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Effects of *N*-*n*-butyl haloperidol iodide on the rat myocardial sarcoplasmic reticulum Ca^{2+}–ATPase during ischemia/reperfusion

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ABSTRACT

We have previously shown that *N*-*n*-butyl haloperidol iodide (F_2), a newly synthesized compound, reduces ischemia/reperfusion (I/R) injury by preventing intracellular Ca^{2+} overload through inhibiting L-type calcium channels and outward current of Na^+/Ca^{2+} exchanger. This study was to investigate the effects of F_2 on activity and protein expression of the rat myocardial sarcoplasmic reticulum Ca^{2+}–ATPase (SERCA) during I/R to discover other molecular mechanisms by which F_2 maintains intracellular Ca^{2+} homeostasis. In an *in vivo* rat model of myocardial I/R achieved by occluding coronary artery for 30–60 min followed by 0–120 min reperfusion, treatment with F_2 (0.25, 0.5, 1, 2 and 4 mg/kg, respectively) dose-dependently inhibited the I/R-induced decrease in SERCA activity. However, neither different durations of I/R nor different doses of F_2 altered the expression levels of myocardial SERCA2a protein. These results indicate that F_2 exerts cardioprotective effects against I/R injury by inhibiting I/R-mediated decrease in SERCA activity by a mechanism independent of SERCA2a protein levels modulation.

1. Introduction

N-*n*-butyl haloperidol iodide (F_2) is a novel compound synthesized in our laboratory that showed to attenuate myocardial ischemia/reperfusion (I/R) injury, as evidenced by amelioration of hemodynamics and myocardial enzyme activity, reduction in myocardial infarction size, and decrease in myocardial inflammation [1,2]. The cardioprotective mechanism of F_2 is thought to be associated with calcium homeostasis maintenance and intracellular Ca^{2+} overload prevention by inhibiting cardiocyte L-type calcium channels and outward current of Na^+/Ca^{2+} exchanger (NCX) [3–6]. However, it is not clear whether other potential mechanisms are involved in F_2-mediated Ca^{2+} regulation.

Ca^{2+} overload is one of the major mechanisms of myocardial I/R injury [7,8]. Ca^{2+} overload results from excessive influx of calcium through L-type calcium channels and NCX (outward current) combined with the inability to rapidly extrude from the cytoplasm via the sarcoplasmic reticulum Ca^{2+}–ATPase (SERCA), NCX (inward current), and plasma membrane Ca^{2+}–ATPase (PMCA). SERCA levels are the highest in cardiocytes, and its major responsibility is to pump Ca^{2+} back into the sarcoplasmic reticulum (SR). SERCA plays a crucial role in maintaining the intracellular calcium balance of cardiac myocytes. Decreases in SERCA activity will reduce the speed and quantity of SR Ca^{2+} reuptake and thereby result in increased, potentially toxic cytoplasmic Ca^{2+} levels. Ca^{2+} overload concomitant with reduced expression and/or activity of SERCA plays a pivotal role in myocardial I/R injury, and thus SERCA plays a pivotal role in preventing the transition from reversible to irreversible injury during I/R [7,9]. Three SERCA genes encoded three homologues: SERCA1, SERCA2 and SERCA3, with SERCA2a being the cardiac-specific isoform [7]. The aim of the present study was to investigate the effects of F_2 on the activity and protein expression of SERCA2a during I/R in order to delineate the underlying molecular mechanism by which F_2 maintains intracellular Ca^{2+} homeostasis and antagonizes myocardial I/R injury.

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2. Materials and methods

2.1. Surgical preparation of animals and experimental protocol

Male adult Sprague–Dawley rats weighing 250–300 g were purchased from Sino British SIPPR/BK Lab Animal Lt [Shanghai License No: SCXK (Shanghai) 2008–0016]. Rats were anesthetized with an intraperitoneal injection of 10% hydration chlorine aldehyde (400 mg/kg). The research protocol was approved by the Medical Animal Care and Welfare Committee of Shantou University Medical College, and complied with the Guide for the Care and Use of Laboratory Animals (NIH Publication, 1996).

The animals were ventilated with oxygen-enriched room air using a rodent respirator with 60 breaths per minute and the tidal volume was set to 9 ml. The chest was opened via a left thoracotomy through the fourth intercostal space. After pericardiotomy, a silk suture was placed under the left anterior descending coronary artery (LADCA), 2–3 mm from its origin, and the ends of the tie were threaded through a small plastic tube to form a snare for reversible LADCA occlusion.

