

Differential MAP kinase activation and Na⁺/H⁺ exchanger phosphorylation by H₂O₂ in rat cardiac myocytes

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Wei, Shan, Emily C. Rothstein, Larry Fliegel, Louis J. Dell'Italia, and Pamela A. Lucchesi. Differential MAP kinase activation and Na⁺/H⁺ exchanger phosphorylation by H₂O₂ in rat cardiac myocytes. *Am J Physiol Cell Physiol* 281: C1542–C1550, 2001.—Bursts in reactive oxygen species production are important mediators of contractile dysfunction during ischemia-reperfusion injury. Cellular mechanisms that mediate reactive oxygen species-induced changes in cardiac myocyte function have not been fully characterized. In the present study, H₂O₂ (50 μM) decreased contractility of adult rat ventricular myocytes. H₂O₂ caused a concentration- and time-dependent activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38, and c-Jun NH₂-terminal kinase (JNK) mitogen-activated protein (MAP) kinases in adult rat ventricular myocytes. H₂O₂ (50 μM) caused transient activation of ERK1/2 and p38 MAP kinase that was detected as early as 5 min, was maximal at 20 min (9.6 ± 1.2- and 9.0 ± 1.6-fold, respectively, vs. control), and returned to baseline at 60 min. JNK activation occurred more slowly (1.6 ± 0.2-fold vs. control at 60 min) but was sustained at 3.5 h. The protein kinase C inhibitor chelerythrine completely blocked JNK activation and reduced ERK1/2 and p38 activation. The tyrosine kinase inhibitors genistein and PP-2 blocked JNK, but not ERK1/2 and p38, activation. H₂O₂-induced Na⁺/H⁺ exchanger phosphorylation was blocked by the MAP kinase kinase inhibitor U-0126 (5 μM). These results demonstrate that H₂O₂-induced activation of MAP kinases may contribute to cardiac myocyte dysfunction during ischemia-reperfusion.

reactive oxygen species; extracellular signal-regulated kinase; c-Jun NH₂-terminal kinase; p38 mitogen-activated protein kinase

REPERFUSION OF AN ISCHEMIC myocardium leads to a burst in the production of reactive oxygen species (ROS) that is associated with contractile dysfunction, Ca²⁺ overload, and myocardial stunning (15). Ischemia-reperfusion injury also initiates activation of several signaling cascades, including mitogen-activated protein (MAP) kinases and tyrosine kinases such as Src (1, 7, 31, 38). However, the exact contribution of ROS to the activation of these signaling events remains unknown.

MAP kinases regulate a number of cellular events that are altered during the reperfusion of an ischemic myocardium, including phosphorylation of heat shock proteins 20 and 27 (13), regulation of apoptotic signaling cascades (see Ref. 37 for a recent review), enhanced gene expression (8), and phosphorylation of the Na⁺/H⁺ exchanger (NHE) to increase its activity (39). Activation of NHE is responsible for the postischemic restoration of intracellular pH (pH_i). The increased Na⁺ influx that arises from NHE activation leads to a secondary increase in intracellular Ca²⁺ levels via an indirect inhibition of Na⁺/Ca²⁺ exchange (12, 19, 40).

Three subfamilies of MAP kinases have been clearly identified in the heart: the extracellular signal-regulated kinases (ERK1/2), the c-Jun NH₂-terminal kinases (JNKs), and the p38 MAP kinases. We and others have investigated the ability of ROS to regulate MAP kinase signaling in primary cultured neonatal cardiac myocytes (3, 31). Physiological concentrations of H₂O₂ (5–100 μM) activated NHE1 in an ERK1/2-dependent manner in cultured neonatal cardiac myocytes (31). However, extrapolation of these results to adult cells is difficult, because there are marked phenotypic differences between neonatal and adult cardiac myocytes, including age-dependent changes in protein kinase C (PKC) expression (30), tyrosine kinase-dependent inhibition of L-type Ca²⁺ channels (20), and β-adrenergic receptor signaling (23). In addition, alterations in ERK signaling between neonatal and adult cardiac myocytes in response to angiotensin II have been reported (11, 32). Therefore, identification of the cellular signaling events involved in H₂O₂-induced MAP kinase activation in primary cultured adult rat ventricular myocytes (ARVM) is important for an understanding of in vivo changes in cardiac function during the ischemia-reperfusion injury.

Although several studies have demonstrated the effects of high doses of H₂O₂ on adult cardiac myocyte function (17, 18), little is known about the effect of low, physiological concentrations of H₂O₂ on intracellular signal transduction. The present study demonstrates a

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significant decrease in ARVM contractility by low doses of H₂O₂. Exposure of primary culture of ARVM to these low doses of H₂O₂ activates ERK1/2, p38 MAP kinase, and JNK members of the MAP kinase superfamily. Of these three MAP kinases, the ERK1/2 pathway mediated H₂O₂-induced phosphorylation of NHE1.

METHODS

Isolation of ARVM. ARVM were isolated from male Sprague-Dawley rats (250–350 g) by cardiac retrograde aortic perfusion, as previously described (35), with minor modifications. Briefly, rats were anesthetized with pentobarbital sodium (50 mg/kg ip). The hearts were rapidly dissected and perfused with Krebs buffer (mM: 118 NaCl, 4.7 KCl, 25 NaHCO₃, 1.8 CaCl₂, 1.2 Mg₂SO₄, 1.2 KH₂PO₄, and 11 glucose), Ca²⁺-free Krebs buffer, and Ca²⁺-free Krebs buffer containing 100 U/ml type II collagenase (Worthington). Ventricular tissue was minced and further digested in collagenase-BSA Krebs buffer containing 25 μM CaCl₂. The resulting cells were resuspended in perfusion buffer with 3% BSA, in which the concentration of CaCl₂ was increased in a stepwise manner to 1 mM. Cells were resuspended in medium 199 (M199) supplemented with 5 mM creatine, 2 mM L-carnitine, and 5 mM taurine and then plated on laminin-coated dishes at a density of 4 × 10⁵/100 mm. After 1 h, the medium was changed to remove loosely attached cells. Rod-shaped myocytes (>90%) were cultured for an additional 1 h before the start of the experiments.

