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ORIGINAL RESEARCH

N-n-butyl haloperidol iodide inhibits H_2O_2-induced Na^+/Ca^{2+}-exchanger activation via the Na^+/H^+ exchanger in rat ventricular myocytes

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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_2O_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_2O_2 increased both NHE and NCX activities, as well as levels of phosphorylated MEK and ERK. The H_2O_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[*o*-aminophenylmercapto] 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_2O_2-induced I_{NCX} increase in a concentration-dependent manner. To determine whether pathway inhibition was H_2O_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_2O_2-induced and EGF-induced MEK/ERK activation, but also completely blocked both H_2O_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_2O_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.[1] 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.[2]

ROS, produced as by-products of oxidative metabolism, are easily managed under normal conditions by reactive oxygen scavengers.[3,4] Several forms of ROS are generated during I/R, including superoxide (O_2^-), H_2O_2, and the highly reactive hydroxyl radical (·OH), which cause lipid peroxidation and myocardial injury and trigger the contractile dysfunction observed during reperfusion.[5,6] It has also been suggested that the burst in

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ROS upon reperfusion may contribute to Ca^{2+} overload in cardiomyocytes.[7,8] Rothstein et al[9] and Sabri et al[10] found that low doses of H_2O_2 (50 μM, similar to those generated during I/R) cause Ca^{2+} overload in cultured neonatal rat ventricular myocytes, which is associated with activation of the Na^+/H^+ 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_2O_2 and diastolic Ca^{2+} overload in neonatal rat ventricular myocytes was proposed. Exposure to H_2O_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^+ concentrations. The resulting rise in intracellular Na^+ decreases the activity of the Na^+/Ca^{2+} exchanger (NCX), leading to an increase in diastolic Ca^{2+} levels.[9] Therefore, pharmacological approaches to decrease MAPK, NHE-1, and/or NCX activity may ameliorate the alterations in Ca^{2+} homeostasis that contribute to myocardial tissue injury following I/R.

N-n-butyl haloperidol iodide (F_2), a novel quaternary ammonium salt derivative of haloperidol synthesized in our laboratory, can maintain the effects of coronary artery relaxation without adverse extrapyramidal reactions.[11] Our previous studies show that F_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.[11–14] The cardioprotective mechanism of F_2 was thought to be associated with calcium-homeostasis maintenance against intracellular Ca^{2+} overload by inhibiting cardiocyte L-type Ca^{2+} channels.[12,13] However, intracellular Ca^{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^+ in exchange for an influx of Na^+. Upon reperfusion, loss of extracellular H^+ causes further extrusion of H^+ in exchange for Na^+. The subsequent elevation in intracellular Na^+ promotes an increase Ca^{2+} influx into the cytosol via the reverse mode of NCX, resulting in Ca^{2+} overload.[15] It is suggested that the mechanism of F_2 antagonizing myocardial I/R injury might not be only related to suppression of the L-type Ca^{2+} channel. In this study, we used rat ventricular myocytes to investigate the effects of F_2 on the MEK/ERK/NHE/NCX signal-transduction pathway involved in H_2O_2-induced Ca^{2+} overload in order to probe the underlying molecular mechanism by which F_2 maintains intracellular calcium homeostasis and antagonizes myocardial I/R injury.

Materials and methods

Materials

F_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-ethanesulphonic 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)-5(6)-carboxyfluorescein-acetoxymethyl 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 antirabbit 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).[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.[17,18] Single ventricular myocytes were harvested after filtration through a nylon mesh (pore size 200 mm).

I_{NCX} recording

Myocytes were perfused with extracellular solution (140 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.33 mM

NaH_2PO_4, 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 BAPTA, 10 mM $CaCl_2$ [free Ca^{2+} concentration of 226 nM], 120 mM CsOH, 3 mM $MgCl_2$, 50 mM aspartic acid, 5 mM Mg-adenosine triphosphate, and 10 mM HEPES, pH 7.2).[19] NCX current (I_{NCX}) 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 I_{NCX}, the extracellular solution contained ouabain, nifedipine, Cs^+, and ryanodine to block Na^+/K^+ pump current, I_{Ca}, I_K, and Ca^{2+} release channels of the sarcoplasmic reticulum, respectively. I_{NCX} 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.[20] I_{NCX} was measured as the N_i^{2+}-sensitive current that could be selectively inhibited by 5 mM $NiCl_2$.

