Original Article

Peroxynitrite-Induced Intracellular Ca^{2+} Depression in Cardiac Myocytes: Role of Sarco/Endoplasmic Reticulum Ca^{2+} Pump

(intracellular Ca^{2+} / SERCA2a / myocytes / peroxynitrite)

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Abstract. Several studies have shown that peroxynitrite ($ONOO^-$), formed upon the reaction of •NO and O^{2-}, is increased in many cardiovascular diseases and is detrimental to myocardial function. Proteins associated with Ca^{2+} homeostasis regulation in the heart may be involved in these effects. Thus, the aim of this study was to elucidate the mechanisms associated with $ONOO^-$-induced effects. We evaluated $[Ca^{2+}]_i$ regulation, sarco/endoplasmic reticulum Ca^{2+}-binding proteins, and phosphorylation levels of the ryanodine receptor in isolated rat myocytes. Electrical field-induced intracellular Ca^{2+} transients and contractions were recorded simultaneously. Myocytes superfused with 3-morpholinosydnonimine N-ethylcarbamide (SIN-1), an $ONOO^-$ donor, decreased the amplitude of Ca^{2+} transients and contraction in a dose-response (1–200 μM) manner. Similarly, SIN-1 increased half-time decay in a concentration-dependent manner. Co-infusion of the $ONOO^-$ donor with FeTMPyP (1 μM), an $ONOO^-$ decomposition catalyst, inhibited the effects induced by $ONOO^-$. Impaired sarcoplasmic reticulum Ca^{2+} uptake caused by $ONOO^-$ (SIN-1 200 μM) was confirmed by a reduction of caffeine-evoked Ca^{2+} release along with prolongation of the half-time decay. Surprisingly, $ONOO^-$ induced a spontaneous Ca^{2+} transient that started at the beginning of the relaxation phase and was inhibited by tetracaine. Also, reduced phosphorylation at the ryanodine receptor 2 (RyR2)-Ser-2814 site was observed. In conclusion, deficient sarco/endoplasmic reticulum Ca^{2+}-ATPase-mediated Ca^{2+} uptake concomitant with augmented Ca^{2+} release by RyR2 in myocytes may be associated with modification of myocyte Ca^{2+} handling by $ONOO^-$. Thus, development of cardiac failure in diabetes, nephropathy, or hypertension may be related with elevated $ONOO^-$ in cardiac tissue.

Introduction

The imbalance between reactive oxygen species (ROS) levels and the antioxidant defence system present in the body leads to dysregulation of several cell functions (Matschke et al., 2019). Several studies have demonstrated the role of ROS in the development of cardiomyopathy (Madamanchi and Runge, 2013; Kanaan and Harper, 2017). Recently, we demonstrated that decreased nitric oxide (•NO) synthesis concomitant with increased superoxide anion radical (O^{2-}) and peroxynitrite ($ONOO^-$) levels were associated with coronary endothelium dysfunction in obese mice (Gamez-Mendez et al., 2015). These data suggested the possibility that $ONOO^-$ may play a role in cardiovascular pathology. Indeed, several studies have shown that $ONOO^-$, formed upon the reaction of •NO and O^{2-}, is increased in many cardiovascular diseases and is detrimental to myocardial function. Mungrue et al. (2002) demonstrated that increased $ONOO^-$ levels in mice caused cardiomyopathy and sudden cardiac death. Furthermore, $ONOO^-$ decomposition catalyst, FP15, improved the cardiac function of isolated cardiomyocytes in a rodent model of DOX-induced cardiac dysfunction (Pacher et al., 2003). This result added in the idea that $ONOO^-$ may be relevant for

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Abbreviations: $[Ca^{2+}]_i$ – intracellular calcium, EGTA – ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid, FeTMPyP – Fe(III) tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride, IL-6 – interleukin 6, L-NAME – N(ω)-nitro-L-arginine methyl ester, NCX – Na^+/Ca^{2+} exchanger, •NO – nitric oxide, O^{2-} – superoxide, $ONOO^-$ – peroxynitrite, ROS – reactive oxygen species, RT – room temperature, RyR2 – ryanodine receptor, SERCA2a – sarco/endoplasmic reticulum Ca^{2+}-ATPase, SIN-1 – 3-morpholinosydnonimine N-ethylcarbamide, SR – sarco/endoplasmic reticulum, TnI – troponin I.

the development of cardiac diseases, such as heart failure and myocardial ischaemia/reperfusion injury.

The effect of $ONOO^-$ on cardiac cells has been explored in different experimental models via direct exposure or using $ONOO^-$ donors (Pacher et al., 2007; Kohr et al., 2012). Thus, exogenous addition of $ONOO^-$ induced reduction in cardiomyocyte spontaneous contraction (Ishida et al., 1996) and irreversible reduction in diastolic and systolic heart function (Digerness et al., 1999). Moreover, in isolated perfused rat heart, $ONOO^-$ administration potentiated myocardial reperfusion injury as well as reduction in left ventricular pressure (Ma et al., 1997; Brunner and Wolkart, 2003). Several possible targets of $ONOO^-$ have been proposed in cardiac and vascular dysfunction. Matrix metalloproteases and the nuclear enzyme polymerase were activated by increased $ONOO^-$ in various types of heart disease (Pacher and Szabo, 2005). Also, increased levels of $ONOO^-$ have been associated with reduced levels of troponin I phosphorylation, stimulation of cGMP production, and triggering of cardiomyocyte apoptosis (Stojanovic et al., 2001; Brunner and Wolkart, 2003; Levrand et al., 2006). Several authors have demonstrated the $ONOO^-$-induced nitration of several cardiac proteins. Tyrosine nitration inhibited myofibrillar creatine kinase, the critical energetic controller of cardiomyocyte contractility (Mihm et al., 2002). Also, increased nitration of the sarco/endoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA2a) has been associated with impaired relaxation (Lokuta et al., 2005), and α-actin nitration was related to altered Ca^{2+}-activated force production in human myocytes (Borbely et al., 2005). Thus, the Ca^{2+} transport system and many other cellular processes may be involved in the cardiac dysfunction evoked by $ONOO^-$. Therefore in the present study, we further explored the effect of $ONOO^-$ on intracellular calcium ($[Ca^{2+}]_i$) fluxes and the contractile function in isolated rat cardiomyocytes. We hypothesized that the $ONOO^-$ effect could be related to depression of SR Ca^{2+} content and explored the possible synergistic effect of SR Ca^{2+}, and RyR2. 3-morpholinosydnonimine N-ethylcarbamide (SIN-1), an O^{2-} and •NO free radical donor, was employed to generate $ONOO^-$. To elucidate the mechanisms associated with $ONOO^-$-induced effect, we evaluated $[Ca^{2+}]_i$ regulation, SR Ca^{2+}-binding proteins, and phosphorylation levels of the ryanodine receptor (RyR2).

