H$_2$O$_2$-induced Ca$^{2+}$ overload in NRVM involves ERK1/2 MAP kinases: role for an NHE-1-dependent pathway

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Rothstein, Emily C., Kenneth L. Byron, Ryan E. Reed, Larry Fliegel, and Pamela A. Lucchesi. H$_2$O$_2$-induced Ca$^{2+}$ overload in NRVM involves ERK1/2 MAP kinases: role for an NHE-1-dependent pathway. Am J Physiol Heart Circ Physiol 283: H598–H605, 2002. First published April 11, 2002; 10.1152/ajpheart.00198.2002.—Generation of reactive oxygen species (ROS) and intracellular Ca$^{2+}$ overload are key mechanisms involved in ischemia-reperfusion (I/R)-induced myocardial injury. The relationship between I/R injury and Ca$^{2+}$ overload has not been fully characterized. The increase in Na$^+/H^+$ exchanger (NHE-1) activity observed during I/R injury is an attractive candidate to link increased ROS production with Ca$^{2+}$ overload. We have shown that low doses of H$_2$O$_2$ increase NHE-1 activity in an extracellular signal-regulated kinase (ERK)-dependent manner. In this study, we examined the effect of low doses of H$_2$O$_2$ on intracellular Ca$^{2+}$ in fura-2-loaded, spontaneously contracting neonatal rat ventricular myocytes. H$_2$O$_2$ induced a time- and concentration-dependent increase in diastolic intracellular Ca$^{2+}$ concentration that was blocked by inhibition of ERK1/2 activation with 5 μM U-0126 (88%) or inhibition of NHE-1 with 5 μM HOE-642 (50%). Increased NHE activity was associated with phosphorylation of the NHE-1 carboxyl tail that was blocked by U-0126. These results suggest that H$_2$O$_2$ induced Ca$^{2+}$ overload is partially mediated by NHE-1 activation secondary to phosphorylation of NHE-1 by the ERK1/2 MAP kinase pathway.

ischemia-reperfusion; reactive oxygen species; myocardium

Reperfusion of ischemic myocardium leads to myocardial stunning, which is characterized by changes in the myocardial metabolic and contractile states. Alterations in Ca$^{2+}$ homeostasis and cardiac myofilament responsiveness to Ca$^{2+}$ are thought to be responsible for the contractile dysfunction in the stunned myocardium. Cardiomyocyte cytosolic Ca$^{2+}$ overload causes numerous potentially degenerative states, including alterations in Ca$^{2+}$ transport processes, altered contraction, arrhythmogenesis, and cell death. It has been suggested that the burst in reactive oxygen species (ROS) on reperfusion may contribute to Ca$^{2+}$ overload (14).

ROS are by-products of oxygen consumption that are easily managed under normal conditions with reactive oxygen scavengers (16, 26). Several forms of ROS are generated during I/R, including superoxide (O$_2^-$), H$_2$O$_2$, and the highly reactive hydroxyl radical (•OH). ROS have been demonstrated to cause lipid peroxidation and myocardial injury, and are thought to trigger the contractile dysfunction observed during reperfusion (9). Increased levels of ROS production have been shown in the isolated perfused rabbit heart peaking 10–30 s after reperfusion after an ischemic episode (41, 42). Scavengers of free radicals such as catalase and superoxide dismutase can reduce myocardial stunning and reperfusion arrhythmias (3). Finally, exposure of nonischemic myocardium or myocytes to ROS can produce cell injury similar to that seen in ischemia-reperfusion (I/R) (14).

Indirect measures of oxidant stress have also been studied in humans after myocardial I/R. For example, electron spin resonance has been used to show peak production of ROS at 5 and 25 min after reperfusion in patients undergoing coronary artery bypass graft surgery (10) and an increase in oxidative stress during transient ischemia in patients undergoing elective coronary angioplasty (6).

The cellular mechanisms of oxidant injury and its relationship to Ca$^{2+}$ overload in the cardiomyocyte have not been elucidated. High concentrations of H$_2$O$_2$ (1–10 mM) have been shown to produce Ca$^{2+}$ overload via regulation of L-type Ca$^{2+}$ channels, Na$^+$/Ca$^{2+}$ exchanger (NCX), and sarcoplasmic reticulum (SR) Ca$^{2+}$ release (13, 17, 39). However, these concentrations of H$_2$O$_2$ are ~10- to 100-fold higher than those observed for the burst in ROS production during I/R.

