using a two-way analysis of variance (ANOVA) followed by Duncan's test. Differences were considered to be statistically significant for $P<0.05$.

3. Results

3.1. Nitrite potently modulates inotropy

In order to assess the effects of nitrite on cardiac function the well-characterized Langendorff perfused rat heart model was used. Basal parameters of this preparation are shown in Table 1 and performance variables measured every 10 min showed that the heart is stable for up to 180 min (see Fig. 1C).

Exposure of the perfused heart to nitrite (10 nM) had a significant negative inotropic effect on the rat heart characterized by a decrease in LVP. The effects of nitrite reached a maximum at 5 min after initial exposure and remained stable for up to 15 min, after which they gradually decreased with time (see Fig. 1B).

Since repeated exposures of the heart to the same concentration of nitrite did not result in pharmacological tolerance in control experiments (data not shown), a cumulative dose response was performed in the rat heart preparation. The nitrite-dependent inotropic effect was concentration-dependent and we showed a significant (20%) decrease in LVP at nitrite concentrations as low as 1 nM (Fig. 1A and D). This decrease in LVP was followed by an increase in coronary pressure, which became significant only at higher concentrations of nitrite (Fig. 1E).

3.2. Nitrite-induced inotropy and lusitropy in rat heart

To determine both inotropic and lusitropic effects of nitrite, the heart was exposed to increasing concentrations of nitrite (0.1 nM to

Table 1
Baseline cardiac parameters in Langendorff rat heart model

Left ventricular pressure	Heart rate	Rate pressure product	Coronary pressure	LV dp/dt max	LV dp/dt min	Time to peak tension	Half time relaxation	$T/-t$
89 ± 2.8 mm Hg	281 ± 7 beats/min	2.5 ± 0.2 10^4 mm Hg beats/min	62 ± 3.1 mm Hg	2491 ± 128 mm Hg/s	1663 ± 70 mm Hg/s	0.08 ± 0.02 s	0.05 ± 0.01 s	−1.48 ± 1.85