280 rats were randomly assigned into one of 35 groups (8 rats in per group): (1) sham, subjected to surgical manipulation without LADCA occlusion; (2) I/R, ischemia for 30 min followed by reperfusion for 0, 30, 60 and 120 min or ischemia for 60 min followed by reperfusion for 30 min; (3) F_2, treatment with F_2 at dose of 0.25,0.5,1,2 or 4 mg/kg [dissolved in 33% (V/V)polyethylene glycol 400] [2] for each I/R duration. Different doses of F_2 were intravenously injected before ischemia. Equal volumes of 0.9% NaCl were administered to the sham and I/R groups. At the end of experiment, the ischemic myocardium in apex area of heart was excised for the identification, activity, and protein expression of SERCA.

2.2. Separation and identification of SERCA

SR vesicles were obtained as described previously with some modifications [10]. Briefly, 100 mg of ischemic myocardium was homogenized in 1 ml of lysis buffer (in mM, pH 7.0): 15 Tris HCl, 10 $NaHCO_3$, 5 NaN_3 and 250 Sucrose. The homogenate was centrifuged at 13,000×*g* for 10 min at 4 °C. Cellular debris mixed in 500 μL of the above buffer was centrifuged at 13,000×*g* for 10 min at 4 °C. The above supernatants were combined and further centrifuged at 13,000×*g* for 20 min at 4 °C. Then, the supernatant was centrifuged at 43,000×*g* for 30 min at 4 °C, and the supernatant was discarded. The pellet was suspended in 1 ml of a mixture of 0.6 mM KCl and 20 mM Tris HCl (pH 7.0), and then centrifuged at 43,000×*g* for 30 min at 4 °C. The final pellet was dissolved in 1 ml of a mixture of 250 mM sucrose and 40 mM imidazole–HCl and SERCA was obtained for identification and measurement of its activity.

Samples were fixed with 2.5% glutaraldehyde for 2 h followed by postfixation with 1% osmium tetroxide for 30 min. Samples were then sequentially dehydrated in a graded series of ethanol, a mixture of dehydrated alcohol and acetone (V:V, 1:1), acetone, a mixture of acetone and epoxypropane (V:V, 1:1), and epoxypropane. After infiltration and polymerization, samples were cut into ultrathin sections. Lastly, sections were stained with lead citrate and aqueous uranyl acetate, and observed in a transmission electron microscope (JEM-1400, Japan). The samples were irradiated with a microwave in a microwave rapid histoprocessor (BioWave@ Pro, USA) when postfixated, dehydrated, infiltrated and polymerized.

2.3. SERCA activity measurement

SERCA activity was measured with an Ultramicro-ATPase Assay Kit (Jiancheng Bioengineering Inst., China) following the manufacturer's instructions. Included were the enzymatic reaction and phosphorus reaction. The test principle was as follows: ATPase can decompose ATP into ADP and Pi. The ATPase activity was determined by measuring the quantity of Pi. One unit of ATPase activity is defined as the amount of Pi which was decomposed by ATPase per milligram tissue protein per hour. That is umolPi/mgprot/hour. The protein content in the SERCA extract was determined by a bicinchoninic acid assay (Shanghai ShengGong Biological Technology Service Company, China).

Formula:

$$\text{ATPase activity}(U/\text{mgprot}) = \frac{\text{OD of Assay} - \text{OD of Control}}{\text{OD of Standard} - \text{OD of Blank}} \times \text{standard concentration} \times 6 \times 10(\text{dilution multiple of sample}) / \text{protein content of SERCA extract}$$

2.4. Western-blot for SERCA2a protein

Total protein extracts were prepared from myocardial tissue using a cell lysis buffer containing a protease inhibitor cocktail (aprotinin, leupeptin, pepstatin A and PMSF). The protein concentration was also determined by the bicinchoninic acid assay. Equal amounts of total protein (100 μg) was subjected to SDS–PAGE (10%) followed by electrophoretic transfer to nitrocellulose membranes. Nonspecific binding was blocked by incubation of membranes with milk at room temperature for 1 h. The blot was incubated with mouse anti-rat SERCA2a, diluted 1:1000 (Santa Cruz Biotechnology Inc., USA) and mouse anti-rat β-actin, diluted 1:10000 (Wuhan Boster Biotechnology Limited Company, China) overnight at 4 °C. Blots were then washed with TBS–T, and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:2000) at room temperature for 1 h. Detection of immunoreactive bands was performed by Western blotting chemiluminescence luminol reagents (Santa Cruz Biotechnology Inc., USA). The band relative densities of protein bands were analyzed by Fluorchem™ software (Alpha Innotech, USA). Levels of SERCA2a protein expression were expressed as the SERCA2a/β-actin ratio of densitometric values.