Preparation of cell lysates for MAP kinase experiments. ARVM were incubated at 37°C in M199 containing H₂O₂, inhibitors, or vehicle for various times, lysed in ice-cold buffer containing 50 mM NaCl, 50 mM NaF, 50 mM sodium pyrophosphate, 5 mM EDTA, 5 mM EGTA, 2 mM Na₃VO₄, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM HEPES, pH 7.4, 0.1% Triton X-100, and 10 μg/ml leupeptin, and immediately frozen on ethanol-dry ice. Cell lysates were then thawed on ice, scraped, sonicated, and centrifuged at 10,000 g at 4°C for 30 min. Supernatants were used immediately or stored at -80°C. Protein concentrations were determined using a bicinchoninic acid protein assay (Pierce) according to the manufacturer's protocol.

Western blot analysis. Western blot analysis was performed as described elsewhere (31). Cell lysates (35 μg) were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The primary antibodies were anti-total ERK1 and ERK2 antibodies (1:5,000; Santa Cruz), antiactive ERK1/2 (1:3,000; Promega), anti-total p38 (1:1,000; New England Biolabs), and anti-phospho-p38 MAP kinase (1:2,000; New England Biolabs). Immunoreactive bands were visualized using enhanced chemiluminescence (Amersham). Autoradiograms exposed in the linear range of film density were scanned, and densitometric analysis was performed with NIH Image software.

JNK immune complex kinase assays. ARVM were lysed by the addition of RIPA buffer (150 mM NaCl, 1.5 mM MgCl₂, 10 mM NaF, 10 mM sodium pyrophosphate, 1 mM EGTA, 0.1 mM Na₃VO₄, 24 mM sodium deoxycholate, and 25 mM HEPES, pH 7.4, 1% Triton X-100, 0.1% SDS, and 10% glycerol). Cell lysates containing 600 μg of protein were incubated with 4 μg of anti-JNK1/SAPK antibody (UBI) overnight at 4°C. Lysates were then incubated with 40 μl of protein A-agarose beads for 90 min at 4°C, and the beads were collected by centrifugation. The beads were washed once with 500 μl of RIPA buffer, once with 500 μl of wash buffer A (0.5 mM LiCl and 20 mM Tris), once with wash buffer B (1 mM EDTA and 20 mM Tris), and once with kinase

buffer (10 mM MnCl₂, 1 mM dithiothreitol, and 20 mM Tris). Immunoprecipitates were resuspended in 10 μl of kinase buffer supplemented with 5 μM ATP, 5 mM MgCl₂, 10 μCi of [γ -³²P]ATP, and 4 μg of glutathione *S*-transferase (GST)-c-Jun(1–79) (Calbiochem). After incubation for 20 min at 20°C, the reaction was terminated by adding Laemmli sample buffer and heating at 100°C for 10 min. c-Jun was resolved by SDS-PAGE. Incorporation of ³²P into GST-c-Jun(1–79) was determined by densitometric analysis of the c-Jun band identified by autoradiography.

In-gel kinase assays. NHE1 kinase activity and MAP kinase activity were analyzed by the in-gel kinase assay as described previously (31). Cell lysates (35 μg) were fractionated by SDS-PAGE in a gel in which 0.15 mg/ml of NHE1 fusion protein (amino acids 501–815 of NHE1 coupled to GST) or 0.1 mg/ml of myelin basic protein (MBP) had been copolymerized. The phosphorylation assay was performed by placing the gel in 10 ml of buffer containing 50 μM ATP with 100 μCi of [γ -³²P]ATP and incubating it for 1 h at 30°C. The reaction was terminated by immersing the gel in fixative solution (5% trichloroacetic acid and 10 mM sodium pyrophosphate). The radioactivity was quantified by densitometric analysis of scanned images.

Measurement of single-cell contractility. Myocytes were cultured for 1–2 h in M199 on laminin-coated glass coverslips. The coverslip was placed in a perfusion chamber with internal stimulating platinum electrodes (Warner) and imaged using an inverted microscope (model IX50, Olympus) and charge-coupled device camera (Philips). Rod-shaped cells with no blebbing or spontaneous contractions were chosen for imaging. The chamber was perfused with Tyrode's basic salt solution for 2 min, and cells were paced at 2 Hz (3-ms duration). This equilibration period was followed by a 10-min perfusion with 50 μM H₂O₂ in Tyrode's basic salt solution and a 10-min washout period with continued stimulation throughout the experiment. Cell contractility was recorded with a video edge detection system (Crescent Electronics) and analyzed using the Digi-Med System Integrator (model 2001) and the Cell Length Analysis program (MicroMed). The average resting cell length was 123 ± 6 μm (*n* = 11). Percent contraction was defined as the change in cell length divided by the resting length and averaged 9.7 ± 0.4% in control cells at 2 Hz. Changes in contractile amplitude were expressed relative to the percent contraction of control cells (set at 100%).

Data analysis. Data were analyzed using InStat statistical software (Graphpad). Values are means ± SE. One-way analysis of variance with a Dunnett's post test was used to compare control with treated groups. Differences between groups were considered statistically significant at *P* < 0.05.

RESULTS

Low concentration of H₂O₂ elicited a significant decrease in ARVM contractility. A video edge detection system was used to monitor the effects of H₂O₂ on single ARVM contractility (Fig. 1). ARVM were paced at 2 Hz for a 2-min equilibration period. Exposure to 50 μM H₂O₂ for 10 min induced a significant, 35% decrease in the contraction amplitude of ARVM compared with control. This contractile impairment was sustained throughout the 10-min washout period.