There has been considerable interest in determining whether the effects of ROS at pathophysiological doses similar to those observed during I/R could lead to myocardial tissue damage. Utilizing cultured neonatal rat ventricular myocytes (NRVM) as an in vitro model,
we (31) previously found that low doses of H2O2 (similar to those generated during I/R) cause contractile dysfunction, which was associated with activation of the Na+/H+ exchanger (NHE). Activity of NHE-1, the only NHE isoform in the myocardium (8), is low during normal physiological conditions. Increased NHE-1 activity during reperfusion after an ischemic episode, although protective against acidosis, paradoxically contributes to the subsequent myocardial injury. NHE-1 inhibition has been shown to protect the I/R myocardium (18).

The relationship among ROS, Ca2+ overload, and enhanced NHE-1 activity during I/R injury remains to be determined. One possibility is that the increased NHE-1 activity results in excess intracellular Na+, leading to an alteration in the activity of the NCX, favoring Ca2+ accumulation within the cell (19). Thus NHE-1 could have deleterious effects on myocardial tissue during I/R by contributing to the Ca2+ overload in the cardiomyocyte.

The extracellular signal-regulated kinase 1/2 (ERK1/2) mitogen-activated protein kinases (MAPK) represent attractive candidate kinases for regulating NHE-1 in response to I/R and or ROS. ERK1/2 are activated during I/R in conscious rabbits (29), and H2O2 and hypoxia activate members of the MAPK family, including p38, c-jun NH2-terminal kinase, and ERK1/2 (1, 23, 31). We have also shown that ERK1/2 are responsible for H2O2-stimulated NHE-1 activation in NRVM (31) and for NHE-1 phosphorylation in the I/R rat myocardium (26).

The present study investigates the role of ERK1/2 MAPK and NHE-1 in H2O2-induced Ca2+ overload in spontaneously beating NRVM. Short-term exposure to low levels of H2O2 (50 μM) caused a dramatic diastolic Ca2+ overload that was reduced by ERK1/2 inhibition and NHE-1 blockade. Our results are consistent with the hypothesis that H2O2 may cause Ca2+ overload in the cardiomyocyte partially through ERK1/2-mediated phosphorylation of NHE-1.

METHODS

Cell preparation and culture. Primary cultures of rat ventricular myocytes were obtained from 1- to 2-day-old Sprague-Dawley rats by enzymatic dissociation of ventricular tissue (32). Myocytes were further purified with differential preplatings for 5 min on collagen-coated plates. The remaining cells were then plated onto collagen-coated glass coverslips (Warner) in 12-well plates at a density of 1,600 per mm2 in complete serum-free medium (BioWhittaker) supplemented with antibiotic-antimycotic solution (GIBCO) and 10 μM arabinosidase C and cultured for 24 h at 37°C. These dense cultures beat spontaneously within 24 h and exhibit <10% contamination by nonmuscle cells. NRVM media was changed after 24 h and then maintained for 2–24 h in a 2:1 mixture of serum-free Dulbecco’s modified Eagle’s-Ham’s F-12/PC-1 media supplemented with an antibiotic-antimycotic solution.

Measurement of intracellular Ca2+. NRVM were loaded with 3 μM fura 2-acetoxyethyl ester in Tyrode basic salt solution (Sigma), supplemented with 0.1% bovine serum albumin for 20 min at 37°C, followed by a 30-min unloading period in Tyrode basic salt solution at room temperature, minimizing dye compartmentalization. The coverslip was placed on an inverted microscope (model IX50, Olympus) secured in an imaging chamber (Warner) and perfused with Tyrode basic salt solution for 5 min (with or without pharmacological inhibitors). This equilibration period was followed by 5 min of perfusion of Tyrode basic salt solution with 50 μM H2O2 and a subsequent 5- to 10-min washout period in Tyrode basic salt solution (with or without pharmacological inhibitors). For all inhibitor studies, the drug was used at the same concentration during the unloading, equilibration, H2O2 perfusion, and washout periods.

