SVOA Medical Research

e-ISSN 2977-3644

ScienceVolks Open Access

Original Article

Nitric Oxide Increases Myocardial Cross-Bridge Cycling Rate and Reduces the Loss of Rapid Relaxation During Its Inhibition of β-Adrenergic Stimulation

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DOI: https://doi.org/10.58624/SVOAMR.2026.04.013

Received: May 08, 2026

Published: May 28, 2026

Citation: Hoh JFY, Turnbull L, Rossmanith GH. Nitric Oxide Increases Myocardial Cross-Bridge Cycling Rate and Reduces the Loss of Rapid Relaxation During Its Inhibition of β-Adrenergic Stimulation. *SVOA Medical Research* 2026, 4:3, 124-135. doi: 10.58624/SVOAMR.2026.04.013

Abstract

Objective: The positive inotropy induced by β-agonist on the myocardium is associated with enhanced relaxation rate due increased cross-bridge cycling rate resulting from cAMP-induced phosphorylation of cardiac troponin I (c-TnI) by protein kinase A (PKA). Nitric oxide (NO) at a high level is negatively inotropic and inhibits β-agonist induced positive inotropy. We investigated the effects of NO and a β-agonist, and both agents acting concurrently, on cross-bridge cycling rate in rat papillary muscle.

Methods: We used dynamic stiffness analysis in the frequency domain to investigate cross-bridge cycling kinetics in rat papillary muscle during a sustained contracture induced by Ba^{2+} ions. This technique yields a characteristic parameter, f_{min}, the frequency at which stiffness is a minimum which reflects the cross-bridge cycling rate. We use S-nitroso-N-acetyl-penicillamine (SNAP) as NO donor and isoprenaline as a β-agonist.

Results: The mean control f_{min} at 25^0C was 1.83 ± 0.08 (SEM, n=4) Hz. After SNAP (60 μM) treatment, f_{min} significantly increased to 2.27 ± 0.04 Hz, an increase of 24% over the control value. After pre-treatment with methylene blue, a guanylyl cyclase inhibitor, SNAP had no influence on f_{min}. Treatment with β-agonist isoprenaline (2 μM) led to a 65% increase in f_{min}. However, after pre-treatment with both isoprenaline and SNAP, f_{min} enhancement was significantly reduced to 43%.

Conclusions: In the light of the current literature, we propose that (i) NO/guanylyl cyclase generated cGMP activated protein kinase G (PKG) to phosphorylate c-TnI, leading to the enhancement of f_{min} and cross-bridge cycling rate, (ii) the effect of NO and isoprenaline acting concurrently on f_{min} is due to two opposing mechanisms: cGMP activated cAMP phosphodiesterase (PDE2) hydrolysing cAMP, thereby reducing PKA phosphorylation of c-TnI, and cGMP activated PKG enhancing the phosphorylation of c-TnI, (iii) The functional significance of NO/cGMP/PKG-induced cross-bridge cycling rate enhancement is that, during endogenous NO induced inhibition of β-agonist mediated positive inotropy, which occurs in failing hearts, it moderates the cross-bridge cycling rate reduction due to PDE2 hydrolysis of cAMP, thereby enhancing myocardial relaxation rate and improving diastolic function.

Keywords: *Nitric oxide, β-agonist, Myocardium, Protein kinase G, Cross-bridge cycling, cAMP phosphodiesterases*

Introduction

The action of nitric oxide (NO) on cardiac contractility is highly complex, having a biphasic effect, a mild positive inotropy at a low concentration but a negative inotropy at a high concentration [1-5]. NO stimulates guanylyl cyclase to generate cyclic guanidine monophosphate (cGMP) [6]. The biphasic response of the myocardium to NO is due in part to the fact that cGMP acts on two distinct cyclic adenosine monophosphate (cAMP) phosphodiesterases with opposing physiological effects [7]: (i) cGMP-activated cAMP phosphodiesterase (PDE2), by which cGMP enhances the hydrolysis of cAMP, thereby reducing the intracellular cAMP level and (ii) cGMP-inhibited cAMP phosphodiesterase (PDE3) by which cGMP inhibits the hydrolysis of cAMP, leading to the enhancement of cAMP level. A high concentration of NO activates PDE2 to lower cAMP level, leads to a negative inotropy associated with the inhibition of the L-type Ca^{2+} channel [8,9] and reduced Ca^{2+} sensitivity [2,10]. A low level of NO activates PDE3, the resulting elevation of cAMP leading to a positive inotropic response [11,12]. The elevation of cAMP may in part be due to NO activating adenylyl cyclase by a mechanism independent of cGMP [2]. Further, a low level of NO may also generate positive inotropism by stimulating the ryanodine receptor, the Ca^{2+} release channel, by S-nitrosylating it [5].