2.5. Statistical analysis

All statistical analyses were performed using SPSS 15.0 software. Data are shown as the mean ± S.E.M. The significance of differences was determined by using one-way ANOVA, followed by the Student–Newman-Keuls test. A $P < 0.05$ was considered statistically significant.

3. Results

3.1. Identification of SERCA by transmission electron microscope

Other than different sized of terminal cisternae vesicles, no other organelles were observed in the transmission electron microscope, indicating that the extracted SERCA was of high purity. The lumen of the terminal cisternae vesicles contained electron-opaque contents. The morphological features of SERCA were consistent with previous literature [11] (Fig. 1).

3.2. Effect of F_2 on change of SERCA activity caused by I/R

The SERCA activities of all sham groups, for each I/R treatment, were expressed as 100%, and then the SERCA activities of other groups (I/R, I/R + 0.25 mg/kg F_2, I/R + 0.5 mg/kg F_2, I/R + 1 mg/kg F_2, I/R + 2 mg/kg F_2 and I/R + 4 mg/kg F_2 in turn) were normalized

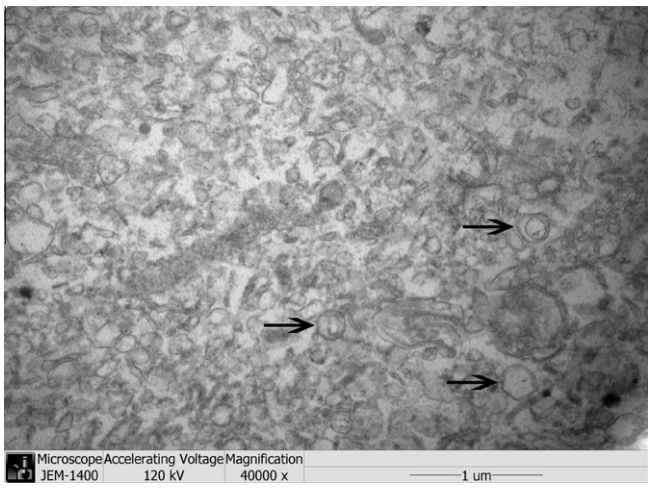

Fig. 1. A SERCA preparation as viewed under transmission electron microscope. The different sizes of vesicles were observed and other organelles were not seen. The lumen of the terminal cisternae vesicles contained electron-opaque contents (arrows show the vesicle of SR).

to the corresponding sham group. The SERCA values obtained were as follows (Table 1).

As shown in Table 1, compared with the respective sham groups, SERCA activities of all I/R groups were decreased. The 30/60 min and 60/30 min I/R groups displayed the least decreases in SERCA activity compared with the other I/R groups, and both decreased to a similar extent. SERCA activities observed in the 30/0 min, 30/30 min, and 30/120 min I/R groups all showed a greater than 55% decline in activity, and extent of decline was similar among these groups ($P > 0.05$). Administration of F_2 under each I/R condition ameliorated the I/R-induced decrease in SERCA activity in a dose-dependent manner ($P < 0.05$).

3.3. Effect of F_2 on I/R-induced changes in SERCA2a protein expression

Protein levels of SERCA did not change after ischemia for 30 min followed by reperfusion for 0, 30, 60 or 120 min as compared with the respective sham group (Fig. 2) (SERCA2a protein expression in the 30/0 min I/R group was shown in our previous article [12]). There was also no change after ischemia for 60 min followed by reperfusion for 30 min. F_2 at different dosages had no effects on SERCA2a protein expression of myocardium in different duration I/R ($P > 0.05$). Therefore, neither I/R nor F_2 appear to mediate their effects by altering protein levels of SERCA2a.