For each experiment, intracellular Ca2+ concentration ([Ca2+]i) was measured in 5–10 cells. Cell fluorescence was measured using alternating excitation wavelengths (340 and 380 nm) controlled by a Spectromaster monochromatic light source (Olympus). Video images of fluorescence at 510-nm emissions were obtained using a LSR AstroCam CCD camera (Olympus) and the digitized measurement of emitted fluorescence was recorded using Merlin for Windows software (Olympus). The autofluorescence of unloaded myocytes was negligible compared with the fura 2-loaded myocytes and therefore was not subtracted. Rmax (fluorescence ratio at saturating Ca2+) and Rmin (fluorescence ratio at 0 Ca2+) were determined empirically at the end of each experiment by treating cells with 10 μM ionomycin in a CaCl2 containing solution or with 10 mM EGTA in nominally Ca2+-free solution, respectively. The equation [Ca2+]i = Kd × (R – Rmin)/(Rmax – R) (15) was used to construct a standard curve relating the fluorescence ratio to [Ca2+]i for each experiment using the Merlin program. In this equation, the R value is the ratio of fluorescence with excitation at 340 and 380 nm, and β is the ratio of fluorescence with excitation at 380 nm in 0 Ca2+ to that saturating Ca2+. Kd is the effective dissociation constant for fura 2 and was used as 224 nM (4, 7, 20). The data were analyzed with Merlin software and graphed using Delta Graph version 4.5 software for Macintosh.

Measurement of intracellular pH. NRVMs were plated at a density of 3 × 105 cells per 35-mm dish containing two 9 × 22-mm collagen-coated glass coverslips. Forty-eight hours after being plated, the cells were loaded with 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) by incubation with BCECF-acetoxyethyl ester (2 μM), 1 mg/ml bovine serum albumin, and 0.02% Pluronic F127 in Tyrode basic salt solution (Sigma) for 15 min in the dark at room temperature. The cells were then washed three times with Krebs solution and incubated in Krebs solution and 5 μM HOE-642 for ~1 h in the dark before BCECF fluorescence was recorded. BCECF fluorescence was recorded using a Perkin-Elmer LS550B fluorescence spectrophotometer, as previously described (31). A ratio of fluorescence emitted at 515 nm from excitation at 490 nm to that at 440 nm was converted to intracellular pH using the nigericin high-K+ protocol of Thomas et al. (32).

Preparation of cell lysates for MAPK experiments with Western blot analysis. Cell lysates (25 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and transferred to nitrocellulose (Hybond, Amersham). The blots were incubated for 1 h with primary antibody and 1 h with secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG). Immunoreactive bands were visualized with the use of enhanced chemiluminescence (Amersham). Autoradiographs were scanned with an AFGA scanner and densitometric analysis performed with NIH Image version 1.6.

In-gel kinase assay. NHE-1 kinase activity and MAPK activity were analyzed by the in-gel kinase assay as de-
scribed previously (37). Cell lysates (30 μg) were fractionated by SDS-PAGE in a gel in which 0.15 mg/ml of NHE-1 fusion protein [amino acids 639–815 of NHE-1 coupled to glutathione S-transferase (GST)] had been copolymerized. The phosphorylation assay was performed by placing the gel in 10 ml of buffer containing 50 μM ATP with 100 μCi [γ-32P]ATP and incubating for 1 h at 30°C. The reaction was terminated by immersion of the gel in a fixative solution (5% trichloroacetic acid and 10 mM sodium pyrophosphate). The radioactivity was quantified by densitometric analysis of scanned images.

Data analysis. Data were analyzed using InStat statistical software (GraphPad). All results are expressed as means ± SE. One-way analysis of variance with Dunnett’s posttest was used to compare control versus treated groups. Differences between groups were considered statistically significant at P < 0.05.

RESULTS

H2O2 causes diastolic [Ca2+]i overload in NRVM. We (31) reported that exposure of spontaneously beating NRVM to low concentrations of H2O2 resulted in a sustained decrease in contractility. To determine whether this contractile dysfunction was associated with intracellular Ca2+ overload, we examined the effects of H2O2 on diastolic [Ca2+]i. Acute exposure to low levels of H2O2 caused a profound increase in diastolic [Ca2+]i during the washout period, similar to the Ca2+ overload observed during I/R. Compared with baseline, exposure to 50 μM H2O2 for 5 min led to a significant 1,210 ± 325 nM (from 74.8 ± 43.7 to 1,280 ± 314 nM) increase in diastolic [Ca2+]i, during the subsequent 10-min washout period (Fig. 1). NRVM responded to H2O2 with both a time- and dose-dependent increase in resting [Ca2+]i during washout. A significant alteration in Ca2+ overload was detected during washout after a 2.5-min exposure to 50 μM H2O2 (273 ± 97 nM, P < 0.05) and was maximum at 5 min (Fig. 1B). As shown in Fig. 1C, H2O2 elicited a concentration-dependent diastolic Ca2+ overload on washout that was observed at 10 μM, significant at 25 μM (584 ± 179 nM, P < 0.05) and maximum at 50 μM H2O2.