Material and Methods

Reagents

All buffers and solutions were prepared daily. SIN-1 was dissolved in 0.05% dimethyl sulphoxide. All other chemicals were dissolved in distilled water. Fe(III) tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride (FeTMPyP) was used as a specific $ONOO^-$ decomposition catalyst. The nitric oxide synthase enzyme inhibitor N(ω)-nitro-L-arginine methyl ester (L-NAME) was used as a negative control of nitrosylation. To obtain 0 Na^+/0 Ca^{2+} Tyrode solution, we replaced sodium chloride (NaCl) by lithium chloride, calcium (Ca^{2+}) was removed and replaced with Ca^{2+}-chelating agent ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA). Caffeine was used for SR Ca^{2+} depletion. Caffeine and tetracaine hydrochloride were dissolved in 0 Na^+/0 Ca^{2+} Tyrode solution. Unless otherwise stated, all reagents were obtained from Sigma-Aldrich (St Louis, MO). FeTMPyP was purchased from Cayman Chemical (Ann Arbor, MI). All antibodies were from Abcam (Cambridge, MA) except anti-RyR-p-Ser2814, which was purchased from Badrilla (Leeds, UK).

Isolation of rat ventricular myocytes

Male Wistar rats (250–300g) were obtained from the Experimental Animal Care Center from CINVESTAV-I.P.N., México. Rats were fed standard chow and maintained on a 12 h light – 12 h dark cycle. All procedures were handled strictly in accordance with the 8th edition of National Institutes of Health "Guide for the Care and Use of Laboratory Animals" (2011) and were approved by the Institutional Ethics Review Committee for Animal Experimentation of Cinvestav-IPN (Approval No. 479-10). Animals were injected with heparin (500U/Kg, i.p.) 20 min before obtaining their hearts for perfusion. Rats were anaesthetized with isoflurane inhalation (2.5%, EZ- Anesthesia 108SA, Palmer, PA).

The heart was rapidly excised from a deeply anaesthetized rat, and placed in ice-cold Tyrode solution (in mM: 140 NaCl, 5.4 KCl, 1 NaH_2PO_4, 1 $MgCl_2$, 10 glucose, and 10 HEPES, pH 7.4) supplemented with 2 mM Ca^{2+}. The excised heart was cannulated and equilibrated with 2 mM Ca^{2+} Tyrode solution for 5 min using a modified Langendorff perfusion system, followed by perfusion for 5 min with Ca^{2+}-free Tyrode solution until contractions ceased. This was followed by 20–30 min perfusion with 50 μM Ca^{2+} Tyrode solution containing 0.4 mg/ml collagenase (Worthington Biochemical Corp., Freehold, NJ) and 0.06 mg/ml protease from *Bacillus sp.* After enzymatic perfusion, ventricles were removed, minced, and filtered (nylon mesh, 300 μm). The cell suspension was centrifuged (25 *g* for 5 min) and myocytes were resuspended in Tyrode solution with 200 μM Ca^{2+} supplemented with 0.1% bovine serum albumin. Ca^{2+} was slowly added back up to 1 mM. Isolated myocytes yielded 80 % viable cells and were always used for experiments within 6 h after isolation.

Confocal Ca^{2+} imaging

Isolated myocytes were loaded with 10 μM Fluo 4-AM (Life Technologies, Carlsbad, CA), a fluorescent dye-based Ca^{2+} indicator, for 30 min at room temperature (RT). Excess dye was removed by washout with 1 mM Ca^{2+} Tyrode solution. Fluo 4-loaded myocytes were plated on laminin-covered glass coverslips mounted in a perfusion chamber (Warner Instruments, LLC, Hamden, CT) and allowed to adhere for 10 min prior to perfusion. Myocytes were continuously superfused with

1 mM Ca^{2+} Tyrode solution. Ca^{2+} transients were elicited by pacing the myocytes at 0.5 Hz and recording for a minimum time window of 15 s. Immediately after reaching a steady state, cells were superfused for 30 min in the absence or presence of increasing concentrations of SIN-1 (1, 20, and 200 μM). At the end of this period, myocytes were superfused again with 1 mM Ca^{2+} Tyrode solution, and Ca^{2+} transients were elicited and recorded as described above.

SIN-1-generated $ONOO^-$ was scavenged with the $ONOO^-$ decomposition catalyst FeTMPyP. Myocytes were co-infused with 1 μM FeTMPyP and with either 20 or 200 μM SIN-1 for 30 min. Ca^{2+} transients were elicited and recorded as described above.

Data were acquired using the fluorescence channel of a laser scanning inverted confocal microscope (Leica TCS SP5, Leica Microsystems, Mannheim, Germany), equipped with a D-apochromatic 63×, 1.2 NA, oil objective. An argon laser was used to excite the fluorophore at 488 nm and emission collected at 500–600 nm. Line scans were drawn along the longitudinal axis of selected cells at 530 Hz (1.8 ms per line scan) with a pixel size of 0.14 μm. Ca^{2+} transients are expressed as $\Delta F/F_0$ $((F_{max} - F_{rest})/F_{rest})$, where F is the fluorescence intensity. All measurements were recorded at room temperature (RT). The transient maximal signal amplitude and time to 50% relaxation are the parameters reported here.

