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N-n-Butyl haloperidol iodide inhibits the augmented Na^+/Ca^{2+} exchanger currents and L-type Ca^{2+} current induced by hypoxia/reoxygenation or H_2O_2 in cardiomyocytes

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ABSTRACT

N-n-butyl haloperidol iodide (F_2), a novel quaternary ammonium salt derivative of haloperidol, was reported to antagonize myocardial ischemia/reperfusion injuries. To investigate its mechanisms, we characterized the effects of F_2 on Na^+/Ca^{2+} exchanger currents (I_{NCX}) and the L-type Ca^{2+} channel current ($I_{Ca,L}$) of cardiomyocytes during either hypoxia/reoxygenation or exposure to H_2O_2. Using whole-cell patch-clamp techniques, the I_{NCX} and $I_{Ca,L}$ were recorded from isolated rat ventricular myocytes. Exposure of cardiomyocytes to hypoxia/reoxygenation or H_2O_2 enhanced the amplitude of the inward and outward of I_{NCX} and $I_{Ca,L}$. F_2 especially inhibited the outward current of Na^+/Ca^{2+} exchanger, as well as the $I_{Ca,L}$, in a concentration-dependent manner. F_2 inhibits cardiomyocyte I_{NCX} and $I_{Ca,L}$ after exposure to hypoxia/reoxygenation or H_2O_2 to antagonize myocardial ischemia/reperfusion injury by inhibiting Ca^{2+} overload.

1. Introduction

Ischemic heart disease is the leading cause of morbidity and mortality worldwide. Subsequent reperfusion of acutely ischemic myocardium is essential for myocardial rescue, but also leads to a unique type of injury known as myocardial ischemia/reperfusion (I/R) injury [1]. Such an injury is often related to endothelial and microvascular dysfunction, impaired blood flow, metabolic dysfunction, and cellular necrosis [2], and its mechanism is associated with cytosolic and mitochondrial calcium overload, release of reactive oxygen species (ROS), and an acute inflammatory response [3,4]. As one of the important mechanisms of I/R injury, much research has focused on the precise intracellular signaling pathways and elements responsible for calcium overload in ischemia/reperfusion. Ca^{2+} influx via both activation of L-type calcium channel and reversal of the Na^+/Ca^{2+} exchanger (NCX) have been reported to occur in cardiocytes following I/R [1,5,6]. Simultaneously, ROS, including superoxide radicals, hydroxyl radicals, and oxidants such as H_2O_2 are generated in significant amounts during reperfusion and could contribute to intracellular Ca^{2+} overload in the heart through reversal or inhibition of the NCX [7,8]. Calcium overload may lead to deleterious consequences such as stunning, apoptosis, and necrosis, which contribute to infarct formation [9–12]. Due to the pivotal role of calcium overload in I/R injury, attenuation of cellular calcium overload remains an important therapeutic goal.

N-n-butyl haloperidol iodide, a novel quaternary ammonium salt derivative of haloperidol, was found to maintain the cardiac and vascular effects without adverse extrapyramidal reactions. Our previous studies showed that F_2 could block L-type calcium channels in ventricular myocytes under physiological conditions [13–15]. Subsequently, we demonstrated that F_2 could antagonize myocardial I/R injury in different animal models [13,16]. So, we inferred that the mechanism by which F_2 antagonizes myocardial I/R injury might be related to the inhibition of Ca^{2+} overload via suppression of cardiomyocyte Na^+/Ca^{2+} exchanger (I_{NCX}) currents and L-type Ca^{2+} channel ($I_{Ca,L}$) during I/R. In this study, we established a model of cardiomyocyte hypoxia/reoxygenation (H/R) and exposure to H_2O_2, to simulate heart I/R conditions, and characterized the changes of I_{NCX} and $I_{Ca,L}$ during H/R and exposure to H_2O_2. We further characterized the effects of F_2 on I_{NCX} and $I_{Ca,L}$ during H/R and exposure to H_2O_2 to elucidate the mechanisms and ability of F_2 to block myocardial I/R injury.