Measurement of intracellular pH and NHE activity

Intracellular pH (pH_i) was measured by monitoring the fluorescence of the pH-sensitive dye BCECF.[9,10] 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 $CaCl_2$, 1.2 mM $MgCl_2$, 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 pH_i 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 NH_4Cl prepulse technique.[21,22] After determination of basal pH_i, cells were exposed to Krebs solution containing 25 mM NH_4Cl for 5 minutes to cause rapid alkalinization as NH_3 diffused into the cells and titrated intracellular H^+. Then, perfusion with Na^+-free Krebs solution (Na^+ isosmotically replaced with *N*-methylglucamine) removed NH_4^+ from the external medium to cause a rapid decrease in pH_i. There was no recovery from this acid load in the absence of Na^+. pH_i 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 pH_i recovery, the slope of a straight line fitted to the initial 60 seconds after the onset of recovery was measured.[10]

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 antirat) at 4°C overnight, followed by secondary antibody (horseradish peroxidase-conjugate goat antirabbit IgG) for 2 hours at room temperature. The bound antibodies were 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's *t*-tests or one-way analysis of variance followed by the Student–Newman–Keuls test, with $P<0.05$ considered statistically significant.

Results

F_2 inhibits the H_2O_2-induced increase of I_{NCX}

Currents were recorded when myocytes were perfused in sequence with the control extracellular solution, and solutions containing H_2O_2 (100 μM), $H_2O_2 + F_2$ (0.1, 1.0, or 10 μM),

and $NiCl_2$ (5 mM) for 10 minutes, respectively. Bidirectional outward and inward I_{NCX} were 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, the reversal potential of I_{NCX} with a $3Na^+$:$1Ca^{2+}$ stoichiometry was calculated to be −65 mV at room temperature according to the equation $E_{Na/Ca}=3E_{Na}-2E_{Ca}$.[23,24] Figure 1A illustrates the *I–V* relation of control myocytes (a), and myocytes exposed to H_2O_2 (b), H_2O_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_{NCX}. Both outward and inward I_{NCX} increased after perfusion with 100 μM H_2O_2. F_2 diminished the increase of I_{NCX} in a concentration-dependent manner, with reverse-mode NCX being greater than forward-mode inhibition (Figure 1C).

U0126 and DMA inhibit H_2O_2-induced I_{NCX} increases

To confirm the involvement of the MAPK pathway and the NHE in H_2O_2-induced NCX activation, we tested the effects of U0126, a highly selective inhibitor of MEK, and DMA, an NHE inhibitor, on the H_2O_2-induced increase in I_{NCX}. We initially determined the minimal effective concentrations that completely blocked H_2O_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_2O_2-mediated induction of I_{NCX} activity. Results showed that perfusion of 5 μM U0126 for 10 minutes, which alone did not affect I_{NCX},[25] significantly inhibited the H_2O_2-induced increase in I_{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_2O_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_2O_2-induced increase in I_{NCX} was primarily mediated by the MEK/MAPK pathway, and partially through activation of NHE-1.

F_2 inhibits H_2O_2-induced MEK/ERK activation and EGF-induced I_{NCX} increases

To investigate whether F_2 modulates MEK activity, we examined the effect of F_2 on H_2O_2-induced and EGF-induced MEK/ERK activation. As shown in Figure 3A, H_2O_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_2O_2-induced and EGF-induced MEK and ERK activation. We then observed the effect of F_2 on the I_{NCX} increase induced by EGF. I_{NCX} was increased by EGF, and treatment with 1 μM F_2 resulted in a significant reduction in EGF-induced I_{NCX} rise at 60 mV by 72.88%±5.76% and at −150mV by 71.14%±3.19% (n=8) (Figure 3B).

F_2 inhibits H_2O_2-induced and Ang II-induced NHE activity

To investigate the effects of F_2 on NHE activity, we examined its effects on H_2O_2-induced 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_4Cl from the external medium caused a rapid rise and decrease in pH_i. Cells were unable to recover from this acid load in Na^+-free medium. Reintroduction of Na^+ led to a rapid recovery of pH_i that approached resting values (Figure 4A). This Na^+-dependent recovery was completely blocked by DMA (25 μM) (Figure 4B). Exposure to

