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RESEARCH PAPER

Inhibition of matrix metalloproteinases prevents peroxynitrite-induced contractile dysfunction in the isolated cardiac myocyte

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Background and purpose: The potent oxidant peroxynitrite ($ONOO^-$) induces mechanical dysfunction in the intact heart in part through activation of matrix metalloproteinase-2 (MMP-2). This effect may be independent of the proteolytic actions of MMPs on extracellular matrix proteins. The purpose of this study was to examine the effects of $ONOO^-$ on contractile function at the level of the single cardiac myocyte and whether this includes the action of MMPs.
Experimental approach: Freshly isolated ventricular myocytes from adult rats were superfused with Krebs–Henseleit buffer at 21 °C and paced at 0.5 Hz. Contractility was measured using a video edge-detector. $ONOO^-$ or decomposed $ONOO^-$ (vehicle control) were co-infused over 40 min to evaluate the contraction cease time (CCT). The effects of $ONOO^-$ on intracellular $[Ca^{2+}]$ were determined in myocytes loaded with calcium green-1 AM. MMP-2 activity was measured by gelatin zymography.
Key results: $ONOO^-$ (30-600 μM) caused a concentration-dependent reduction in CCT. Myocytes subjected to 300 μM $ONOO^-$ had a shorter CCT than decomposed $ONOO^-$ ($14.9 + 1.5$ vs $32.2 + 3.5$ min, $n = 7$–8; $P < 0.05$) and showed increased MMP-2 activity. The MMP inhibitors doxycycline (100 μM) or PD 166793 (2 μM) reduced the decline in CCT induced by 300 μM $ONOO^-$. $ONOO^-$ caused shorter calcium transient cease time and significant alterations in intracellular $[Ca^{2+}]$ homoeostasis which were partially prevented by doxycycline.
Conclusions and implications: This is the first demonstration that inhibition of MMPs protects the cardiac myocyte from $ONOO^-$-induced contractile failure via an action unrelated to proteolysis of extracellular matrix proteins.
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Keywords: peroxynitrite; matrix metalloproteinases; calcium homoeostasis; cardiac myocyte; doxycycline

Abbreviations: CCT, contraction cease time; MMP-2, matrix metalloproteinase-2; MMPs, matrix metalloproteinases

Introduction

The overproduction of reactive oxygen species including the potent cellular oxidant peroxynitrite ($ONOO^-$) can outstrip cellular antioxidant defences leading to oxidative stress (Cai and Harrison, 2000; Ferdinandy and Schulz, 2003; Ferdinandy, 2006; Pacher *et al.*, 2007). This condition has been implicated in the pathogenesis of many cardiovascular diseases such as hypertension, atherosclerosis and the complications resulting from diabetes and aging, and heart-specific defects in myocarditis, heart failure (Ide *et al.*, 2000; Pacher *et al.*, 2005) and ischaemia–reperfusion injury (Wang and Zweier, 1996; Yasmin *et al.*, 1997; Crestanello *et al.*, 1998).

Overproduction of reactive oxidant species can activate matrix metalloproteinases (MMPs), enzymes best known for their action in remodelling the extracellular matrix (Rajagopalan *et al.*, 1996; Okamoto *et al.*, 2001; Siwik *et al.*, 2001). However, recent studies have unravelled new roles of MMPs, particularly for matrix metalloproteinase-2 (MMP-2), which is ubiquitous to most cell types including cardiac myocytes (Coker *et al.*, 2001; Wang *et al.*, 2002b). These include both physiological and pathological processes such as platelet aggregation, inflammation and neurodegenerative disease (Sawicki *et al.*, 1997; McQuibban *et al.*, 2000; Zhang *et al.*, 2003). In each of these conditions, the biological effect of MMP-2, which is a rapid and early response of the cell to enhanced oxidative stress, occurs by its proteolytic action on novel substrates unrelated to extracellular matrix proteins (Chow *et al.*, 2007; Schulz, 2007). MMPs are implicated in the pathogenesis of many cardiovascular diseases for their

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actions on both intracellular and extracellular targets in both animal models and in human disease including myocardial infarction (Rohde *et al*., 1999; Hayashidani *et al*., 2003; Villarreal *et al*., 2003), heart failure (Rouet-Benzineb *et al*., 1999; Spinale *et al*., 2000; Wilson *et al*., 2002), ischaemia–reperfusion injury (Fujimura *et al*., 1999; Cheung *et al*., 2000; Wang *et al*., 2002b; Schulze *et al*., 2003; Sawicki *et al*., 2005), cytokine-induced cardiac dysfunction (Gao *et al*., 2003) and sepsis (Yassen *et al*., 2001; Lalu *et al*., 2006). In addition, cardiac preconditioning has shown to decrease the deleterious effects caused by MMP-2 in cardiac ischaemia–reperfusion injury, an effect which is lost in hyperlipidemic conditions (Lalu *et al*., 2002; Giricz *et al*., 2006).

