Oxidative Stress Activates Extracellular Signal–regulated Kinases through Src and Ras in Cultured Cardiac Myocytes of Neonatal Rats

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Abstract

A growing body of evidence has suggested that oxidative stress causes cardiac injuries during ischemia/reperfusion. Extracellular signal–regulated kinases (ERKs) have been reported to play pivotal roles in many aspects of cell functions and to be activated by oxidative stress in some types of cells. In this study, we examined oxidative stress–evoked signal transduction pathways leading to activation of ERKs in cultured cardiomyocytes of neonatal rats, and determined their role in oxidative stress–induced cardiomyocyte injuries. ERKs were transiently and concentration-dependently activated by hydrogen peroxide (H_2O_2) in cardiac myocytes. A specific tyrosine kinase inhibitor, genistein, suppressed H_2O_2–induced ERK activation, while inhibitors of protein kinase A and C or Ca^{2+} chelators had no effects on the activation. When CSK, a negative regulator of Src family tyrosine kinases, or dominant-negative mutant of Ras or of Raf-1 kinase was overexpressed, activation of transfected ERK2 by H_2O_2 was abolished. The treatment with H_2O_2 increased the number of cells stained positive by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling, and induced formation of DNA ladder and activation of CPP32, suggesting that H_2O_2 induced apoptosis of cardiac myocytes. When H_2O_2–induced activation of ERKs was selectively inhibited by PD98059, the number of cardiac myocytes which showed apoptotic death was increased. These results suggest that Src family tyrosine kinases, Ras and Raf-1 are critical for ERK activation by hydroxyl radicals and that activation of ERKs may play an important role in protecting cardiac myocytes from apoptotic death following oxidative stress. (*J. Clin. Invest.* 1997. 100:1813–1821.) Key words: oxidative stress • ERK • p38MAPK • apoptosis • cardiomyocytes

Introduction

Many studies have suggested that both neutrophils and reactive oxygen intermediates (ROI)[1] play important roles in ischemia/reperfusion–induced cardiac abnormalities (1–4). Low levels of ROI are regularly produced during a process of physiological metabolism, and every cell contains several enzymes such as catalase, glutathione peroxidase, and superoxide dismutase, which scavenge ROI from the cell. High levels of ROI are generated from a variety of sources such as the xanthine oxidase system (1), the leakage of electrons from mitochondria (2), the cyclooxygenase pathway of arachidonic acid metabolism (3), and the respiratory burst of phagocytic cells (4), and induce a variety of tissue damages (5). In the heart, it has been reported recently that ROI evoke a variety of abnormalities including cytotoxicity (5), cardiac stunning (6), arrhythmia (7), reduction of the Ca^{2+} transient and contractility (8), elevated diastolic Ca^{2+} levels (8), and intracellular ATP depletion (9). Administration of oxygen free-radical scavengers such as superoxide dismutase and catalase results in a significant decrease in infarct size in canines after 90 min of coronary artery occlusion (10). Furthermore, Horwitz et al. showed that *N*-(2-mercaptopropionyl)-glycine, an endogenous antioxidant, markedly reduced cytotoxicity caused by hydrogen peroxide in cultured cardiac myocytes (11). However, the molecular mechanism by which ROI induce cardiac injuries remains largely unknown.

The mitogen-activated protein kinases (MAPKs) are serine/threonine protein kinases, which play pivotal roles in a variety of cell functions in many cell types (12, 13). Three subfamilies of MAPKs consisting of extracellular signal–regulated kinase (ERK), c-Jun NH_2-terminal protein kinase (JNK) and p38-MAPK, have been isolated to date in mammals. They are regulated by three distinctive signal transduction pathways and show different functions (14). ERKs are activated by a variety of growth factors, cytokines and phorbol esters, and play pivotal roles in proliferation and differentiation in many types of cells (12, 13). In cardiac myocytes, the activation of ERKs has been reported to be critical for the development of the morphological feature of hypertrophy and specific gene expression (15, 16). Stimulation of receptor tyrosine kinases often activates the Raf-1-MAPK/ERK kinase (MEK)-ERK cascade through Ras (17). It has been reported that angiotensin II (Ang II) activates Src family tyrosine kinases and Ras in cardiac myocytes and smooth muscle cells through G protein–coupled Ang II type 1 receptor (18, 19). Activation of Src fam-