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2. Materials and methods

2.1. Cell isolation

Adult male Sprague–Dawley rats (180–250 g) were obtained from the Laboratory Animal Breeding and Research Center of Shantou University Medical College. The investigation was in compliance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Cardiomyocytes were isolated by collagenase type 2 and protease (Sigma, Type XIV) perfusion as previously described [15,17]. All experiments were performed at 37 ± 0.5 °C. Cardiac ventricular tissue was cut into small pieces. Single myocytes were harvested and stored at 4 °C and the myocytes were used for experiments within 6 h.

2.2. Patch clamp recordings

Membrane currents were recorded by whole cell patch-clamp method using pCLAMP 8.2 software (Axon Instruments, Foster City, CA, USA). Single cardiac ventricular cells were placed in a 1 ml recording chamber attached to an inverted microscope (OLYMPUS, Tokyo) and were perfused with Tyrode solution at a rate of 1 ml/min. The temperature of the bath solution was maintained at room temperature (22–25 °C). Patch pipettes were forged from 1.5-mm-diameter glass capillaries with a two-stage microelectrode puller (pp-83; Narishige Scientific Instrument Lab, Tokyo). Pipette resistance was 2–4 MΩ when filled with the pipette solution. The electrode was connected to a patch-clamp amplifier (Axopatch-200B, Axon Instruments, Foster City, CA, USA). Recording signals were filtered at 2.5 kHz bandwidth.

2.3. Measurement of I_{NCX}

After establishing the whole-cell configuration in Tyrode solution, the cell was perfused with a special K^+-free bath solution (140 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.2). To block Na^+/K^+ pump currents and currents flowing through K^+ or Ca^{2+} channels, 0.02 mM ouabain, 2 mM CsCl and 0.01 mM nifedipine were added to the solution. After recording the control current, the external solution was switched from the special K^+-free bath solution to the simulated hypoxic solution including 0.02 mM ouabain, 2 mM CsCl and 0.01 mM nifedipine. The pipette solution contained 120 mM CsOH, 50 mM aspartic acid, 20 mM NaCl, 10 mM $CaCl_2$ (free Ca^{2+} concentration 226 nM), 20 mM BAPTA, 3 mM $MgCl_2$, 5 mM Mg ATP, and 10 mM HEPES, pH 7.2). The ramp pulse was initially depolarized from a holding potential of −60 to +60 mV, then hyperpolarized to −150 mV, and depolarized back to the holding potential at a speed of 680 mV/s [18]. The descending limb of the ramp was used to plot current–voltage (*I–V*) curves without capacitative current compensation. I_{NCX} was identified as a Ni^{2+}-sensitive current because 5 mM Ni^{2+} selectively inhibits I_{NCX} under these ionic conditions and the Ni^{2+}-insensitive current was not affected by H/R.

2.4. Measurement of $I_{Ca,L}$

$I_{Ca,L}$ was recorded using a whole-cell patch clamp configuration. The pipette solution contained 150 mM CsCl, 15 mM EGTA, 1 mM $MgCl_2$, 5 mM MgATP, and 5 mM HEPES, adjusted to pH 7.2 with CsOH). After establishing a high resistance seal by gentle suction, the cell membrane beneath the tip of the electrode was disrupted by further suction to obtain the whole-cell patch-clamp configuration. $I_{Ca,L}$ was elicited by 300 ms pulses to potentials ranging from −30 to +70 mV in 10 mV increments from a holding potential of −40 mV (to inactivate I_{Na} and T-type Ca^{2+} currents) at 0.2 Hz [15]. Peak outward K+ current (I_{to}) was suppressed by 3 mM 4-aminopyridine added to Tyrode solution. Representative current traces and the *I–V* relationships were obtained from a ventricular myocyte.

