Peroxynitrite-induced cardiac depression: role of myofilament desensitization and cGMP pathway

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Abstract

Objective: The oxidant species peroxynitrite, the reaction product of nitric oxide (NO·) and superoxide, has been implicated in several pathophysiological conditions of the heart. Here, we studied the mechanism of peroxynitrite-induced cardiac depression using specific drugs and simultaneous analyses of myocardial function and intracellular Ca2+ ([Ca2+]i). Methods: Rat hearts were perfused retrogradely and left ventricular function (balloon method) and [Ca2+]i transients were recorded on a beat-to-beat basis using the aequorin bioluminescence method. Peroxynitrite was infused at 10.8 ± 0.93 μM via sideline for 10 min, followed by a 15-min recovery period to monitor irreversible effects. Test drugs were infused prior to and during peroxynitrite application. Results: Peroxynitrite depressed left ventricular developed pressure (LVDevP; 40%), yet increased systolic and diastolic [Ca2+]i (1.3 and 2.3 fold, respectively; n = 12). When Ca2+ entry through Ca2+ channels or Na+/Ca2+ exchange transport was blocked using nicardipine (1 μM, n = 3) or dichlorobenzamil (30 μM, n = 5), respectively, cells showed lower [Ca2+]i and accentuated negative inotropic action in response to peroxynitrite. Peroxynitrite slowed left ventricular relaxation and [Ca2+]i transients, both of which were not affected by extracellular Ca2+ restriction. Importantly, the oxidant greatly depressed myofilament responsiveness to Ca2+, which was partly antagonized by Rp-8-(4-chlorophenylthio)guanosine-3′,5′-cyclic monophosphorothioate (Rp-cGMPS), an inhibitor of cGMP-dependent protein kinase. Also, the inhibitor partially restored left ventricular contractility (n = 6). Peroxynitrite stimulated cardiac cGMP production and coronary cGMP efflux (n = 6). Decomposed peroxynitrite had no effect in any of the tests. Conclusions: Peroxynitrite depresses myocardial contractility by decreasing the ability of Ca2+ to trigger contraction, and this effect is partly mediated by the cGMP/cGMP-dependent protein kinase pathway. © 2003 European Society of Cardiology. Published by Elsevier B.V. All rights reserved.

Keywords: Peroxynitrite; Contractility; Free radicals; Calcium

1. Introduction

Several types of cells of the cardiovascular system continuously generate superoxide and nitric oxide (NO·), two free radicals that, when occurring together, react at exceedingly high rate to form peroxynitrite anion [1]. Peroxynitrite is a strong oxidant that is thought to play a key role in several cardiovascular pathologies, including disorders resulting from enhanced production of NO- through inducible NO- synthase (NOS). The cytotoxicity of peroxynitrite appears to result from the conversion of peroxynitrite to peroxynitrurous acid (HOONO) which then reacts (via an activated state: HOONO·) [2] with biological substrates such as enzymes, lipids and nucleic acids in a hydroxyl radical-like manner. These properties make peroxynitrite a potent tissue-damaging species that exerts its cytotoxicity by interacting with various biomolecules, resulting in cellular dysfunction.

In spite of these findings, the physiological effects of peroxynitrite on the heart are controversial. Exogenous peroxynitrite was found to impair cardiac contractile function [3], to contribute to cytokine-induced myocardial failure [4] and to disrupt myofibrillar energetics [5]. Cardiospecific overexpression of inducible NOS in transgenic mice led to enhanced production of peroxynitrite, which was associated with cardiac fibrosis, hypertrophy and dilatation [6]. In studies of myocardial ischemia/reperfusion, the formation of (endogenous) peroxynitrite was found to be increased as evident from enhanced...
nitrotyrosine staining in ischemic tissue [7], and exogenous peroxynitrite aggravated reperfusion injury [8]. In contrast to these studies, peroxynitrite exerted cardioprotective effects under in vivo conditions at relatively low concentrations, possibly secondary to detoxification and formation of NO-donating nitrosothiols [9]. In line with this notion, peroxynitrite impaired functional recovery in hearts subjected to crystalloid cardioplegia, whereas it increased functional recovery in blood cardioplegia [10].