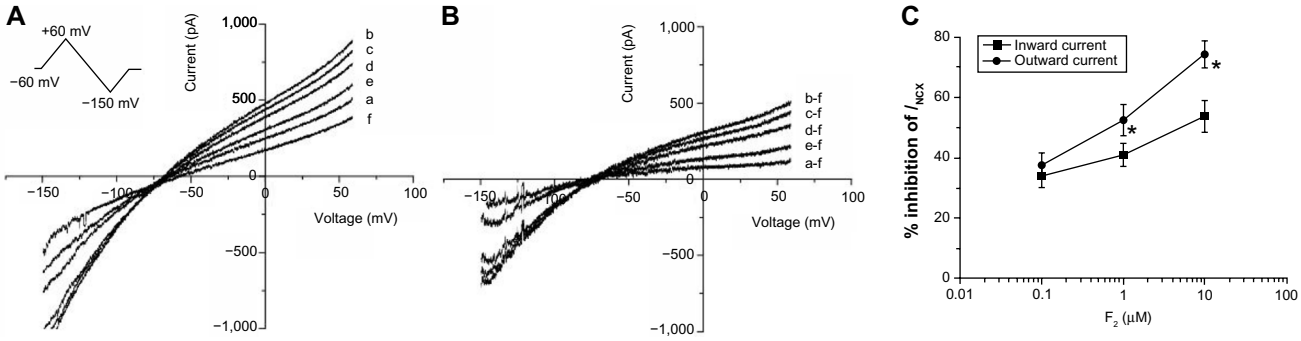

Figure 1 Effect of F_2 on the H_2O_2-induced increase in I_{NCX}.
Notes: (**A**) *I–V* curves of control (a), or in the presence of 100 μM H_2O_2 (b), H_2O_2 + F_2 (0.1, 1.0 or 10 μM) (c–e) and 5 mM $NiCl_2$ (f). Inset: ramp-pulse protocol. (**B**) *I–V* curves of net Ni^{2+}-sensitive currents, obtained by subtracting the corresponding *I–V* curves in (**A**). (**C**) Concentration–response relationships of the inhibitory effect of F_2 on I_{NCX}. Outward currents were measured at +60 mV, inward currents were measured at −150 mV (n=9 cells/group). *$P<0.05$ outward currents versus inward currents.
Abbreviations: F_2, *N*-n-butyl haloperidol iodide; I_{NCX}, current of Na^+/Ca^{2+} exchanger; *I–V*, current–voltage; H_2O_2, hydrogen peroxide; $NiCl_2$, nickel chloride; Ni^{2+}, nickel ion.

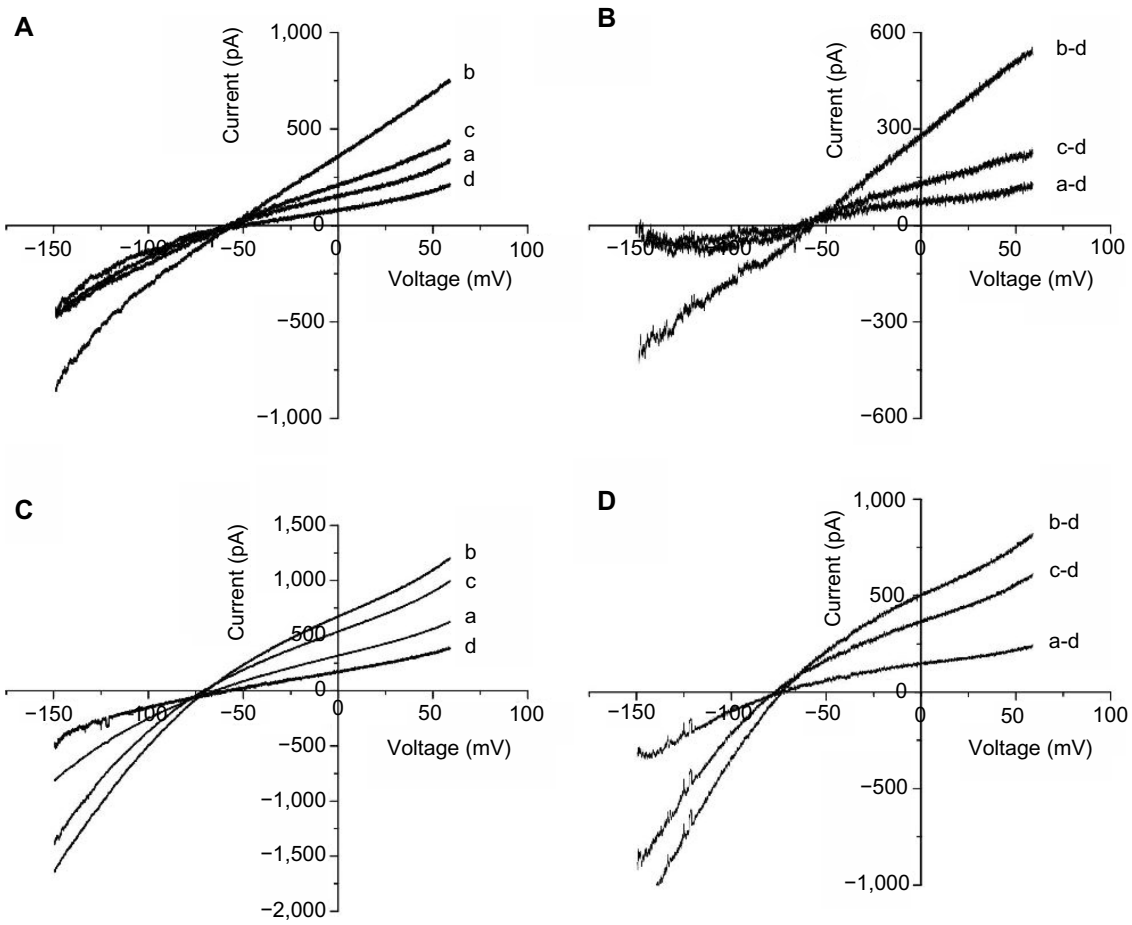

Figure 2 Effects of U0126 and DMA on the H_2O_2-induced I_{NCX} increase.