Activation of ERK1/2, p38 MAP kinase, and JNK by low concentrations of H₂O₂. ARVM were exposed to H₂O₂ (5–200 μM) for 20 min. Activation of ERK1/2 was detected by Western blot analysis with antibodies that

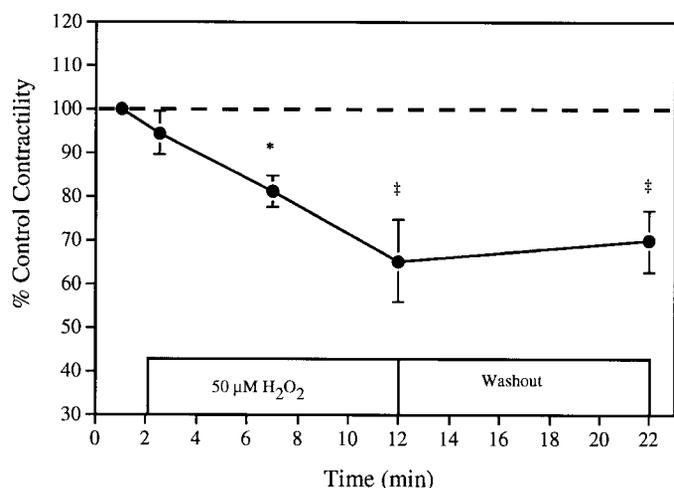


Fig. 1. Effect of H₂O₂ on adult rat ventricular myocyte contractility. Amplitude of contraction was measured using video edge detection. Cells stimulated at 2 Hz were perfused for 2 min with Tyrode's basic salt solution, exposed to 50 μ M H₂O₂ for 10 min, and subjected to a 10-min washout period. Values are means \pm SE from 5 experiments. Data are expressed relative to control values measured at the start of every experiment. * P < 0.05; ‡ P < 0.01 vs. control.

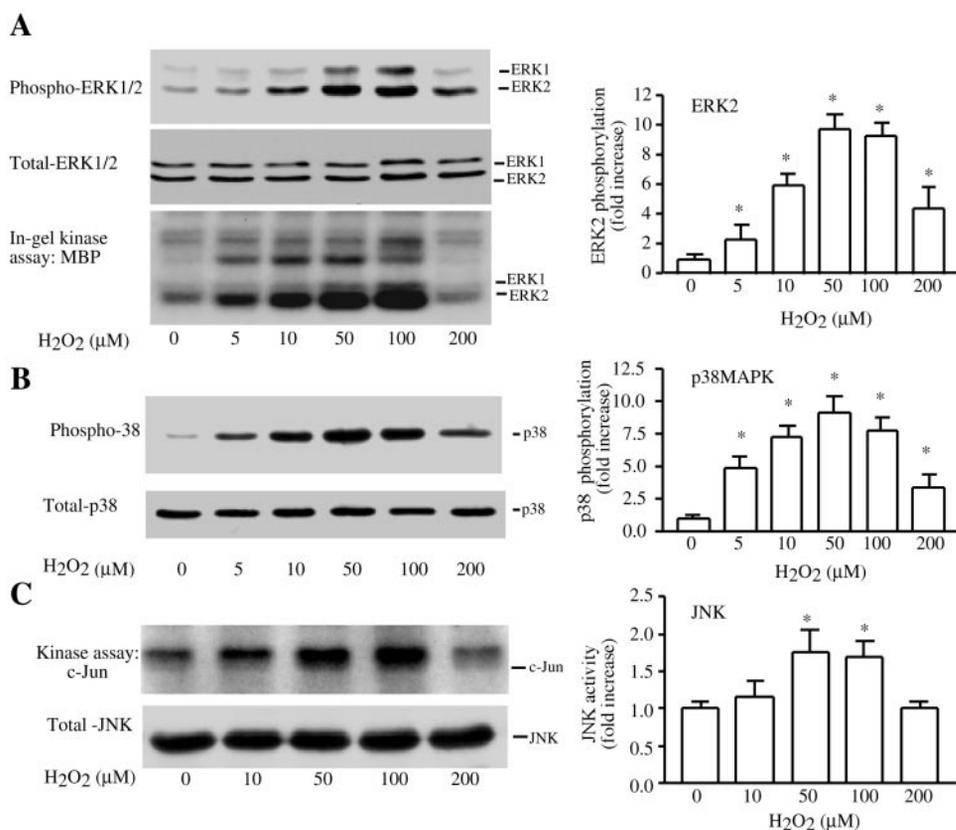
recognize phosphorylated, active ERK1/2. H₂O₂ caused a dose-response-dependent phosphorylation of ERK2 that was detected at 5 μ M H₂O₂ and was maximal at 50 μ M H₂O₂ (9.6 \pm 1.2-fold vs. control; Fig. 2A, top blot). H₂O₂ at \geq 200 μ M resulted in a decreased ERK1/2 activation. There was no difference in ERK1/2 expression as detected by Western blot analysis with

anti-total ERK1/2 antibodies (Fig. 2A, middle blot). ERK1/2 activation was confirmed using in-gel kinase assays with MBP as a substrate. Compared with control, treatment with H₂O₂ (5–200 μ M) resulted in a concentration-dependent increase in MBP phosphorylation by 42- and 44-kDa kinases, the molecular weights of ERK2 and ERK1, respectively (Fig. 2A, bottom blot). Thus both measures of ERK1/2 MAP kinase activation yielded similar results.

A similar concentration-dependent activation of p38 MAP kinases and JNK by H₂O₂ was observed (Fig. 2, B and C). For p38 MAP kinase, phosphorylation was detected at 5 μ M H₂O₂ and was maximal at 50 μ M H₂O₂ (9.0 \pm 1.6-fold vs. control). JNK activation was measured by immune complex kinase assays with GST-c-Jun as a substrate. Significant JNK activation was detected at 50 μ M H₂O₂ (2.0 \pm 0.3-fold vs. control), increased at 100 μ M H₂O₂, and declined at 200 μ M H₂O₂.