A prominent feature of the action of NO on cardiac myocytes [13] and working hearts [14] is the acceleration of relaxation. In this respect it resembles the action of β-agonists except for the fact that the lucitropy due to NO is associated with negative inotropism whereas lucitropy due to β-agonists is associated with positive inotropism. This raises interesting questions about their molecular bases and functional significance. The lucitropic action of β-agonists is generally thought to be due to the accelerated uptake of Ca^{2+} by the sarcoplasmic reticulum and the reduced Ca^{2+} sensitivity of myofibrillar proteins [15]. However, the Ca^{2+} transient at the peak of the cardiac isometric twitch is reduced to a very low level [16], suggesting that the rate of cross-bridge detachment, the final step in the relaxation process, may play a significant role during relaxation.

The role of cross-bridge cycling in influencing the rate of cardiac muscle relaxation had been highlighted by mechanical analysis of cardiac muscle in the frequency domain using pseudorandom binary noise (PRBN) perturbations of muscle length during sustained contracture activated by Ba^{2+} ions [17,18]. This analysis yields a mechanical parameter, f_{min}, the stiffness minimum frequency, at which the cross-bridges are cycling in phase with the perturbing length oscillation frequency. This parameter represents the mean cross-bridge cycling frequency during sustained contraction [18] and has been shown to be correlated with the rate at which cross-bridges perform the power stroke and the rate of detachment of cross-bridges from the high-tension state, the final and rate-limiting step in the relaxation process [19]. Using the Q_{10} of f_{min} to extrapolate the value of f_{min} at room temperature to the value at body temperature yields the heart rate of the animal, so that during an isometric twitch, there is only time for the cross-bridges to undergo a single cycle [18]. It is thus important to adjust cross-bridge cycling rate to changes in heart rate to allow adequate diastolic ventricular filling. With an increase in heart rate, a proportional increase in cross-bridge cycling rate would maintain the ratio of the duration of systole and diastole, leading to optimal diastolic ventricular filling and cardiac function.

Adrenaline increases the f_{min} of cardiac muscle to match its enhancement of the heart rate, the enhanced cross-bridge cycling rate playing an important role in the lucitropic action of adrenaline [17]. This enhancement of cross-bridge cycling rate is implemented by the phosphorylation of cardiac troponin I (c-TnI) by protein kinase A (PKA) activated by the elevated cAMP level in response to β-adrenergic stimulation [20,21]. Since the positive inotropic action of low level of NO is at least in part due to elevation of cAMP, it is expected that the cross-bridge cycling rate would be elevated by a low level of NO.

The accelerated relaxation rate of cardiac muscle by NO is associated with a rise of intracellular cGMP [13] which is known to activate protein kinase G (PKG) that is also capable of phosphorylating c-TnI [22]. Both PKA and PKG phosphorylate c-TnI at the same sites (serine 22/23 in mouse) [23,24] and have the same effect of reducing Ca^{2+} sensitivity of cardiac myofilaments [25], which helps to promote the lucitropic effect of high-level NO exposure. Of great significance is the fact that treatment of cardiac myocytes with a NO donor at a high level results in an increase in c-TnI phosphorylation mediated by cGMP activated PKG [26]. The PKG phosphorylation of c-TnI should lead to an enhancement of cross-bridge cycling kinetics, but this has not been verified experimentally.

NO has been reported to inhibit β-agonist induced positive inotropy [27-29]. Treatment of cardiac myocytes with NO donor which results in a three-fold increase in intracellular cGMP decreases the contractile response to β-agonist isoprenaline by half [29]. This effect is mediated by PDE2 which is compartmentalized with adenylyl cyclase to efficiently target the rise in cAMP [30]. The reduction of β-agonist-induced rise of cAMP by NO is expected to reduce β-agonist induced enhancement of cross-bridge cycling rate, but this has also not been demonstrated experimentally.

At present there has apparently been no study on the action of NO on cardiac myosin cross-bridge cycling kinetics. In the present work, we use mechanical analysis of cardiac muscle in the frequency domain during Ba^{2+} contractures to determine (i) whether NO influences cross-bridge cycling rate, and if so, (ii) whether it is mediated by cGMP and (iii) whether NO influences the β-agonist induced enhancement of cross-bridge cycling kinetics. The molecular mechanisms underlying the observations and their functional significance will be discussed.