Notes: (**A**) *I–V* curves of control (a), or in the presence of 100 μM H_2O_2 (b), H_2O_2 + U0126 (5 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**). (**C**) *I–V* curves of control (a), or in the presence of 100 μM H_2O_2 (b), H_2O_2 + DMA (20 μM) (c), and 5 mM $NiCl_2$ (d). (**D**) *I–V* curves of net Ni^{2+}-sensitive currents, obtained by subtracting the corresponding *I–V* curves in (**C**).

Abbreviations: U0126, 1,4-diamino-2,3-dicyano-1,4-bis(*o*-aminophenylmercapto)butadiene; DMA, 5-(*N*,*N*-dimethyl)-amiloride; H_2O_2, hydrogen peroxide; $NiCl_2$, nickel chloride; I_{NCX}, current of Na^+/Ca^{2+} exchanger; $NiCl_2$, nickel chloride; *I–V*, current–voltage; Ni^{2+}, nickel ion.

100 μM H_2O_2 caused an increase in Na^+-dependent recovery of pH_i from acid load ($4.8\pm0.6\times10^{-3}$ ΔpH/second [n=5] versus $2.5\pm0.3\times10^{-3}$ ΔpH/minute in controls [n=5], $P<0.05$) that was again completely blocked by DMA, indicating that H_2O_2-mediated enhancement of recovery from acid load is mediated by the NHE (Figure 4C and D). Similar to DMA, pretreatment with the MEK inhibitor U0126 abolished H_2O_2-induced NHE activity ($2.4\pm0.4\times10^{-3}$ ΔpH/minute [n=4], $P<0.05$ versus control) (Figure 4E). Perfusion with 1 μM F_2 completely blocked both Na^+-dependent recovery in the presence of H_2O_2 (Figure 4F) and NHE activity in the presence of 1 nM Ang II (Figure 4G). These results suggest that F_2 exerted its cardioprotective effects by blocking NHE activity.

F_2 inhibits Ang II-induced I_{NCX} increases

Ang II at a low concentration stimulates NHE-1 activity to elevate intracellular Na^+ levels,[26,27] 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.[28] Addition of 1 μM F_2 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 F_2 in blocking NHE activation.

Effects of F_2 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.[29–33] Therefore, we examined the effects of F_2 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