Time course for MAP kinase activation by H₂O₂. Exposure of ARVM to 50 μ M H₂O₂ resulted in a rapid but transient phosphorylation of ERK1/2 and p38 (Fig. 3, A and B). Activation of both MAP kinases was detected at 5 min and was maximal at 20 min (6.2 \pm 1.2-fold vs. control for ERK1/2 and 6.3 \pm 0.9-fold vs. control for p38) but returned to values close to baseline at 60 min. In contrast, significant JNK activation, as assessed by immune complex kinase assays, occurred much more slowly but was prolonged (Fig. 3C). Significant JNK activation by 50 μ M H₂O₂ did not occur until

Fig. 2. Concentration dependence of mitogen-activated protein (MAP) kinase (MAPK) activation by H₂O₂. Adult rat ventricular myocytes (ARVM) were treated with various concentrations of H₂O₂ for 20 or 60 min [c-Jun NH₂-terminal kinase (JNK)]. Cell lysates were size-fractionated by SDS-PAGE and subjected to Western blot analysis. Left, A: extracellular signal-regulated kinase (ERK) 1 and 2 activation was assessed with anti-active ERK1/2 antibodies (top blot) and by in-gel kinase assays with myelin basic protein (MBP) as a substrate (bottom blot). ERK1/2 expression was measured with anti-total ERK1/2 antibodies (middle blot). Left, B: p38 activation was assessed with anti-phospho-p38 (top blot) and p38 expression by blotting with anti-total p38 antibodies (bottom blot). Left, C: JNK activity was assessed by immune complex kinase assays with glutathione S-transferase (GST)-c-Jun as a substrate (top blot). JNK expression was detected using anti-JNK antibodies (bottom blot). Right: cumulative data from 3–5 experiments. * P < 0.05 vs. control.



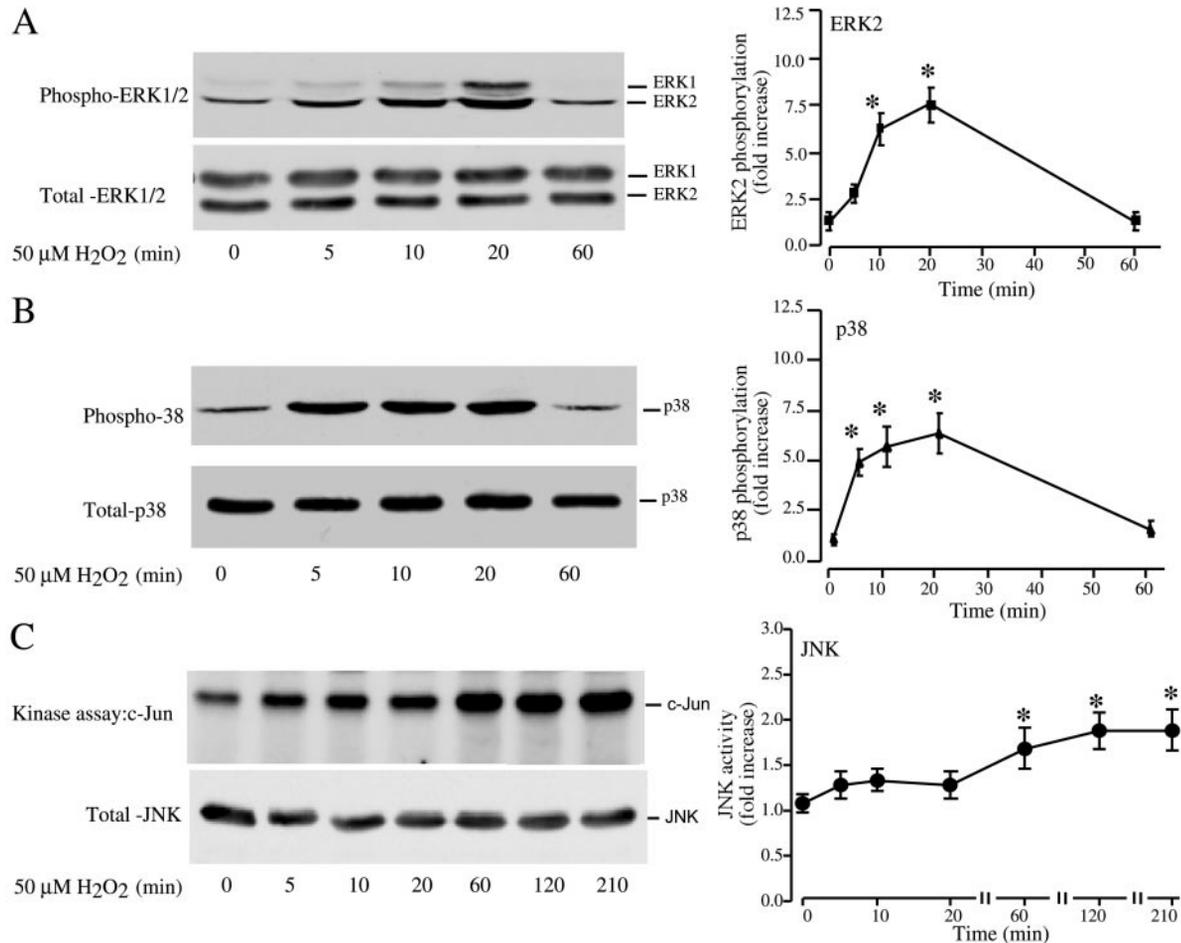


Fig. 3. Time course of MAP kinase activation by H₂O₂. ARVM were exposed to 50 μ M H₂O₂ for indicated times. *Left*: activation was determined by Western blot analysis with anti-active ERK1/2 (A) and anti-active p38 MAP kinase (B) antibodies or by immune complex kinase assays for JNK (C). MAP kinase expression was detected by Western blot analysis with anti-total MAP kinase antibodies. *Right*: cumulative data from 3–5 experiments. **P* < 0.05 vs. control.