Myocyte shortening measurement

Sarcomere shortening was measured in line scan images obtained with the transmitted light channel from a laser scanning inverted confocal microscope (Leica TCS SP5). Each line scan was analysed using a computer algorithm developed in MATLAB r2016a, in which the fast Fourier transform was used. Sarcomere length was normalized as $-\Delta L/L_0$, where ΔL is the sarcomere length as a function of time in negative, and L_0 is the sarcomere length at rest. Sarcomere shortening and Fluo 4-AM fluorescence measurements were simultaneously recorded.

Ca^{2+} transport system evaluation

The participation of L-type Ca^{2+} channels, SERCA2a transporter, and Na^+/Ca^{2+} exchanger (NCX) in the $ONOO^-$-dependent effect on intracellular Ca^{2+} and myocyte contraction was investigated. After myocytes were stimulated (0.5 Hz) in the presence or absence of SIN-1 (200 μM), the solution was rapidly changed to a 0 Na^+/0 Ca^{2+} Tyrode solution for 60 s without field electrical stimulation. Na^+/Ca^{2+} free solution eliminates the contribution of L-type Ca^{2+} channels and NCX over the SR response. Thus, the SR Ca^{2+} release elicited by caffeine can be evaluated. Myocytes were rapidly superfused with 10 mM caffeine in 0 Na^+/0 Ca^{2+} Tyrode solution in the presence or absence of SIN-1 (200 μM). $[Ca^{2+}]_i$ changes were recorded for 4 min. The maximum amplitude of caffeine-evoked Ca^{2+} transients was normalized to the average maximum amplitude of electrically evoked Ca^{2+} transients elicited before 30 min superfusion and recorded previously in the same cell (Amplitude Caffeine $[Ca^{2+}]_i$/Twitch $[Ca^{2+}]_i$).

To explore the role of RyR2 in the $ONOO^-$-dependent effect on intracellular Ca^{2+} response, myocytes were superfused with tetracaine (2 mM) and caffeine (10 mM) in 0 Na^+/0 Ca^{2+} Tyrode solution in the presence or absence of SIN-1 (200 μM). $[Ca^{2+}]_i$ changes were recorded for 4 min.

RyR2 nitrosylation

Freshly isolated myocytes were plated in poly-L-lysine-coated cover glasses and incubated at RT with vehicle (control), SIN-1 (200 μM), L-NAME (100 μM), and SIN-1 + L-NAME. L-NAME was incubated for 3 h, whereas SIN-1 was added the last hour of incubation. Subsequently, cells were fixed with 10% formalin solution for 10 min at RT, rinsed, and permeabilized with 0.5% Triton X-100 for 10 min at RT. Cells were blocked afterwards with 0.1% BSA for 30 min at RT. Incubation with mouse monoclonal anti-3-nitrotyrosine (1 : 250, Cat. AB61392) and rabbit polyclonal anti-ryanodine receptor (1 : 250, Cat. AB90629) antibodies was carried out overnight. Then, Alexa Fluor 488-conjugated goat anti-mouse IgG (1 : 250, Cat. AB150113) and Alexa Fluor 594-conjugated goat anti-rabbit (1 : 250, Cat. AB150080) antibodies, respectively, were incubated for 2 h at RT. Images were obtained using a laser scanning confocal imaging system (Leica TCS SP5) with excitation/emission wavelengths of 488/530 and 543/610, respectively. Images were analysed with ImageJ software (NIH).

Western blot for phosphorylated RyR2

Freshly isolated myocytes were incubated at RT with SIN-1 (200 μM) for 30 min or isoproterenol (ISO, 1 μM) for 5 min. DMSO-incubated cells were used as a vehicle control and ISO-incubated cells were used as positive control for phosphorylation. After incubation, myocytes were placed in a direct current electrical stimulation chamber and allowed to rest for 5 min in 1 mM Ca^{2+} Tyrode solution. Then, myocytes were electrically stimulated (0.5 Hz; Tyrode solution) for 2.5 min. Immediately after stimulation, cells were centrifuged at 25 *g* for 30 s and the pellet was quickly frozen in liquid N_2. Frozen cell pellets were homogenized with lysis buffer (50 mM Tris-HCl pH = 7.4, 137 mM NaCl, 2 mM EDTA, 1% NP-40, 5% glycerol, 0.1 mM EGTA, 0.1% deoxycholic acid) containing protease (COmplete Ultra TM, Roche Diagnostics, Indianapolis, IN) and phosphatase (PhosStop, Roche Diagnostics, Indianapolis, IN) inhibitors. The homogenate was centrifuged at 200 *g* for 20 min at 4 °C, and the supernatant was collected. Protein was measured by the BCA method, and 20 μg of protein was resolved in 3% SDS-PAGE. Gels were transferred to PVDF membrane (Hybond-P, Amersham, Buckinghamshire, UK) at 100 V for 2 h. Blots were incubated with anti-RyR2 phospho-Ser-2814 antibody (rabbit polyclonal RyR-p-Ser2814, 1 : 10,000, Cat. A010-31). After incubation with the secondary antibody (Alexa

Fluor 594-conjugated goat anti-rabbit, 1 : 2,000, Cat. AB150080), blots were reprobed twice. First, they were incubated with anti-RyR2 antibody (mouse monoclonal, 1 : 10,000, Cat. AB2827) and then with the corresponding secondary antibody (Alexa Fluor 488-conjugated goat anti-mouse IgG, 1 : 2,000, Cat. AB150113) to normalize the loads. Afterwards, blots were incubated with anti-RyR2 phospho-Ser2808 antibody (rabbit polyclonal, 1 : 9,000, Cat. AB59225) followed by Alexa Fluor 594-conjugated goat anti-rabbit antibody (1 : 2,000, Cat. AB150080). Primary antibodies were incubated for 2 h and secondary antibodies for 1 h, at RT. Immunoreactivity was detected by enhanced chemiluminescence (ECL, Amersham Biosciences, Freiburg, Germany) and analysed using Image J software (NIH, Bethesheda, MD).