Materials and Methods

1. Muscle preparation

All experiments were performed in accordance with guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Male Sprague Dawley rats (3-6 weeks old) weighing 150-200g were euthanised by cervical dislocation and the hearts immediately removed and placed in Krebs-Henseleit Solution at 25°C. Using a dissecting binocular microscope for visualisation, the left ventricle was opened, and a papillary muscle was isolated. The average papillary muscle length was 3.0 mm and average diameter was 0.9 mm. The papillary muscle was dissected by tying and cutting the tendinous end and tying and removing a portion of the ventricular wall at the other end. The muscle was attached to a force gauge and length driver, the details of the procedure and equipment have been previously described [17].

2. Solutions

The standard solution used was a modified Krebs-Henseleit Solution (KHS) containing (in mM), 120 NaCl, 4.69 KCl, 1.5 $CaCl_2$, 0.54 $MgCl_2$, 1.02 KH_2PO_4, 25 $NaHCO_3$ and 10 D-glucose. A calcium-free KHS was prepared by omitting $CaCl_2$ from KHS. Barium contracture solutions were prepared by adding $BaCl_2$ to calcium free-KHS to a concentration of 0.5 mM. All solutions were kept at pH 7.4 by bubbling continuously with carbogen (95% O_2 and 5% CO_2).

The drugs used, S-nitroso-N-acetyl-penicillamine (SNAP), isoprenaline and methylene blue (MB) were obtained from Sigma-Aldrich, St Louis, Missouri, USA. Stock solutions of all drugs were made by dissolving in calcium-free KHS.

3. Length-tension dynamics and measurement of f_{min}

Small-amplitude changes to muscle length together with the resulting changes to muscle tension were recorded during steady Ba^{2+} contractures. The imposed changes in muscle length took the form of a sequence of pseudo-random length reversals, referred to here as pseudo-random binary noise (PRBN) perturbations. Fourier analysis of these length oscillations and the resulting interrupted tension transients gave rise to complex stiffness values similar to those resulting from small-amplitude sinusoidal changes to muscle length [31], that is, a minimum in stiffness accompanied by a minimum/maximum feature in the phase response. The frequency at which stiffness assumes a minimum value is termed f_{min}.

As reported before [17,18,32] the sequence of pseudo-random length reversals was software generated and introduced to the muscle via the length driver (Ling Dynamic System, Royston, England). Muscle force was measures by means of a piezoresistive strain gauge (Model 801, Akers, Horten, Norway). The amplitude of the length steps did not exceed 0.1% of the operating length. The length signal and the resulting force responses were recorded on a computer (Hewlett-Packard Co., Palo Alto, Calif). Fast Fourier transforms of the length and tension signals yielded the complex stiffness values which were subsequently displayed on a digital plotter (model 7225A, Hewlett-Packard). Two successive and identical PRBN sequences were generated, each lasting twenty seconds, where the first sequence ensured that dynamic steady state had been attained. The second sequence and the corresponding changes in tension yielded complex stiffness values at 350 equally spaced frequencies in the range 0.05Hz to 17 Hz. From the stiffness versus frequency plot, the stiffness minimum frequency f_{min} is obtained. Details of the PRBN and the analytic procedures that generates the stiffness and phase spectra are given in earlier works [17,31].

4. Experimental Protocol

All experiments were done with the muscle bath maintained at 25^{0}C. After mounting, the muscle was stretched by 30% of its resting length to arrive at the operating length. The muscle was initially stimulated with single pulses via platinum wire electrodes. The stimulating pulses were of 40V at a pulse frequency of 0.3 Hz.

Cross-bridge kinetics was analysed during Ba^{2+} activated contractures. To induce Ba^{2+} contracture, the muscle was first incubated in calcium-free KHS until twitch force stabilized at 10% of peak-force. The muscle was activated with the solution containing 0.5 mM $BaCl_2$ and the stimulator turned off. A contracture tension developed and reached a steady state within about 5 minutes. Once the contracture tension had stabilised, the control computer delivered the PRBN length signals, and the dynamic length and tension signals recorded. This recording formed the control response. The muscle bath was then replaced with solution containing isoprenaline and/or SNAP and perturbation analysis performed at 15 minutes after solution change to monitor the effect of the agent or agents on f_{min}. Data are presented as mean ± SEM. Student's t-test was used to determine significance differences between means, values of $P<0.05$ were considered significant.