60 min (1.7 ± 0.2 -fold vs. control) and was sustained at 3.5 h (1.8 ± 0.2 -fold vs. control).

To determine that H₂O₂ was the oxidant responsible for activation of MAP kinases, the ability of the H₂O₂ scavenger catalase to block the effect of H₂O₂ was studied. Pretreatment with catalase (400 U/ml) for 10 min completely abolished the activation of all three MAP kinases by 50 μ M H₂O₂ (Fig. 4). In contrast, pretreatment with the superoxide scavenger superoxide dismutase (1,000 U/ml) for 30 min failed to inhibit MAP kinase activation by H₂O₂ (Fig. 4).

To mimic the free radical burst that occurs during ischemia-reperfusion injury, we tested the effect of a brief exposure of ARVM to H₂O₂. ARVM were treated with 50 μ M H₂O₂ for 5 min. The medium was then replaced with M199, and ARVM were incubated for an additional 5, 15, and 55 min (205 min for JNK activation) in the absence of H₂O₂. Brief exposure to H₂O₂ was sufficient to cause a transient activation of ERK1/2 and p38 MAP kinase and a sustained activation of JNK (Fig. 5) that persisted after the removal of H₂O₂.

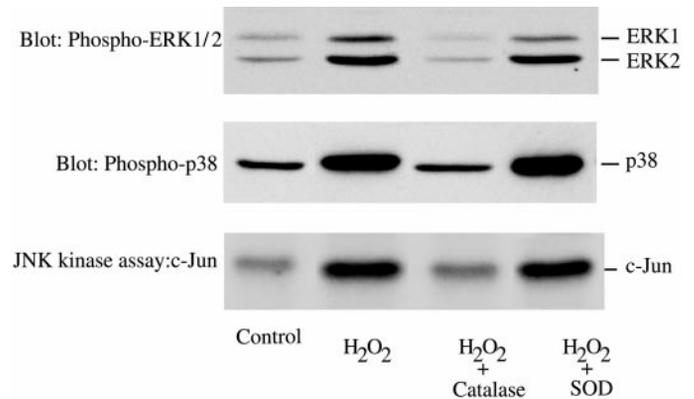


Fig. 4. Catalase, but not superoxide dismutase (SOD), blocks H₂O₂-induced MAP kinase activation. ARVM were pretreated with 400 U/ml of catalase for 10 min or 1,000 U/ml of SOD for 30 min and then exposed to 50 μ M H₂O₂ for 20 min. Activated MAP kinases were detected by Western blot analysis with anti-active ERK1/2 (*top*) and anti-active p38 MAP kinase (*middle*) antibodies or by immune complex kinase assays for JNK (*bottom*). Blots are representative of 3 experiments.

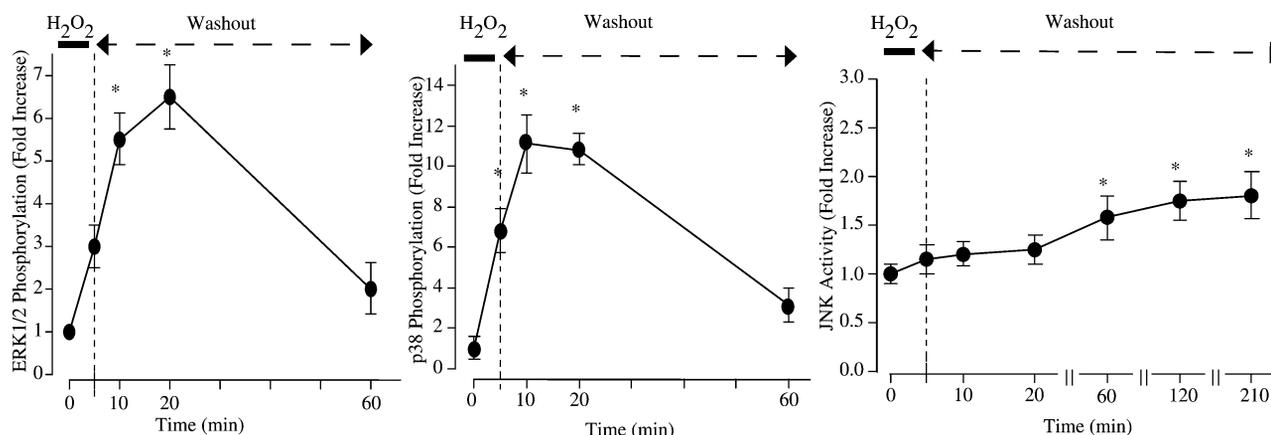


Fig. 5. Brief exposure to H₂O₂ results in prolonged MAP kinase activation. For experiments assessing the acute effects of H₂O₂, ARVM were treated with 50 μ M H₂O₂ for 5 min. Medium was replaced with fresh medium 199, cells were incubated for the indicated times, and MAP kinase activation was detected by Western blot analysis with anti-active ERK1/2 (*left*) and anti-active p38 MAP kinase (*middle*) antibodies or by immune complex kinase assays for JNK (*right*). Cumulative data from 4 experiments are presented. * $P < 0.05$ vs. control.

Role of tyrosine kinases and PKC in H₂O₂-induced MAP kinase activation. To determine whether one or more upstream tyrosine kinases were involved in the H₂O₂-induced MAP kinase activation in ARVM, cells were pretreated with the tyrosine kinase inhibitor genistein (100 μ M) or the selective Src family tyrosine kinase inhibitor PP-2 (10 μ M) for 45 min. Neither genistein nor PP-2 had an effect on the phosphorylation of ERK1/2 and p38 MAP kinase by 50 μ M H₂O₂ (Fig. 6, A and B). In contrast, genistein and PP-2 caused a 40 and 70% inhibition of H₂O₂-induced JNK activation, respectively (Fig. 6C). We next examined whether PKC was involved in the H₂O₂-induced MAP kinase activation in ARVM. Pretreatment with the PKC inhibitor chelerythrine (5 μ M, 45 min) caused a partial inhibition of H₂O₂-induced activation of ERK1/2 (50%) and p38 MAP kinase (45%) and a complete inhibition of JNK activation (Fig. 6).