Statistical analysis

Data are presented as mean ± SEM, except for data that are presented as percentage. Unpaired two-tailed student *t*-test, χ^2 test, and one-way analysis of variance (ANOVA) followed by post hoc Fisher-LSD were used to determine statistical significance. $P < 0.05$ was considered statistically significant.

Results

Effect of SIN-1 on Ca^{2+} transients and myocyte contraction

We evaluated the effect of $ONOO^-$ generator SIN-1 on Ca^{2+} transients and contraction responses in isolated single myocytes from the rat heart. Figure 1 shows typical traces of Ca^{2+} transients and myocyte contraction evoked by 15 s of electrical field stimulation under basal conditions (0.84 ± 0.04 and 0.93 ± 0.08, Fig. 1 A and C, respectively). After 30 min of superfusion with SIN-1 (200 μM), myocytes were field stimulated again. The continuous presence of SIN-1 resulted in a reduction of both Ca^{2+} transient amplitude (0.41 ± 0.07) and myocyte contraction (0.40 ±.0.07, Fig. 1 B and D, respectively) compared to the control. Additionally, after 25 s of field stimulation, SIN-1-superfused myocytes showed alterations in the Ca^{2+} transient shape with premature Ca^{2+} release, compared to control cells.

Superfusion with SIN-1 (1 μM – 200 μM) evoked a dose-response effect in Ca^{2+} transient amplitude (Fig. 2A), myocyte contraction (Fig. 2B), and Ca^{2+} transient half-time decay (Fig. 2C).

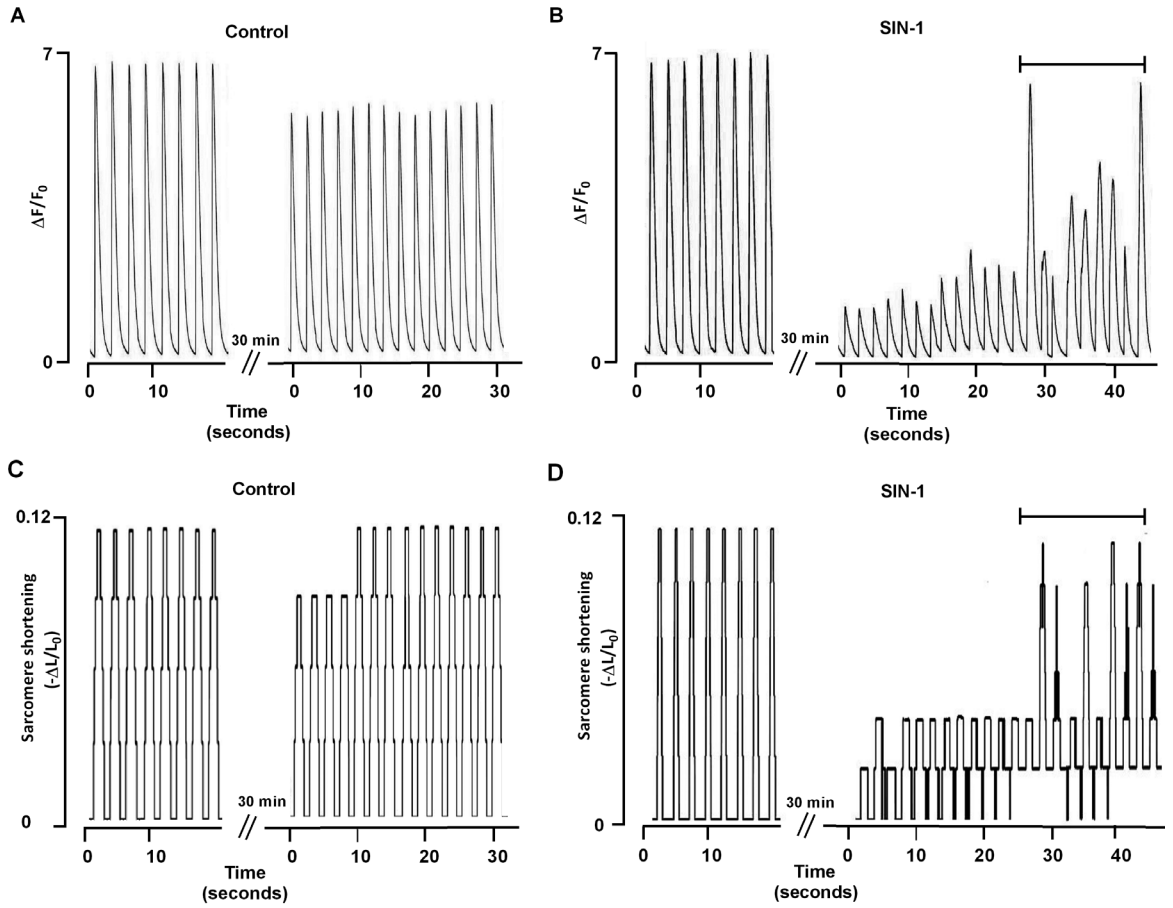

Fig. 1. SIN-1 effect on myocyte $[Ca^{2+}]_i$ and contraction. (**A)** and (**C**), typical traces of control steady-state Ca^{2+} transients and contraction, respectively. (**B)** and (**D**), representative traces of steady-state Ca^{2+} transients and contraction, respectively, after 30 min of SIN-1 superfusion (200 μM). Each trace represents five different experiments.