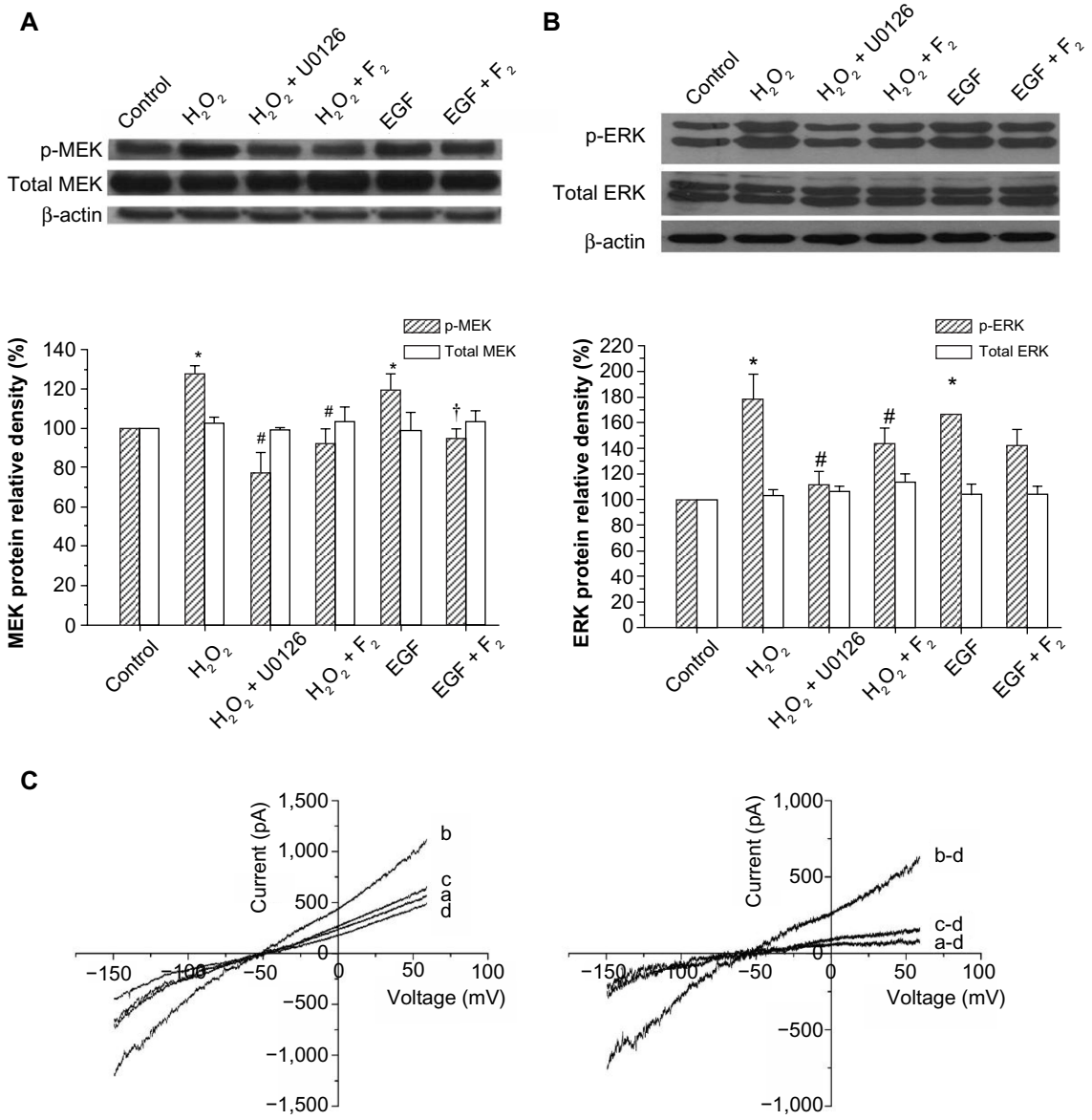

Figure 3 Effects of F_2 on H_2O_2-induced MEK/ERK activation and EGF-induced I_{NCX} increase.

Notes: (A) Western blot analysis of MEK phosphorylation and total MEK protein. Upper, representative blot of three independent experiments; lower, quantitative densitometric data were normalized as a percentage of those in the control group, which was plotted as 100%. (**B**) Western blot analysis of ERK phosphorylation and total ERK protein. Upper, representative blot of three independent experiments; lower, quantitative densitometric data were normalized as a percentage of those in the control group, which was plotted as 100%. (**C**) Left, *I–V* curves of control (a), or in the presence of 50 ng/mL EGF (b), EGF + F_2 (1 μM) (c), and 5 mM $NiCl_2$ (d); right, *I–V* curves of net Ni^{2+}–sensitive currents, obtained by subtracting the corresponding *I–V* curves in (C) (left). $^*P<0.05$ versus control group, $^{\#}P<0.05$ versus H_2O_2 group, $^{\dagger}P<0.05$ versus EGF group.

Abbreviations: H_2O_2, hydrogen peroxide; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(*o*-aminophenylmercapto)butadiene; $NiCl_2$, nickel chloride; F_2, *N*-n-butyl haloperidol iodide; ERK, extracellular signal-related kinase; I_{NCX}, current of Na^+/Ca^{2+} exchanger; *I–V*, current–voltage; MEK, extracellular signal-regulated kinase kinase; p-MEK, phosphorylated-MEK; p-ERK, phosphorylated-ERK, β-actin, beta-actin; EGF, epidermal growth factor.

with H_2O_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_2O_2-induced signal-transduction pathway for I_{NCX} increase in rat ventricular myocytes. F_2 can inhibit the signal-transduction pathway involved in H_2O_2-induced I_{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+}.[34–36] Ca^{2+} can enhance ROS generation.[37] ROS can activate MAPKs