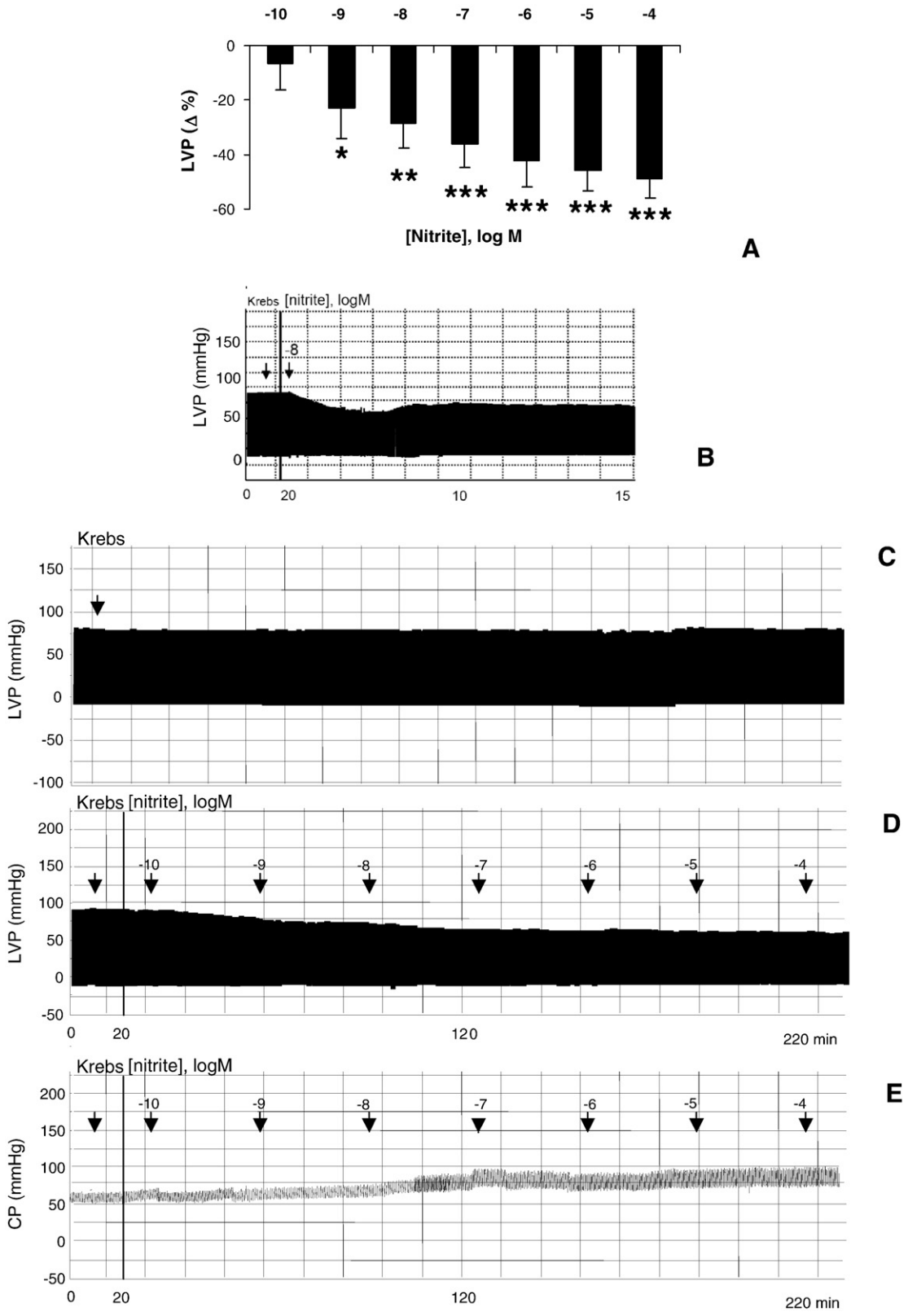

Fig. 1. Nitrite modulates cardiac inotropy. (A) Bar graph showing the dose–response nitrite effect on LVP ($n=7$). (B) Representative LVP trace showing the effect of single concentration of nitrite (10^{-8} M). (C) Representative LVP trace showing the time course obtained in the presence of vehicle alone. (D) Representative LVP trace showing the effects of increasing concentrations (10^{-10} to 10^{-4} M) of nitrite (each arrow represents the administration of a single concentration). (E) Representative CP trace showing the effects of increasing concentrations (10^{-10} to 10^{-4} M) of nitrite (each arrow represents the administration of a single concentration). Percentage changes were evaluated as means ± S.E.M. Asterisks indicate values significantly different from the control value: *$P<0.05$, **$P<0.025$, ***$P<0.01$.

100 μM) and changes in basal cardiac parameters were measured. Nitrite mediated negative inotropic and lusitropic responses as demonstrated by a significant decrease in LVP, RPP, +(LVdP/dt)max, and −(LVdP/dt)max, in parallel with a significant increase in T/−t. A small, but significant decrease in HTR was also observed when nitrite concentration reached 100 μM (Fig. 2). Taken together, these data suggest that physiological concentrations of nitrite mediate negative inotropy and lusitropy even at basal conditions (i.e. perfusion with normoxic buffer).

3.3. A comparison of nitrite-dependent negative inotropic effects with other NO donors suggests an NO-dependent mechanism of activity

A number of studies have shown that nitrite mediates its effects through its conversion to NO. Hence, we compared the inotropic effects of nitrite and authentic NO. To determine the effects of endogenously NO synthase (NOS)-generated NO on cardiac function, we treated the isolated heart preparations with the physiological substrate for NOS, L-arginine. Routinely [26], we use L-lysine as an amino acid control instead of D-arginine because, as stated by Amrani et al. [27], the latter is not taken up by amino acid uptake mechanism and therefore cannot be used as a control. L-arginine (10^{-8} M) induced a significant decrease in LVP (−17%), similar to the effects observed with nitrite. The L-arginine-dependent effect was inhibited by the NOS inhibitor L-NMMA (10 μM), confirming that the inotropic effects of L-arginine were indeed NOS-dependent (see Fig. 3A). These data suggest that NOS-derived NO has a similar negative inotropic effect as nitrite-derived NO.

We then tested the effects of the NOS-independent NO donors, sodium nitroprusside (SNP) (0.1 nM to 100 μM), S-nitrosoglutathione (GSNO) (0.1 nM to 100 μM), and S-nitrosopenacillamine (SNAP) (0.1 nM to 100 μM), as well as SIN-1 (0.1 nM to 100 μM), a simultaneous donor of NO and superoxide (O_2^-) (Fig. 3A and B). Similar to nitrite, SNP, which is a direct NO donor, was a negative inotrope, mediating a decrease in LVP at nanomolar concentrations.

In contrast, GSNO, SNAP and SIN-1 exerted a biphasic effect, with low concentrations (0.1–1 nM) resulting in a significant (20%) increase in LVP and higher concentrations resulting in a decrease in LVP and (LVdP/dt)max (Fig. 3A and B). It is of physiological interest that in rat isolated hearts GSNO, generated by a nitrosative pathway, was shown to activate sGC through Cu^{2+}-catalysed release of free NO [28]. When SIN-1 is converted to a pure NO donor by the removal of superoxide with superoxide dismutase, the drug exhibited negative inotropy even at a lower concentration (10^{-10} M) (Fig. 3D), consistent with the observed effects of L-arginine and SNP and effects reported by Paolocci et al. [16].

