N-n-butyl haloperidol iodide inhibits H$_2$O$_2$-induced Na$^+$/Ca$^{2+}$-exchanger activation via the Na$^+$/H$^+$ exchanger in rat ventricular myocytes

Abstract: N-n-butyl haloperidol iodide (F$_2$), a novel compound, has shown palliative effects in myocardial ischemia/reperfusion (I/R) injury. In this study, we investigated the effects of F$_2$ on the extracellular signal-regulated kinase kinase (MEK)/extracellular signal-regulated kinase (ERK)/Na$^+$/H$^+$ exchanger (NHE)/Na$^+$/Ca$^{2+}$ exchanger (NCX) signal-transduction pathway involved in H$_2$O$_2$-induced Ca$^{2+}$ overload, in order to probe the underlying molecular mechanism by which F$_2$ antagonizes myocardial I/R injury. Acute exposure of rat cardiac myocytes to 100 μM H$_2$O$_2$ increased both NHE and NCX activities, as well as levels of phosphorylated MEK and ERK. The H$_2$O$_2$-induced increase in NCX current (I$_{NCX}$) was nearly completely inhibited by the MEK inhibitor U0126 (1,4-diamino-2,3-dicyano-1,4-bis[(aminomethyl)mercaptop]butadiene), but only partly by the NHE inhibitor 5-(N,N-dimethyl)-amiloride (DMA), indicating the I$_{NCX}$ increase was primarily mediated by the MEK/mitogen-activated protein kinase (MAPK) pathway, and partially through activation of NHE. F$_2$ attenuated the H$_2$O$_2$-induced I$_{NCX}$ increase in a concentration-dependent manner. To determine whether pathway inhibition was H$_2$O$_2$-specific, we examined the ability of F$_2$ to inhibit MEK/ERK activation by epidermal growth factor (EGF), and NHE activation by angiotensin II. F$_2$ not only inhibited H$_2$O$_2$-induced and EGF-induced MEK/ERK activation, but also completely blocked both H$_2$O$_2$-induced and angiotensin II-induced increases in NHE activity, suggesting that F$_2$ directly inhibits MEK/ERK and NHE activation. These results show that F$_2$ exerts multiple inhibitions on the signal-transduction pathway involved in H$_2$O$_2$-induced I$_{NCX}$ increase, providing an additional mechanism for F$_2$ alleviating intracellular Ca$^{2+}$ overload to protect against myocardial I/R injury.

Keywords: N-n-butyl haloperidol, hydrogen peroxide, Na$^+$/Ca$^{2+}$ exchanger, Na$^+$/H$^+$ exchanger

Introduction

Reperfusion of an ischemic myocardium leads to heart dysfunction and cardiomyocyte injury. Such myocardial ischemia/reperfusion (I/R) injury is characterized by impaired blood flow, metabolic dysfunction, contractile dysfunction, dysrhythmias, cellular necrosis, and apoptosis. I/R injury is a complex process involving numerous mechanisms, including cytosolic and mitochondrial Ca$^{2+}$ overload, release of reactive oxygen species (ROS), acute inflammatory response, and shift in substrate use.

ROS, produced as by-products of oxidative metabolism, are easily managed under normal conditions by reactive oxygen scavengers. Several forms of ROS are generated during I/R, including superoxide (O$_2^-$), H$_2$O$_2$, and the highly reactive hydroxyl radical (-OH), which cause lipid peroxidation and myocardial injury and trigger the contractile dysfunction observed during reperfusion. It has also been suggested that the burst in...
ROS upon reperfusion may contribute to Ca\textsuperscript{2+} overload in cardiomyocytes.\textsuperscript{7,8} Rothstein et al\textsuperscript{9} and Sabri et al\textsuperscript{10} found that low doses of H\textsubscript{2}O\textsubscript{2} (50 μM, similar to those generated during I/R) cause Ca\textsuperscript{2+} overload in cultured neonatal rat ventricular myocytes, which is associated with activation of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger (NHE) in part through extracellular signal-regulated kinase (ERK)-1/2-mediated phosphorylation of NHE-1, the only NHE isoform in the myocardium. A link between H\textsubscript{2}O\textsubscript{2} and diastolic Ca\textsuperscript{2+} overload in neonatal rat ventricular myocytes was proposed. Exposure to H\textsubscript{2}O\textsubscript{2} results in the alteration of signaling proteins involved in the mitogen-activated protein-kinase (MAPK) pathway, ultimately leading to extracellular signal-regulated kinase kinase (MEK) activation, which then phosphorylates and activates ERK1/2. Activated ERK1/2 subsequently phosphorylates the COOH tail of NHE-1, increasing its exchanger activity to elevate intracellular Na\textsuperscript{+} concentrations. The resulting rise in intracellular Na\textsuperscript{+} decreases the activity of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX), leading to an increase in diastolic Ca\textsuperscript{2+} levels.\textsuperscript{9} Therefore, pharmacological approaches to decrease MAPK, NHE-1, and/or NCX activity may ameliorate the alterations in Ca\textsuperscript{2+} homeostasis that contribute to myocardial tissue injury following I/R.