Several lines of evidence suggest that reactive oxygen species cause intracellular Ca\(^{2+}\) (Ca\(^{2+}\)) overload and that preventing generation or scavenging of reactive oxygen during several cardiac pathologies reduces both Ca\(^{2+}\) overload and attendant injury. On the other hand, an increase in [Ca\(^{2+}\)], e.g., during experimental ischemia/reperfusion, induces the conversion of xanthine dehydrogenase to xanthine oxidase and subsequently results in the generation of superoxide radicals. Whether peroxynitrite, formed in a further step from NO\(^{-}\) and superoxide, affects cellular Ca\(^{2+}\) homeostasis is not known, nor have the mechanisms underlying peroxynitrite-induced changes in Ca\(^{2+}\) dynamics been characterized.

In the present study, the mechanism of peroxynitrite-induced cardiac dysfunction was investigated. The effects of the oxidant were documented in parallel measurements of cardiac function and [Ca\(^{2+}\)], in isolated beating rat hearts using previously established methods [11]. In addition, we determined the origin of changes in [Ca\(^{2+}\)], and the role of the guanylyl cyclase/cGMP/cGMP-dependent protein kinase pathway in the negative inotropic action of peroxynitrite using specific antagonists.

2. Methods

2.1. Materials

Dichlorobenzamil was from Molecular Probes (Leiden, Netherlands), nicardipine was from Yamanouchi Pharmaceutical (Tokyo, Japan), ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) was from Tocris Cookson (Bristol, UK), Rp-8-(4-chlorophenylthio)guanosine-3′,5′-cyclic monophosphorothioate (Rp-cGMPS) was from BioLog (Bremen, Germany) and 3-isobutyl-1-methyl-xanthine (IBMX) and \(N^{\text{G}}\)-nitro-L-arginine (L-NNA) were from Sigma (Vienna). Peroxynitrite was synthesized from sodium nitrite (NaNO\(_2\)) and acidified hydrogen peroxide (H\(_2\)O\(_2\)) essentially as previously described [1]. The concentration of the solution was determined (~80–120 mM; see below) and the solution stored in aliquots of 1.5 ml at ~70 °C. Decomposed peroxynitrite was obtained by allowing peroxynitrite to decompose (5 min) before the NaOH solution was added. The final solution was filtered to remove excess H\(_2\)O\(_2\) and aliquots were stored at ~70 °C. Both peroxynitrite and decomposed peroxynitrite solutions had a pH close to 14.

2.2. Heart perfusion

Adult Sprague–Dawley rats of either sex were anesthetized with diethyl ether and heparinized (1000 U/kg, ip). The hearts were rapidly excised, retrogradely perfused at constant flow (13 ml/min per gram of heart wet weight) using Krebs–Henseleit buffer of standard composition (HCO\(_3\)\(^{-}\) concentration: 25 mM), and functional parameters were obtained as described in detail previously [11]. Hearts were paced at 5 Hz. The investigation conforms with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1985).

2.3. Exposure of hearts to peroxynitrite

Peroxynitrite or decomposed peroxynitrite solution (3 ml of each) was thawed in a small beaker and titrated with 1 N HCl to pH 12.5 (~1 ml). This decrease in pH was necessary to obtain a stable pH value inside the aortic cannula after mixing with HCl (see below). To obtain the nominal final concentration of 10 \(\mu\)M (mainly used in this paper), both peroxynitrite and decomposed peroxynitrite solutions were then diluted 1:3 with 10 mM NaOH (pH 12.0) and drawn into a chilled 5-ml glass syringe which was shielded from light and covered in ice to prevent peroxynitrite degradation. The concentration of peroxynitrite in the syringe was routinely monitored before use by measuring the extinction coefficient at 303 nm after the addition of 5 \(\mu\)l of peroxynitrite solution to 995 \(\mu\)l of 1 N NaOH. Hearts were exposed to peroxynitrite essentially as previously described [12]. Briefly, the peroxynitrite solution was delivered into the aortic cannula via a catheter (~10 cm long) attached to a port. Hydrochloric acid (0.01 N, pH 2.0) was delivered through a second catheter attached to a second port located at the opposite side on the aortic cannula (flow rate in both cases, 100 \(\mu\)l/min). Mixing of the two components resulted in a pH of 7.4–7.5 at the orifice of the aortic cannula as determined in separate experiments without heart attached. Because the two ports were located less than 1 cm from the heart, peroxynitrite immediately entered the coronary arteries. The concentration of peroxynitrite entering the heart was determined using dichlorofluorescein fluorescence [13]. The standard curve was constructed from 2.5 to 100 \(\mu\)M authentic peroxynitrite (\(r=0.999, n=4\)). Reagent blanks (perfusion buffer containing no peroxynitrite) were subtracted from readings. We never observed any change in the fluorescence signal of the blank during the 10 min of measurements. The target concentration of 10 \(\mu\)M was reached within 2 min after onset of application and maintained throughout the 10 min of application. The mean measured concentration, determined every 2 min, was 10.8 ± 0.93 \(\mu\)M (n = 8 determinations per time point). In several experiments, a lower (nominally 3 \(\mu\)M; measured: 3.7 ± 0.3 \(\mu\)M) and a higher (nominally 40 \(\mu\)M; measured: 40.0 ± 5.1 \(\mu\)M) concentration of peroxynitrite were used that were prepared analogously using higher and lower initial dilutions, respectively.
2.4. Effect of peroxynitrite on myocardial function