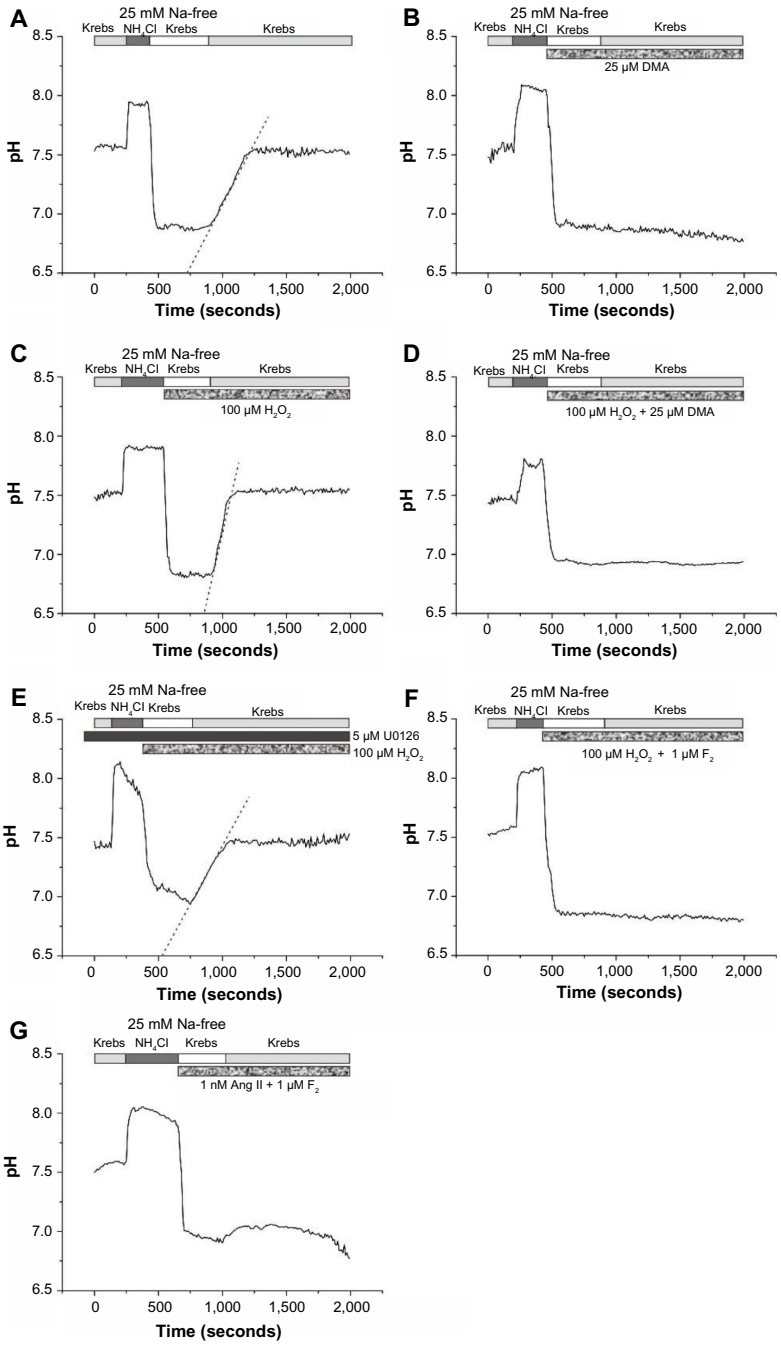

Figure 4 Effects of F_2 on H_2O_2-induced and Ang II-induced NHE activity.

Notes: (**A**) Intracellular pH (pH_i) was measured with BCECF in rat ventricular myocytes during exposure to NH_4Cl, followed by removal of external Na^+ (to induce an acid load) and reintroduction of Na^+ (Krebs solution). The rapid removal of NH_4Cl caused an immediate decrease in pH_i. Recovery of pH_i did not occur until the cells were perfused with Na^+-containing Krebs solution. (**B**) Na^+-dependent pH_i 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 H_2O_2 caused an increase in Na^+-dependent recovery of pH_i from acid load. (**D**) DMA blocked Na^+-dependent pH_i recovery in the presence of H_2O_2. (**E**) Pretreatment with U0126 inhibited the H_2O_2-induced increase in Na^+-dependent recovery of pH_i. (**F**) Perfusion with 1 μM F_2 completely blocked Na^+-dependent pH_i recovery in the presence of H_2O_2. (**G**) Perfusion with F_2 completely blocked Na^+-dependent pH_i recovery in the presence of 1 nM Ang II.

Abbreviations: F_2, *N*-n-butyl haloperidol iodide; H_2O_2, 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(*o*-aminophenylmercapto)butadiene; DMA, 5-(N,N-dimethyl)-amiloride; Na^+, sodium ion ; NH_4Cl, ammonium chloride.

(ERK, Jun N-terminal kinase [JNK], p38),[38] which are activated during ischemia, and to a greater extent on reperfusion.[39,40] Activated ERK1/2 leads to phosphorylation and activation of NHE-1,[9,41] and this may contribute to a feed-forward activation loop ($Ca^{2+} \rightarrow$ ROS $\rightarrow$ ERK $\rightarrow$ more $Na^+ \rightarrow$ more Ca^{2+}), enhancing Ca^{2+}overload in I/R injury.[37] The ability to disrupt this vicious cycle will exert beneficial effects on recovery from I/R injury. In this study, we