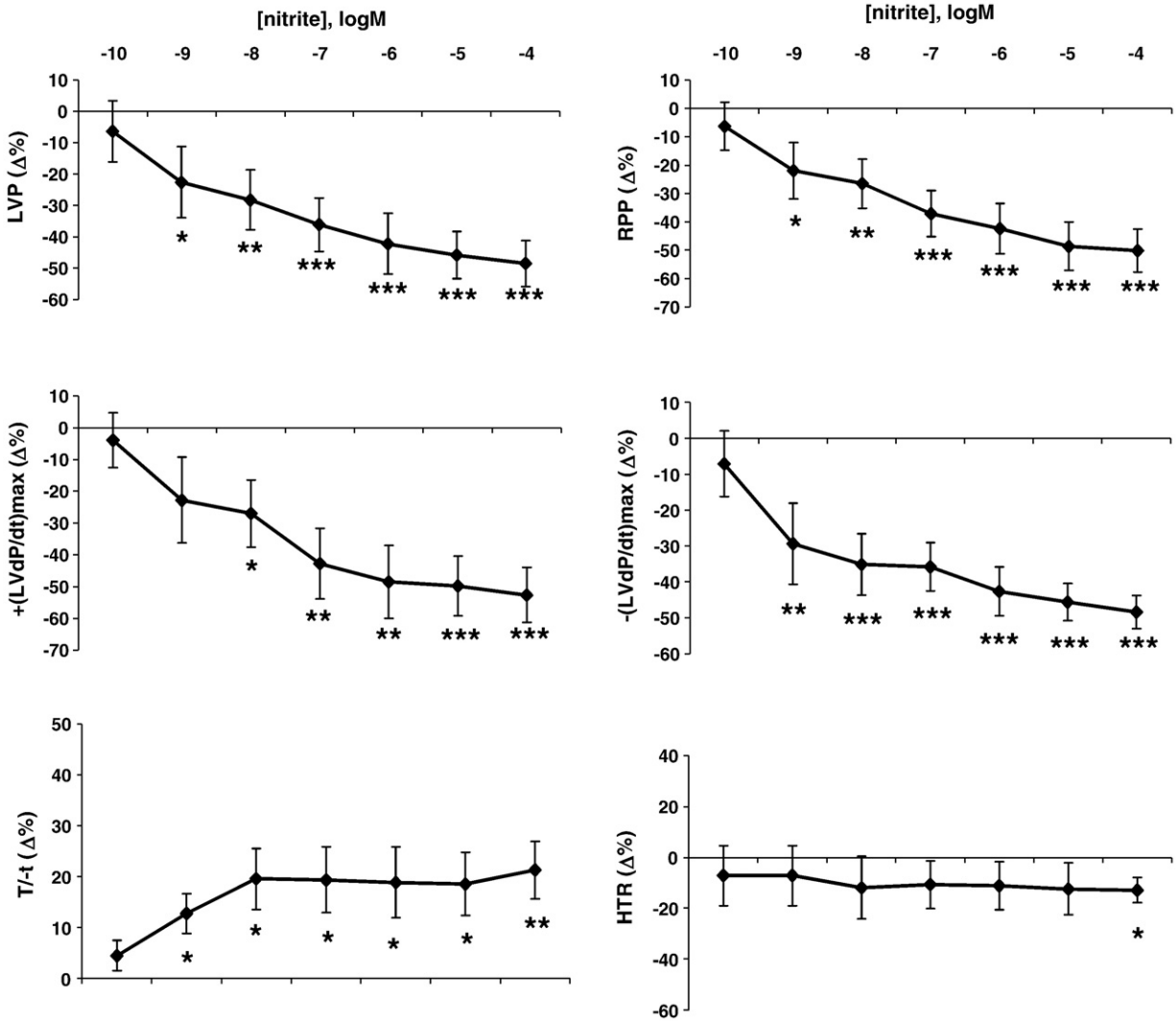

Fig. 2. Nitrite modulates lusitropic parameters. Cumulative dose–response curves showing nitrite effect on inotropic and lusitropic parameters in isolated and perfused rat hearts ($n=7$). Percentage changes were evaluated as means ± S.E.M. Asterisks indicate values significantly different from the control value: *$P<0.05$, **$P<0.025$, ***$P<0.01$.