4. Discussion

Myocardial I/R injury has always been one of the most attractive fields in experimental and clinical studies. Although the causes of myocardial I/R injury are multifactorial, it is well known that intracellular Ca^{2+} overload plays a prominent role [13,14]. Ca^{2+} balance is co-regulated by plasma membrane and intracellular transport systems, which are responsible for Ca^{2+} entry or exit from the cytoplasm. For Ca^{2+} removal, the major fraction of increased Ca^{2+} (between 70 and 90%, depending on species) is sequestered by SERCA into the SR, while a minor fraction is extruded from the cell via the NCX and PMCA [8,10,15,16]. When the heart undergoes I/R, a huge influx of Ca^{2+} via L-type Ca^{2+} channel causes high level in cytoplasmic Ca^{2+} concentration, Meanwhile, reduced expression and/or activity of SERCA reduces prompt removal of cytoplasmic Ca^{2+} into the SR and at last contributes to an imbalance in Ca^{2+} homeostasis [7,9,17].

We demonstrate that myocardial SERCA activity decreases after 30–60 min ischemia followed by 0–120 min reperfusion, consistent with prior studies [7,15]. Moreover, prolongation of reperfusion time did not cause a further decrease in SERCA. Interestingly, the SERCA activities both in the 30/60 min and 60/30 min I/R groups recovered to some degree compared with other I/R group durations, although the recovery of SERCA activity in 60/30 min I/R group was not lower than that of 30/60 min I/R group as would normally be expected. These results indicate that: (*1*) during the study period, no matter how long the reperfusion time, ischemia for 30 min is sufficient to cause a maximal decline in myocardial SERCA activity; (*2*) regardless of the durations of ischemia and reperfusion, provided their total time is 90 min, the decrease in SERCA activity was much less than other I/R combinations. We deduce that it is within this time frame that compensatory mechanisms can predominate. For example, macrophage migration inhibitory factor (MIF) might be partially compensate for the decrease in SERCA activity by its oxidoreductase activity since it can be induce at 90 min of I/R and reach a maximum at 120 min of I/R [18–20]; (*3*) as the duration of I/R is prolonged, the body might adapt, especially after 90 min of I/R when intrinsic protective mechanisms reach a limit. However, decreases in SERCA activity could become further aggravated due to continuous noxious stimulation.

This study showed that alteration of I/R duration did not alter expression levels of SERCA2a protein expression, but did alter levels of SERCA activity. SERCA is a Ca^{2+}–ATPase in essence and second-biggest consumer of ATP after myoglobulin. Therefore it is likely that, the SERCA activity decreased first because ATP is in short supply during I/R, and second because SERCA2a is under direct control of an intrinsic SR protein, phospholamban (PLB). The nonphosphorylated form of PLB inhibits SERCA2a activity. It was

Table 1
SERCA activity values in myocardial tissues of different I/R groups.

Group	30/0 min I/R	30/30 min I/R	30/60 min I/R	30/120 min I/R	60/30 min I/R
sham	100%	100%	100%	100%	100%
I/R	(44.29 ± 7.42)%*	(37.75 ± 1.44)%*	(67.13 ± 2.13)*,§	(37.46 ± 1.73)%*	(57.70 ± 4.74)%*,§
F_2(0.25)	(58.41 ± 5.78)%*	(55.22 ± 4.26)%$^{*,\#}$	(76.68 ± 2.45)%$^{*,\#}$	(48.92 ± 2.20)%$^{*,\#}$	(67.09 ± 4.11)%$^{*,\#}$
F_2(0.5)	(66.49 ± 6.10)%$^{*,\#}$	(63.70 ± 5.57)%$^{*,\#,\dagger}$	(79.54 ± 1.41)%$^{*,\#}$	(54.25 ± 2.44)%$^{*,\#}$	(72.84 ± 4.12)%$^{*,\#}$
F_2(1.0)	(69.49 ± 5.91)%$^{*,\#}$	(73.43 ± 2.59)%$^{*,\#,\dagger,\ddagger}$	(83.42 ± 2.67)%$^{*,\#}$	(61.35 ± 2.71)%$^{*,\#,\dagger}$	(77.39 ± 4.72)%$^{*,\#}$
F_2(2.0)	(72.04 ± 5.69)%$^{*,\#}$	(77.33 ± 2.77)%$^{*,\#,\dagger,\ddagger}$	(90.05 ± 3.05)%$^{*,\#,\dagger,\ddagger}$	(63.48 ± 3.83)%$^{*,\#,\dagger,\ddagger}$	(84.14 ± 5.13)%$^{*,\#,\dagger,\ddagger}$
F_2(4.0)	(83.24 ± 4.77)%$^{*,\#,\dagger}$	(80.32 ± 3.25)%$^{*,\#,\dagger,\ddagger}$	(91.09 ± 3.02)%$^{*,\#,\dagger,\ddagger}$	(69.55 ± 3.21)%$^{*,\#,\dagger,\ddagger}$	(86.73 ± 3.86)%$^{*,\#,\dagger,\ddagger}$