N-n-butyl haloperidol iodide (F\textsubscript{2}), a novel quaternary ammonium salt derivative of haloperidol synthesized in our laboratory, can maintain the effects of coronary artery relaxant.\textsuperscript{11} Laboratory studies show that F\textsubscript{2} can attenuate myocardial I/R injury, as evidenced by amelioration of hemodynamics and myocardial enzyme activity, reduction in myocardial infarction size, prevention of ventricular arrhythmias, and decreases in myocardial inflammation.\textsuperscript{12–14} The cardioprotective mechanism of F\textsubscript{2} was thought to be associated with calcium-homeostasis maintenance against intracellular Ca\textsuperscript{2+} overload by inhibiting cardiocyte L-type Ca\textsuperscript{2+} channels.\textsuperscript{12,13} However, intracellular Ca\textsuperscript{2+} overload during I/R results primarily from the functional coupling of NHE and NCX. Ischemic hearts develop intracellular acidosis, which activates NHE to extrude H\textsuperscript{+} in exchange for an influx of Na\textsuperscript{+}. Upon reperfusion, loss of extracellular H\textsuperscript{+} causes further extrusion of H\textsuperscript{+} in exchange for Na\textsuperscript{+}. The subsequent elevation in intracellular Na\textsuperscript{+} promotes an increase Ca\textsuperscript{2+} influx into the cytosol via the reverse mode of NCX, resulting in Ca\textsuperscript{2+} overload.\textsuperscript{15} It is suggested that the mechanism of F\textsubscript{2}, antagonizing myocardial I/R injury might not be only related to suppression of the L-type Ca\textsuperscript{2+} channel. In this study, we used rat ventricular myocytes to investigate the effects of F\textsubscript{2} on the MEK/ERK/NHE/NCX signal-transduction pathway involved in H\textsubscript{2}O\textsubscript{2}-induced Ca\textsuperscript{2+} overload in order to probe the underlying molecular mechanism by which F\textsubscript{2} maintains intracellular calcium homeostasis and antagonizes myocardial I/R injury.

Materials and methods

Materials

F\textsubscript{2} (synthesized by our lab and identified 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 dimethyl sulfoxide and diluted to the desired concentration with extracellular solution before each experiment. HEPES (4-[2-hydroxyethyl]-1-piperazine-ethanesulfonic acid), CsCl, 1,2-bis(2-aminophenoxy)-ethane-N,N,N',N''-tetraacetic acid (BAPTA), ouabain, nifedipine, ryanodine, epidermal growth factor (EGF), angiotensin (Ang) II and 5-(N,N-dimethyl)-amiloride (DMA) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). U0126 (1,4-diamino-2,3-dicyano-1,4-bis[o-aminophenylmercapto]butadiene) was from Merck Millipore (Billerica, MA, USA), 2,7-bis(2-carboxyethyl)-(6)-carboxyfluorescein-acetoxyethyl ester (BCECF-AM), and Pluronic® F127 were from Thermo Fisher Scientific (Waltham, MA, USA). Anti-MEK, anti-phosphorylated (p)-MEK, anti-ERK, and anti-p-ERK antibodies were from Cell Signaling Technology (Danvers, MA, USA), anti-NCX from Santa Cruz Biotechnology Inc., (Dallas, TX, USA), anti-NHE-1 antibody from Abcam (Cambridge, UK), anti-β-actin from Sigma-Aldrich, and secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Ig)G) from BosterBio (Pleasanton, CA, USA).