$ONOO^-$ is a potent oxidant that activates MMPs by *S*-glutathiolation of a critical cysteine residue in their propeptide domain resulting in a full-length active MMP (Okamoto *et al*., 2001). We have previously shown that infusion of $ONOO^-$ into the isolated rat heart causes cardiac contractile dysfunction through MMP-2, which is prevented by MMP inhibition (Wang *et al*., 2002a). However, this previous study did not directly address whether the detrimental effect of MMP-2 occurred via its proteolytic actions on extracellular matrix proteins or on targets specifically associated with the cardiac myocyte (Schulz, 2007).

In this regard, Ishida *et al*. (1996) showed that the direct administration of $ONOO^-$ to isolated neonatal cardiac myocytes caused severe contractile dysfunction, evaluated as the time taken for contractions to cease (contraction cease time, CCT). However, whether MMPs are involved in $ONOO^-$-induced contractile failure and alterations of calcium handling in isolated adult cardiac myocytes is unknown. Therefore, we hypothesized that $ONOO^-$ produces contractile dysfunction through activation of MMPs independent of their action on extracellular matrix proteins.

Methods

All the experiments were performed according to the recommendations given by the *Guide to the Care and Use of Experimental Animals* published by the Canadian Council on Animal Care (revised 1993).

Isolation of cardiac myocytes

Calcium-tolerant ventricular myocytes were obtained by enzymatic dissociation as described previously (Bouchard *et al*., 1993; Light *et al*., 1998). Briefly, adult male Sprague–Dawley rats (250–350 g) were anaesthetized with an injection of sodium pentobarbital (60 mg kg^{-1}, i.p.). The hearts were isolated and perfused through the aorta in a modified Langendorff perfusion system at constant flow and at 37 °C with oxygenated (95% O_2: 5% CO_2) Krebs–Henseleit solution containing (in mM): NaCl (121), KCl (5), CH_3COONa (1.7), $MgCl_2$ (0.1), Na_2HPO_4 (0.4), $NaHCO_3$ (20.8), glucose (5.5), taurine (1) and $CaCl_2$ (1) for 5 min to clear any blood from the coronary circulation. The solution was then switched to a Ca^{2+}-free Krebs–Henseleit solution for 9 min. After this, the solution was replaced for the first digestion process with Krebs–Henseleit solution containing 40 μM Ca^{2+} and 13.3 μg ml^{-1} *Streptomyces* collagenase (Yalkut Pharmaceutical, Tokyo, Japan) and 13.3 μg ml^{-1} *Streptomyces* protease (Sigma, Oakville, Ontario, Canada) for approximately 10 min. The ventricles were then separated from the atria and great vessels using scissors and chopped into small pieces. For the second digestion process, the chopped tissue was placed into a flask with Krebs–Henseleit solution containing 5.5 mM HEPES, 3% BSA, 100 μM Ca^{2+}, and 83.3 μg ml^{-1} collagenase and 83.3 μg ml^{-1} protease. The cells were further dissociated by incubation at 37 °C under gentle agitation. A 2 ml aliquot of cell suspension was removed after 10, 20, 30 and 40 min incubation and centrifuged for 25 s at 2000 × *g*. A volume of 2 ml of the second digestion buffer was replaced in the flask each time after sampling. Each cell suspension was immediately centrifuged and the cells were resuspended in storage buffer (Krebs–Henseleit solution containing 5.5 mM HEPES, 3% BSA and 100 μM Ca^{2+}). The aliquots were observed under a light microscope for morphology and aliquots that showed more than 70% rod-shaped cells (typically after 30 min digestion) were used for experiments.

Synthesis of $ONOO^-$

Active and decomposed $ONOO^-$ were synthesized, and the concentration was verified on the same day of experiments using ultraviolet spectroscopy as described previously (Yasmin *et al*., 1997). Both active and decomposed $ONOO^-$ were diluted with 1 mM NaOH immediately prior to the experiment to achieve the desired working concentration.

Measurement of myocyte contractility

To maintain a constant $ONOO^-$ to myocyte ratio, approximately 5000 cardiac myocytes were placed into a perfusion chamber in each experiment. Experiments were performed at 21 °C to allow us to directly compare to the conditions used by Ishida *et al*. (1996) and to facilitate the accurate temporal measurement of cellular contracture. Freshly isolated rat ventricular myocytes were continuously superfused at 1 ml min^{-1} with Krebs–Henseleit solution containing 2 mM Ca^{2+} using an infusion pump (Cole-Palmer, Barrington, IL, USA) and electrically paced at 0.5 Hz. Single myocyte contractility was measured using a video edge-detector (Crescent Electronics, Salt Lake City, UT, USA) and data were recorded using pClamp 8.0 software. After 3 min, an infusion of either decomposed or authentic $ONOO^-$ was begun (using a microinfusion pump (Baxter, Deerfield, IL, USA) linked via a side arm to the perfusion line delivering Krebs–Henseleit solution), so that the final concentrations of $ONOO^-$ in the Krebs–Henseleit solution reached either 30, 100 or 300 μM. The infusion was continued for an experimental observation period of up to a maximum of 40 min or until the time at which the cells ceased to contract (CCT). A second series of cells were perfused in an identical manner except that the MMP inhibitors doxycycline (100 μM; Sigma) or PD 166793 (2 μM; a kind gift from Pfizer, New York, NY, USA), dissolved in Krebs–Henseleit solution, were used to superfuse the cells after the 3 min baseline evaluation period. These MMPs inhibitors at their respective concentrations were chosen