Hearts were equilibrated for 30 min and peroxynitrite (or decomposed peroxynitrite) was applied for 10 min, followed by 15 min of perfusion without peroxynitrite. Cardiac parameters were monitored throughout and included heart rate, coronary perfusion pressure, LVDevP (difference between left ventricular peak systolic pressure and end-diastolic pressure), maximal rate of rise and fall of LVDevP (+ dP/dt and − dP/dt, respectively) and left ventricular end-diastolic pressure (LVEDP). The effects of nicardipine (1 μM), dichlorobenzamil (30 μM), Rp-cGMPS (5 μM) and ODQ (2 μM) were tested in separate experiments. These drugs were applied starting 5 min before peroxynitrite administration and discontinued at the same time as peroxynitrite. Perfusion was in non-recirculating mode.

2.5. Effect of peroxynitrite on intracellular Ca2+ homeostasis

[Ca2+]i was measured using aequorin bioluminescence as previously described [11,14]. After 25 min of perfusion at ambient temperature to eliminate nonspecific luminescence, the heart was immersed in perfusion buffer and its temperature was gradually raised to 37 °C while the aequorin light signal reached a steady state. At this point, the experimental protocol was started. [Ca2+]i was estimated from aequorin luminescence using the method of fractional luminescence (L/Lmax) which relates the recorded light signals (L) to the amount of light emitted after lysis of the muscle cell membranes at the end of the experiment (Lmax) [15]. [Ca2+]i was calculated using the L/Lmax value and an in vitro calibration curve (37 °C) as described previously [16]. Light (and pressure) signals were wave-averaged at the time of interest (64–256 cycles) and values converted to μM [Ca2+]i using the triton X-100 method [16].

2.6. Effect of peroxynitrite on myofilament responsiveness to [Ca2+]i

To determine whether peroxynitrite affected the concentration–myocardial response relation to Ca2+, the peroxynitrite experiment was completed (at 55 min), increasing concentrations of CaCl2 (0.5–3 mM) were added to the perfusion buffer, each over 10 min, and light signals and cardiac parameters were recorded as above. After recording the effects of 3-mM extracellular calcium, triton X-100 was added and Lmax determined as described [15].

2.7. cGMP measurements

A separate set of hearts was perfused in the presence of IBMX (0.1 mM to inhibit degradation of cGMP by phosphodiesterases), and cGMP was determined in coronary effluent and cardiac homogenate using standard methods [17].

2.8. Data analysis

Data are presented as absolute effect values and reported as arithmetic mean ± S.E.M. Differences were analyzed for statistical significance using Student’s t-test or two-way analysis of variance (ANOVA) as appropriate. A P value < 0.05 was considered as significant.

3. Results

3.1. Myocardial function and [Ca2+]i

The effect of peroxynitrite on myocardial contractility and [Ca2+]i was documented in simultaneous measure-
The oxidant depressed LVDevP (Fig. 1A) reduced +dP/dt (data not shown) and /C0 dP/dt (Fig. 1B) and raised LVEDP, reflecting diastolic dysfunction (Fig. 1C) (P < 0.05 in all cases, n = 3 and 12, respectively), whereas decomposed peroxynitrite had no effect (P = NS vs. baseline; n = 9). The three concentrations exerted significantly different systolic effects (P < 0.05 for 10 vs. 3 and 40 vs. 10 or 3 AM; Fig. 1A and B), whereas the rise in LVEDP was not different for 3 and 10 AM peroxynitrite (Fig. 1C). During exposure to peroxynitrite, the size of individual Ca²⁺ transients increased significantly, reflecting increased intracellular Ca²⁺ level. The resulting time courses of systolic and diastolic Ca²⁺ concentrations are shown in Fig. 2. At 10 μM, the oxidant raised peak systolic and diastolic [Ca²⁺]i 1.3- and 2.3-fold, respectively (open symbols in Fig. 2A and B; P < 0.05 vs. baseline, n = 12). Both levels remained significantly elevated during the recovery period. The lower and the higher concentration of peroxynitrite raised [Ca²⁺]i to different degrees (n = 3), whereas decomposed peroxynitrite had no effect on systolic or diastolic [Ca²⁺]i, (n = 9) (see legend to Fig. 2).