Results

1. Effect of isoprenaline on f_{min}

Analysis of the PRBN length changes and the resulting force changes gave rise to the characteristic dynamic stiffness and phase data. Figure 1 shows the stiffness and phase plots of a papillary muscle following PRBN length perturbations under control conditions, superimposed on the result showing the effect of adding isoprenaline (2 μM). It clearly shows that isoprenaline shifts the dynamic stiffness and phase curves to higher frequencies, with a clear increase in the value of f_{min}, signifying that isoprenaline enhances cross-bridge cycling kinetics. The mean values of f_{min} for the control condition and after isoprenaline treatment, based on the results from 4 papillary muscles are 2.08 ± 0.14 (SEM) Hz and 3.43 ± 0.25 (SEM) Hz respectively and are statistically significant, with $P < 0.01$. Isoprenaline increased f_{min} by 65% above the mean control value. These results are substantially the same as that reported earlier for adrenaline on hearts from rats of similar age [17].

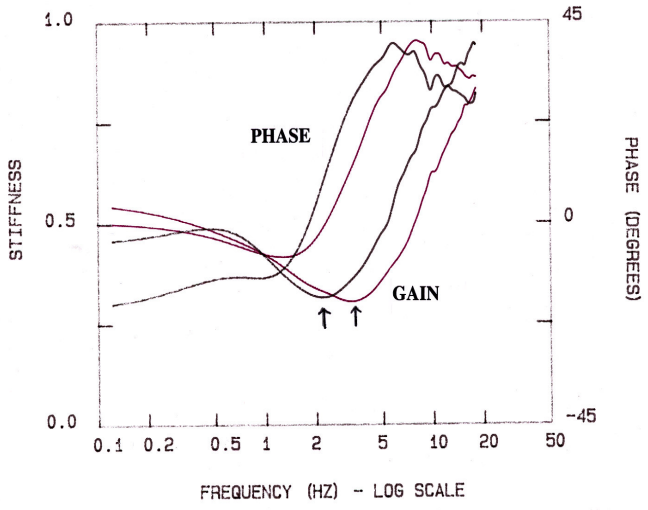

Figure 1. Stiffness (force/length or gain) and phase plots of a rat papillary muscle in response to PRBN length perturbations during Ba^{2+} induced contracture. The black traces indicate control responses with the f_{min} indicated by the left arrow. The red traces indicate responses 15 minutes after the addition of isoprenaline (2 μM), with the f_{min} indicated by the arrow on the right, clearly showing an upward shift in f_{min} in response to isoprenaline. Stiffness value of 1 is equivalent to 134 Nm^{-1}. Abbreviation: f_{min}: stiffness minimum frequency.

2. Effect of SNAP on f_{min}

To determine the effect of SNAP on cross-bridge kinetics, PRBN perturbation analyses were performed under control conditions and successively with 5 μM and 60 μM of SNAP on the same papillary muscle. Figure 2 shows an upward shift in f_{min} occurred at both these concentrations of SNAP. The mean values of f_{min} for control and after treatment with 60 μM isoprenaline for 4 papillary muscles are 1.83 ± 0.08 (SEM) Hz and 2.27 ± 0.04 (SEM) Hz respectively, and are statistically significant, with $P < 0.05$. SNAP at 60 μM induced a significant increase in f_{min} of 24% above the mean control value.

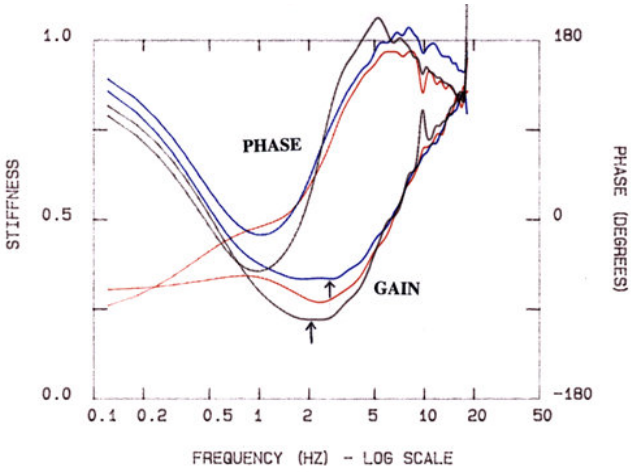

Figure 2. Dynamic stiffness (labelled gain) and phase plots of control and the effects of SNAP at 5 μM and 60 μM. The black traces are control responses with f_{min} indicated by the left arrow. The blue traces show the effect of SNAP at 60 μM with the enhanced f_{min} indicated by the right arrow. The responses to 5 μM SNAP (red traces) lie in between, showing an intermediate increase in f_{min}. Abbreviations: f_{min}: stiffness minimum frequency; SNAP: S-nitroso-N-acetyl-penicillamine.