based on previous data from isolated rat hearts subjected to either ischaemia–reperfusion (Cheung *et al*., 2000; Wang *et al*., 2002b; Schulze *et al*., 2003; Sawicki *et al*., 2005), proinflammatory cytokines (Gao *et al*., 2003) or exogenous $ONOO^-$ (Wang *et al*., 2002a). Parallel experiments were performed using their respective vehicles (water for doxycycline and 0.05% dimethylsulphoxide v/v for PD 166793). Although the previous studies (Cheung *et al*., 2000; Wang *et al*., 2002b; Schulze *et al*., 2003; Sawicki *et al*., 2005) indicated that doxycycline or PD 166793 does not have any direct effect on myocardial contractile function in intact rat hearts exposed to these drugs for up to 120 min, we also evaluated whether these compounds could have any effect on the contractile function of isolated cells. Infusion of the inhibitors or their vehicles had no effect on contractile function as observed over a 10 min period (data not shown).

Evaluation of MMP activity

The cell storage buffer was removed from an aliquot of freshly isolated cardiac myocytes and replaced with Krebs–Henseleit buffer containing 100 μM Ca^{2+} (oxygenated with 95% O_2: 5% CO_2) to reduce hypercontracture by three separate wash and centrifugation steps ($2000 \times g$ for 25 s). Cardiac myocytes (~200 000 cells) in 3 ml buffer were then exposed to the bolus addition of either 300 μM $ONOO^-$ or decomposed $ONOO^-$ followed by 5 min incubation at 21 °C. The addition of $ONOO^-$ or decomposed $ONOO^-$ had no significant effect on buffer pH. Cells were separated from the media by centrifugation ($2000 \times g$ for 40 s) and the latter was concentrated 20 times using Amicon Ultra-4 centricon tubes (Millipore, Bedford, MA, USA). Cell homogenates were prepared in 350 μl of homogenization buffer containing 50 mM Tris-HCl (pH 7.4), 3.1 mM sucrose, 1 mM dithiothreitol (Fisher Scientific, Ottawa, Ontario, Canada), 1:1000 protease cocktail inhibitor (P-8340; Sigma) and 0.1% Triton X-100 and homogenized on ice using a sonicator (Misonix, Farmingdale, NY, USA, 3×10 s with 60 s between each cycle). Gelatin zymography was performed as described by Cheung *et al*. (2000). Samples from cell homogenates and media were applied to 8% polyacrylamide gels co-polymerized with 2 mg ml^{-1} gelatin. After electrophoresis, gels were rinsed three times for 20 min each in 2.5% Triton X-100 to remove sodium dodecyl sulphate. Then the gels were washed twice in incubation buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 150 mM NaCl and 0.05% NaN_3) for 20 min each at room temperature and then incubated in a fresh aliquot of the same at 37 °C. The gels were stained in 2% Coomassie Brilliant Blue G/25% methanol/10% acetic acid for 2 h and then destained for 1 h in 2% methanol/4% acetic acid. Gels were scanned using a GS-800-calibrated densitometer (Bio-Rad, Mississauga, Ontario, Canada) and band densities were measured using SigmaGel software (Jandel, San Rafael, CA, USA).

Evaluation of intracellular [Ca^{2+}]

Cardiac myocytes placed in storage buffer were loaded for 30 min at room temperature and then for 30 min at 37 °C with the Ca^{2+}-sensitive fluorescent probe calcium green-1 AM (4 μM, dissolved in a mixture of dimethyl sulphoxide and pluronic acid, 1:1 v/v; Molecular Probes, Eugene, OR, USA). After loading, cells were washed and centrifuged twice ($2000 \times g$ for 25 s) with storage buffer and placed on coverslips for observation at $\times 200$ with an inverted microscope (CK40; Olympus, Center Valley, PA, USA) while being superfused with the same Krebs–Henseleit buffer used for contractility measurements, and paced at 0.5 Hz at 21 °C. An infusion of either decomposed or 300 μM $ONOO^-$ was started after the baseline recording over a period of 20 min via a side arm using a microinfusion pump. Some cardiac myocytes were exposed to doxycycline (100 μM) dissolved in Krebs–Henseleit buffer 5 min before starting the infusion of 300 μM $ONOO^-$ and maintained during the 20 min exposure period. A Photon Technology International (Model 814; Lawrenceville, NJ, USA) photomultiplier detection system and Clampex software (version 8.1) was used for data acquisition and analysis. Calcium Green-1 AM was excited at 480 nm and the emitted light intensity at 520 nm was digitized and stored. The parameters evaluated were the percentage of change in the calcium transient amplitude vs baseline, the time to cease calcium transients and the time for maximal increase in diastolic calcium.