3.2. Peroxynitrite-induced rise in [Ca²⁺]i and systolic function

The mechanism(s) underlying reduced contractility despite increased [Ca²⁺]i, was examined using nicardipine, a L-type Ca²⁺ channel blocker, and dichlorobenzamil, an inhibitor of myocardial Na⁺/Ca²⁺ exchange. Nicardipine (1 μM) and dichlorobenzamil (30 μM) by themselves depressed LVDevP from 90 (baseline) to 27 ± 2 mm Hg (−70%) and 45 ± 3 mm Hg (−50%), respectively. Following addition of peroxynitrite (10 μM), LVDevP was further reduced to ~15 mm Hg in the presence of

![Fig. 2. Effect of peroxynitrite on systolic (A) and diastolic (B) [Ca²⁺]i. For experimental details, see legend to Fig. 1.* P<0.05 vs. decomposed peroxynitrite for the entire curve (ANOVA analysis). In both panels, 3 and 40 μM exerted significantly different effects (P<0.05), whereas the effect of 10 μM was similar to that of 40 μM in A and similar to that of 3 μM in B (P=NS) (not indicated).](https://academic.oup.com/cardiovascres/article-abstract/60/2/355/339016)

![Fig. 3. Effect of nicardipine (1 μM) or dichlorobenzamil (DCB; 30 μM) on peroxynitrite-induced changes in peak systolic (A) and diastolic (B) [Ca²⁺]i. Both agents (administered from 25 to 55 min of the protocol) by themselves significantly depressed [Ca²⁺]i and significantly reduced peak Ca²⁺ levels during (bar) and after peroxynitrite (10 μM) administration. Data are means ± S.E.M. of 12 (peroxynitrite), 3 (nicardipine), 5 (DCB) or 9 (decomposed peroxynitrite) experiments, respectively. *P<0.05 vs. 10 μM peroxynitrite at 30, 40 and 55 min, respectively (t-test) (significance of difference for 10 μM peroxynitrite vs. decomposed form not indicated; see Fig. 2).](https://academic.oup.com/cardiovascres/article-abstract/60/2/355/339016)
nicardipine, and to ~30 mm Hg in the presence of dichlorobenzamil (n = 3 and 5, respectively; not shown). The effect of the two test compounds on \([\text{Ca}^{2+}]_i\) during systole is shown in Fig. 3A. Nicardipine depressed baseline \([\text{Ca}^{2+}]_i\) by ~32% and dichlorobenzamil by ~22%, respectively (n = 3 and 5, \(P < 0.05\) vs. baseline). In the presence of either inhibitor, peroxynitrite still raised \([\text{Ca}^{2+}]_i\), but the levels attained were significantly less, particularly in the presence of nicardipine, indicating that L-type \(\text{Ca}^{2+}\) channels are the major pathway for peroxynitrite-induced \(\text{Ca}^{2+}\) mobilization. Therefore, the very low remaining basal contractility after \(\text{Ca}^{2+}\) channel blockade likely results from lack of activator \(\text{Ca}^{2+}\). Of note, the dichlorobenzamil data illustrate an additional point. Whereas the peroxynitrite-induced peak systolic \([\text{Ca}^{2+}]_i\) is only marginally less ( ~0.1 \(\mu\)M at termination of peroxynitrite administration) when the Na\(^+\)/Ca\(^{2+}\) exchanger is blocked (Fig. 3A), LVDevP is still only ~29 mm Hg at this time point, indicating that the mobilization of \([\text{Ca}^{2+}]_i\) in systole is intact, while left ventricular function remains depressed, pointing to peroxynitrite-induced myofilament desensitization. This is also supported by the fact that during the recovery phase, systolic \([\text{Ca}^{2+}]_i\), remained significantly above 0.68 \(\mu\)M (baseline after dichlorobenzamil), whereas pressure declined further (compared to its baseline after equilibration of hearts with the blocker).