3. Effect of SNAP and isoprenaline on f_{min}

We investigated the effect of exposing the papillary muscle to both SNAP (60 μM) and isoprenaline (2 μM) on f_{min}. Figure. 3 shows superimposed stiffness and phase plots of a papillary muscle under control conditions and with SNAP (60 μM) + isoprenaline (2 μM). It reveals that the agents caused an upward shift in f_{min} compared with control. The mean values for f_{min} of control and SNAP + isoprenaline derived from 4 papillary muscles are 1.92 ± 0.3 (SEM) Hz and 2.75 ± 0.11 (SEM) Hz respectively, and are statistically significantly different, with $P < 0.05$. The combination of SNAP and isoprenaline significantly shifted f_{min} up by 43% over the mean control value.

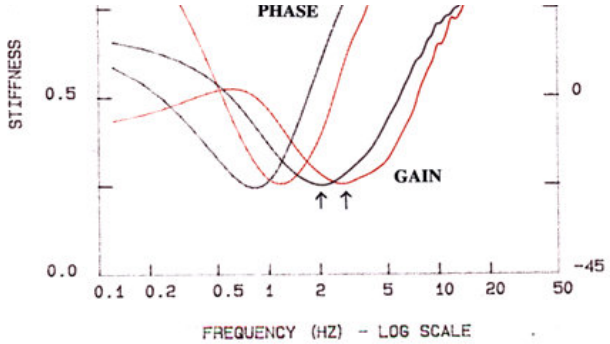

Figure 3. Stiffness and phase plots of a papillary muscle showing the effect of exposing the muscle simultaneously to both SNAP (60 μM) and isoprenaline (2 μM). The f_{min} of the control response (black traces) is indicated by the arrow on the left, the arrow on the right indicates the f_{min} of responses (red traces) 5 minutes after the exposure of the muscle to SNAP + isoprenaline. Abbreviation: SNAP: S-nitroso-N-acetyl-penicillamine.

4. Effects of MB on SNAP induced increase in f_{min}

The major pathway by which NO influences cardiac function is mediated by cGMP generated by NO stimulation of guanylyl cyclase. To investigate whether the enhancement of f_{min} by SNAP is mediated by this pathway, we studied the effect of SNAP after pre-treatment with MB which blocks the synthesis of cGMP by guanylyl cyclase. Papillary muscles were exposed to 10 μM MB for 15 minutes. SNAP (100 μM) was then added and the muscles were perturbed 15 minutes later. Fig. 4 shows a representative result which reveals that SNAP no longer has any significant effect on f_{min}. Analysis of 4 muscles give mean f_{min} values of 2.10 ± 0.18 (SEM) Hz and 2.05 ± 0.05 (SEM) Hz for control and MB + SNAP, respectively, the difference being not significantly different, with P > 0.79. Thus, MB completely blocks the effect of SNAP on f_{min}, indicating that the SNAP induced f_{min} enhancement is mediated by cGMP.

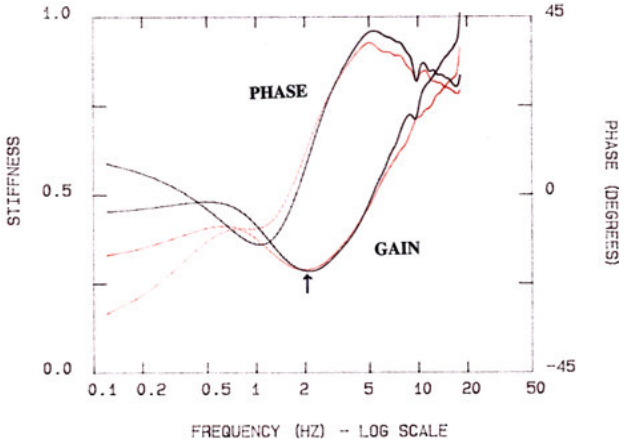

Figure 4. Representative stiffness and phase plots of a papillary muscle in the presence of SNAP (100 μM) and MB (10 μM). The black traces are control responses, and the red traces show the effects of incubating the muscle with MB + SNAP. The f_{min} values for control and MB + SNAP indicated by the arrow are identical. Abbreviations: MB: methylene blue; SNAP: S-nitroso-N-acetyl-penicillamine.