ERK1/2-dependent NHE1 phosphorylation by H₂O₂. We previously showed that low doses of H₂O₂ cause a rapid, ERK1/2-dependent activation of NHE1 (31). The primary sequence of NHE1 contains several sites that are suitable phosphorylation sites for proline-directed MAP kinases (12). Because NHE1 activity is regulated by phosphorylation, we determined whether H₂O₂-induced MAP kinase activation mediates NHE1 phosphorylation in ARVM. In-gel kinase assays using a recombinant NHE1-GST fusion protein as a substrate were performed. H₂O₂ induced a concentration-dependent increase in NHE1 phosphorylation by two proteins that corresponded to ERK1 (44 kDa) and ERK2 (42 kDa; Fig. 7A). The time course of H₂O₂-induced MAP kinase-dependent NHE activation was nearly identical to that of H₂O₂-induced ERK1/2 phosphorylation (compare Fig. 7B with Fig. 3A). To confirm that the ERK pathway is a critical step in H₂O₂-mediated stimulation of NHE1, the effects of the specific MAP kinase kinase (MEK) inhibitor U-0126 and the p38 MAP kinase inhibitor SB-203580 were studied. Pretreatment with U-0126 (5 μ M, 45 min) completely

abolished the ERK1/2-dependent phosphorylation of NHE1 induced by 50 μ M H₂O₂, whereas pretreatment with SB-203580 (10 μ M, 45 min) had no effect (Fig. 7C). U-0126 completely inhibited H₂O₂-induced ERK1/2 activation (data not shown).

DISCUSSION

The most important findings of the present study are that low physiological concentrations of H₂O₂ result in activation of multiple MAP kinases in adult ventricular myocytes that are associated with altered contractile function and NHE phosphorylation. The H₂O₂ concentrations (5–100 μ M) are similar to those observed during a burst in free radical production that occurs on reperfusion of an ischemic myocardium (15). However, ≥ 200 μ M H₂O₂ actually decreased MAP kinase activity, suggesting that these higher concentrations may exert cytotoxic effects. H₂O₂ induced a rapid but transient phosphorylation in ERK1/2 and p38 MAP kinase in ARVM but a slower, more sustained JNK activation. These MAP kinase activations required only a brief exposure to H₂O₂.

The physiological roles of MAP kinase activation during ischemia-reperfusion injury in the intact myocardium are somewhat controversial. For example, ischemia-reperfusion elicited an increase in ERK1/2 activation in conscious rabbits (27), isolated guinea pig hearts (39), and isolated rat hearts (21). In contrast, Bogoyevitch et al. (6) reported that 10–20 min of ischemia with or without reperfusion failed to activate ERK1/2. However, Ping et al. (27) examined MAP kinase activity in the nuclear and cytosolic fractions of heart tissue and found a significant elevation of ERK1/2 only in the nuclear compartment. On the other hand, other groups determined MAP kinase activity only on the cytosolic fraction (6, 7, 21, 39), and in the study by Bogoyevitch et al., ERK activity was measured in the cytosolic fraction after fast protein liquid chromatography on Mono Q. Thus the conflicting re-