2.5. H/R model

H/R conditions were induced by switching the Tyrode solution to the hypoxic solution and then to control extracellular solution. After perfusing with normal Tyrode solution, to mimic hypoxic conditions, isolated ventricular cardiac myocytes were perfused for 15-min with the simulated hypoxic solution (123 mM NaCl, 6 mM $NaHCO_3$, 0.9 mM NaH_2PO_4, 8 mM KCl, 0.5 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 20 mM sodium lactate, pH 6.8; gassed with 90% N_2-10% CO_2) [19–21]. The method is convenient and severe enough to produce an H/R single cell model. Perfusion with buffer was controlled by gravity to maintain a flow rate of 6 ml/min.

2.6. Drugs

F_2 (synthesized by our lab and assayed by the Shanghai Organic Chemistry Institute of the Chinese Academy of Sciences; purity greater than 98%) was prepared as a 0.1 M stock solution in DMSO and diluted to the desired drug concentration with extracellular solution before each experiment. A DMSO of less than 0.1% did not affect the I_{NCX} and $I_{Ca,L}$ at the highest F_2 concentration used. Ouabain, nifedipine, CsCl, HEPES, and BAPTA were purchased from the Sigma Chemical Co., St. Louis, MO. All chemicals used were the highest grade available.

2.7. Analysis of statistics

All values presented are arithmetic means ± SEM. Statistical significance was determined using a paired Students' *t*-test. Differences were considered significant when the *P* value was less than 0.05.

3. Results

3.1. F_2 reduces both outward and inward I_{NCX} under physiological conditions

Bi-directional I_{NCX} was induced by 1 mM Ca^{2+} and 140 mM Na^+ in the external solution and 20 mM Na^+ and 226 nM free Ca^{2+} in the pipette solution. Under these ionic conditions, following establishment of the whole-cell clamp configuration, the external solution was switched from the control external solution to Tyrode solution, while monitoring the increase in current until a steady state was reached. I_{NCX} was recorded under conditions in which we selectively blocked various ion channel currents, such as Na^+/K^+ pump currents, K^+ current, sarcoplasmic reticulum Ca^{2+} release channels, and Ca^{2+} currents. After recording the control current, 1.0 μM F_2 was added to the extracellular solution. Upon stabilization of current, 5 mM Ni^{2+}, a selective NCX inhibitor under these ionic conditions, was added to the extracellular solution to block I_{NCX} (Fig. 1B and C). F_2 inhibited outward I_{NCX} at +60 mV by 39.51 ± 2.62% ($n = 4$) and inward I_{NCX} at −150 mV by 10.68 ± 0.62% ($n = 4$).

3.2. F_2 inhibits I_{NCX} during H/R

To examine the effect of F_2 on I_{NCX} during H/R, current traces were recorded in the presence and absence of H/R exposure and with and without F_2. The *I–V* relationship recorded in the presence