3.3. Effects of nicardipine and dichlorobenzamil on mechanical function and \([\text{Ca}^{2+}]_i\)

As shown above (Fig. 1C), LVEDP slightly rose (by ~4 mm Hg at end of recovery period) in response to peroxynitrite while the net gain in \([\text{Ca}^{2+}]_i\), was ~0.39 \(\mu\)M (Fig. 2B). Perfusion of hearts with nicardipine per se had no effect on LVEDP, but starting with infusion of peroxynitrite LVEDP began to rise and reached 22.3 ± 2.2 mm Hg at the end of the recovery period (\(P < 0.05\) vs. control, \(n = 3\)). Dichlorobenzamil did not affect LVEDP at any time (8–9 mm Hg, \(n = 5\); \(P = \text{NS}\) vs. peroxynitrite alone). The corresponding \([\text{Ca}^{2+}]_i\) data during diastole are shown in Fig. 3B. Both \(\text{Ca}^{2+}\) agents depressed baseline \([\text{Ca}^{2+}]_i\), (~30% and ~20%, respectively), yet peroxynitrite effectively raised \([\text{Ca}^{2+}]_i\), above control level of 0.3 \(\mu\)M (\(P < 0.05\) vs. control), albeit the attained levels were
significantly lower than for peroxynitrite alone. Importantly, diastolic [Ca\(^{2+}\)] returned to predrug baseline level (0.3 \(\mu\)M) both after nicardipine and dichlorobenzamil, while it remained significantly elevated after treatment with peroxynitrite alone, indicating a major role for extracellular Ca\(^{2+}\) influx in LVEDP rise.

### 3.4. Time courses of pressure and Ca\(^{2+}\) signals

Raised diastolic Ca\(^{2+}\) and increased LVEDP suggested diastolic dysfunction following peroxynitrite treatment. Further, diastolic Ca\(^{2+}\) was raised by peroxynitrite to a greater extent than systolic Ca\(^{2+}\) (net amplitude: \(\sim 0.4\) vs. \(\sim 0.6\) \(\mu\)M at baseline, i.e., in absence of peroxynitrite; see Fig. 2), which might indicate reduced Ca\(^{2+}\) flux during the cardiac cycle. Therefore, the time courses of pressure and aequorin light signals were examined (Fig. 4). Peroxynitrite had no effect on time-to-peak-pressure (TPP) or time-to-peak-light (TPL) (Fig. 4A and C) but significantly lengthened both the time necessary for 90% decline in LVEDP and aequorin light (\(T_{90P}\) and \(T_{90L}\), respectively) and these protracted time courses were maintained during the recovery phase (\(P<0.05\) vs. decomposed peroxynitrite; Fig. 4B and D). Therefore, the increase in diastolic [Ca\(^{2+}\)], likely results from impaired Ca\(^{2+}\) removal, thus contributing to the increase in LVEDP. The significantly lower [Ca\(^{2+}\)] during the recovery compared to the peroxynitrite infusion phase reflects the halted Ca\(^{2+}\) influx after cessation of peroxynitrite infusion. Nicardipine and dichlorobenzamil had no effect on TPP or TPL but significantly elevated \(T_{90P}\) and \(T_{90L}\) both before, during and after peroxynitrite infusion (\(P<0.05\) vs. decomposed peroxynitrite; \(n=3\) and 5, respectively). Thus, although blockade of Ca\(^{2+}\) entry (by nicardipine or dichlorobenzamil), or sequestration impaired the relaxation time course, these interventions had no consequences on relaxation times above and beyond those resulting from peroxynitrite itself.

### 3.5. Effect of peroxynitrite on myofilament responsiveness to Ca\(^{2+}\)

To test whether peroxynitrite blunts myofilament sensitivity to [Ca\(^{2+}\)], we increased the concentration of Ca\(^{2+}\) in the perfusion buffer stepwise from 0.5 to 3 mM and recorded ventricular function and aequorin light signals. These are shown in Fig. 5A and B. LVEDP (rather than peak systolic pressure) is shown in order to include systolic as well as diastolic pressure. The resulting relationship between systolic pressure and [Ca\(^{2+}\)] is shown in Fig. 5C (peak systolic pressure rather than LVDevP is depicted to correspond to peak systolic [Ca\(^{2+}\)]). It is clear that peroxynitrite greatly impaired contractility compared to controls (i.e., hearts infused with decomposed peroxynitrite), e.g., at 1 \(\mu\)M [Ca\(^{2+}\)], peak systolic pressure was \(\sim 110\) mm Hg in control but only \(\sim 65\) mm Hg after peroxynitrite (\(-40\%; P<0.05, n=6\)).