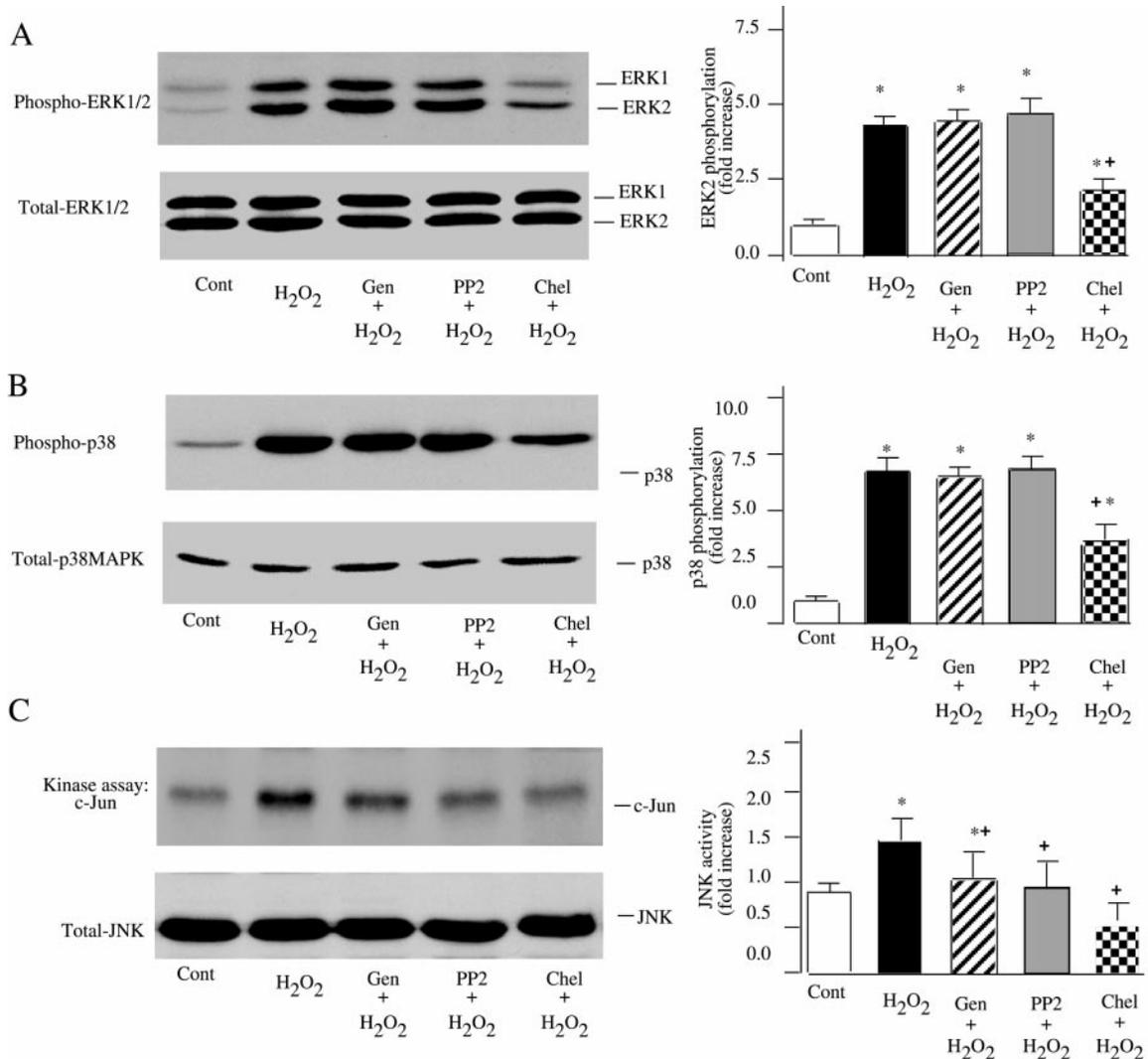


Fig. 6. Effects of tyrosine kinase and protein kinase C (PKC) inhibitors on H₂O₂-induced MAP kinase activation. ARVM were pretreated with 100 μ M genistein (Gen), 10 μ M PP-2, or 5 μ M chelerythrine (Chel) for 45 min before the addition of 50 μ M H₂O₂ for 20 min (60 min in JNK activation). *Left*: activation was determined by Western blot analysis with anti-active ERK1/2 (A) and anti-active p38 MAP kinase (B) antibodies or by immune complex kinase assays for JNK (C). MAP kinase expression was detected by Western blot analysis with anti-total MAP kinase antibodies. *Right*: cumulative data from 3–5 experiments. * P < 0.05 vs. control; + P < 0.05 vs. H₂O₂.

sults may be attributed to species-related differences or variation in the preparation of myocardium samples. In contrast, our results provide direct evidence that ROS directly activate ERK1/2 MAP kinases in cardiac myocytes.

Our results also demonstrate that low concentrations of H₂O₂ induced a rapid but transient activation of p38 MAP kinase and a slower, sustained activation of JNK in ARVM. These data are in agreement with previous studies by Ping et al. (28), who reported a sustained activation of JNK, but only a transient activation of p38 MAP kinase, triggered by ischemic preconditioning in the hearts of conscious rabbits. Schneider et al. (34) also reported a transient activation of p38 MAP kinase, whereas Bogoyevitch et al. (6) reported a sustained activation of p38 MAP kinase in isolated rat hearts. Besides the differences in sample preparation for MAP kinase activity assays, these dif-

ferences may also reflect discrepancies between animal models or ischemic protocols. Our study is the first to demonstrate, on the cellular level, that H₂O₂ induces a rapid but transient activation of p38 in ARVM.

The activation of MAP kinase by ROS, which contribute to cardiac myocyte dysfunction, appears at first glance to be in contradiction to the well-documented MAP kinase activation by growth factors and neurohormones. However, recent studies suggested the importance of the ERK1/2 pathway for myocyte survival in ischemic myocardium on the basis of the results that pretreatment with the MEK1 inhibitor PD-98059 reduced the number of apoptotic cells on isolated rat hearts subjected to global ischemia (44) and H₂O₂-treated rat neonatal myocytes (2, 3). Our results do not exclude the possibility of protective roles of MAP kinase activation in ischemic myocardium. For example, it is possible that MAP kinases could regulate the

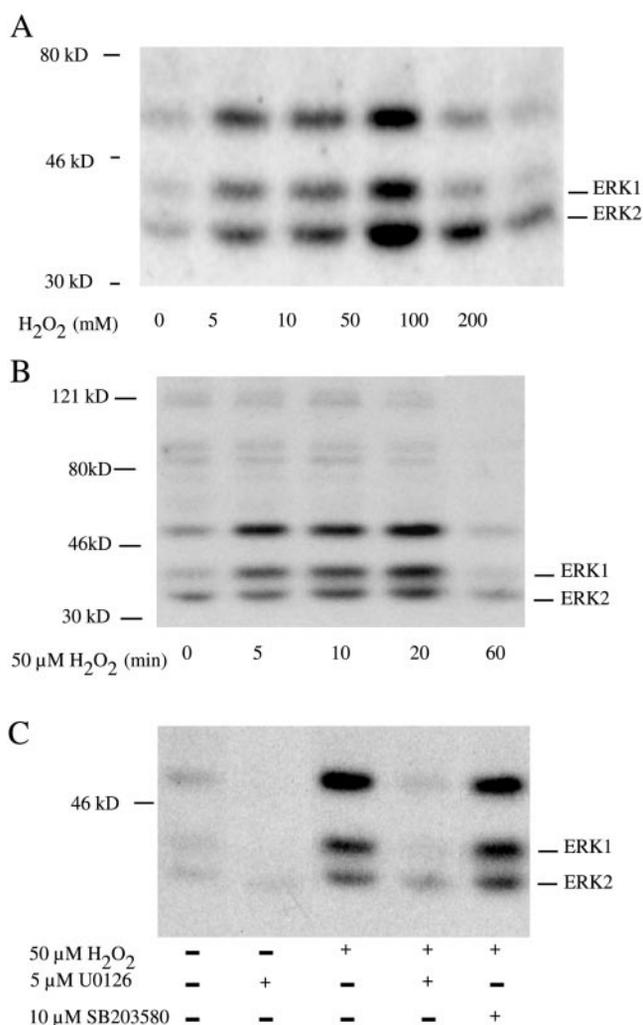


Fig. 7. ERK1/2-dependent phosphorylation of Na⁺/H⁺ exchanger isoform 1 (NHE1) by H₂O₂. ARVM were treated with H₂O₂, and NHE1 phosphorylation was determined by in-gel kinase assays using a carboxy tail of NHE1 coupled to GST as a substrate. *A*: concentration dependence for H₂O₂ was determined at 20 min. *B*: time course with 50 μM H₂O₂. *C*: effect of pretreatment with the MAPK kinase inhibitor U-0126 (5 μM, 45 min) or the p38 inhibitor SB-203580 (10 μM, 45 min) on H₂O₂-induced NHE1 phosphorylation.

expression and activation of heat shock proteins or the expression of genes that would prevent further myocardial damage (8, 13).