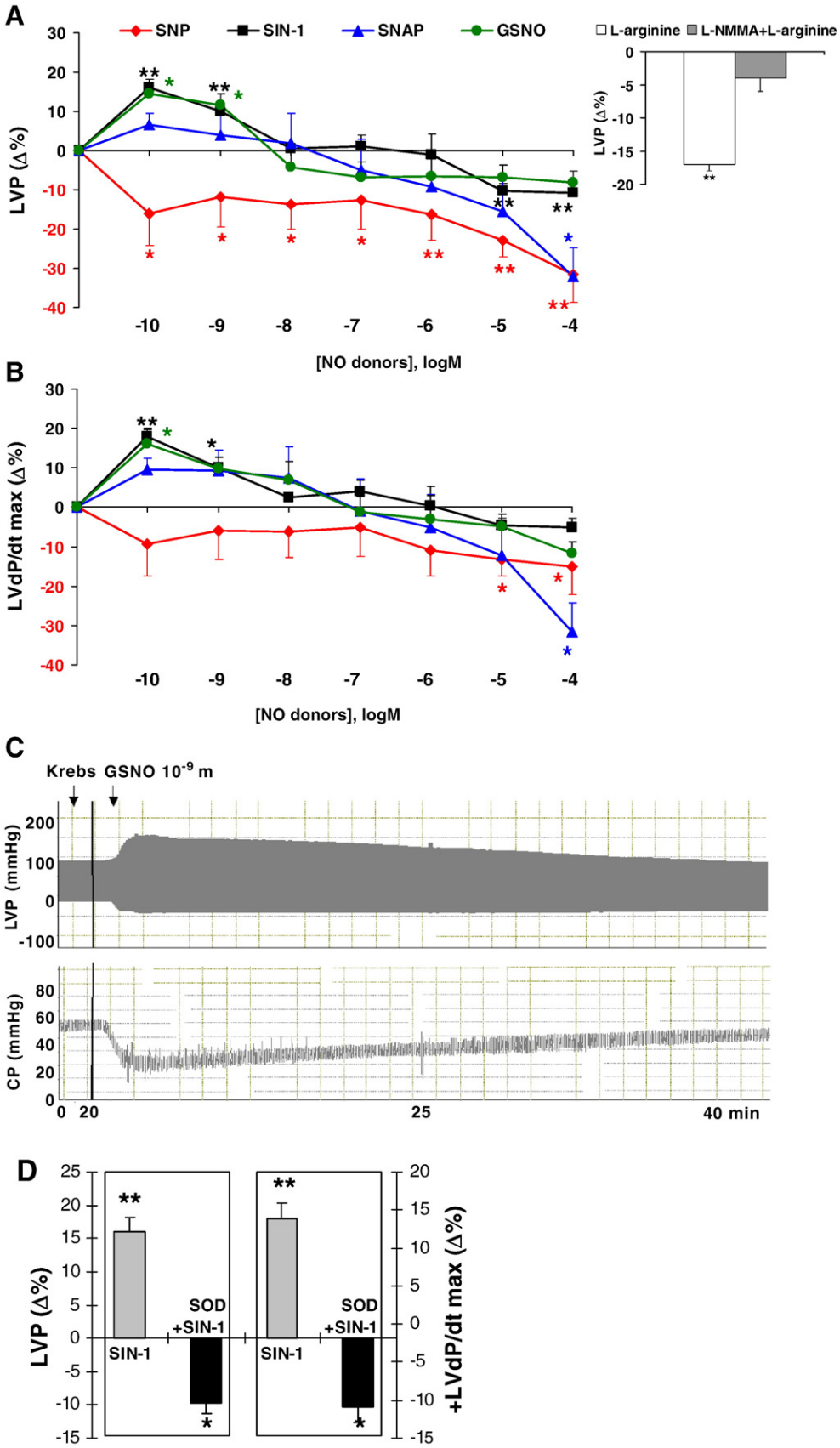

Fig. 3. Endogenously produced NO modulates cardiac inotropy. (A, B) The effect of NO donors on isolated and perfused rat hearts ($n=5$–7). On the right the effect of the authentic NO donor L-arginine (10^{-8} M) is depicted ($n=4$) (C) Representative LVP and CP traces showing the effects of GSNO (10^{-9} M) on isolated and perfused rat hearts. (D) Effect of SIN-1 (10^{-10} M) on isolated and perfused rat hearts before and after treatment with SOD (10 IU/ml) ($n=4$–5 for each group). Percentage changes were evaluated as means ± S.E.M. Asterisks indicate values significantly different from the control value: *$P<0.05$, **$P<0.025$, ***$P<0.01$.

The biological response to S-nitrosothiol NO donors was unique from that of nitrite, L-arginine, SNP, and SIN-1 with SOD, showing a biphasic response similar to SIN-1 without SOD. This differential effect could be secondary to unique effects of nitrosonium NO donors or secondary to potent coronary perfusion effects that modulate inotropy. A typical coronary dilation trace elicited by GSNO is shown in Fig. 3C.

These data demonstrate that in this system, NO generated by a number of sources (both endogenous and exogenous) and nitrite behave similarly. The divergent effects of GSNO, compared with nitrite, L-arginine, SIN-1 with SOD, and SNP, suggest that nitrite does not behave in the heart as an S-nitrosothiol donor, but as a direct NO radical donor.

3.4. Nitrite-dependent negative inotropy is NO-dependent but NOS independent

Since NO and nitrite mediate similar effects on the heart, we investigated whether NOS was involved in the negative inotropic effect of nitrite. To determine whether nitrite may be stimulating NO production from NOS or whether NOS reduces nitrite to NO as previously reported [5], we exposed the isolated rat heart to nitrite (0.1 nM–100 μM) in the presence and absence of the NOS inhibitors L-NMMA or L-NIO (Fig 4A and B).

Treatment with L-NMMA (0.1 nM–100 μM) did not modify the nitrite-induced decrease in LVP and +(LVdP/dt)max (Fig. 4A). Although the selective inhibition of eNOS by L-NIO (0.1 nM–100 μM) appears to enhance the nitrite-dependent decrease in LVP, this can be accounted for by the decrease in LVP observed in the presence of L-NIO alone (Fig. 4A).

To test whether the reduction of nitrite to NO is involved in the mechanism of nitrite-dependent negative inotropy, rat hearts were treated with nitrite in the presence and absence of the NO scavengers C-PTIO (100 nM), PTIO (100 nM), or oxyhemoglobin (10 μM) (Fig. 4C and D). In this system the heart is perfused with oxygenated buffer and hence hemoglobin will remain oxygenated to react with and inactivate NO but will not reduce nitrite to NO. The nitrite-dependent decrease in LVP and +(LVdP/dT)max was significantly attenuated by all three NO scavengers suggesting that NO formation from nitrite mediates the negative inotropic effects.

The response of the hearts to pharmacological agents (L-NMMA, L-NIO, C-PTIO, PTIO, Hb) was obtained by perfusing the cardiac preparations with the buffer containing the specified pharmacologic agent at the desired concentration in the presence or absence of increasing concentrations of sodium nitrite. Under basal conditions, the LVP value was $4.94 \pm 1.83\%$ in the presence of L-NMMA alone; $5.10 \pm 2.47\%$ in the presence of L-NIO alone; $-5.98 \pm 3.04\%$ in the presence of C-PTIO alone; $-4.65 \pm 3.71\%$ in the presence of PTIO alone; $4.81 \pm 2.5\%$ in the presence of Hb alone. However, these variations were not significantly different from the control values and did not affect the analysis of effect on nitrite signalling.