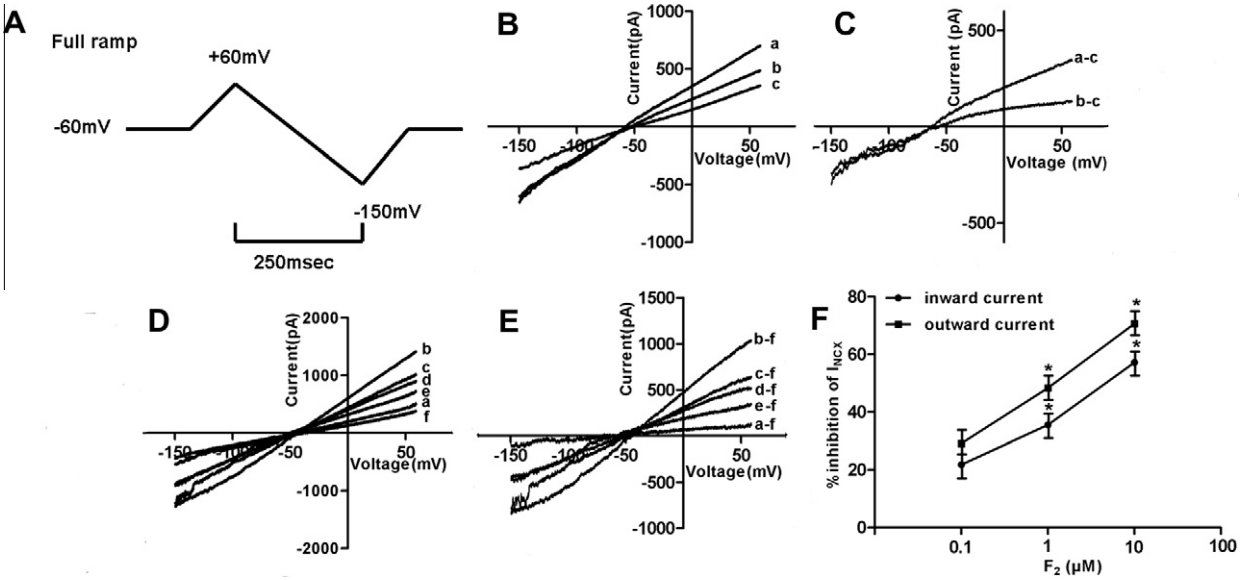

Fig. 1. Effect of F_2 on I_{NCX}. (A) Shape of a "full" ramp pulse. The holding potential is −60 mV. (B) *I–V* curves of control (a), in the presence of F_2 1.0 μM (b), and 5 mM Ni^{2+} (c). (C) *I–V* curves of net Ni^{2+}-sensitive currents obtained by subtracting the corresponding *I–V* curves in panel B. (D) Current–voltage relationship before control (a) and after H/R (b), respectively. Trace c, d, e, and f after application of F_2 and Ni^{2+}, respectively ($n = 13$). (E) Difference between the *I* and *V* curves in panel D. (F) Concentration–response relationships of the inhibitory effect of F_2 on NCX currents. The outward current was achieved at +60 mV, inward current was achieved at −150 mV.

of F_2 intersected with the control *I–V* curve at −60 mV. After the effect of F_2 reached a steady state, 5 mM Ni^{2+} was applied to completely block I_{NCX}. Fig. 1D and E illustrates the net *I–V* curves with 5 mM Ni^{2+} from those before and after F_2 application. The percent inhibition of F_2 on outward and inward currents was determined at +60 mV to be 29.18 ± 2.49%, 53.38 ± 5.01%, and 70.68 ± 3.93%, and at -150 mV to be 21.83 ± 1.58%, 38.56 ± 5.52%, and 57.25 ± 7.39% ($n = 9$) ($P < 0.05$) . Percent inhibition was calculated assuming that 5 mM Ni^{2+} completely inhibited each direction of I_{NCX}. Therefore, F_2 inhibited the H/R-induced increase in I_{NCX}, and inhibition was greater for the outward current compared to the level of inhibition of the inward current.

3.3. F_2 blocks the H_2O_2-induced I_{NCX} increases in inward and outward currents

In order to test whether H_2O_2 regulates I_{NCX}, we established the whole-cell clamp configuration, then switched the external solution from the control external solution to Tyrode solution with 100 μM H_2O_2 and monitored the increase in current until it reached a steady state. Immediately after cells were exposed to H_2O_2, the current began to increase. When current peaked, 5 mM Ni^{2+} was added to the extracellular solution to block I_{NCX}. H_2O_2 at 100 μM increased outward I_{NCX} at +60 mV by 91.61 ± 10.55% ($n = 9$) and inward I_{NCX} at −150 mV by 58.33 ± 4.18% ($n = 9$) (Fig. 2A and B). Thus, we investigated the effects of F_2 on H_2O_2-induced increases in I_{NCX} (Fig. 2C and D). F_2 (0.1, 1.0, 10 μM) caused a decrease in I_{NCX} and inhibited the outward I_{NCX} to a greater extent than the inward current in a concentration-dependent manner (Fig. 2D and E) outward I_{NCX} at +60 mV by 37.17 ± 7.45%, 56.16 ± 7.54%, 73.81 ± 7.13% ($n = 5$) and inward I_{NCX} at −150 mV by 34.23 ± 9.19%, 41.48 ± 8.72%, 53.54 ± 10.10% ($n = 5$) ($P < 0.05$). Similar to results obtained with H/R, F_2-mediated inhibition of I_{NCX} was greater for the outward current.