Data analysis and statistical procedures

Data are expressed as means ± s.e.mean. Student's *t*-test (unpaired) or one-way ANOVA followed by *post hoc* analysis using Tukey's multiple comparison test was used for statistical analysis, as appropriate. $P<0.05$ was the criterion for significance. The n refers to the number of individual cardiac myocytes tested. In all experiments, cells were tested from $\geqslant 4$ separate isolations of myocytes.

Results

Concentration-dependent contractile dysfunction by $ONOO^-$

To directly evaluate the effects of $ONOO^-$ on contractile dysfunction in the isolated cardiac myocyte, we used the CCT as a measure of severely impaired contractility. This measure was previously used in neonatal cardiac myocytes exposed to $ONOO^-$ (Ishida *et al*., 1996), although the effects of $ONOO^-$ on adult rat ventricular myocytes have not been documented. The administration of decomposed $ONOO^-$ did not affect the CCT, compared to control conditions (32.5 ± 3.5 vs 35.1 ± 3.0 min; $P>0.05$, $n=8$ and $n=9$, respectively) (Figure 1a). However, in the presence of $ONOO^-$, there was a concentration-dependent decrease in CCT (Figure 1b). As pH is a potential variable in these experiments due to the alkaline $ONOO^-$ buffer, we monitored the effects of up to 300 μM $ONOO^-$ or its vehicle, decomposed $ONOO^-$, on superfusate pH and observed no change in pH.

MMP inhibitors do not alter baseline contractile properties of isolated cardiac myocytes

Cardiac myocytes were treated with two different MMP inhibitors (doxycycline or PD 116793) as well as their respective vehicle controls (water for doxycycline and 0.05% dimethylsulphoxide for PD 166793; $n=5$ per group from four independent isolations). During the 10-min perfusion period with MMP inhibitors, the cells contracted

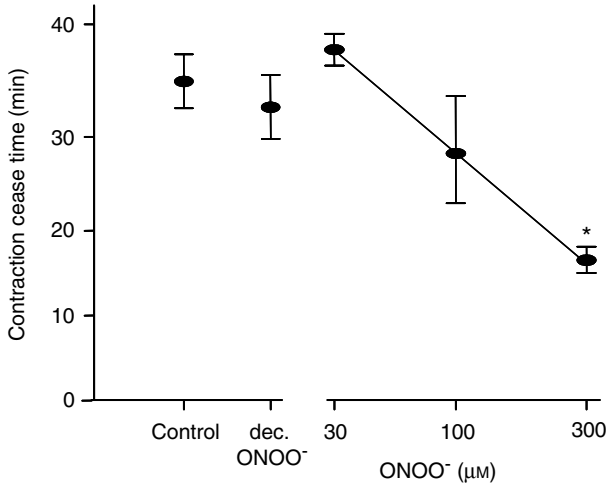

Figure 1 Concentration-dependent effect of a continuous infusion of $ONOO^-$ for 40 min on contraction cease time (CCT) in isolated cardiac myocytes. There was no effect of infusions of decomposed $ONOO^-$ (dec. $ONOO^-$) on the CCT. *$P<0.05$ vs control, $n=4$–9 cells from a total of 6 independent preparations. $ONOO^-$, peroxynitrite.

regularly and none of them ceased to contract. Moreover, there was no evidence of early depolarizations or after-depolarizations, as shown by the lack of alterations of the contractile waveform of the traces or absence of significant differences in cell shortening or the time to 50% relaxation (data not shown).

MMP inhibitors prevent contractile dysfunction caused by $ONOO^-$

Cardiac myocytes were exposed to a continuous infusion of 300 μM $ONOO^-$ in the presence or absence of the MMP inhibitors (doxycycline or PD 166793) or their respective vehicles. Administration of 300 μM $ONOO^-$ caused a rapid onset in contractile dysfunction in myocytes, which was revealed as irregular contractions with early afterdepolarizations and hypercontracture as the infusion of $ONOO^-$ progressed (Figure 2a). Myocytes challenged with 300 μM $ONOO^-$ in the presence of doxycycline or PD 166793 exhibited a significantly delayed onset of contractile dysfunction in comparison to $ONOO^-$ alone (Figures 2b and c). These data are summarized in Figure 2d.

Impairment in Ca^{2+} homoeostasis caused by $ONOO^-$ is attenuated by MMPs inhibitors

Alteration in calcium homoeostasis is a common mechanism for dysfunctional excitation coupling in cardiac myocytes. Therefore, to evaluate whether the protective effect of MMP inhibition was related to possible effects on Ca^{2+} homoeostasis, we measured calcium transients in cardiac myocytes subjected to 300 μM $ONOO^-$. The generation, amplitude and shape of Ca^{2+} transients in cardiac myocytes exposed to decomposed $ONOO^-$ were unaltered over the duration of the experimental protocol (Figure 3a). In contrast, 300 μM $ONOO^-$ caused a progressive increase in diastolic $[Ca^{2+}]$ as well as a time-dependent reduced peak systolic transient amplitude (Figure 3b). Doxycycline treatment delayed the onset of changes in Ca^{2+} homoeostasis but did not prevent