Isolation of ventricular myocytes

Adult male Sprague Dawley rats (180–250 g) were obtained from the Laboratory Animal Breeding and Research Center (Shantou, People’s Republic of China). All experiments were conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (publication 85-23, revised 1996).\textsuperscript{16} The protocol was approved by the Medical Animal Care and Welfare Committee of Shantou University Medical College (permit SUMC2010-093). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. Single ventricular myocytes were isolated by an enzymatic dissociation method described previously.\textsuperscript{17,18} Single ventricular myocytes were harvested after filtration through a nylon mesh (pore size 200 mm).

I\textsubscript{NCX} recording

Myocytes were perfused with extracellular solution (140 mM NaCl, 1 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 0.33 mM
NaH₂PO₄, 10 mM glucose, 10 mM HEPES, 0.02 mM ouabain, 0.01 mM nifedipine, 2 mM CsCl, and 0.01 mM ryanodine, pH 7.2) at a rate of 1 mL/minute in a recording chamber. Patch pipettes were forged from 1.5 mm diameter glass capillaries with a two-stage microelectrode puller (pp-830; Narishige, Tokyo, Japan). The pipette resistance was 2–3 MΩ when filled with the pipette solution (20 mM NaCl, 20 mM BaCl₂, 10 mM CaCl₂ [free Ca²⁺ concentration of 226 nM], 120 mM CsOH, 3 mM MgCl₂, 50 mM aspartic acid, 5 mM Mg-adenosine triphosphate, and 10 mM HEPES, pH 7.2). NCX current (Iₙcx) was recorded by a tight-seal whole-cell voltage clamp with the use of an Axopatch™ 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) with low-pass filtering at 2 kHz, digitized with a DigiData 1322A interface, and processed by pCLAMP® 8.2 software (Molecular Devices, Sunnyvale, CA, USA). The electrode capacitance was maximally compensated by use of the amplifier. No compensation was made for membrane capacitance or series resistance.

For recording Iₙcx, the extracellular solution contained ouabain, nifedipine, Cs⁺, and ryanodine to block Na⁺/K⁺ pump current, Iₚ, and Ca²⁺ release channels of the sarcoplasmic reticulum, respectively. Iₙcx was induced by ramp-voltage pulses from a holding potential of −60 mV to +60 mV, and then hyperpolarizing to −150 mV before ramping back to the holding potential at a rate of 600 mV/second. The descending limb (from +60 to −150 mV) was plotted as the current–voltage (I–V) relationship without capacitance compensation. Iₙcx was measured as the Nₐ⁺-sensitive current that could be selectively inhibited by 5 mM NiCl₂.

**Measurement of intracellular pH and NHE activity**

Intracellular pH (pHᵢ) was measured by monitoring the fluorescence of the pH-sensitive dye BCECF. Myocytes placed in a petri dish were loaded with BCECF by incubation for 15 minutes in the dark at room temperature with the acetoxymethyl ester form (BCECF-AM, 2 μM) in modified Krebs solution (135 mM NaCl, 5.9 mM KCl, 1.5 mM CaCl₂, 1.2 mM MgCl₂, 11.5 mM glucose, 11.6 mM HEPES, pH 7.4) supplemented with 0.1% bovine serum albumin and 0.02% Pluronic F127. The cells were then washed three times and incubated for an additional 45 minutes in fresh Krebs solution in the presence or absence of the MEK inhibitor U0126 (5 μM). BCECF fluorescence was recorded using confocal microscopy (FluoView FV1000; Olympus, Tokyo, Japan). A ratio of fluorescence emitted at 515 nm from excitation at 490 nm to that at 440 nm was converted to intracellular pHᵢ using the nigericin high-K⁺ protocol of Thomas et al.²¹

NHE activity was measured by monitoring the recovery rate from rapid acidification using the NH₄Cl prepulse technique.²¹,²² After determination of basal pHᵢ, cells were exposed to Krebs solution containing 25 mM NH₄Cl for 5 minutes to cause rapid alkalization as NH₃ diffused into the cells and titrated intracellular H⁺. Then, perfusion with Na⁺-free Krebs solution (Na⁺ isomotically replaced with N-methylglucamine) removed NH₄⁺ from the external medium to cause a rapid decrease in pHᵢ. There was no recovery from this acid load in the absence of Na⁺. pHᵢ recovered when the perfusate was switched to an Na⁺-containing Krebs solution. This Na⁺-dependent recovery was operationally defined as NHE activity. To quantify the rate of pHᵢ recovery, the slope of a straight line fitted to the initial 60 seconds after the onset of recovery was measured.²⁰