3.5. Nitrite-dependent formation of cardiomyocyte iron-nitrosylated hemoproteins (Fe^{+2}-NO)

Since nitrite-dependent negative inotropy appears to be NO mediated, but not dependent on NOS, we hypothesized that nitrite is reduced to NO by hemoproteins in the heart. While several mechanisms of nitrite reduction have previously been described, we and others have previously shown that myoglobin is the predominant nitrite reductase in the rat and mouse hearts [21,22]. However, other investigators have reported a role of XOR in the heart as a nitrite reductase [14]. The balance of data suggests that both pathways contribute to NO formation from nitrite in the heart. Reduction of nitrite by myoglobin and the concomitant NO production from this reaction has been correlated with an increase in the concentration of

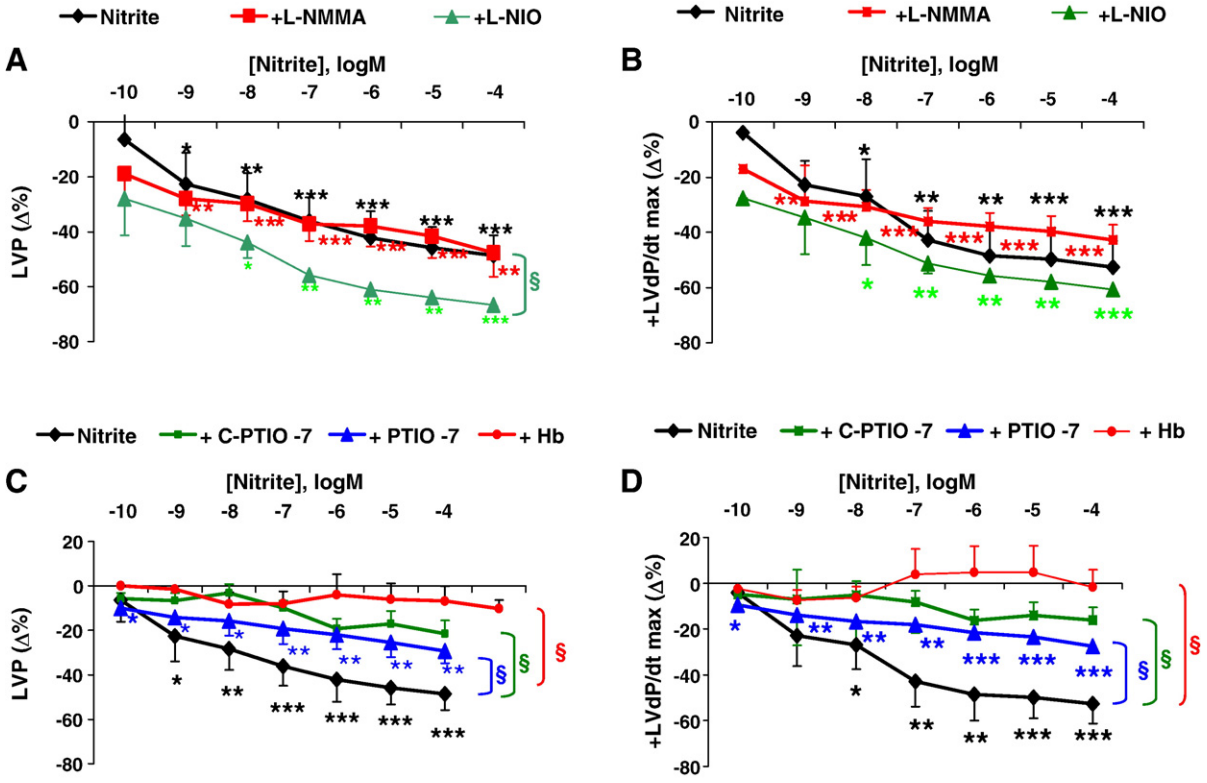

Fig. 4. Nitrite-dependent negative inotropy is NO-dependent. (A, B) The effect of nitrite of LVP and LVdP/dt max alone and in the presence of L-NMMA (10^{-5} M) and L-NIO (10^{-5} M). (C, D) The effect of nitrite alone and in the presence of C-PTIO (10^{-7} M), PTIO (10^{-7} M) and Hb (10^{-5} M) on LVP and LVdP/dt max in isolated and perfused rat hearts.

NO-modified proteins in the heart [1,21,22]. To determine whether nitrite is reduced by hemoproteins in the perfused heart, levels of iron–nitrosyl and S-nitrosated proteins were measured by reductive chemiluminescence in perfused, paced rat hearts after treatment with either nitrite (0.1 nM–10 μM) or saline (Fig. 5).

As expected, nitrite-treated hearts contained higher concentrations of nitrite (187 ± 16 pmol/mg) than control hearts (50 ± 22 pmol/mg) (Fig. 5A – C). Hearts treated with nitrite also had significantly higher concentrations of S-nitrosothiols (103 ± 17 pmol/mg) (Fig. 5D) and iron-nitrosylated proteins (165 ± 19 pmol/mg) (Fig. 5D) in comparison to control hearts (1.8 ± 0.8 and 0.7 ± 0.1 pmol/mg respectively). These data are consistent with the reduction of nitrite to NO by heme containing proteins, most probably myoglobin and xanthine oxidoreductase, in the heart.