I/R, ischemia/reperfusion. F_2, *N*-*n*-butyl haloperidol iodide (mg/kg). All values are expressed as mean ± S.E.M ($n = 8$).
* $P < 0.05$ *vs.* sham group.
$^{\#}$ $P < 0.05$ *vs.* I/R group.
† <0.05 *vs.* I/R + 0.25 mg/kg F_2 group.
‡ $P < 0.05$ *vs.* I/R + 0.5 mg/kg F_2 group.
§ $P < 0.05$ *vs.* 30 min/0 min, 30 min/30 min and 30 min/120 min I/R groups.

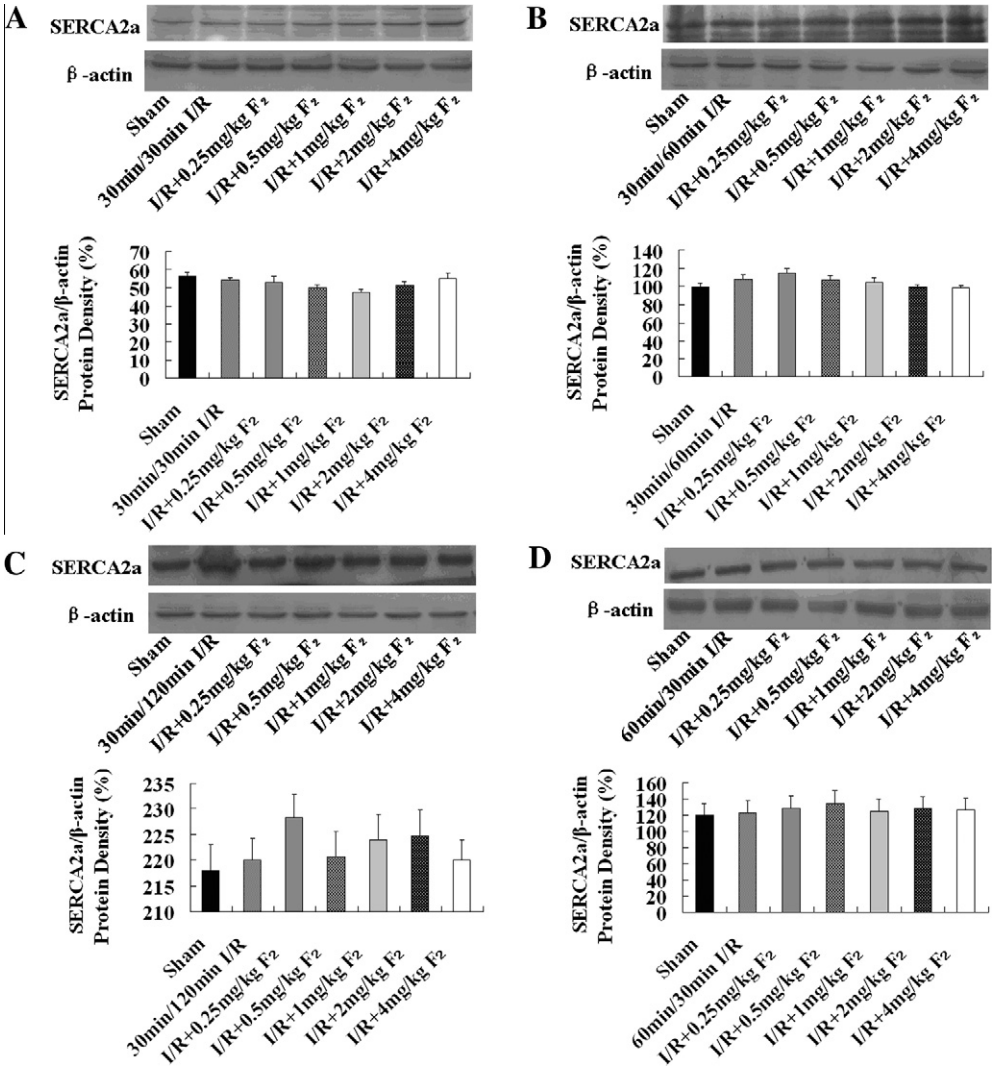

Fig. 2. Detection of SERCA2a protein expression in myocardial tissues by Western blot. (A) 30 min of ischemia followed by 30 min reperfusion; (B) 30 min of ischemia followed by 60 min reperfusion; (C) 30 min of ischemia followed by 120 min reperfusion; (D) 60 min of ischemia followed by 30 min reperfusion. Quantitative densitometric data were expressed as a percentage of β-actin. All values are expressed as mean ± SEM of at least 5 separate experiments.

reported that PLB phosphorylation decrease after 30 min of ischemia [9]. Moreover, oxidative stress with large amounts of reactive oxygen species (ROS) is another critical mechanism of I/R injury, and ROS is know to damage the SERCA pump [8,21]. For instance, Xu KY et al. reported that increased ROS inhibits SERCA function by oxidizing cysteine thiols within the SERCA molecule, interfering with the ATP binding site, and making it unable to hydrolyze the ATP [22]. Tang WH et al. found that in hearts following I/R, increased levels of peroxynitrite (a type of ROS) led to increased tyrosine nitration of SERCA [15]. Therefore, these data suggest that the nonphosphorylated form of PLB and oxidized form of SERCA2a [15,23], rather than decreased SERCA2a protein level, account for the inactivation of SERCA during I/R. Of course, we considered that the time frame of our model might be too short to detect any changes in protein levels, but our results are supported by the study of Yeung HM et al. [24], who found that it was chronic hypoxia (being exposed to 10% oxygen for 4 week) led to attenuated SERCA2a protein expression, and the study by Seehase M et al. [16], who found that the change in SERCA2a mRNA expression (which should be an early indicator of changes) was not represented at the protein level (which is the functionally relevant form) during a 60/120 min I/R. What is more, Lüss H et al. [25] reported that a 90/120 min I/R induced neither SERCA2a protein expression nor SERCA2a mRNA expression. In sum, change in SERCA2a protein expression lag behind changes in signal transduction and gene transcription, cannot be chiefly responsible for decreased SERCA activity in the early phases of I/R.