**Western blotting**

Total protein extracts were prepared from cells using cell-lysis buffer containing a protease-inhibitor cocktail (aprotinin, leupeptin, pepstatin A, and phenylmethylsulfonyl fluoride). The protein concentration was determined by a Bradford protein-assay kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). Equal amounts of total protein (40 μg) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (10%), followed by electrophoretic transfer to nitrocellulose membranes (GE Healthcare UK Ltd., Little Chalfont, UK). The blots were incubated with primary antibody (rabbit antibody) and horseradish peroxidase-conjugate goat antirabbit IgG and detected by the use of a SuperSignal Western blotting kit (Thermo Fisher Scientific). Densitometric analysis of protein bands was performed with Quantity One® software (version 4.5.2; Bio-Rad Laboratories Inc.).

**Statistical analysis**

All values are presented as means ± standard error of the mean. Statistical analysis was carried out using paired Students’ t-tests or one-way analysis of variance followed by the Student–Newman–Keuls test, with P<0.05 considered statistically significant.

**Results**

F₂ inhibits the H₂O₂-induced increase of Iₙcx

Currents were recorded when myocytes were perfused in sequence with the control extracellular solution, and solutions containing H₂O₂ (100 μM), H₂O₂ + F₂ (0.1, 1.0, or 10 μM),
and NiCl$_2$ (5 mM) for 10 minutes, respectively. Bidirectional outward and inward $I_{\text{NCX}}$ were induced by 1 mM Ca$^{2+}$ and 140 mM Na$^+$ in the external solution, and 20 mM Na$^+$ and 226 mM free Ca$^{2+}$ in the pipette solution. Under these ionic conditions, the reversal potential of $I_{\text{NCX}}$ with a 3Na$^+:1$Ca$^{2+}$ stoichiometry was calculated to be $-65$ mV at room temperature according to the equation $E_{\text{NCX}}=3E_{\text{Na}}-2E_{\text{Ca}}$.

Figure 1A illustrates the $I-V$ relation of control myocytes (a), and myocytes exposed to H$_2$O$_2$ (b), H$_2$O$_2$ + 0.1, 1.0, or 10 µM F$_2$ (c–e), and NiCl$_2$ (f). The net Ni$^{2+}$-sensitive currents all crossed the voltage axis at about $-65$ mV (Figure 1B), confirming that the Ni$^{2+}$-sensitive currents were $I_{\text{NCX}}$. Both outward and inward $I_{\text{NCX}}$ increased after perfusion with 100 µM H$_2$O$_2$. F$_2$ diminished the increase of $I_{\text{NCX}}$ in a concentration-dependent manner, with reverse-mode NCX being greater than forward-mode inhibition (Figure 1C).

**U0126 and DMA inhibit H$_2$O$_2$-induced $I_{\text{NCX}}$ increases**

To confirm the involvement of the MAPK pathway and the NHE in H$_2$O$_2$-induced NCX activation, we tested the effects of U0126, a highly selective inhibitor of MEK, and DMA, an NHE inhibitor, on the H$_2$O$_2$-induced increase in $I_{\text{NCX}}$. We initially determined the minimal effective concentrations that completely blocked H$_2$O$_2$-induced MEK activation and NHE-1 activity, and used those concentrations to examine the roles of MEK and NHE in F$_2$-mediated inhibition of H$_2$O$_2$-mediated increase of $I_{\text{NCX}}$. Results showed that perfusion of 5 µM U0126 for 10 minutes, which alone did not affect $I_{\text{NCX}}$, significantly inhibited the H$_2$O$_2$-induced increase in $I_{\text{NCX}}$ at 60 mV by 81.13%±3.63% and at $-150$ mV by 93.64%±4.52% (n=5) (Figure 2A and B). In contrast, perfusion of 20 µM DMA for 10 minutes only inhibited the H$_2$O$_2$-induced increase by 39.98%±3.00% at 60 mV, and by 32.42%±1.78% at $-150$ mV (n=5) (Figure 2C and D). This result indicates that the H$_2$O$_2$-induced increase in $I_{\text{NCX}}$ was primarily mediated by the MEK/MAPK pathway, and partially through activation of NHE-1.