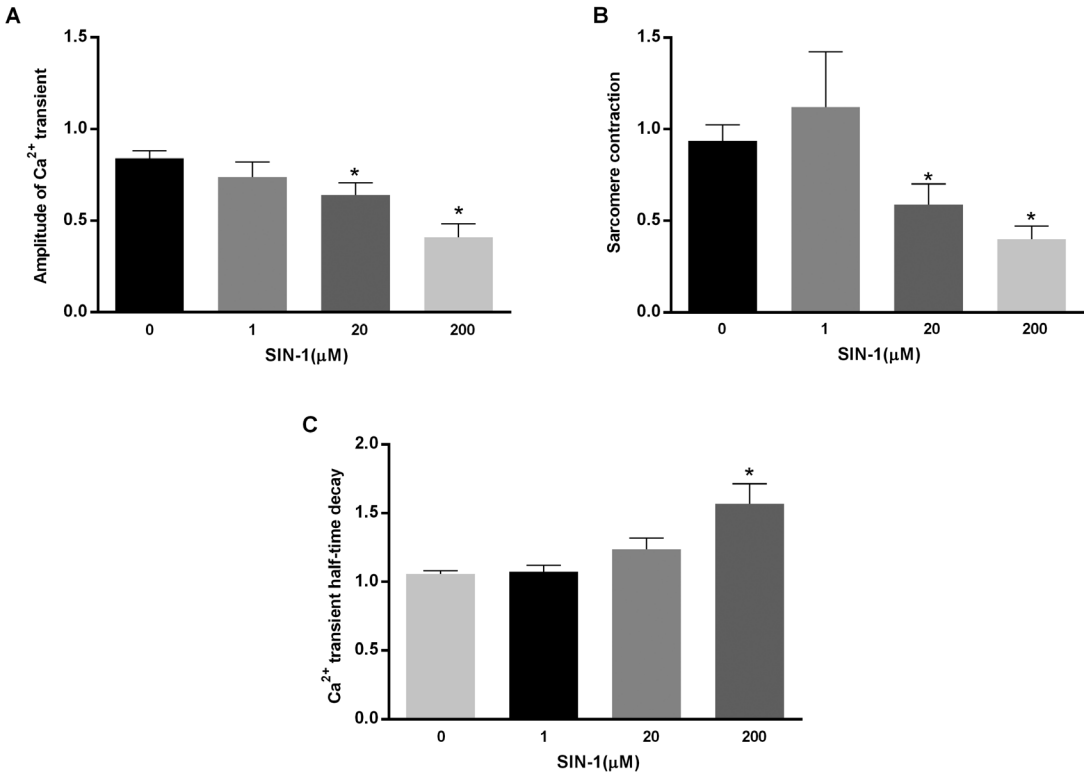

Fig. 2. Rat myocytes were superfused with increasing concentrations of SIN-1 (1–200 μM). (**A)** SIN-1 dose-dependent effect on $[Ca^{2+}]_i$ amplitude, (**B)** contraction, (**C)** half-time decay. Responses were normalized to Ca^{2+} transients elicited electrically 30 min before myocytes were superfused with SIN-1. Each bar represents the mean ± SEM of 25 myocytes from five different rats. *P < 0.05 *vs* control without SIN-1.

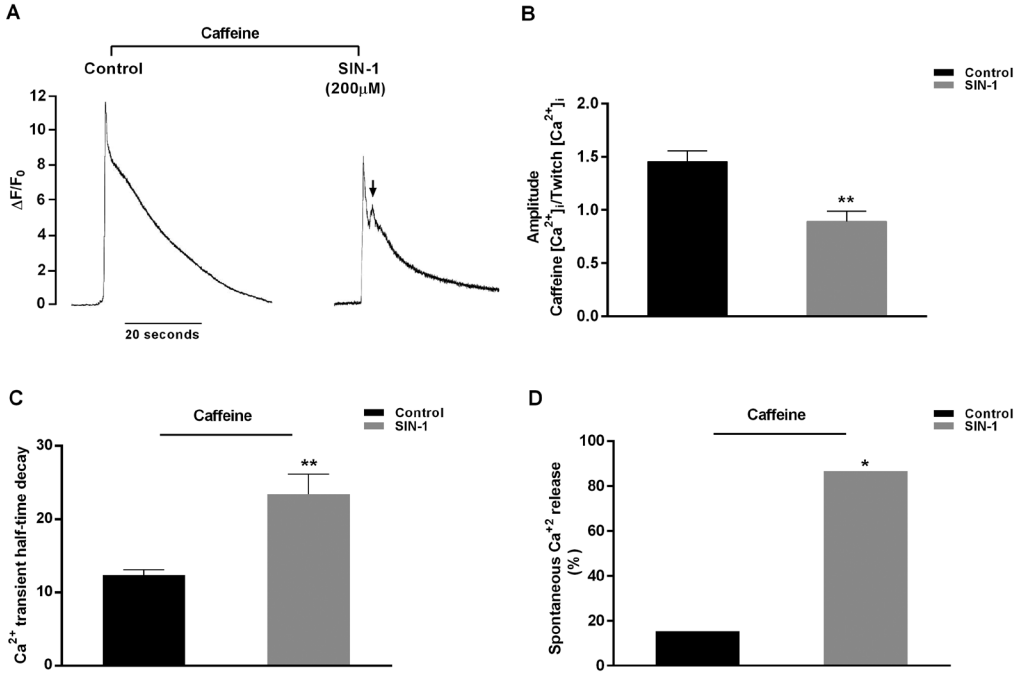

Fig. 3. Effect of SIN-1 on caffeine-mediated Ca^{2+} release in rat cardiac myocytes. (**A**) Representative caffeine (10 mM)-induced Ca^{2+} transients, control and in the presence of SIN-1 (200 μM). (**B**) Fractional Ca^{2+} release (Amplitude Caffeine $[Ca^{2+}]_i$/Twitch $[Ca^{2+}]_i$) after stimulation at 0.5 Hz and a pulse of 10 mM caffeine in control and SIN-1 (200 μM)-superfused myocytes. (**C**) Data for caffeine-induced Ca^{2+} transient half-time decay, control and in the presence of SIN-1 (200 μM). (**D**) Data for spontaneous Ca^{2+} release [(**A**) arrow] were expressed as the percentage in control and SIN-1 (200 μM)-superfused myocytes. Each bar represents the mean ± SEM of 15 myocytes from five different rats (**B**, **C**). χ^2 test was used to evaluate the statistical significance of spontaneous Ca^{2+} release data. *P < 0.05 *vs* control. **P < 0.01 *vs* control.