3.4. F_2 inhibits H/R-induced $I_{Ca,L}$

To activate $I_{Ca,L}$, we delivered 300 ms pulses to potentials ranging from −30 to +70 mV in 10 mV increments from a holding potential of −40 mV (to inactivate I_{Na} and T-type Ca^{2+} current) at 0.2 Hz. The peak $I_{Ca,L}$ was elicited at the potential of +10 mV. Fig. 3 shows a typical trace of $I_{Ca,L}$ during H/R. Representative current traces obtained from a ventricular myocyte and the current–voltage relationships in the absence and presence of F_2 are shown. The $I_{Ca,L}$ amplitude increased by 15.89 ± 3.22%, then decreased progressively during perfusion with F_2 over the perfusion period. F_2 inhibited $I_{Ca,L}$ by 44.35 ± 5.52%, 61.06 ± 2.99%, and 71.88 ± 4.45% at concentrations of 0.1, 1.0 and 10 μM, respectively. F_2 shifted the current–voltage curve of $I_{Ca,L}$ upward, without affecting the voltage-dependent properties of $I_{Ca,L}$.

3.5. F_2 inhibits the H_2O_2-induced $I_{Ca,L}$ increase

To examine the effect of F_2 on the voltage-dependent $I_{Ca,L}$ exposure to H_2O_2. Ventricular myocytes were superfused with H_2O_2 and the $I_{Ca,L}$ was recorded under a whole cell configuration. Fig. 4 shows the effects of F_2 on the H_2O_2-induced $I_{Ca,L}$ increase. H_2O_2 induced an increase in inward current. Fig. 4A shows the *I–V* relationships measured using the peak inward current. H_2O_2 did not significantly change the 33.65 ± 1.90% increase in magnitude of the peak current. However, "rundown" of calcium currents is always a concern in whole-cell patch-clamp recordings. In our study, conventional whole-cell recording with ATP and EGTA in the patch-pipette led to stable recordings of L-type currents. The $I_{Ca,L}$ amplitude decreased progressively during perfusion with F_2 over the perfusion period. F_2, at concentrations of 0.1, 1.0 and 10 μM, inhibited H_2O_2-mediated increases in $I_{Ca,L}$ amplitude by 37.50 ± 2.81%, 54.83 ± 2.93%, and 70.21 ± 2.03%, respectively.

4. Discussion

The present study demonstrated that exposure to either H/R or to H_2O_2 increased both I_{NCX} and $I_{Ca,L}$ in cardiomyocytes, and that F_2 could inhibit these increases in a concentration-dependent manner during H/R or exposure to H_2O_2. This is maybe the mechanism of F_2 to inhibit Ca^{2+} overload and antagonize myocardial I/R injury.

NCX is an important regulator in maintaining Ca^{2+} homeostasis and is responsible for Ca^{2+} removal from the cytoplasm. Under