**F$_2$ inhibits H$_2$O$_2$-induced MEK/ERK activation and EGF-induced $I_{\text{NCX}}$ increases**

To investigate whether F$_2$ modulates MEK activity, we examined the effect of F$_2$ on H$_2$O$_2$- and EGF-induced MEK/ERK activation. As shown in Figure 3A, H$_2$O$_2$ (100 µM) and EGF (50 ng/mL) led to a significant increase in the level of phosphorylated MEK and ERK, and 1 µM F$_2$ inhibited both H$_2$O$_2$- and EGF-induced MEK and ERK activation. We then observed the effect of F$_2$ on the $I_{\text{NCX}}$ increase induced by EGF. $I_{\text{NCX}}$ was increased by EGF, and treatment with 1 µM F$_2$ resulted in a significant reduction in EGF-induced $I_{\text{NCX}}$ rise at 60 mV by 72.88%±5.76% and at $-150$ mV by 71.14%±3.19% (n=8) (Figure 3B).

**F$_2$ inhibits H$_2$O$_2$-induced and Ang II-induced NHE activity**

To investigate the effects of F$_2$ on NHE activity, we examined its effects on H$_2$O$_2$- and Ang II-induced, Na$^+$-dependent recovery from acid load in rat ventricular myocytes. The mean resting pH of ventricular myocytes in bicarbonate-free Krebs solution at room temperature was 7.48±0.13 (n=10). The addition and removal of NH$_4$Cl from the external medium caused a rapid rise and decrease in pH. Cells were unable to recover from this acid load in Na$^+$-free medium. Reintroduction of Na$^+$ led to a rapid recovery of pH that approached resting values (Figure 4A). This Na$^+$-dependent recovery was completely blocked by DMA (25 µM) (Figure 4B). Exposure to

![Figure 1](https://example.com/figure1.png)

**Notes:** (A) $I-V$ curves of control (a), or in the presence of 100 µM H$_2$O$_2$ (b), H$_2$O$_2$ + F$_2$ (c–e) and NiCl$_2$ (f). Inset: ramp-pulse protocol. (B) $I-V$ curves of net Ni$^{2+}$-sensitive currents, obtained by subtracting the corresponding $I-V$ curves in (A). (C) Concentration–response relationships of the inhibitory effect of F$_2$ on $I_{\text{NCX}}$. Outward currents were measured at +60 mV, inward currents were measured at $-150$ mV (n=7 cells/group). *P<0.05 outward currents versus inward currents.

**Abbreviations:** $F_2$, N-n-butyl haloperidol iodide; $I_{\text{NCX}}$, current of Na$^+$/Ca$^{2+}$ exchanger; $I-V$, current–voltage; H$_2$O$_2$, hydrogen peroxide; NiCl$_2$, nickel chloride; Ni$^{2+}$, nickel ion.
100 µM H₂O₂ caused an increase in Na⁺-dependent recovery of pH from buffer load (4.8±0.6×10⁻³ ΔpH/second [n=5] versus 2.5±0.3×10⁻³ ΔpH/minute in controls [n=5], P<0.05) that was again completely blocked by DMA, indicating that H₂O₂-mediated enhancement of recovery from buffer load is mediated by the NHE (Figure 4C and D). Similar to DMA, pretreatment with the MEK inhibitor U0126 abolished H₂O₂-induced NHE activity (2.4±0.4×10⁻³ ΔpH/minute [n=4], P<0.05 versus control) (Figure 4E). Perfusion with 1 µM F₂ completely blocked recovery of buffer load in the presence of H₂O₂ (Figure 4F) and NHE activity in the presence of 1 nM Ang II (Figure 4G). These results suggest that F₂ exerted its cardioprotective effects by blocking NHE activity.

**F₂ inhibits Ang II-induced I_{NCX} increases**

Ang II at a low concentration stimulates NHE-1 activity to elevate intracellular Na⁺ levels, which reverses NCX activity and leads to I_{NCX} increases. We observed that 1 nM Ang II increased outward I_{NCX} at 60 mV by 26.92%±4.40% and inward I_{NCX} at −150 mV by 14.26%±2.95% (n=5), consistent with a prior report. Addition of 1 µM F₂ resulted in a significant reduction in the Ang II-induced I_{NCX} rise at 60 mV by 62.27%±3.42% and at −150 mV by 46.19%±3.36% (n=5) (Figure 5), consistent with a role for F₂ in blocking NHE activation.