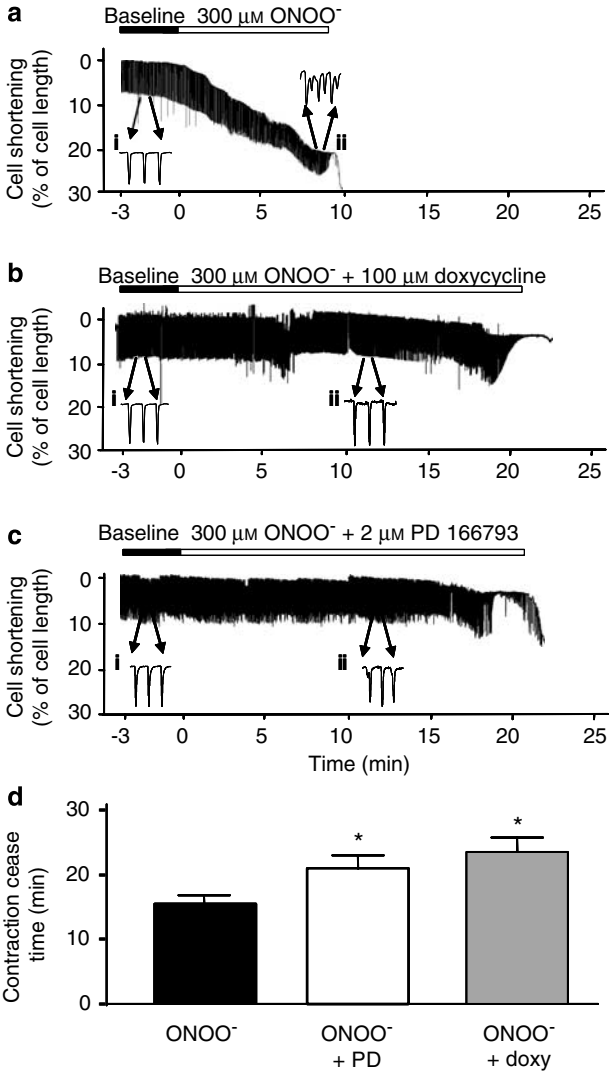

Figure 2 Effects of MMP inhibitors on contraction cease time (CCT) during 300 μM $ONOO^-$ exposure. (**a–c**) Representative traces of cell shortening in cardiac myocytes subjected to $ONOO^-$ alone (**a**) or with 100 μM doxycycline (**b**) or 2 μM PD 166793. (**c**) In each panel, the expanded time scale of the cell-shortening traces are shown at (i) baseline recording and (ii) 10 min after infusion of 300 μM $ONOO^-$. (**d**) Summary analysis in terms of the CCT. $n=5$–6 cells per group from a total of five independent preparations. *$P<0.05$ vs $ONOO^-$. MMP, matrix metalloproteinase; $ONOO^-$, peroxynitrite.

their occurrence, as alterations of Ca^{2+} transient shape in the form of premature Ca^{2+} release were observed during the experiment (Figure 3c), although at a later time. However, in the presence of doxycycline, the peak Ca^{2+} transient amplitude was better maintained in comparison to $ONOO^-$ alone.

Cardiac myocytes exposed to 300 μM $ONOO^-$ displayed a significant increase in Ca^{2+} transient amplitude at 5 min in comparison to decomposed $ONOO^-$. Doxycycline treatment normalized this variable to values equal to those obtained with exposure to decomposed $ONOO^-$ (Figure 4a). These data are consistent with early alteration in Ca^{2+} homoeostasis induced by $ONOO^-$. Furthermore, cardiac myocytes challenged with $ONOO^-$ had a significant reduction in the time to cease calcium transients that was attenuated by doxycycline treat-