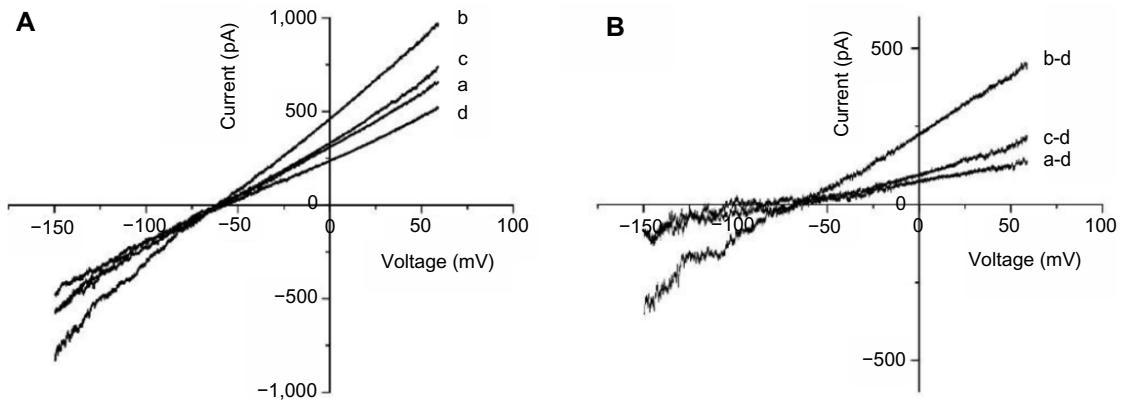

Figure 5 Effect of F_2 on Ang II-induced I_{NCX} increase.
Notes: (**A**) *I–V* curves of control (a), or in the presence of 1 nM Ang II (b), Ang II + F_2 (1 μM) (c), and 5 mM $NiCl_2$ (d). (**B**) *I–V* curves of net Ni^{2+}-sensitive currents, obtained by subtracting the corresponding *I–V* curves in (**A**).
Abbreviations: F_2, *N*-n-butyl haloperidol iodide; Ang, angiotensin; *I–V*, current–voltage; $NiCl_2$, nickel chloride; Ang II, angiotensin II; I_{NCX}, NCX current; Ni^{2+}, nickel ion.

demonstrated that F_2 can inhibit H_2O_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_2O_2 causes the I_{NCX} to increase, along with a rapid activation of MEK and an increase in NHE activity. The H_2O_2-induced I_{NCX} increase was blocked almost completely by the MEK inhibitor U0126, but only partly by the NHE inhibitor DMA (Figure 2), indicating the I_{NCX} increase was primarily mediated by the MEK MAPK pathway and partially through activation of NHE, consistent with prior reports.[9,25] Furthermore, the H_2O_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_2O_2-induced I_{NCX} increase. The present study shows that F_2 blocks MEK activation-induced by not only H_2O_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.[31] However, in our experiments, acute exposure to H_2O_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_2O_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_{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.[42] H_2O_2 increases NCX activity, leading to Ca^{2+} overload via activation of the MEK/ERK/NHE pathway.[4,9,10,25,43] 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.[12,14,18,44] 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_2O_2-induced I_{NCX} increase, but we do not rule out inhibition by F_2 on NCX activity. Figure 7 illustrates the possible signaling pathways from H_2O_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