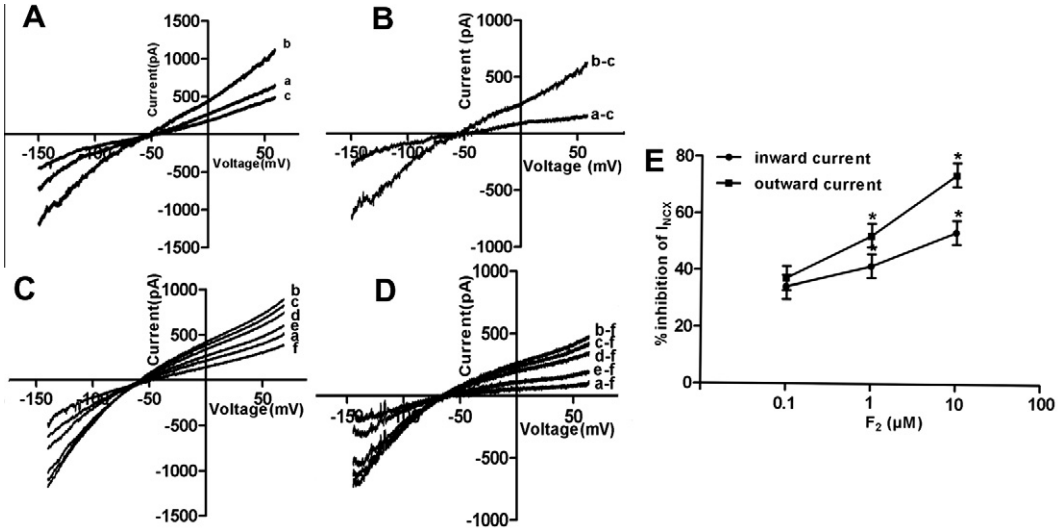

Fig. 2. Effect of F_2 on the I_{NCX} increase caused by 100 μM H_2O_2. (A) *I–V* curves of control (a), in the presence of 100 μM H_2O_2 (b) and 5 mM Ni^{2+} (c). (B) *I–V* curves of net Ni^{2+}-sensitive currents obtained by subtracting the corresponding *I–V* curves in panel A. (C) *I–V* curves of control (a), in the presence of 100 μM H_2O_2 (b) and 0.1 μM, 1 μM and 10 μM F_2 (c, d, e), 5 mM Ni^{2+} (f). (D) *I–V* curves of net Ni^{2+}-sensitive currents obtained by subtracting the corresponding *I–V* curves in panel C. (E) Concentration–response relationships of the inhibitory effect of F_2 on NCX currents. The outward current was achieved at +60 mV, inward current was achieved at −150 mV ($n = 9$).

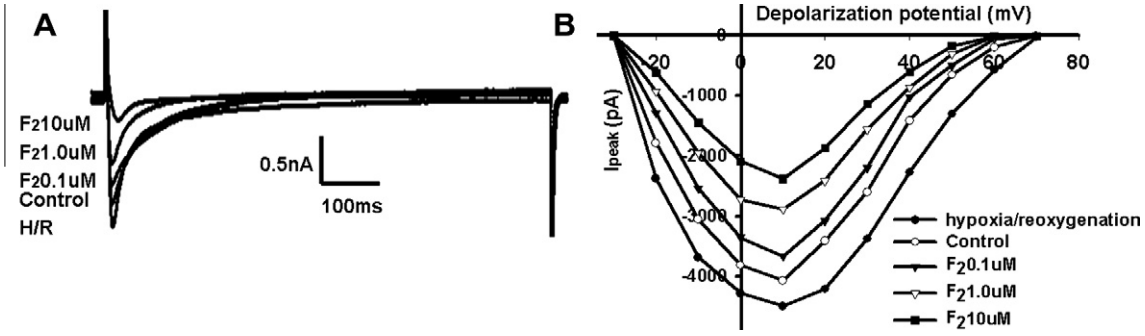

Fig. 3. Effect of F_2 on $I_{Ca,L}$ during H/R. (A) The original recordings of $I_{Ca,L}$ under control conditions, H/R, and after superfusion with F_2. (B) *I–V* relationship for $I_{Ca,L}$ in rat ventricular myocytes ($n = 7$).