![Fig. 5. Relationship between contractile function and peak systolic [Ca\(^{2+}\)].](image-url)

(A) LVDevP as function of the Ca\(^{2+}\) concentration in the perfusate. (B) Peak systolic intracellular Ca\(^{2+}\) concentration as function of the Ca\(^{2+}\) concentration in the perfusate. (C) Peak systolic pressure as function of systolic [Ca\(^{2+}\)]. The [Ca\(^{2+}\)]–force relationship was significantly depressed in hearts previously perfused with peroxynitrite. Data are means ± S.E.M. of six measurements. *\(P<0.05\) vs. decomposed peroxynitrite for the entire curve (ANOVA analysis). For experimental protocol, see Section 2.6.

### 3.6. cGMP-dependent protein kinase and myofilament desensitization

We tested whether modulation of cGMP-dependent protein kinase activity would affect peroxynitrite-induced myo-
filament desensitization [18]. We found that Rp-cGMPS, an inhibitor of cGMP-dependent protein kinase, significantly attenuated the peroxynitrite-induced negative inotropic effect (Fig. 6A) without affecting systolic and diastolic \([Ca^{2+}]_i\) (\(P=NS\) vs. peroxynitrite alone; \(n=4\), data not shown). This protective effect was particularly evident at higher \(Ca^{2+}\) concentrations (Fig. 6B). Likewise, plotting left ventricular peak systolic function against systolic \([Ca^{2+}]_i\), in the presence of Rp-cGMPS also showed improved contractile responsiveness at all levels of systolic \([Ca^{2+}]_i\) (Fig. 6C) while \([Ca^{2+}]_i\) levels themselves were unchanged (Fig. 6D). As these data implicate activation of cGMP-dependent protein kinase in peroxynitrite-induced cardiodepression, we finally determined the effect of the oxidant on cardiac cGMP that is necessary for kinase activation.

3.7. Effect of peroxynitrite on myocardial cGMP

As shown in Fig. 7, cGMP content in coronary effluent was significantly (2-fold) increased during peroxynitrite infusion and declined to baseline thereafter. Decomposed peroxynitrite was without effect. Inhibition of soluble guanylyl cyclase with ODQ (2 \(\mu\)M) completely abolished peroxynitrite-induced cGMP efflux. The tissue content of cGMP, determined in homogenates of left ventricle, was \(33 \pm 3.4\) fmol/mg in response to decomposed peroxynitrite and \(132 \pm 6.4\) fmol/mg after peroxynitrite infusion (4-fold increase; \(P<0.05\)). ODQ depressed cGMP content to \(19 \pm 2\) fmol/mg protein (\(P<0.05\), \(n=6\) in all cases). As expected [19], the inhibitor constricted coronary arteries.

Fig. 6. Effects of inhibition of cGMP-dependent protein kinase with Rp-cGMPS (5 \(\mu\)M) (Rp, bar). Rp-cGMPS diminished peroxynitrite (P, bar)-induced decrease in left ventricular developed pressure (LVDevP; A) and partly restored \(Ca^{2+}\) responsiveness of contractile elements (B and C), whereas it had no effect on systolic \([Ca^{2+}]_i\) (D). *\(P<0.05\) vs. peroxynitrite alone (ANOVA analysis). Data are expressed as means \(\pm\) S.E.M., \(n=6\) (peroxynitrite) and 4 (Rp-cGMPS), respectively.

Fig. 7. Stimulation of cGMP formation by peroxynitrite (bar) and inhibition by ODQ (2 \(\mu\)M), a specific inhibitor of soluble guanylyl cyclase. Peroxynitrite, but not the decomposed form, stimulated cGMP production, and ODQ reduced it to control level. *\(P<0.05\) vs. decomposed peroxynitrite (\(t\)-test) and \(P=NS\) for ODQ vs. decomposed peroxynitrite. Data are means \(\pm\) S.E.M., \(n=6\).
(increase in coronary perfusion pressure \( \sim 50 \text{ mm Hg} \)) and depressed LVDevP (by \( \sim 20 \text{ mm Hg}; P<0.05 \) in both cases, \( n=6 \)). Inhibition of NOS using l-NNa (200 \( \mu \text{M} \)) reduced basal contractility as we have observed previously [20] but had no effect on peroxynitrite-induced negative inotropy (relative to baseline after l-NNa; \( n=5 \); not shown).