3.6. Nitrite mediates negative inotropy through activation of sGC

Several studies have shown that activation of the NO–sGC pathway can mediate negative inotropy in the mammalian heart [27,39]. To determine whether this is the mechanism of nitrite-dependent negative inotropy, hearts were treated with ODQ (0.1 nM–100 μM), a potent oxidant and soluble guanylate cyclase inhibitor, and then subjected to nitrite treatment. While ODQ treatment significantly attenuated the negative inotropic effect of nitrite (Fig. 6A and B), it remains possible that ODQ will also oxidize heme proteins necessary for nitrite reduction. Therefore to further test a role for the cGMP pathway we inhibited protein kinase G (PKG). PKG is a downstream target of sGC and cGMP. Inhibition of PKG by KT5823 (100 nM) abolished nitrite-dependent negative inotropy supporting the involvement of the NO–sGC pathway (Fig. 6C and D). Inhibition of the nitrite-dependent negative inotropic effect by Rp-8-Br-cGMPs, another specific inhibitor of PKG, confirmed the involvement of PKG in the mechanism of nitrite's actions (Fig. 6C and D). In addition, we have tested the effects of the cGMP analogue 8-Br-cGMP in the presence and absence of the specific PKG inhibitors, KT5823 and Rp-8-Br-cGMPs. These data show that 8-Br-cGMP induces a dose-dependent negative inotropism which is abolished in presence of both PKG inhibitors (Fig. 6E and F).

Under basal conditions, the LVP value was −4.99 ± 2.64% in the presence of ODQ alone; 2.48 ± 1.77% in the presence of KT5823 alone; −2.08 ± 1.68% in the presence of Rp-8-Br-cGMPs alone. However, these variations were not significantly different from the control values.

Taken together, these data demonstrate that nitrite-dependent negative inotropy is dependent on the formation of NO, but independent

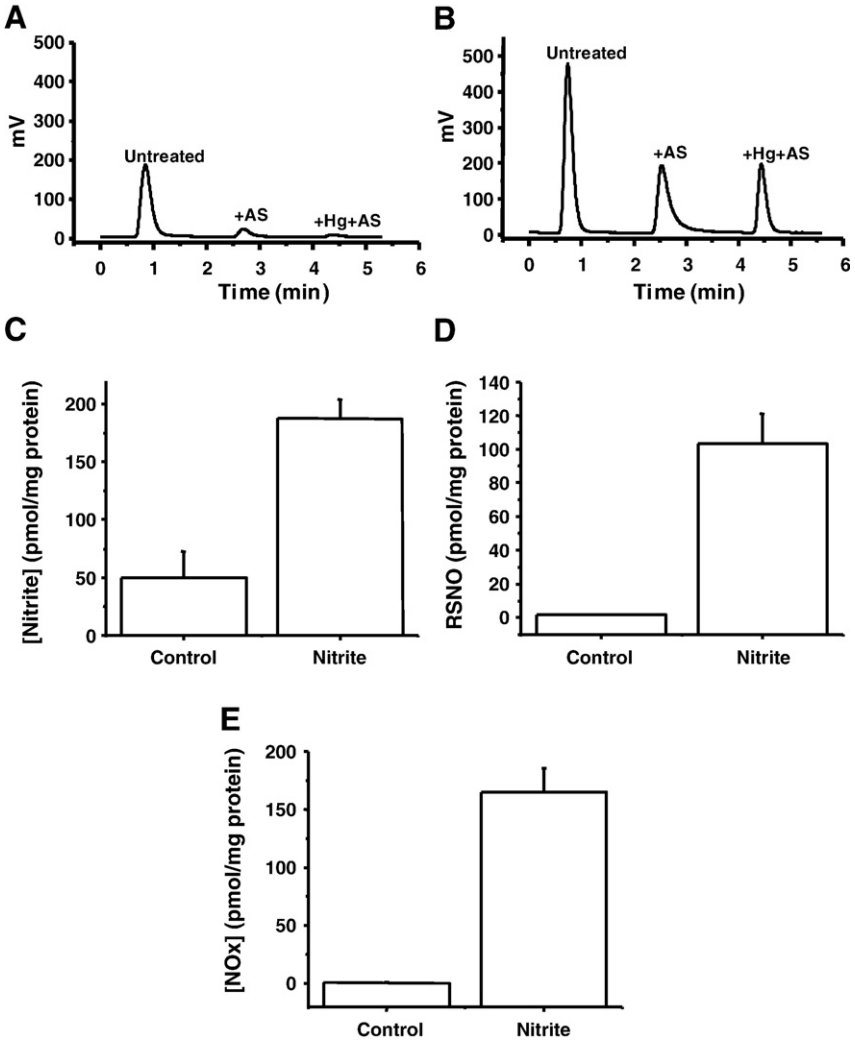

Fig. 5. Nitrite perfusion generates S-nitrosothiol and iron–nitrosyl in the heart. Rat hearts were perfused with either nitrite (10^{-10} to 10^{-4} M) or saline and tissue was analyzed by chemiluminescence. (A, B) Representative raw trace of saline (A) and nitrite (B) perfused heart subjected to tri-iodide based chemiluminescence with no pretreatment (untreated), treatment with acidified sulfanilamide (AS) and acidified sulfanilamide and mercuric chloride (Hg + AS). (C–E) Quantification of nitrite (C), S-NO (D), and Fe-NO (E) from traces such as those in panels A and B. Data are means ± SEM of at least 5 independent hearts.