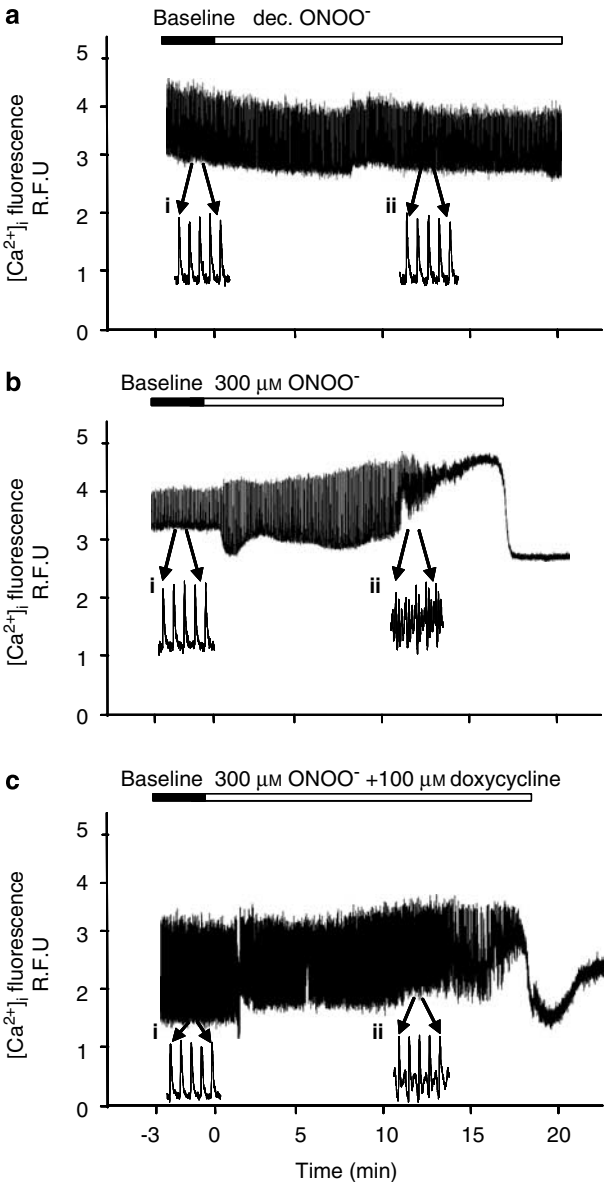

Figure 3 Effects of $ONOO^-$ and doxycycline on calcium homoeostasis. Representative traces of cells exposed to either decomposed $ONOO^-$ (**a**) or 300 μM $ONOO^-$ alone (**b**) or with addition of 100 μM doxycycline. (**c**) Expanded time scale traces of calcium transients are shown at (i) baseline recording and (ii) 10 min after infusion of $ONOO^-$. $ONOO^-$, peroxynitrite.

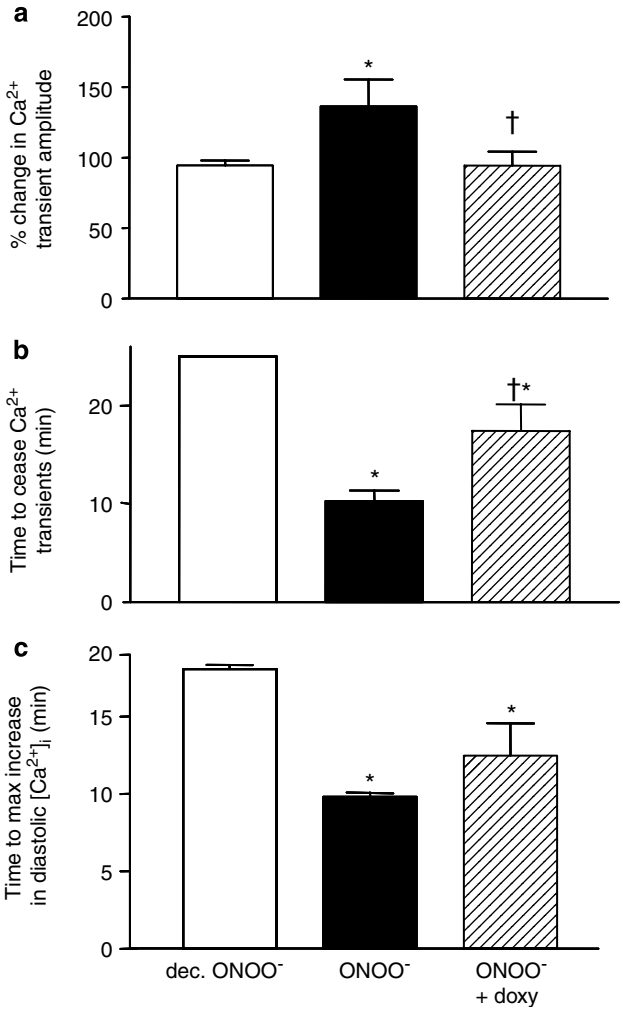

Figure 4 Quantitative analysis of the effects of $ONOO^-$ and doxycycline (doxy) on calcium homoeostasis. (**a**) Percent change in calcium transient amplitude at 5 min $ONOO^-$ infusion. (**b**) Time to cease calcium transients. (**c**) Time to reach the maximum increase in diastolic calcium level. $*P<0.05$ vs decomposed $ONOO^-$ (dec. $ONOO^-$), $\dagger P<0.05$ vs $ONOO^-$, $n=4$ cells per group from a total of four independent preparations. $ONOO^-$, peroxynitrite.

ment (Figure 4b). Represented in terms of the time to reach maximum diastolic $[Ca^{2+}]$ during the experimental protocol, cardiac myocytes challenged with 300 μM $ONOO^-$ showed a reduced time to maximum increase in diastolic $[Ca^{2+}]$ level compared with decomposed $ONOO^-$. Doxycycline, however, did not alter this variable (Figure 4c). Furthermore, we performed an analysis of the decay rates by fitting to a monoexponential function and found no differences in the values of half-life (data not shown).

$ONOO^-$ activates myocyte MMP-2

To evaluate whether $ONOO^-$ was able to activate MMPs when given as a bolus to an aliquot of cardiac myocytes, gelatin zymography was performed on the concentrated incubation media from cardiac myocytes to examine changes in MMP-2 or MMP-9 activities. After 5 min exposure to 300 μM $ONOO^-$, there was a significant increase in 62 kDa MMP-2 activity in the media, compared with the effects of exposure to decomposed $ONOO^-$ and no evidence of 72 kDa activity (Figure 5). We did not observe any evidence of MMP-9 activity (data not shown).