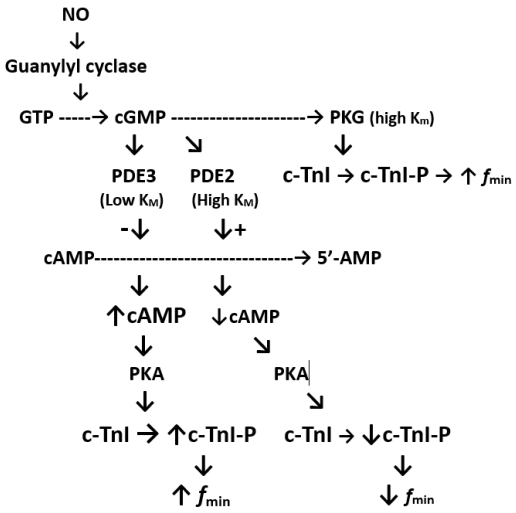

Figure 5. Molecular pathways through which NO generated cGMP may influence c-TnI phosphorylation and f_{min}. At low levels of NO, cGMP activates PDE3 with low K_m to inhibit the hydrolysis of cAMP, the elevated cAMP level activates PKA to phosphorylate c-TnI into c-TnI-P, enhancing f_{min}. At high NO levels, the elevated cGMP activates PDE2 with high K_m to hydrolyse cAMP, leading to reduced c-TnI-P and lowered f_{min}. High level NO and cGMP also activates high K_m protein kinase G (PKG) to phosphorylate c-TnI, enhancing f_{min}, compensating for the PDE2 induced reduction of f_{min}. Abbreviations: cAMP: cyclic adenosine monophosphate; cGMP: cyclic guanosine monophosphate; c-TnI; cardiac troponin I; cTnI-P: phosphorylated cTnI; NO: nitric oxide; PDE2: phosphodiesterase 2; PDE3: phosphodiesterase 3; PKA: protein kinase A; PKG: protein kinase G.

Table 1. Summary of the effects of isoprenaline, SNAP, and SNAP + isoprenaline and MB + SNAP on f_{min}.

Reagents	f_{min} (Hz)	f_{min} enhancement %
Control Isoprenaline (2 μM)	2.08 ± 0.14 3.43 ± 0.25**	65%
Control SNAP (60 μM)	1.83 ± 0.08 2.27 ± 0.04*	24%
Control Isoprenaline + SNAP	1.92 ± 0.3 2.75 ± 0.11*	43%
Control MB + SNAP	2.10 ± 0.18 2.05 ± 0.05***	-2%

Discussion

The present work demonstrates a novel aspect of the influence of NO on cardiac contractility, namely, the enhancement of cross-bridge cycling rate which plays an important role during cardiac relaxation. We first show that the β-adrenergic agent isoprenaline significantly enhanced f_{min} relative to the control value by 65%. This effect on f_{min} is essentially the same as the previously reported effect of another β-adrenergic agent, adrenaline [17]. Treatment of rat papillary muscles with NO donor SNAP at 60 μM leads to a 24% enhancement of f_{min} relative to the control value. These enhancements of f_{min} by isoprenaline and by SNAP signify that these agents increase the rate of cross-bridge cycling, the cross-bridges detaching earlier, accelerating the cardiac relaxation process [18].

Even though isoprenaline and SNAP enhanced f_{min} by 65% and 24% respectively, in the presence of both isoprenaline and SNAP, their effects on f_{min} did not summate. Rather, NO appeared to have reduced the effect of isoprenaline on f_{min} enhancement from 65% to 43%, a moderate reduction of 22% considering that under similar circumstances, the contractile response to β-adrenergic stimulation is halved [29]. Thus, when NO inhibits β-adrenergic induced positive inotropy, a substantial amount of cross-bridge cycling rate enhancement due to β-adrenergic stimulation is retained. Since NO itself enhances f_{min}, this result suggests that the inhibitory effect of NO on the β-adrenergic induced cross-bridge cycling enhancement is moderated by the NO induced enhancement of cross-bridge cycling.

An important way that NO influences cardiac contractility is by its modulation of the intracellular level of cAMP. cAMP activates PKA to phosphorylate various myocardial myofibrillar proteins and structures in the excitation-contraction coupling pathway, leading to a wide range of phenomena that accelerate and intensify excitation-contraction coupling, enhancing contractile force and accelerating relaxation [33]. Relevant to the current study, PKA phosphorylates c-TnI, causing a decrease in Ca^{2+} sensitivity, [34] which would tend to reduce contractile force, and an increase in f_{min} which leads to an enhanced relaxation rate [20,21]. Concurrently, PKA phosphorylates cardiac myosin binding protein-C, causing cross-bridges to swing out towards thin filaments [35]. This increases cross-bridge attachment rate and the enhancement of contractile force, thereby compensating the effect of the decrease in Ca^{2+} sensitivity due to c-TnI phosphorylation. PKA also phosphorylates titin, which reduces myocardial passive tension [36], thereby facilitating diastolic filling in a working heart. The overall result of an increase in cAMP level on a working heart is an increase in heart rate and cardiac output by enabling a strong systolic contraction and a rapid relaxation to allow the heart to complete the cardiac cycle in less time to accommodate the β-agonist induced tachycardia. The rapid relaxation and reduced passive tension promote an increase in end-diastolic volume to take advantage of the Frank-Starling's law [15].