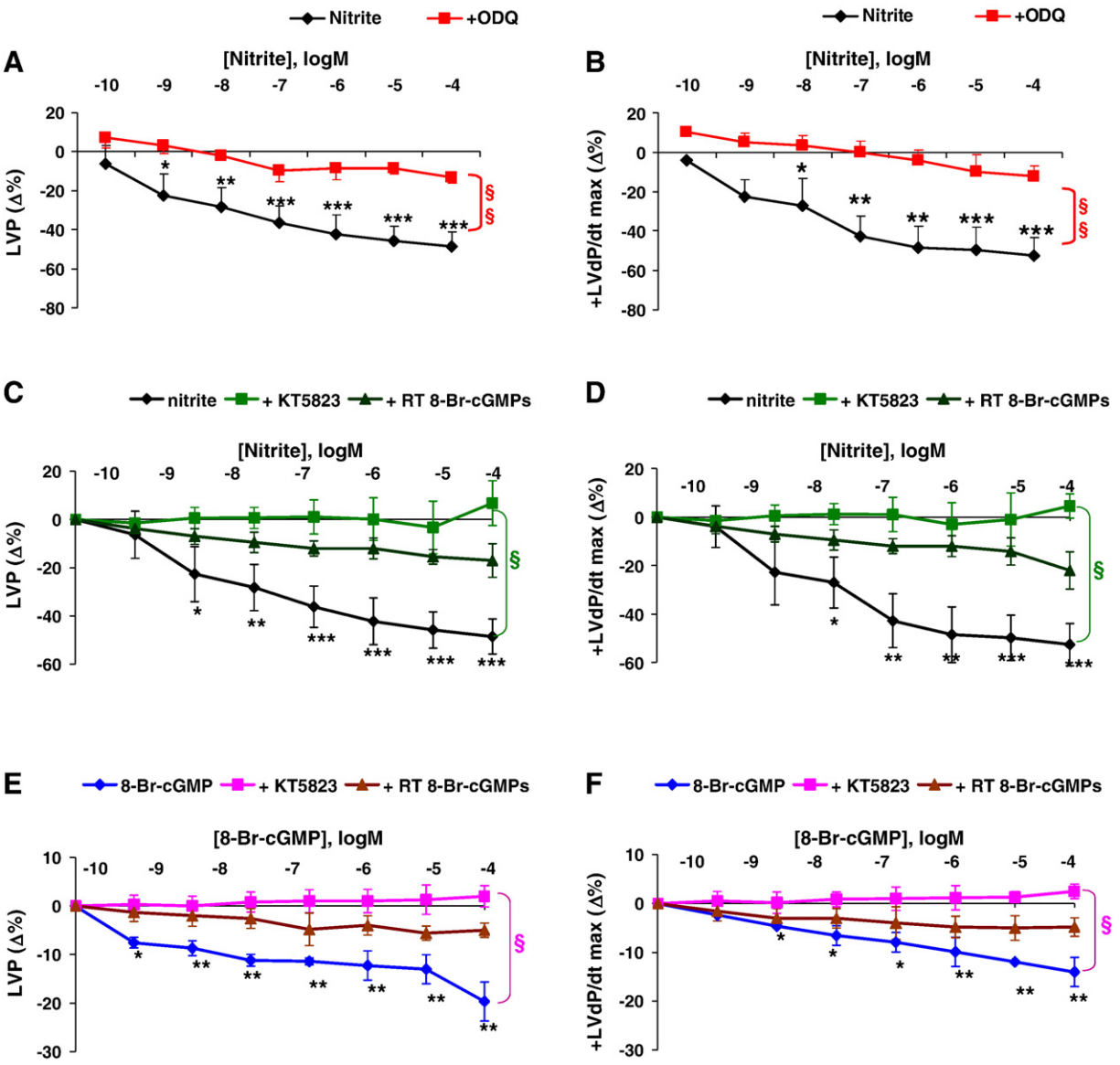

Fig. 6. Nitrite-dependent negative inotropy is cGMP–PKG-dependent. The effect of nitrite alone and in the presence of ODQ (10^{-5} M) (A, B) or KT5823 (10^{-7} M) or Rp-8-Br-cGMPs (10^{-7} M) (C, D) and the effect of 8-Br-cGMP alone and in the presence of KT5823 (10^{-7} M) or Rp-8-Br-cGMPs (10^{-7} M) (E, F) on inotropic parameters in isolated and perfused rat hearts ($n=5$–7). Percentage changes were evaluated as means ± S.E.M. Asterisk indicates values significantly different from the control value: *$P<0.05$, **$P<0.025$, ***$P<0.01$.

of NOS and also indicate that the generated NO activates soluble guanylate cyclase–PKG cascade.

4. Discussion

In this study, we have shown that physiological concentrations of nitrite potently affect cardiac mechanical performance by modulating contractility in the beating heart. In fact, nitrite elicited a concentration-dependent negative inotropic and lusitropic effects, decreasing LVP and (LVdP/dt)max. Furthermore, we show that the nitrite-dependent negative inotropic effect parallels the effects of authentic NO in this system and is inhibited by NO scavengers but not NO synthase inhibition, suggesting that nitrite is reduced to NO in the beating heart via NOS-independent mechanisms. Mechanistically, we show evidence that this negative inotropic effect is mediated via activation of the NO–sGC–PKG pathway.

In the rat Langendorff heart, after more than an hour of activity the heart gradually develops coronary constriction. However, the nitrite-dependent negative inotropy clearly precedes this increased vaso-pressure response demonstrating that nitrite-elicited inotropy is independent from other vascular effects of the anion. In another study using avascular endoluminally perfused hearts of fish (*Anguilla anguilla*) and frog (*Rana esculenta*), we showed similar nitrite-induced negative inotropic effects thus consistent with direct myocardial (and coronary-independent) action [29].