To confirm that $ONOO^-$ was the reactive nitrogen species responsible for the altered responses described in the presence of SIN-1, myocytes were treated with $ONOO^-$ decomposition catalyst FeTMPyP. Co-infusion of myocytes with SIN-1 and FeTMPyP prevented the SIN-1 effect on Ca^{2+} transient amplitude and myocyte contraction. Thus, SIN-1 (200 μM) reduced the Ca^{2+} transient amplitude from 0.84 ± 0.04 to 0.41 ± 0.07, while in the presence of FeTMPyP, the amplitude was 0.99 ± 0.10. Similarly, SIN-1 reduced myocyte contraction from 0.93 ± 0.08 to 0.40 ± 0.07, and in the presence of FeTMPyP the contraction was 0.88 ± 0.10.

Role of Ca^{2+} transporters in $ONOO^-$-induced response in cardiac myocytes

The role of Ca^{2+} transporters, SERCA2a and sarcolemmal NCX, in $ONOO^-$-induced effects was explored in cardiac cells. NCX was blocked with superfusion of 0 Na^+/0 Ca^{2+} Tyrode solution. Total Ca^{2+} release from SR was stimulated by caffeine (10 mM), and the effect of $ONOO^-$ under these conditions was evaluated.

Caffeine-evoked Ca^{2+} release amplitude was clearly reduced in SIN-1-superfused myocytes compared with the caffeine-evoked Ca^{2+} release from control myocytes (Fig. 3A). Fractional Ca^{2+} release, portrayed as Amplitude Caffeine $[Ca^{2+}]_i$/Twitch $[Ca^{2+}]_I$, showed a decrease of 60 % in the presence of SIN-1 (1.45 ± 0.20 in control myocytes *vs* 0.88 ± 0.19 in the presence of SIN-1, Fig. 3B). Half-time decay was increased in SIN-1-superfused myocytes compared to control cells (23.47 ± 2.7 s and 12.4 ± 0.70 s, respectively; Fig. 3C). This effect was similar to that observed in electrically stimulated myocytes (Fig. 2C).

Also, $ONOO^-$ induced a second Ca^{2+} transient that started at the beginning of the relaxation phase (Fig. 3 A,