Involvement of NHE-1 and ERK1/2 MAPK in H2O2-induced diastolic Ca2+ overload. We have previously demonstrated that H2O2 activates NHE-1 in an ERK1/2 MAPK-dependent manner. To link these
events to H$_2$O$_2$-induced diastolic Ca$^{2+}$ overload, we examined the effects of the NHE-1 blocker HOE-642 and the ERK kinase (MEK) inhibitor U-0126 on H$_2$O$_2$-induced increases in resting [Ca$^{2+}$]$_i$ (Fig. 2A). The concentrations of the inhibitors used were based on preliminary experiments that determined the minimal effective concentration that blocked H$_2$O$_2$-induced NHE-1 activity and ERK1/2 activation, respectively (data not shown). Pretreatment with HOE-642 (5 μM) led to a 50% reduction (from 1,208 ± 132 to 639 ± 92 nM, P < 0.05) in H$_2$O$_2$-induced diastolic Ca$^{2+}$ overload (Fig. 2B). To confirm that this concentration of HOE-642 blocked NHE-1 activity, we examined its effects on H$_2$O$_2$-induced, Na$^+$-dependent recovery from an acid load in spontaneously beating NRVM. As shown in Fig. 3, NRVM treated with 50 μM H$_2$O$_2$ in the absence of pharmacological inhibitors exhibited complete recovery from an acid load. Treatment with HOE-642 completely abolished NHE-1 activity. These results are consistent with HOE-642 blunting H$_2$O$_2$-induced diastolic Ca$^{2+}$ overload secondary to NHE-1 inhibition.

We then examined the involvement of the ERK1/2 MAPK pathway in diastolic Ca$^{2+}$ overload and NHE-1 activation in response to H$_2$O$_2$. Treatment with the MEK inhibitor U-0126 (5 μM) resulted in an 88% decrease in the rise in diastolic Ca$^{2+}$ levels after H$_2$O$_2$ treatment. Similar to our previous study (31) with the MEK inhibitor PD-90859, U-0126 (5 μM) blocked
H₂O₂-stimulated recovery from an acid load (data not shown). U-0126 also caused a dose-dependent decrease in H₂O₂-stimulated ERK1/2 phosphorylation, with complete inhibition at concentrations ≥2.5 μM (Fig. 4A).

To gain insight into the mechanisms by which ERK1/2 MAPKs mediate the activation of NHE-1 by H₂O₂, we examined the ability of ERK1/2 to phosphorylate NHE-1 using in-gel kinase assays with the carboxytyplasmic tail of NHE-1 (amino acids 639–815) coupled to GST. Exposure to 100 μM H₂O₂ led to a significant increase in NHE-1 phosphorylation by proteins that correspond to ERK1 (44 kDa), ERK2 (42 kDa), and 90-kDa ribosomal S6 kinase (p90rsk) (Fig. 4B). Both NHE-1 phosphorylation and ERK1/2 activation by H₂O₂ were significantly blocked (~85%) by 5 μM U-0126. In a series of separate experiments, U-0126 also blocked H₂O₂-induced p90rsk phosphorylation (data not shown). On the other hand, the p38 MAPK inhibitor SB-203580 (10 μM), a concentration that completely inhibits H₂O₂-induced p38 MAPK activation (data not shown), was without effect. Together, these data show that ERK1/2 MAPK phosphorylate NHE-1 and activate NHE-1 activity in vitro.

**DISCUSSION**

It is widely accepted that reperfusion of ischemic myocardium leads to contractile dysfunction and injury. Although the pathogenesis of this injury is complex, Ca²⁺ overload, ROS, and the NHE-1 have been implicated as key mechanisms that are responsible for the deleterious effects of I/R. The goal of the present study was to further elucidate the mechanisms that link ROS to altered Ca²⁺ homeostasis in cardiac myocytes. Previously, our laboratory (31, 37) showed H₂O₂-induced activation of MAPKs, increased NHE-1 activity, and decreased contractility in NRVM and adult RVM. The results from the current study provide direct evidence that low concentrations of H₂O₂ caused a significant diastolic Ca²⁺ overload that was reduced by NHE-1 blockade and abolished by MEK inhibition. This is the first evidence that directly links low levels of H₂O₂ to increases in cardiomyocyte resting [Ca²⁺]ₐ through NHE-1 activation.