Discussion and conclusions

This study is the first demonstration that inhibition of MMPs reduces $ONOO^-$-induced contractile dysfunction in isolated cardiac myocytes, independent of the canonical actions of MMPs on extracellular matrix proteins. Previously, we had demonstrated that MMP-2 is activated in isolated rat hearts challenged with $ONOO^-$ and that a MMP inhibitor prevented this dysfunction (Wang *et al.*, 2002a). However, this

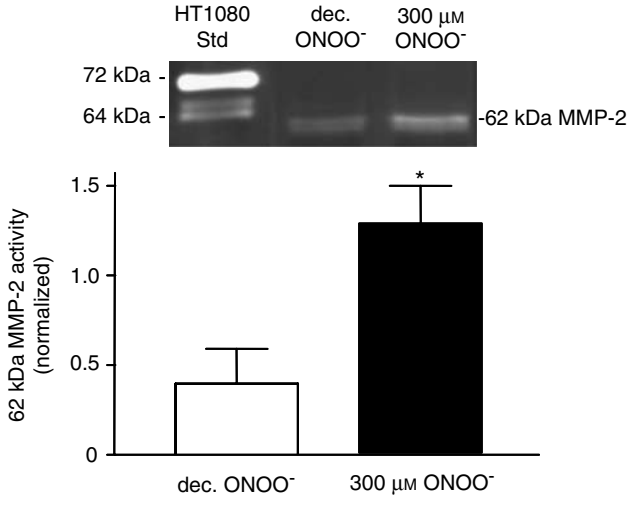

Figure 5 Gelatin zymography of media from cardiac myocytes exposed to either decomposed $ONOO^-$ (dec. $ONOO^-$) or 300 μM $ONOO^-$. Upper panel shows a representative zymogram. Lower panel shows summary data of the densitometric analysis of 62 kDa MMP-2 activity. Note that the HT1080 cell supernatant used as a control is human MMP-2 which consists of both 72 and 64 kDa forms, the latter which is slightly higher in molecular weight than the 62 kDa rat MMP-2. *$P<0.05$ vs dec. $ONOO^-$, $n=3$ experiments. $ONOO^-$, peroxynitrite.

study did not determine whether the effect of the activation of MMP-2 was independent of proteolysis of extracellular matrix proteins.

During myocardial ischaemia–reperfusion injury, $ONOO^-$ is rapidly generated during the first few minutes of reperfusion, which precedes the activation and release of MMP-2 from the heart (Yasmin *et al*., 1997; Cheung *et al*., 2000). In the current study, we observed an increase in 62 kDa MMP-2 activity in the conditioned cell medium following exposure to $ONOO^-$. The release of MMP-2 from heart muscle is understood to be a consequence of its activation by oxidative stress (Cheung *et al*., 2000; Wang *et al*., 2002a, b) and may be a means for the cell to protect itself from further proteolytic stress. MMP-2 activation as a result of ischaemia–reperfusion injury in the intact heart results in the cleavage of troponin I and myosin light chain 1 (Wang *et al*., 2002b; Sawicki *et al*., 2005). Whether the same event occurs during $ONOO^-$ exposure to the isolated myocytes is unknown. Our data suggest that the effect of $ONOO^-$ was in part mediated by increased MMP activity as the contractile dysfunction was diminished by two structurally independent MMPs inhibitors, doxycycline or PD 166793, and was independent of an action of MMPs to cleave extracellular matrix proteins in the isolated myocytes studied here. We found that 100 μM doxycycline was superior to 3 μM PD 166793 in preventing the contractile dysfunction caused by $ONOO^-$. This could be, to some extent, due to proposed ROS scavenging properties of some tetracyclines at high concentrations (Whiteman and Halliwell, 1997), in comparison to the lower concentration used here at which it has marked MMP inhibitor activity in heart tissue (Cheung *et al*., 2000). To the best of our knowledge, ROS scavenging properties have not been reported for PD 166793.

We focused our study on the effects of $ONOO^-$ and activation of MMPs on the contractile function of isolated cardiac myocytes to rule out effects of MMPs on extracellular matrix proteins. Our experimental model and the functional parameters measured have some resemblance to the situation in myocardial ischaemia–reperfusion injury, which is characterized by an increased $[Ca^{2+}]_i$ implicated in contractile dysfunction. As reported in this study, alterations in calcium handling and contractile function were observed in isolated cardiac myocytes. In addition, in isolated perfused rat hearts subjected to ischaemia–reperfusion injury (endogenous increase of $ONOO^-$), we found enhanced MMP-2 activity and degradation of troponin I and myosin light chain-1 (Yasmin *et al*., 1997; Cheung *et al*., 2000; Wang *et al*., 2002b; Sawicki *et al*., 2005) as also observed in isolated guinea pig hearts exposed to $ONOO^-$ (Rork *et al*., 2006). Therefore, our results from single cardiomyocytes suggest a possible intracellular action of MMPs and provide a plausible mechanism for the deleterious effects of $ONOO^-$-mediated MMPs activation during ischaemia–reperfusion injury at the whole heart level.