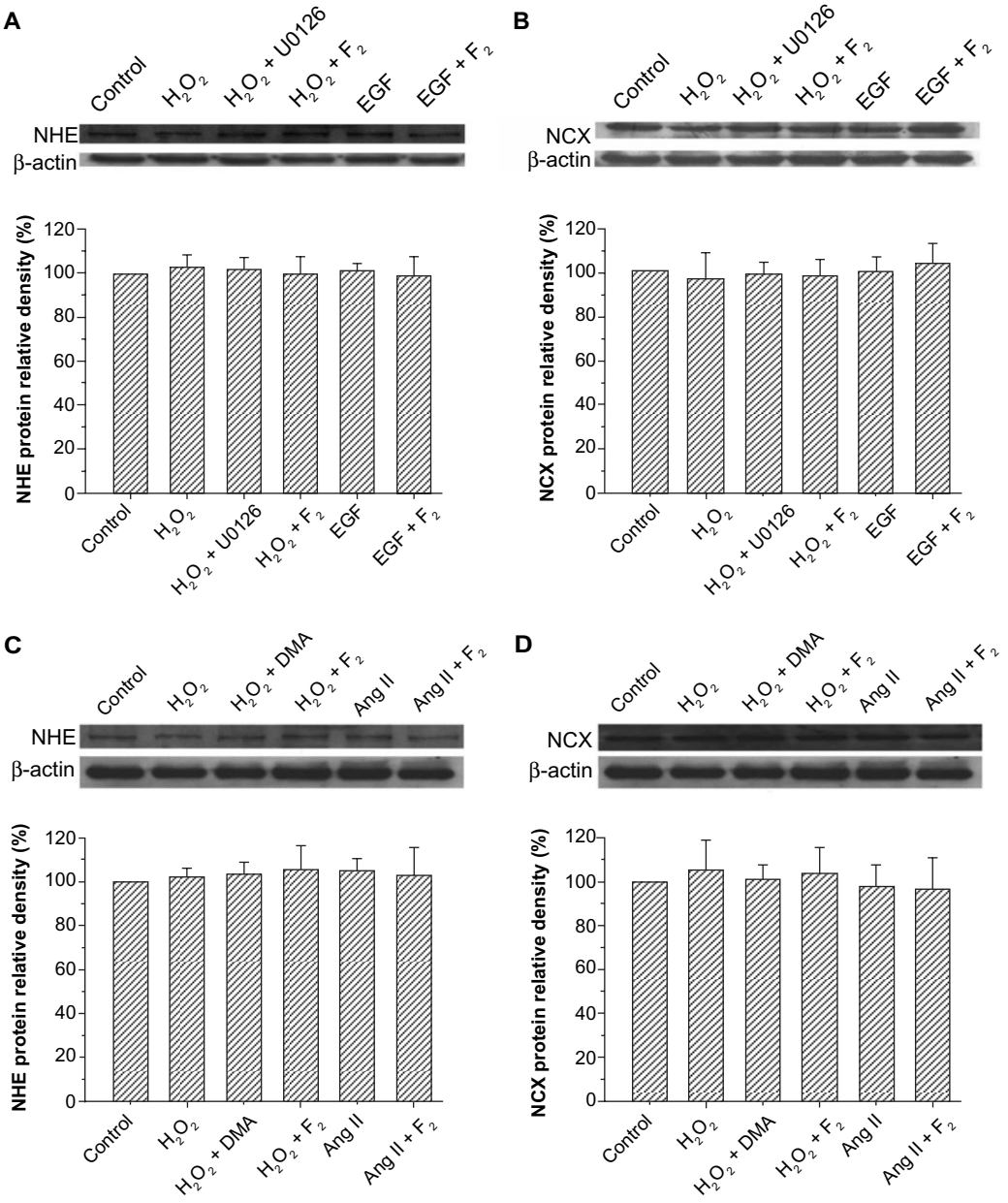

Figure 6 Effect of F_2 on NHE and NCX protein expression.

Notes: (**A** and **C**) Western blot analysis of total NHE protein. (**B** and **D**) Western blot analysis of total NCX protein. Upper, representative blot of three independent experiments; lower, quantitative densitometric data were normalized as a percentage of those of the control group, which was plotted as 100%.

Abbreviations: F_2, *N*-n-butyl haloperidol iodide; NHE, Na^+/H^+ exchanger; NCX, Na^+/Ca^{2+} exchanger; Ang, angiotensin; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(*o*-amino-phenylmercapto)butadiene; DMA, 5-(*N*,*N*-dimethyl)-amiloride; H_2O_2, hydrogen peroxide; EGF, epidermal growth factor.

effects, but the study of its structure–function relationship may help to develop new drugs for the treatment of ischemic heart disease.

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Disclosure

The authors report no conflicts of interest in this work.

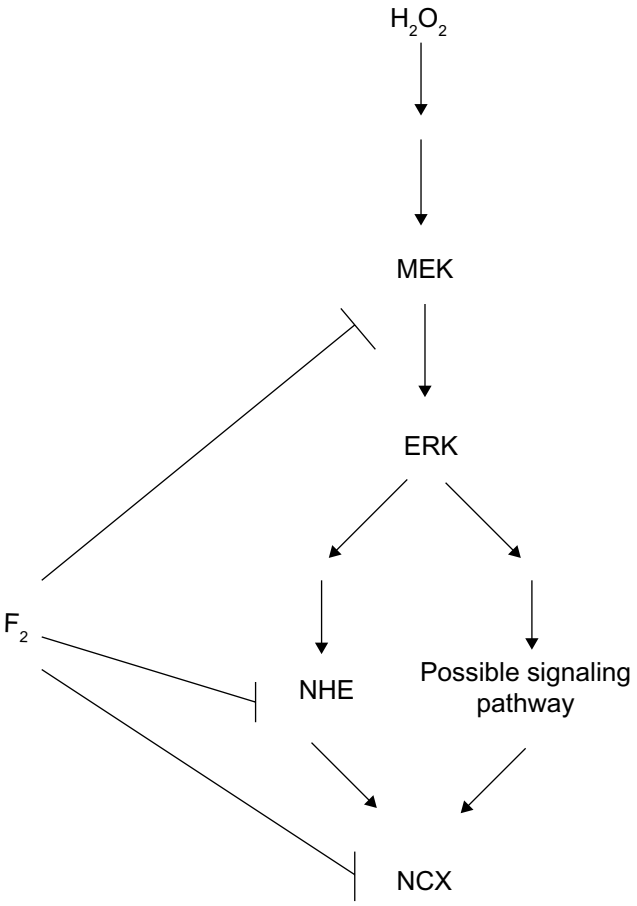

Figure 7 Scheme of possible signaling pathways for NCX activation by H_2O_2 and the target of F_2 action.
Abbreviations: NCX, Na^+/Ca^{2+} exchanger; H_2O_2, hydrogen peroxide; F_2, *N*-n-butyl haloperidol iodide; NHE, Na^+/H^+ exchanger; ERK, extracellular signal-related kinase; MEK, extracellular signal-regulated kinase kinase.

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