4. Discussion

The present investigation has shown that authentic peroxynitrite administered to isolated beating hearts in a moderate concentration (10 \( \mu \text{M} \)) depresses contractility mainly by lowering the Ca\(^{2+} \) sensitivity of the contractile elements and that this action is partly mediated by cGMP-dependent protein kinase. Whereas previous authors frequently used very high concentrations of peroxynitrite (0.1–1 mM), or peroxynitrite sources such as 3-morpholinosydnonimine, which generate unknown concentrations of peroxynitrite in tissues [8], we chose a rather low concentration (10 \( \mu \text{M} \)) that caused significant alterations in cellular homeostasis. Due to its quick decomposition at physiological pH (estimated half-life, \( \sim 1 \text{ s at pH } 7.4 \) [2]), low-micromolar concentrations probably translate into nanomolar concentrations in the vicinity of, or inside, cells. We consistently observed concentration-dependent (3–40 \( \mu \text{M} \)) cardiac depression, but never cardioprotection, with any of the three concentrations used.

An important observation was that peroxynitrite effectively and promptly raised systolic and diastolic [Ca\(^{2+} \)], whereas the simultaneously measured LVDevP and rates of pressure development decreased (Figs. 1A, B and 2). The systolic [Ca\(^{2+} \)], levels reached were similar to those observed during early reperfusion after brief ischemia in ferret (\( \sim 1.5 \mu \text{M} \)) [21] or mouse hearts (\( \sim 0.9 \mu \text{M} \)) [22], while diastolic [Ca\(^{2+} \)], was similar to that observed during short periods of ischemia (\( \sim 0.5–0.7 \mu \text{M} \)). Therefore, in view of the likely generation of peroxynitrite during experimental [7] and clinical [23] ischemia/reperfusion injury, it is possible that the oxidant contributes to the elevation in [Ca\(^{2+} \)], commonly observed in ischemia- and hypoxia-related pathologies. Interestingly, functional deterioration was persistent despite partial normalization of Ca\(^{2+} \) transients. We suspect that the secondary reaction products potentially formed from peroxynitrite, including OH\(^-\) radical, thionitrite, nitrite anion, etc. [24], preferentially interfere with force generation and less with the different Ca\(^{2+} \) transporters and pumps, leading to the observed divergent time courses.

Besides systolic depression, end-diastolic pressure was consistently raised in response to peroxynitrite. As the rise in LVEDP was related to diastolic [Ca\(^{2+} \)], (compare Figs. 1C and 2B), the abnormally high myofilament tension during diastole probably resulted from the excessive cytosolic level of Ca\(^{2+} \), such that significant Ca\(^{2+} \) binding to regulatory proteins continued throughout diastole, resulting in persistent Ca\(^{2+} \)-activated force generation. One possible reason for elevated diastolic [Ca\(^{2+} \)], is reduced Ca\(^{2+} \) cycling back into the sarcoplasmic reticulum as suggested by the significantly prolonged T\(_{90L} \) (1.3-fold; Fig. 4), strongly indicating slowed Ca\(^{2+} \) reuptake induced by peroxynitrite. A similar retardation of Ca\(^{2+} \) sequestration was previously observed in association with the process of myocardial hypertrophy [25] or during experimental ischemia/reperfusion [14]. In the latter case, looking at controls, T\(_{90L} \) remained elevated throughout reperfusion (30 min), indicating considerable impairment of Ca\(^{2+} \) pumping capacity. Additional work is needed to determine whether endogenous peroxynitrite plays a role in these post-ischemic diastolic derangements.

The route of Ca\(^{2+} \) entry induced by peroxynitrite has been somewhat controversial. Experiments with isolated myocytes suggested that both L-type Ca\(^{2+} \) channels and Ca\(^{2+} \) transport via the Na\(^+\)/Ca\(^{2+} \) exchanger were involved [26,27]. The present nicardipine experiments showed that L-type Ca\(^{2+} \) channels are mainly responsible for Ca\(^{2+} \) entry induced by peroxynitrite in the contracting heart because systolic [Ca\(^{2+} \)] fell far below the level of control (decomposed peroxynitrite; see 30-min time point in Fig. 3A) and remained below that level during and after peroxynitrite addition. In contrast, Ca\(^{2+} \) transport by Na\(^+\)/Ca\(^{2+} \) exchange was close to negligible because peroxynitrite still raised [Ca\(^{2+} \)], substantially in the presence of dichlorobenzamil. Interestingly, the exchanger operated in reverse mode, i.e., it transports Ca\(^{2+} \) into cells as dichlorobenzamil reduced basal Ca\(^{2+} \) during systole and diastole, possibly indicating that the transporter has a greater role in Ca\(^{2+} \) influx (reverse mode) than previously considered. Alternatively, amiloride derivatives seem to have multiple sites of action, which also may play a role [28]. Neither nicardipine nor dichlorobenzamil affected relaxation time or aequorin light transients above and beyond the effects of peroxynitrite (Fig. 4B and D for times during and after peroxynitrite administration), while diastolic [Ca\(^{2+} \)] returned close to control level at the end of the recovery period (Fig. 3B). Taken together, these data may indicate that Ca\(^{2+} \) sequestration back into intracellular stores is lastingly impaired by the oxidant, whereas extrusion from the cytosol appears to be unaffected.