$ONOO^-$ can activate MMPs by different mechanisms. One of these is the *S*-glutathiolation of the propeptide domain which has been observed for MMP-1, -8 and -9 (Okamoto *et al*., 2001). Moreover, it was suggested that activation of MMPs by $ONOO^-$ could be mediated by nitration of tyrosine residues in the hinge region and further unfolding of the pro-MMP (Rajagopalan *et al*., 1996) or by *S*-nitrosylation of the cysteine residue in the propeptide domain, as observed with MMP-9 on its activation by either ischaemia–reperfusion or following administration of nitric oxide donors (Gu *et al*., 2002). The effect of $ONOO^-$ on MMP activity is biphasic as higher concentrations of $ONOO^-$ have clearly been shown to inactivate MMPs (Owens *et al*., 1997; Okamoto *et al*., 2001). However, $ONOO^-$ was shown to inhibit tissue inhibitors of MMPs (TIMP-1 and TIMP-2), which could increase net gelatinolytic activity in the cells (Frears *et al*., 1996; Chakraborti *et al*., 2004). Owing to technical limitations of the whole heart experiments, we could not determine the intracellular compartment(s) in which activation of MMPs occurred on exposure to $ONOO^-$. However, $ONOO^-$ may cause cellular injury through several mechanisms including protein modifications and the inactivation of proteins involved in Ca^{2+} handling, such as the nitration and inactivation of sarcoplasmic reticulum Ca^{2+} ATPase in porcine cardiac sarcoplasmic reticulum microsomes, with either 0.3 mM or 1 mM $ONOO^-$ (Lokuta *et al*., 2005). Furthermore, $ONOO^-$ inhibited the Na^+/Ca^{2+} exchanger in microsomes prepared from bovine smooth muscle cells, an event accompanied by the activation of MMP-2 and inhibition of TIMP-2 (Chakraborti *et al*., 2004). Moreover, reactive oxygen species have deleterious effects on Ca^{2+} homoeostasis in cardiac myocytes through their action on a number of proteins including phospholamban and calcium channels (Guerra *et al*., 1996; Sulakhe *et al*., 1997; Eigel *et al*., 2004; Sharikabad *et al*., 2004). In this regard, we observed alterations in both diastolic and systolic calcium homoeostasis in the presence of $ONOO^-$ and this effect was partially reversed by MMPs inhibitors. However, our study cannot determine which structure(s) related to calcium

handling are susceptible to degradation by MMPs. Therefore, these results are consistent with the notion that $ONOO^-$ alters intracellular calcium handling and contractility through mechanisms that involve activation of MMPs.

In addition, $ONOO^-$ is known to alter proteins involved in cardiac contractility including myofibrillar, cytoskeletal and mitochondrial respiratory chain enzymes. These changes include post-translational modifications of several amino acids, such as cysteine oxidation of creatine kinase, tyrosine nitration of cytoskeletal α-actinin and desmin along with modifications in cysteine and tyrosine residues and Fe–S bridges of mitochondrial proteins (see for review Pacher *et al*., 2007). These alterations are likely to contribute to contractile dysfunction that cannot be prevented by MMPs inhibition, as our study reports. Recently, we identified α-actinin as a further target of MMP-2 degradation in isolated hearts infused with $ONOO^-$ (Sung *et al*., 2007) and it is tempting to speculate that $ONOO^-$-induced post-translational modifications may enhance the targeting of susceptible proteins to proteolysis by MMP-2.

Ishida *et al*. (1996) showed that infusion of 200 μM $ONOO^-$ caused contractile dysfunction in isolated neonatal cardiac myocytes and this effect was accompanied by impaired Ca^{2+} homoeostasis. In our model using freshly isolated adult cardiac myocytes, we also observed abnormalities in Ca^{2+} homoeostasis due to $ONOO^-$. The MMP inhibitor doxycycline delayed the onset but did not prevent the impairment in Ca^{2+} homoeostasis caused by $ONOO^-$. However, we observed in our experiments that the time to cease calcium transient was delayed by doxycycline treatment and also that there was a significant increase in Ca^{2+} transient amplitude at 5 min, suggesting that the contractile dysfunction observed in this model is related to systolic Ca^{2+} abnormalities and also to a loss of sensitivity to Ca^{2+} in the contractile machinery. The loss of sensitivity of the cardiac myofibrils for Ca^{2+} upon $ONOO^-$ challenge has been shown in isolated perfused rat hearts (Brunner and Wolkart, 2003).

In conclusion, we have demonstrated that inhibition of MMPs partially reduces contractile dysfunction in isolated cardiac myocytes resulting from their direct exposure to $ONOO^-$. The effect of MMP activation in these cells is independent of a proteolytic action on extracellular matrix proteins. This provides further insight into the pathological mechanisms related to myocardial injury following oxidative stress. Detoxifying $ONOO^-$ or inhibiting the activation of MMPs or their enzymatic activity may be an effective therapeutic strategy to lessen the impact of oxidative stress on cardiomyocyte function in pathological conditions such as ischaemia–reperfusion injury.

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Conflict of interest

The authors state no conflict of interest.

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