Several studies have demonstrated an NO–cGMP-dependent negative inotropy in the mammalian heart [16,30–32]. While NOS has previously been considered as the physiological source of NO in these studies, here we show that physiological stores of nitrite, which exist in both blood (150–300 nM) [33] and tissue (0.5–2 μM) [34], may also be an integral source of NO. This thesis is supported by the observed high potency of nitrite with significant effects on inotropy observed at concentrations (1 nM) well below the physiological level. The effects of nitrite on inotropy cannot be inhibited by NO synthase blockers. The effects are inhibited by direct NO scavenging by PTIO and oxyhemoglobin, as well as by inhibition of the NO–cGMP–PKG pathway. These data support an emerging paradigm that nitrite subserves a function as a NOS independent source of NO [35].

Experiments comparing nitrite to exogenous NO donors also suggest an NO-dependent effect of nitrite on cardiac inotropy. Accordingly, L-arginine increases NO formation from NOS and exerts similar negative inotropic effects as nitrite. The direct NO donors sodium nitroprusside and SIN-1, treated with SOD to scavenge the superoxide, both exert negative inotropy. Note that SOD treatment of SIN-1 converts this donor to an NO donor [36]. The biological response to S-nitrosothiol NO donors appears to be unique from that of nitrite, L-arginine, SNP, and SIN-1 with SOD, since S-nitrosothiol NO donors show a biphasic response similar to SIN-1 without SOD. This differential effect could be secondary to unique effects of nitrosonium NO donors or secondary to potent coronary perfusion effects that modulate inotropy. The comparative NO donor experimental data demonstrate that, in this system, NO generated by a number of sources (both endogenous and exogenous) and nitrite behave similarly. The divergent effects of GSNO, compared with nitrite, L-arginine, SIN-1 with SOD, and SNP, suggest that nitrite does not behave in the heart as an S-nitrosothiol donor, but as a direct NO radical donor.

The inhibition of the nitrite-dependent decrease in LVP in the presence of ODQ suggests the involvement of the soluble guanylate cyclase–cGMP in this mechanism. An important intramyocardial target of cGMP is a cGMP-dependent protein kinase (PKG). The finding that KT5823, an inhibitor structurally unrelated to cGMP, abolished the nitrite-induced inotropy is consistent with a cGMP–PKG-dependent mechanism underlying the nitrite-induced negative inotropy. In fact, it is well acknowledged that in many tissues, including rat ventricular myocytes, NO, by targeting soluble GC, and thus PKG, negatively affects contractility by reducing L-type Ca^{2+} current [37,38] and by phosphorylating troponin I, thus reducing troponin C affinity for calcium, and depressing contractility [39]. Conceivably, both L-type Ca^{2+} current reduction and PKG-mediated myofilament desensitization to Ca^{2+} may account for this nitrite-induced negative inotropy (see [39] for reference).

Several mechanisms by which nitrite can be reduced to NO along a physiological oxygen and pH gradient have previously been described. In this study, by using a hemoglobin-free perfusate and NOS inhibitors, we have demonstrated that hemoglobin and NOS are not involved in nitrite reduction in the myocardial tissue perfused with buffer. We have previously shown that myoglobin is the predominant nitrite reductase in the hypoxic heart, and that NO generation from myoglobin can bind to and inhibit cytochrome *c* oxidase of the mitochondrial electron transport chain to inhibit cellular respiration [21,22]. Indeed it has also been shown that during hypoxia, myoglobin-dependent reduction of nitrite to NO in the heart is responsible for the downregulation of cardiac energy status [22]. Interestingly, in this study, even though the heart was perfused with normoxic buffer, an increase in iron–nitrosyl heme in the nitrite-infused heart tissue was detected, consistent with myoglobin-dependent nitrite reduction. This suggests that in the heart even with adequate oxygen supply, tissue oxygen concentration and/or pH falls low enough to deoxygenate myoglobin and allow the reduction of nitrite to occur. In fact, in the mammalian heart, the presence of a significant transmural oxygen gradient has been reported. For example, in the dog heart, characterized by an abundant collateral coronary circulation and in which the myocardial pO_2 averages around 31 mm Hg, the transmural oxygen gradient is 15–20 mm Hg, the lowest pO_2 values being detected in the innermost myocardial layers (subendocardium) [40]. This situation can be even more exacerbated in those hearts such as the human, pig and rat hearts which are supplied by a terminal type of coronary arterial vascularization.

In our study, nitrite dose-dependently decreases oxygen consumption in the rat heart (data not shown). A negative inotropism, accompanied by a decreased myocardial oxygen consumption, has been recently reported in a Langendorff perfused mice heart [22]. Interestingly, these effects, obtained only at higher concentrations (100 μM/L) than the present study, were not observed in Mb-lacking mice [22].

In conclusion, we have shown that nitrite mediates negative inotropy in the rat heart through a mechanism involving its reduction to NO and consequent cGMP–PKG-dependent modulation of contractility. Although the exact target of nitrite is unknown, it is clear that even during normoxic conditions, nitrite is an important source of NO in the beating heart. This study may have implications for understanding the mechanisms by which nitrite regulates cardiac energetics physiologically and protect the heart during pathological ischemia.

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