As the depression of myocardial contractile function did not result from a decrease in intracellular activator Ca\(^{2+} \), we investigated whether it might be due to decreased myofilament responsiveness to [Ca\(^{2+} \)], similar to observations in states of heart failure [29]. Myofilament Ca\(^{2+} \) responsiveness is generally considered in terms of two determinants: maximum Ca\(^{2+} \)-activated force (defining the efficacy of Ca\(^{2+} \) as an agonist, \( E_{\text{max}} \)) and Ca\(^{2+} \) sensitivity which is usually expressed in terms of the effective dose producing contraction, e.g., 50% of maximum Ca\(^{2+} \)-activated force, and which defines the potency of Ca\(^{2+} \) as an agonist [30]. As shown in Fig. 5A, the relation LVDevP–perfusion Ca\(^{2+} \)
is shifted downward pointing to decreased maximum Ca$^{2+}$-activated force as a result of peroxynitrite application. This view is strengthened by the analysis of the systolic pressure–[Ca$^{2+}$]$_i$ relation (Fig. 5C) which shows that peroxynitrite decreases myofilament sensitivity both by decreasing maximum activated force (peak systolic pressure) and the potency of Ca$^{2+}$ to induce contraction. The mechanism of desensitization, not addressed in this study, may involve intracellular acidification, known to cause depression of myocyte contractility [31], or reduced phosphorylation of target proteins such as troponin I or myosin [32].

We observed that peroxynitrite stimulated cGMP production in ODQ-inhibitable manner (Fig. 7), similar to observations in smooth muscle. Further, the desensitization of the contractile filaments was partially reversed by inhibiting cGMP-dependent protein kinase using Rp-cGMPS (Fig. 6). This indicates that the oxidant’s effects on the cardiac myofilaments may be a regulated process subject to modulation by the NO/cGMP pathway. cGMP-induced myofilament desensitization toward Ca$^{2+}$ is known as important physiological mechanism regulating myocardial relaxation and diastolic tone [33]. Nonetheless, in our experiments, significant depression of function remained, suggesting that additional mechanisms play a role, e.g., reduced myosin phosphorylation previously observed in guinea pig mesenteric arteriole or in rabbit ileal muscle [32], both at constant Ca$^{2+}$. Similar to the present observations with peroxynitrite, we have recently shown that reinforcing the NO/cGMPS pathway by overexpressing endothelial NOS exclusively in cardiac myocytes also depresses contractility mainly via myofilament desensitization [17]. It is conceivable, therefore, that the negative inotropic effect observed in disease states with pathologically increased myocardial NO generation [4,34] may actually be due to peroxynitrite rather than NO itself.

On the other hand, NO/cGMP generation has been associated with improved myocardial preservation in most models of myocardial stunning and ischemia/reperfusion, both at normal and increased NO levels due to endothelial NOS overexpression [14]. The cardioprotective effects of endogenous NO/cGMP are probably multifactorial in origin but may mainly derive from the nucleotide’s well-established energy sparing effects [35] that help to maintain cardiac muscle in better condition during ischemic stress, resulting in more rapid recovery from myocardial stunning or improved overall reperfusion function, consistently demonstrated in almost all reports.

In summary, a moderate concentration of peroxynitrite produces a marked decrease in Ca$^{2+}$ sensitivity of contractile elements, explaining in large part the negative inotropic action of this oxidant despite an increase in [Ca$^{2+}$]$. Because many pathophysiological conditions of the heart are associated with peroxynitrite formation, attempts to reverse myofilament desensitization in these conditions may be a valid therapeutic approach.

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**References**

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