Our previous studies have shown that F_2 has significant protective effects on myocardial I/R injury *in vivo* and *in vitro*, which are mediated by preventing intracellular Ca^{2+} overload through blocking L-type calcium channels and inhibiting outward current of Na^+/Ca^{2+} exchanger in the cell membrane [1–6,26]. We extend these studies to demonstrate that F_2 can prevent I/R-mediated decreases in SERCA activity, another major component responsible for maintaining Ca^{2+} homeostasis and preventing Ca^{2+} overload in the heart. Prior data from our laboratory suggested that cardioprotection by F_2 was also associated with the inhibition of ROS [2,3,27]. Thus, we deduce that the protective effect F_2 on SERCA activity might, in part, be the result of the inhibition of SERCA2a oxidation induced by ROS. However, whether there are other mechanisms involved in its protective action such as supply or redistribution of ATP, as mentioned above, needs further exploration.

Prior data from our laboratory also showed simultaneously that the decay time from 75 to 25% (DT75–25) of calcium transients,

which is an indicator determined by activity of SERCA, NCX and PMCA, was prolonged after hypoxia, whereas treatment with F_2 at different concentrations dose-dependently reduced the prolongation of DT75–25 induced by hypoxia. Furthermore, employing tool agents, we found that SERCA2a is a principal factor for Ca^{2+} removal, and that the protective role of F_2 in Ca^{2+} transients and calcium homeostasis in ventricular myocytes after hypoxia was probably due to changing the function of SERCA2a [12], in agreement with this study.

In conclusion, the present study demonstrates an additional mechanism by which F_2 can alleviate intracellular Ca^{2+} overload, and thus protect against myocardial I/R injury. Protection occurs by a dual mechanism: one by decreasing Ca^{2+} influx via inhibiting L-type channels and outward current of NCX, and the other by preserving SERCA activity, and enabling prompt cytoplasm Ca^{2+} removal into the SR.

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