There are several functional alterations in Ca²⁺ homeostatic proteins that could contribute to the H₂O₂-induced Ca²⁺ overload. These mechanisms include increased Ca²⁺ influx through voltage-sensitive Ca²⁺ channels (either direct modulation of channel activity or indirect through inhibition of K⁺ channels and subsequent membrane depolarization), enhanced SR Ca²⁺ release, inhibition of Ca²⁺ uptake by the SR Ca²⁺ ATPase, or enhanced Na⁺/Ca²⁺ exchange through direct modulation of the NCX or indirect activation secondary to increased NHE-1 activity. Our data are most consistent with the indirect modulation of Na⁺/Ca²⁺ exchange secondary to NHE-1 activation. The following lines of evidence support this contention. First, we have previously shown that low concentrations of H₂O₂ increase NHE-1 activity in NRVM. Second, this study demonstrates that H₂O₂-induced Ca²⁺ overload was
significantly inhibited by NHE-1 blockade with HOE-642 at a concentration that completely blocks NHE-1 activity (Figs. 2 and 3). The results indicating that NHE-1 contributes to the Ca\(^{2+}\) overload that occurs in response to oxidative stress are in close agreement with previous studies (19, 38) that have demonstrated the crucial relationship between NHE activation, NCX modulation, and the development of cardiomyocyte Ca\(^{2+}\) overload. However, we cannot rule out a direct modulation of NCX by H\(_2\)O\(_2\) because HOE-642 did not completely block H\(_2\)O\(_2\)-induced Ca\(^{2+}\) overload at concentrations that completely inhibited H\(_2\)O\(_2\)-induced, Na\(^{+}\)-dependent recovery from an acid load. This possibility is supported by a recent report (33) demonstrating that direct activation of the reverse mode of NCX contributes to reoxygenation-induced cardiomyocyte injury and altered Ca\(^{2+}\) flux. In addition, a recent report (13) shows that high concentrations of H\(_2\)O\(_2\) activate the reverse mode of NCX in guinea pig ventricular myocytes. Taken together, these results suggest that H\(_2\)O\(_2\) causes diastolic Ca\(^{2+}\) overload by modulation of both NHE-1 and NCX.

Our results are in close agreement with the concept that specific NHE-1 inhibitors exert beneficial effects on recovery from I/R injuries and diminish post-I/R Ca\(^{2+}\) overload. Early studies (18, 27) demonstrated that amiloride or its derivatives reduce Na\(^{+}/Ca^{2+}\) loading and enhance postischemic myocardial recovery. These inhibitors, however, are known to affect other sarcolemmal proteins, including NCX (11). Newer, more specific NHE-1 inhibitors have been shown to reduce myocardial damage after I/R injury that is characterized by an improvement in the postischemic recovery of left ventricular developed pressure, decreases in creatine phosphate release, and a reduction in tissue Na\(^{+}\) and Ca\(^{2+}\) content (5, 16, 24, 40).

To elucidate the mechanisms by which H\(_2\)O\(_2\) regulates NHE-1 activity, we examined the effects of MEK inhibition with U-0126. We previously showed that activation of NHE-1 by H\(_2\)O\(_2\) was dependent on ERK1/2 MAPKs (31). Using in-gel kinase assays, we showed that U-0126 inhibited at least three kinases that were able to phosphorylate the NHE-1 COOH tail: ERK1, ERK2, and p90\(^{rsk}\) (Fig. 4B). Moor et al. (25, 26), Snabaitis et al. (34), and our laboratory (37) have shown an important role of ERK MAPKs in the regulation of NHE-1 activity in cardiovascular tissue in response to neurohormones, serum, and I/R. In addition to directly phosphorylating NHE-1, ERK1/2 MAPKs also participate in NHE-1 regulation indirectly through p90\(^{rsk}\) because U-0126 also blocked p90\(^{rsk}\)-dependent NHE-1 phosphorylation. The identification of p90\(^{rsk}\) as a NHE-1 kinase is similar to a report in vascular smooth muscle (28) and is in close agreement with recent data (26) that demonstrated NHE-1 phosphorylation by p90\(^{rsk}\) in response to myocardial I/R injury. In fact, a recent study (22) indicated that although recombinant NHE-1 was a substrate for ERK1/2 and p90\(^{rsk}\), the stoichiometry of phosphorylation observed for p90\(^{rsk}\) was greater than that for ERK1/2. p90\(^{rsk}\) has been shown to phosphorylate NHE-1 at Ser\(^{703}\) but the ERK1/2 site has not been identified (35). Therefore, it is likely that H\(_2\)O\(_2\)-stimulated NHE-1 activation involves phosphorylation by p90\(^{rsk}\) and/or ERK1/2 MAPK pathway.