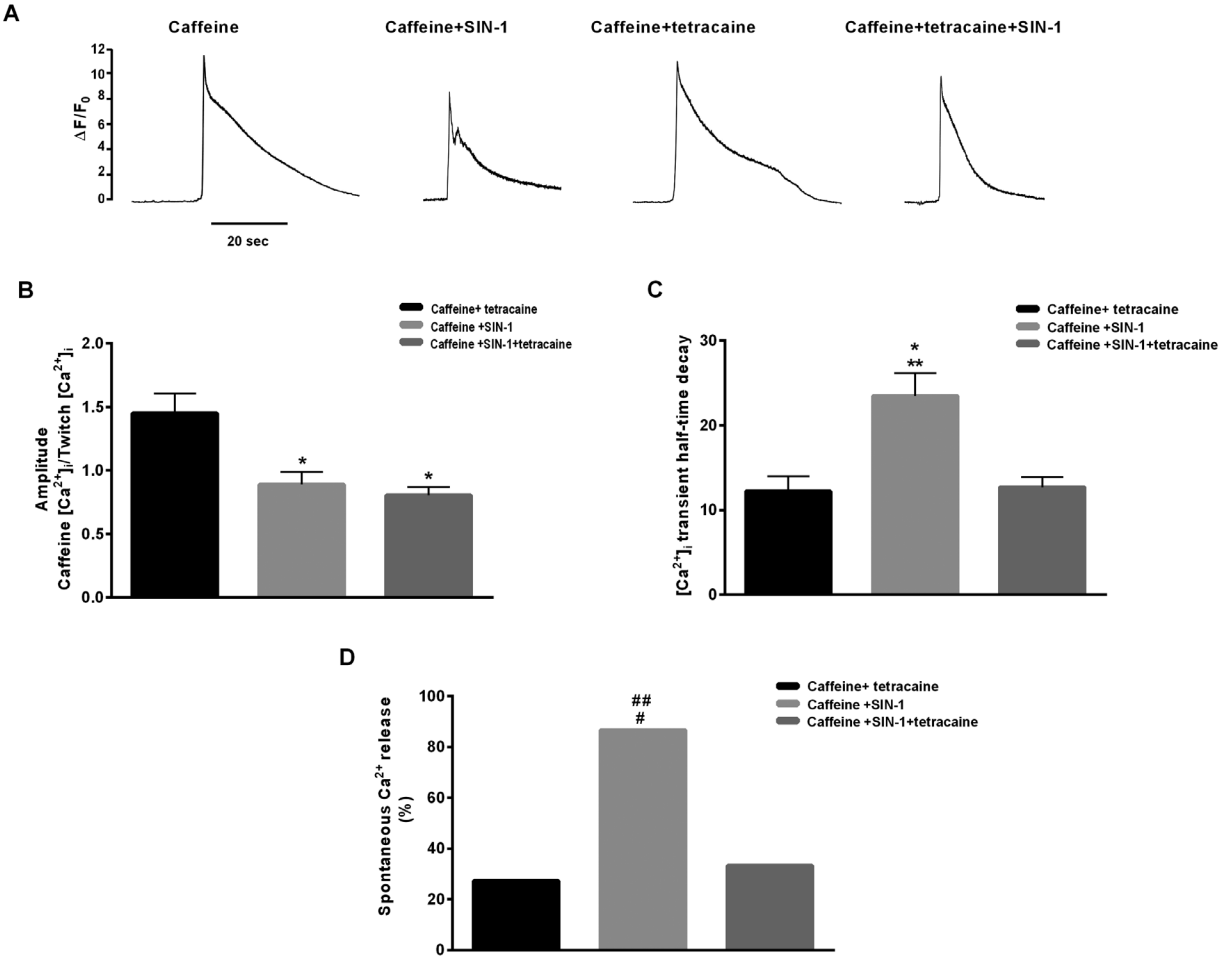

Fig. 4. Effect of tetracaine and SIN-1 on caffeine-induced $[Ca^{2+}]_i$ release. (**A**) Representative caffeine-induced Ca^{2+} transients in the presence of tetracaine (2 mM), SIN-1 (200 μM), and tetracaine + SIN-1 simultaneously. (**B**) Fractional Ca^{2+} release (Amplitude Caffeine $[Ca^{2+}]_i$/Twitch $[Ca^{2+}]_i$) after stimulation at 0.5 Hz and a pulse of 10 mM caffeine in the three types of conditions. (**C**) Data for caffeine-induced Ca^{2+} transient half-time decay in the three types of conditions. (**D**) Data for spontaneous Ca^{2+} release, expressed as the percentage [(**A**) arrow]. Each bar represents the mean ± SEM of 15 myocytes from five different rats (**B**, **C**). χ^2 test was used to evaluate the statistical significance of spontaneous Ca^{2+} release data. *P < 0.01 *vs* caffeine+tetracaine. **P < 0.01 *vs* caffeine+SIN-1+tetracaine. $^{\#}$P < 0.05 *vs* caffeine+SIN-1. $^{\#\#}$P < 0.05 *vs* caffeine+SIN-1+tetracaine.

arrow). This second peak occurred in as many as 86.7 % of total experiments (13/15 total cells from 5 rat hearts) compared to 15.4 % observed in the control group (Fig. 3D). Development of a second peak in a caffeine-stimulated Ca^{2+} transient in the presence of SIN-1 suggested a new Ca^{2+} release event.

Tetracaine (2 mM), RyR2-dependent Ca^{2+} efflux blocker, was used to further explore the mechanisms involved in the increased half-time decay response. Tetracaine did not affect the amplitude in caffeine-induced Ca^{2+} fluxes (Fig. 4A and B). However, the presence of tetracaine prevented the effect of SIN-1 on the transient half-time decay (Fig 4C). Furthermore, tetracaine prevented generation of spontaneous Ca^{2+} release (Fig. 4D).

To investigate the possible downstream targets of $ONOO^-$, we evaluated protein nitrosylation in cardiac myocytes. SIN-1-treated myocytes showed a similar level of protein nitration as control myocytes (data not shown).

We also explored RyR2 phosphorylation at Ser2814 and Ser2808 residues. Incubation of myocytes with SIN-1 resulted in reduced phosphorylation of the Ser2814 residue as observed in Fig. 5A, while the Ser2808 residue was not affected (Fig. 5B). RyR2 expression was used as a control to normalize data.

Discussion

In the present study, we demonstrated that exogenous administration of $ONOO^-$ generator SIN-1 depresses myocyte contractibility associated with decreasing $[Ca^{2+}]_i$ fluxes. This effect may be mediated through changes in $[Ca^{2+}]_i$ stores. Also, we observed a SIN-1 concentration-dependent effect (1 to 200 μM) on both cardiac contraction and Ca^{2+} fluxes. Simultaneous incubation of SIN-1 and FeTMPyP, an $ONOO^-$ decomposition catalyst, prevented these effects and demonstrated that they were specifically driven by $ONOO^-$.

There is some controversy about whether $ONOO^-$ decreases or increases Ca^{2+} transients. Several reports have shown that $ONOO^-$ increases systolic and diastolic intracellular Ca^{2+} (Brunner and Wolkart, 2003). Also, an increased peak amplitude of Ca^{2+} transients in SIN-1-stimulated murine cardiac myocytes has been reported (Kohr et al., 2010). Our observation that $ONOO^-$ decreases Ca^{2+} transients agrees with the inhibition of isoproterenol-elicited increases in Ca^{2+} effluxes by SIN-1 in guinea pig-isolated myocytes (Wahler and Dollinger, 1995). Similar data showed inhibition of isoproterenol effect on Ca^{2+} effluxes and myocyte contraction in single rat myocytes (Stojanovic et al., 2001; Yin et al., 2002). However, our data demonstrated a direct effect of SIN-1, without β-adrenergic stimulation, on Ca^{2+} fluxes in isolated rat myocytes, suggesting a role for SIN-1-generated $ONOO^-$ in Ca^{2+} regulation.

We demonstrated a SIN-1 dose-dependent effect on myocyte contractility. Previous reports have shown similar results. Perfusion with SIN-1 reduced left ventricular pressure in isolated perfused rat heart (Brunner and Wolkart, 2003). Also, decreased spontaneous contraction followed by complete arrest was demonstrated in