The literature reviewed in the Introduction suggests that there are 3 possible signalling pathways by which NO may influence cardiac muscle f_{min}, and thus the kinetics of cross-bridge cycling and myocardial relaxation. These pathways involve the actions of NO/guanylyl cyclase-generated cGMP on PDE3, PDE2 and PKG, as shown in Fig. 5. cGMP stimulates PDE3 to inhibit, and PDE2 to enhance, the hydrolysis of either cAMP or cGMP. PDE3 binds both substrates with high affinity and have low K_m values and is thus active at low substrate levels. The inhibitory action of PDE3 on the hydrolytic activity for cAMP is much higher than that for cGMP so that PDE3 acts as a cGMP-inhibited cAMP-hydrolysing enzyme [37], activation of which allows the level of cAMP to rise.

While PDE2 can hydrolyse both cGMP and cAMP, cGMP binds to an allosteric site or GAF domain in PDE2 with high affinity, resulting in the enhancement of the cAMP hydrolysing activity of the enzyme [7]. Thus, PDE2 acts as a cGMP activated cAMP hydrolysing enzyme which reduces the level of cAMP.

The activity level of these phosphodiesterases is determined by the intracellular level of cGMP. At a low level of NO, the low level of cGMP will activate only PDE3, which has low K_m, to inhibit the hydrolysis of cAMP, leading to the enhancement of cAMP level and thus a positive inotropic response [12]. This positive inotropy is mediated by an enhancement of the voltage gated Ca^{2+} current [38,39], and should lead to the phosphorylation of c-TnI by PKA, which would increase f_{min}. Our preliminary observation (Fig. 2) shows that NO generated by a low level of SNAP (5 μM) does enhance f_{min}. The myocardial endothelium is a normal, physiological source of low NO level which generates a positive inotropic effect. Removal of the endothelium is associated with a negative inotropic effect partly due to the loss of NO from this source [13].

At high levels of NO, the higher level of cGMP generated would additionally activate PKG and PDE2 which have high K_m values. Activation of PKG, which phosphorylates c-TnI at the same sites as that phosphorylated by PKA [22], would raise f_{min}. Our observation that SNAP at 60 μM enhancing f_{min} by 24% may be attributed to this mechanism. PKG also generates a negative inotropic response mediated by a reduced voltage-gated Ca^{2+} current [38,39], the result of PKG phosphorylation of the L-type Ca^{2+} channel [40,41]. It is noteworthy that while enhancement of f_{min} due to elevated cAMP is associated with positive inotropism whereas NO induced enhancement of f_{min} is associated with negative inotropism.

Activation of PDE2 by cGMP enhances the hydrolysis of cAMP, the lowered cAMP would reduce PKA phosphorylation of c-TnI, tending to depress f_{min}. However, in the absence of β-agonist stimulation, cAMP level would already be low and the PDE2 mediated inhibitory effect on f_{min} would be minimal, so that our observation that SNAP at 60 μM moderately raising f_{min} by 24% represents principally, if not exclusively, the effect on f_{min} of PKG phosphorylation of c-TnI. We showed that MB, which inhibits cGMP production by NO, completely inhibited the enhancement of f_{min} by NO, indicating that NO induced f_{min} enhancement is mediated by the NO/cGMP pathway. Blocking PKG specifically should reveal whether the potential decrease in cAMP due to the activation of the cGMP/PDE2 pathway by NO plays a significant role in affecting the value of f_{min} mediated by high level NO.

PKG also phosphorylates titin, leading to reduced passive stiffness [42,43]. This, together with the accelerated cross-bridge cycling indicated by the enhanced f_{min}, underlies the molecular basis for the NO induced hastening of relaxation rate observed in ferret papillary muscle [44], the accelerated ventricular relaxation and enhanced chamber passive relaxation in isolated ejecting guinea pig heart [45] and in humans [46] in response to high level NO.