Although H\(_2\)O\(_2\) activates p38 MAPK in NRVM, it is not a major regulator of NHE-1, because the p38 inhibitor SB-203580 had no effect on H\(_2\)O\(_2\)-induced NHE-1 phosphorylation. A recent report (12) ruled out the involvement of p38 MAPK in NHE-1 activation in a human fibroblast cell line in response to osmotic stress. In agreement, also found was a diminished or insignificant role of p38 in NRVM (26). In contrast, p38 was shown to be a negative regulator of NHE-1 in VSMC treated with angiotensin II (22). Another recent report (21) has shown that p38 can phosphorylate and activate the NHE-1 and induce alkalinization in some tissues, but these effects of p38 may be tissue specific or may vary with the isoform of p38 involved.

Our results also suggest that the ERK1/2 pathway makes a significant contribution to H\(_2\)O\(_2\)-induced diastolic Ca\(^{2+}\) overload independent of NHE-1 phosphorylation, because U-0126, but not HOE-642, completely inhibited H\(_2\)O\(_2\)-induced increases in resting Ca\(^{2+}\) levels (Fig. 2). This suggests that ERK1/2 can regulate other ion transporters independently of NHE-1. The possible targets for ERK1/2 that could contribute to the cytosolic Na\(^{+}\) and Ca\(^{2+}\) load include the Na\(^{+}/K\(^{+}\) -2Cl\(^{-}\) cotransporter, which is activated by \(\alpha_{1}\)-adrenergic agonist in an ERK1/2-dependent mechanism in cardiac myocytes (2), or the Na\(^{+}/\)HCO\(_{3}^{-}\) cotransporter, which is coupled to muscarinic receptor activation by ERK1/2 in renal epithelial cells (30). We cannot rule out the possibility that ERK1/2 can directly modulate NCX or Na\(^{+}/K\(^{+}\) pump activity that could also contribute to alterations in Ca\(^{2+}\) or Na\(^{+}\) homeostasis. However, there is no evidence that ERK1/2 MAPKs actually regulate NCX through phosphorylation. Moreover, H\(_2\)O\(_2\), albeit at high concentrations, actually increased NCX activity (13). On the basis of this study, one would predict that the MEK inhibitor U-0126 would actually...
increase intracellular Ca\textsuperscript{2+} levels by eliminating the increase in forward mode of NCX.

On the basis of our results, we propose that H\textsubscript{2}O\textsubscript{2}-induced Ca\textsuperscript{2+} overload is primarily mediated by ERK1/2 MAPKs and partially through the phosphorylation and activation of NHE-1 (Fig. 5). The subsequent rise in intracellular Na\textsuperscript{+} results in an inhibition of the forward mode and/or activation of reverse mode of NCX to ultimately cause a rise in intracellular Ca\textsuperscript{2+}. The diastolic Ca\textsuperscript{2+} overload may then contribute to the contractile dysfunction that is a hallmark of I/R injury. However, it is likely that other cellular processes participate in oxidative stress-induced myocardial contractile dysfunction. These mechanisms include alterations in excitation-contraction coupling, decreased myofilament sensitivity and/or responsiveness to Ca\textsuperscript{2+}, diminished mitochondrial function, and apoptosis. Further studies are needed to precisely define the signal transduction pathways that contribute to the altered cardiomyocyte function in response to ROS.

In conclusion, our results indicate that low levels of ROS cause a decrease in contractility and Ca\textsuperscript{2+} overload in NRVM. Therapies targeting ERK1/2 kinases and/or NHE-1 activation may ameliorate the alterations in Ca\textsuperscript{2+} homeostasis that contribute to myocardial tissue injury following I/R.

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