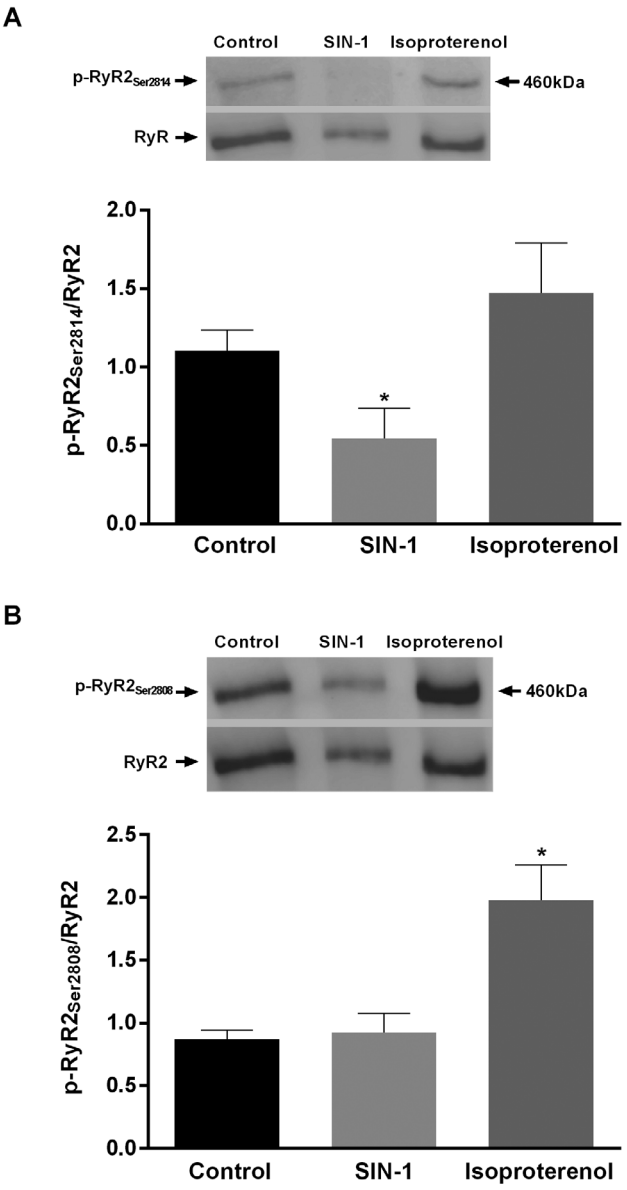

Fig. 5. SIN-1-induced hypo-phosphorylation of RyR2 Ser-2814 residue. Phosphorylation at (**A**) RyR2-Ser-2814 and (**B**) RyR2-Ser-2808 sites was determined by Western blot. Myocytes were incubated with SIN-1 (200 μM) and isoproterenol as a positive control. After incubation with anti-RyR2 phospho-Ser-2814 antibody and the corresponding secondary antibody, blots were reprobed with anti-RyR2 to normalize the blots. Then, the blots were reprobed again with anti-RyR2 phospho-Ser2808 antibody. Graphs show the densitometric analysis of the Western blots analysed with Image J software. Data were normalized to the loading control. Fold differences were obtained for each biological replicate by dividing the normalized density of each sample by normalized density from the control. Each bar represents the mean ± SEM of three different rats. *$P < 0.001$ *vs* control.

cultured rat myocytes (Ishida et al., 1996; Katori et al., 2006).

In isolated rat myocytes, we demonstrated that $ONOO^-$ has a negative inotropic effect. This result is supported by Yu et al. (2005), who demonstrated an $ONOO^-$-modulated negative inotropic effect of interleukin 6 (IL-6) on ventricular myocytes. However, the effect of $ONOO^-$ on cardiovascular tissue has been controversial. Thus, the presence of exogenous $ONOO^-$ aggravated the development of heart damage after reperfusion in studies of myocardial ischaemia (Ma et al., 1997), whereas *in vivo*, $ONOO^-$ administration exerted cardioprotection (Nossuli et al., 1998). Other reports further support our results. Decreased spontaneous myocyte contraction was demonstrated after exposure to $ONOO^-$ in cultured cardiac cells from mouse embryo hearts (Ishida et al., 1996). Also, $ONOO^-$ decreased the positive inotropic effect of β-adrenergic stimulation in rat myocytes (Stojanovic et al., 2001) and reduced by 26 % the developed force of intact papillary rat muscle (Digerness et al., 1999).

There are several possible mechanisms involved in the intracellular Ca^{2+} instability and negative inotropic effects of $ONOO^-$. Interference of contractile proteins in the release and uptake of Ca^{2+} and modification of Ca^{2+} transporters has been reported. Snook et al. (2008) have shown that cardiac myocyte injury induced by $ONOO^-$ may be associated with myosin nitration. The reduced SR contractile function induced by IL-6 has also been associated with $ONOO^-$ (Yu et al., 2005). Thus, $ONOO^-$ may be targeting specific proteins through post-translational modifications such as phosphorylation, S-nitrosylation, or both.

Our data showed SIN-1-dependent reduction of caffeine-evoked Ca^{2+} release in a Na^+/ Ca^{2+}-free Tyrode solution. This result suggests that the SIN-1 effect may be associated with Ca^{2+} release from internal stores, e.g., the SR Ca^{2+} stores. Indeed, previous studies have shown that SIN-1 induces SERCA2a nitration and oxidation along with reduction in SERCA2a expression and activity (Li et al., 2016). Thus, we explored RyR2 as the target for SIN-1-decreased Ca^{2+} fluxes. In our studies, tetracaine prevented SIN-1 effects. This suggested a possible role for RyR2 in the SIN-1 mechanism of action. The reduced phosphorylation at the Ser2814 site by SIN-1 further supports this idea.

Additionally to the reduced Ca^{2+} fluxes induced by SIN-1, we observed prolonged Ca^{2+} decay time in myocytes treated with SIN-1. Moreover, when caffeine was used as a stimulus for Ca^{2+} fluxes, a second peak in Ca^{2+} was observed during the relaxation phase. This observation suggests increased activity of RyR2 leading to spontaneous Ca^{2+} release into the cytosol. Thus, the SR Ca^{2+} store depression would be exacerbated by RyR2 altered behaviour concomitant with a reduction of Ca^{2+} uptake by SERCA2a.

In conclusion, modifications in myocyte Ca^{2+} handling in the presence of $ONOO^-$ may be associated with a reduction of Ca^{2+} uptake mediated by SERCA2a along with an increase in the RyR2 Ca^{2+} release. Thus, under pathological conditions where $ONOO^-$ production is elevated, the impaired Ca^{+2} sequestration into intracellular stores may result in altered Ca^{2+} myocyte processes and cardiac arrhythmogenesis. However, further studies are necessary to elucidate whether cardiac failure developed in diabetes, nephropathy, or hypertension might be related to increased $ONOO^-$ in the cardiac tissue. Therefore, the use of either $ONOO^-$ decomposition catalysts or antioxidants may represent a promising therapeutic approach in cardiac pathologies and related diseases.

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