**Effects of F₂ on the protein expression of NHE and NCX**

Exchanger activity is regulated by changes in protein expression and by phosphorylation of existing exchangers or a closely associated modulatory protein. Therefore, we examined the effects of F₂ on the protein expression of NHE and NCX. The results showed that the total protein expression of NHE and NCX did not change after myocytes were treated.
with H$_2$O$_2$, EGF, and Ang II for 30 minutes, and that F$_2$ had no significant effect on the total protein expression of either NHE or NCX (Figure 6).

**Discussion**

The present study describes the effects of F$_2$ on the H$_2$O$_2$-induced signal-transduction pathway for $I_{\text{NCX}}$ increase in rat ventricular myocytes. F$_2$ can inhibit the signal-transduction pathway involved in H$_2$O$_2$-induced $I_{\text{NCX}}$ increase at multiple sites.

Excess ROS production and intracellular Ca$^{2+}$ overload play a prominent role in I/R injury. Moreover, there is a reciprocal interaction between excess ROS production and accumulation of cytosolic and mitochondrial Ca$^{2+}$ due to the cross talk between ROS and Ca$^{2+}$, which can enhance ROS generation. ROS can activate MAPKs...
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(ERK, Jun N-terminal kinase [JNK], p38), which are activated during ischemia, and to a greater extent on reperfusion. Activated ERK1/2 leads to phosphorylation and activation of NHE-1, and this may contribute to a feed-forward activation loop (Ca²⁺ → ROS → ERK → more Na⁺ → more Ca²⁺), enhancing Ca²⁺ overload in I/R injury. The ability to disrupt this vicious cycle will exert beneficial effects on recovery from I/R injury. In this study, we

Figure 4 Effects of F₂ on H₂O₂-induced and Ang II-induced NHE activity.

Notes: (A) Intracellular pH (pHᵢ) was measured with BCECF in rat ventricular myocytes during exposure to NH₄Cl, followed by removal of external Na⁺ (to induce an acid load) and reintroduction of Na⁺ (Krebs solution). The rapid removal of NH₄Cl caused an immediate decrease in pHᵢ. Recovery of pHᵢ did not occur until the cells were perfused with Na⁺-containing Krebs solution. (B) Na⁺-dependent pHᵢ recovery from acid load was completely blocked when 20 µM DMA was present during Na⁺-free treatment, as well as during recovery in Na⁺-containing Krebs solution. (C) Exposure to 100 µM H₂O₂ caused an increase in Na⁺-dependent recovery of pHᵢ from acid load. (D) DMA blocked Na⁺-dependent pHᵢ recovery in the presence of H₂O₂. (E) Pretreatment with U0126 inhibited the H₂O₂-induced increase in Na⁺-dependent recovery of pHᵢ. (F) Perfusion with 1 µM F₂ completely blocked Na⁺-dependent pHᵢ recovery in the presence of H₂O₂. (G) Perfusion with F₂ completely blocked Na⁺-dependent pHᵢ recovery in the presence of 1 nM Ang II.

Abbreviations: F₂, N-n-butyl haloperidol iodide; H₂O₂, hydrogen peroxide; Ang, angiotensin; NHE, Na⁺/H⁺ exchanger; BCECF, 2,7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-acetoxymethyl; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylmercapto)butadiene; DMA, 5-(n,n-dimethyl)-amiloride; Na⁺, sodium ion; NH₄Cl, ammonium chloride.
Figure 5 Effect of F$_2$ on Ang II-induced $I_{\text{NCX}}$ increase.

Notes: (A) $I-V$ curves of control (a), or in the presence of 1 mM Ang II (b), Ang II + F$_2$ (1 μM) (c), and 5 mM NiCl$_2$ (d). (B) $I-V$ curves of net Ni$^{2+}$-sensitive currents, obtained by subtracting the corresponding $I-V$ curves in (A).

Abbreviations: F$_2$ N-butyl haloperidol iodide; Ang, angiotensin; $I-V$, current–voltage; NiCl$_2$, nickel chloride; Ang II, angiotensin II; $I_{\text{NCX}}$, NCX current; Ni$^{2+}$, nickel ion.