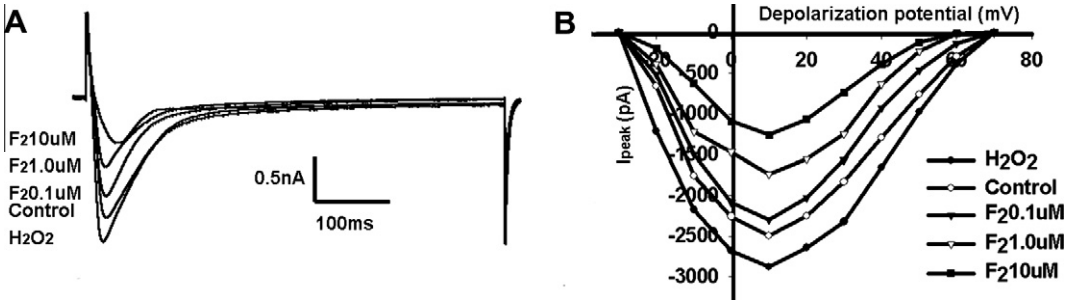

Fig. 4. Effect of F_2 on $I_{Ca,L}$ during H_2O_2 exposure. (A) The original recordings of $I_{Ca,L}$ under control conditions, H_2O_2 exposure and after superfusion with F_2. (B) *I–V* relationship for $I_{Ca,L}$ in rat ventricular myocytes ($n = 10$).

physiological states, the primary function of NCX is the extrusion of Ca^{2+} during diastole. During hypoxemia, anaerobic metabolism results in intracellular acidosis, which activates Na^+/H^+ exchanger to extrude H^+ in exchange for an influx of Na^+. Upon reoxygenation, loss of extracellular H^+ establishes an outward transsarcolemmal H^+ gradient, causing further extrusion of H^+ in exchange for Na^+, and leading to higher intracellular Na^+ levels. The subsequent elevation in intracellular Na^+ promotes an increase Ca^{2+} influx into the cytosol via reverse mode of Na^+/Ca^{2+} exchange, resulting in calcium overload [22]. This is thought to be the principal mechanism of calcium overload induced by I/R. In the present study, treatment with 0.1, 1.0, or 10 μM F_2 during H/R reduced the I_{NCX}, especially the outward component of I_{NCX} in a concentration-dependent manner. This would account for the cardioprotection of F_2 from I/R injury.

We have found that hypoxia enhanced the $I_{Ca,L}$ amplitude of cardiomyocytes (data not shown). During H/R, entry of calcium through the L-type calcium channels was further augmented, which might be connected with changes in membrane depolarization and opening of voltage sensitive L-type Ca^{2+} channel [6]. These

changes promote calcium overload in myocardial I/R. We found that F_2 could also reduce $I_{Ca,L}$ in a concentration-dependent manner during H/R, which provides an additional mechanism for F_2 in the regulation of calcium homeostasis.

As we know, large amounts of ROS, such as H_2O_2, are produced primarily during reperfusion and contribute to myocardial injury. H_2O_2 plays an important role in the pathogenesis of H/R injury and could induce intracellular dysfunction via several signaling pathways. For example, H_2O_2 induces cell apoptosis through signaling pathways mediated by extracellular signal-regulated kinases, protein kinase C, Janus protein kinase, and nuclear factor kB [23]. Our studies show that H_2O_2 causes reversal of NCX activity, resulting in calcium influx, in close agreement with previous studies [24–27]. Activation of the NCX increases intracellular Ca^{2+} concentrations, giving rise to calcium overload. F_2 inhibits I_{NCX} after exposure to H_2O_2, with the outward current of NCX being decreased more than the inward current. Meanwhile, we found that exposure of cardiomyocytes to H_2O_2 alters the function of L-type calcium channel, which leads to a pronounced elevation in $I_{Ca,L}$, consistent with previous studies [28,29]. These combined synergistic interactions would further accelerate calcium overload and dysfunction of Ca^{2+} homeostasis, ultimately resulting in I/R injury. Treatment with F_2 also decreased the enhancement of $I_{Ca,L}$ in a concentration-dependent manner. These results indicate that the inhibition of I_{NCX} and $I_{Ca,L}$ by F_2 could prevent the calcium overload and further attenuate the myocardial injury.

In conclusion, improved efficacy with the use of F_2 in protecting against H/R-mediated calcium overload can be attributed to the combined effects of inhibition of I_{NCX} and $I_{Ca,L}$. F_2, a novel quaternary ammonium salt derivative of haloperidol, has a chemical structure different from other typical Ca^{2+} channel blockers, yet produces strong effects on cardiac dysfunction. Thus F_2 may be a promising drug for the treatment of cardiac dysfunction and improvement of cardiac recovery.

Acknowledgments

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