We also showed that in the presence of both a high level of NO and a β-adrenergic agonist, the f_{min} enhancement due to the latter acting alone was significantly reduced from 65% to 43%, a reduction of 22%. The elevation of cAMP due to the β-adrenergic agonist would be inhibited by the hydrolysis of cAMP by PDE2 activated via the NO/cGMP pathway, reducing the inotropic effects of β-adrenergic stimulation [30]. The reduced cAMP level would also decrease PKA phosphorylation of c-TnI, which would tend to decrease the 65% enhancement of f_{min} seen with β-adrenergic stimulation acting alone. The cGMP activation of PDE2 hydrolysis of cAMP leading to the lowering of f_{min} explains why the positive effects of NO and isoprenaline on f_{min} do not summate. We propose that the observed 22% decrease in f_{min} in the presence of NO and β-adrenergic agonist was moderate relative to the 50% reduction of contractile force response to β-adrenergic stimulation [29] because of the concurrent f_{min} enhancement due to the phosphorylation of c-TnI by cGMP activated PKG. The NO/cGMP/PKG-mediated 24% enhancement of f_{min} is postulated to partially compensate for the decrease in f_{min} due to NO/cGMP/PDE2 hydrolysis of cAMP, thereby reducing the loss of lucitropy associated with the negative inotropy due to the NO/cGMP/PDE2 mediated fall in cAMP.

This hypothesis can be tested by blocking PKG in the presence of β-agonist and high-level NO, which would be expected to result in a fall in f_{min} below the level for NO + β-agonist (43%) by an amount attributable to the effect of PKG phosphorylation of c-TnI (about 24%) to about 19%.

The interaction between NO and β-adrenergic agonist occurs in failing hearts in which circulating catecholamine levels are markedly increased [47] and the heart rate is increased [48]. Failings hearts have enhanced expression of nitric oxide synthase 3 (NOS3) at the mRNA and protein levels [49]. Further, inflammatory cytokines such as tumour necrosis factor (TNF) α, interleukin (IL)-1β and IL-6 are also increased in plasma and circulating leukocytes, as well as in the failing myocardium itself [50]. These cytokines would stimulate nitric oxide synthase 2 (NOS2) expression and release high levels of NO. The negative inotropic action of NO via PDE2 suppression of cAMP level helps to protect the failing heart from being overstimulated by the β-adrenergic agonist. However, this action via NO/cGMP/PDE2 alone would also reduce cross-bridge cycling and relaxation rates due to reduced PKA phosphorylation of c-TnI and would reduce diastolic ventricular filling and reduced stroke work. This is aggravated by the high heart rate reducing diastolic filling time, leading to further reduction of stroke work. However, the high level of NO would concurrently result in enhancing cross-bridge cycling rate via NO/cGMP/PKG-mediated phosphorylation of c-TnI. This would partially compensate for the reduced PKA phosphorylation of c-TnI, reducing the loss of lucitropy. The enhanced cross-bridge cycling rate due to PKG mediated c-TnI phosphorylation would help to complete the systolic contractions in less time, an important function in the face of enhanced heart rate in a failing heart. This leaves more time for filling the ventricle, assisted by the reduced passive stiffness from concomitant titin phosphorylation. The improved ventricular filling augments stroke work via Frank-Starling's law and is beneficial in normal as well as in pathological situations. High expression of NOS2 and NOS3 genes has been reported in myocytes in human athlete's heart and in hearts of patients with hypertrophic cardiomyopathy and is associated with low diastolic left ventricular stiffness and preserved left ventricular stroke work [51].

Conclusion

High level NO increases the rate of cross-bridge cycling of cardiac myocytes, which can be attributed to the NO/guanylyl cyclase generated cGMP activating PKG to phosphorylate c-TnI. This action enhances cardiac relaxation rate and improves diastolic function. Stimulation by a β-agonist increases cross-bridge cycling via cAMP activated PKA phosphorylation of c-TnI. NO, in the presence of β-agonist stimulation, inhibits the rise in cAMP via the NO/cGMP/PDE2 pathway, leading to reduced contractility and reduced PKA phosphorylation of c-TnI and would consequently reduce cross-bridge cycling rate and reduce lucitropy. The reduced lucitropy resulting from reduced PKA phosphorylation of c-TnI is compensated by the concurrent phosphorylation of c-TnI via the NO/cGMP/PKG pathway. This reduces the loss of lucitropy associated with NO inhibition of β-agonist stimulated inotropy and is beneficial in normal and failing hearts by improving myocardial relaxation rate and diastolic function.

Acknowledgement

This work was supported by a grant from the National Health and Medical Research Council of Australia. We thank Mark K Mariathas for his technical assistance in these experiments.

Disclosure and Conflict of Interest

The authors have no conflict of interest to declare.

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