Huang et al demonstrated that F$_2$ can inhibit H$_2$O$_2$-induced increase in NCX activity through inhibiting both MEK/ERK activation and NHE activity, blocking intracellular Ca$^{2+}$ overload to protect against myocardial I/R injury.

Our results show that acute exposure of cardiac myocytes to 100 μM H$_2$O$_2$ causes the $I_{\text{NCX}}$ to increase, along with a rapid activation of MEK and an increase in NHE activity. The H$_2$O$_2$-induced $I_{\text{NCX}}$ increase was blocked almost completely by the MEK inhibitor U0126, but only partly by the NHE inhibitor DMA (Figure 2), indicating the $I_{\text{NCX}}$ increase was primarily mediated by the MEK MAPK pathway and partially through activation of NHE, consistent with prior reports. Furthermore, the H$_2$O$_2$-induced increase in NHE activity was abolished by pretreatment with the MEK inhibitor U0126 (Figure 4E), suggesting that MAPKs act upstream of NHE in H$_2$O$_2$-induced $I_{\text{NCX}}$ increase. The present study shows that F$_2$ blocks MEK activation-induced by not only H$_2$O$_2$ but also EGF (Figure 3A), suggesting that F$_2$ directly inhibits MEK activation.

Dyck et al found an increase in steady-state levels of NHE-1 messenger ribonucleic acid in chronic ischemia in rat myocardium, suggesting that increased activity is due to an increase in protein expression. However, in our experiments, acute exposure to H$_2$O$_2$ caused a rapid activation of NHE and NCX activity in the absence of changes in total NHE and NCX. The most likely explanation is that the exposure to H$_2$O$_2$ in our experiment was too short for changes in protein expression, indicating that posttranslational modification rather than gene expression played the major role in the rapid time course for regulation of exchanger activity. Unfortunately, we could not detect phosphorylation of NHE-1 and NCX due to the absence of antibodies for phospho-NHE-1 and phospho-NCX, which was a limitation of this study.

NHE activation increases $I_{\text{NCX}}$ through increasing intracellular Na$^+$ concentration. NCX is one of the major mechanisms for regulating intracellular Ca$^{2+}$ concentration in cardiac myocytes. Under physiological conditions, the Na$^+$/Ca$^{2+}$ exchanger operates in forward mode, extruding Ca$^{2+}$ from the cell to maintain intracellular Ca$^{2+}$ homeostasis. Conversely, during I/R, a large burst of ROS contributes to Ca$^{2+}$ loading via activation of the NCX Ca$^{2+}$-influx mode, which accelerates intracellular Ca$^{2+}$ overload. H$_2$O$_2$ increases NCX activity, leading to Ca$^{2+}$ overload via activation of the MEK/ERK/NHE pathway.

Our previous studies demonstrate that F$_2$ blocks L-type Ca$^{2+}$ channels and protects the activity of sarco/endoplasmic reticulum Ca$^{2+}$-adenosine triphosphatases to attenuate Ca$^{2+}$ overload against I/R injury in cardiac myocytes. We now show an additional mechanism for F$_2$ in the regulation of calcium homeostasis, demonstrating that F$_2$ inhibits both MEK activation and NHE activity to diminish H$_2$O$_2$-induced $I_{\text{NCX}}$ increase, but we do not rule out inhibition by F$_2$ on NCX activity. Figure 7 illustrates the possible signaling pathways from H$_2$O$_2$ to NCX and the target of F$_2$ action.

In conclusion, we demonstrate an additional mechanism by which F$_2$ can alleviate intracellular Ca$^{2+}$ overload, and thus protect against myocardial I/R injury. F$_2$, a novel quaternary ammonium salt derivative of haloperidol with a different chemical structure from classical Ca$^{2+}$-channel antagonists, seems like an undesirable drug due to its broad, nonspecific...
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was supported by National Natural Science Foundation of China–Guangdong Joint Funds (U0932005), the National Natural Science Foundation of China (81173048 and 81072633), central government special funds supporting the development of local colleges and universities.

Disclosure

The authors report no conflicts of interest in this work.
**Figure 7** Scheme of possible signaling pathways for NCX activation by H2O2 and the target of F2 action.

**Abbreviations:** NCX, Na+/Ca2+ exchanger; H2O2, hydrogen peroxide; F2, N-n-butyl haloperidol iodide; NHE, Na+/H+ exchanger; ERK, extracellular signal-related kinase; MEK, extracellular signal-regulated kinase.

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