It is also possible that differences in the magnitude and duration of MAP kinase activation can result in different physiological responses. For example, transient activation of p38 and JNK by tumor necrosis factor- α is a survival signal, whereas persistent activation induces apoptosis (16, 29). Transient ERK1/2 activation leads to proliferation, whereas sustained activation mediates growth arrest (4, 5). In addition, the simultaneous activations of multiple MAP kinases may result in different responses due to cross talk. Kusuhashi et al. (22) showed that activation of p38 MAP kinase leads to decreased ERK1/2 signaling due to inhibition of MEK activity. Therefore, it is tempting to speculate that different stimuli, such as H₂O₂ and

phenylephrine, may elicit distinct functional responses by eliciting a specific temporal or spatial pattern of activation of a single MAP kinase pathway or by stimulating cross talk between multiple signaling pathways.

The activation of all three MAP kinases by H₂O₂ in ARVM was PKC dependent (Fig. 6). This is consistent with the finding that PKC activates MAP kinases in neonatal myocytes (24, 31, 41), isolated rat hearts (6, 14), and in vivo rabbit hearts (28). Activation of ERK1/2 and p38 by H₂O₂ in ARVM was not tyrosine kinase dependent (Fig. 6). In contrast, the tyrosine kinase inhibitor genistein and the Src inhibitor PP-2 partially inhibited the activation of JNK by H₂O₂. This is consistent with a study in adult rat myocytes that α -adrenoceptor stimulation of ERK1/2 was PKC dependent, but not genistein sensitive (33). Yoshizumi et al. (43) reported an Src- and Cas-mediated activation of JNK, but not ERK1/2 and p38 MAP kinase by H₂O₂ in smooth muscle cells and also showed that JNK activation by H₂O₂ was completely blocked in cells derived from *src*-deficient transgenic mice. Our data suggest a possible mechanism whereby tyrosine kinases are located upstream of JNK activation in response to H₂O₂, but not upstream of ERK1/2 and p38 MAP kinase activation. We previously showed that the activation of ERK1/2 by H₂O₂ is partly modulated by the activity of upstream tyrosine kinases and PKC (31) in neonatal rat ventricular myocytes. Thus a role for tyrosine kinases in H₂O₂-induced MAP kinase activation appears to exhibit age-related differences in cardiac myocytes. Interestingly, Katsube et al. (20) reported that inhibition of basal L-type Ca²⁺ channel currents by genistein in rat ventricular myocytes was greater in neonatal than in adult cells, suggesting the existence of age-related changes in the expression of tyrosine kinases or in their coupling to channel regulation.

Many studies have established a good correlation between myocardial stunning, ROS, and Ca²⁺ overload with ischemia-reperfusion injury. Early studies described a reversible depression of contractility during moderate ischemia-reperfusion, which was thought to be "cardioprotective," since it would serve to maintain myocardial integrity and viability during persistent ischemia (9). Changes in ATP concentration, pHi, or Ca²⁺ handling have been proposed as mechanisms responsible for acute, ischemic contractile dysfunction (15). The data from our study suggest that ROS may contribute to ischemia-reperfusion injury through an ERK1/2-dependent NHE1 phosphorylation (Fig. 7). The ability of the MEK inhibitor U-0126, but not the p38 inhibitor SB-203580, to abolish the H₂O₂-induced increase in NHE1 phosphorylation provides evidence that the ERK1/2, but not the p38, pathway is critical for H₂O₂-mediated stimulation of NHE1 in ARVM.

There is a large body of evidence to suggest that NHE1 activation during cardiac ischemia-reperfusion plays a major role in restoring pHi after an acid load. Phosphorylation of the exchanger is thought to be the primary mechanism responsible for increased NHE activity during ischemia-reperfusion injury (10). How-

ever, the increases in intracellular Na⁺ concentration resulting from enhanced exchanger activity can result in Ca²⁺ overload via the Na⁺/Ca²⁺ exchanger and actually exacerbate the damage to the myocardium (19). Moreover, Myers et al. (26) reported that the NHE inhibitor cariporide prevented H₂O₂-induced impairment of postischemic ventricular function. ERK1/2 (and the downstream kinase p90^{rsk}) regulate NHE activity by phosphorylation in the rat myocardium and in endothelin-stimulated rat neonatal ventricular myocytes (25). Snabaitis et al. (36) demonstrated a similar requirement for ERK1/2, but not p38, in an α_1 -adrenoceptor-mediated NHE activation in ARVM. On the other hand, Kusuvara et al. (22) reported that ERK1/2 and p38 phosphorylate NHE1 in response to angiotensin II in vascular smooth muscle, although p38-mediated phosphorylation was associated with decreased NHE activity. The underlying cellular basis for these discrepancies remains unclear but may be in part due to the differences in agonists used to stimulate NHE1 phosphorylation or in cell type-specific regulatory mechanisms.

In conclusion, the present study is the first to characterize, at the cellular level, the activation of multiple MAP kinases in adult cardiac myocytes by ROS at physiologically relevant concentrations. It is possible that the ROS- and ERK1/2-dependent NHE1 activation may contribute to the contractile dysfunction observed during reperfusion injury. Future studies with antisense or dominant-negative strategies are needed to elucidate the exact pathophysiological roles of each kinase using models of oxidant-induced myocardial injury.

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A preliminary report of these findings has been published in abstract form (42).

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