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1. *Abbreviations used in this paper:* Ang II, angiotensin II; Cal.C, Calphostin C; CSK, C-terminal Src kinase; CSK^-, kinase negative mutant of CSK; D.N.Raf-1, dominant negative mutant of Raf-1; D.N.Ras, dominant negative mutant of Ras; ECL, enhanced chemiluminescence; ERK, extracellular signal–regulated kinase; HA, hemagglutinin; JNK, c-Jun NH_2-terminal protein kinase; MAPKs, mitogen-activated protein kinases; MBP, myelin basic protein; PKA, protein kinase A; ROI, reactive oxygen intermediates; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TUNEL, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling.

ily tyrosine kinases and Ras is required for activation of ERKs in smooth muscle cells (18); however, in cardiac myocytes, protein kinase C (PKC), but not Src family or Ras, is critical for ERK activation (20). Therefore, the signal transduction pathways leading to activation of ERKs may be different among cell types. JNK and p38MAPK are not activated efficiently by growth factors and phorbol esters but markedly activated by proinflammatory cytokine tumor necrosis factor–α, ultraviolet irradiation, and cellular stresses including heat shock, and osmotic stress (21–24). In cardiac myocytes, JNK is activated by ischemia/reperfusion (25), mechanical stretch (26), and Ang II (27). Functions of JNK/p38MAPK are different from those of ERKs. While activation of ERKs functions to protect cells from a variety of cellular stresses, activation of JNK and p38MAPK induce apoptosis (28–30). In this study, we show that oxygen-derived free radicals induce activation of ERKs and p38MAPK in cultured cardiac myocytes and that Src family tyrosine kinases and Ras are essential for H_2O_2–induced ERK activation. In addition, H_2O_2 induces apoptotic death of cardiac myocytes, and selective inhibition of ERK activation further increases the number of apoptotic cells.

Methods

Materials. [γ-^{32}P]ATP was purchased from Du Pont-New England Nuclear Co. (Boston, MA). Dulbecco's modified Eagle's medium (DME), FBS, and genistein were from GIBCO BRL (Gaithersburg, MD). Calphostin C (Cal.C) was from Funakoshi (Tokyo, Japan). An anti-ERK antibody was provided by K. Tobe (University of Tokyo School of Medicine, Tokyo, Japan) (31). An anti-Flag monoclonal antibody was from Kodak Co. (New Haven, CT). An anti-hemagglutinin (HA) polyclonal antibody was from MBL (Japan) and an antiphospho-specific p38MAPK antibody was from New England Biolabs, Inc. (Beverly, MA). An anti-CPP32 monoclonal antibody was purchased from Transduction Laboratories, Inc. (Lexington, Kentucky). H_2O_2 was from Nacalai Tesque, Inc. (Tokyo, Japan). 12-*O*-tetradecanoylphorbol-13-acetate (TPA), myelin basic protein (MBP), and other reagents were from Sigma Chemical Co. (St. Louis, MO).

cDNA plasmids. HA-tagged ERK2 (HA-ERK2) was a kind gift from M. Karin (UCSD, La Jolla, CA) (32). Dominant negative mutant of Ras (D.N.Ras), dominant negative mutant of Raf-1 (D.N.Raf-1), C-terminal Src kinase (CSK) and kinase negative mutant of CSK (CSK$^-$) were provided by Y. Takai (Osaka University, Osaka, Japan) (33), T. Kadowaki (University of Tokyo, Tokyo, Japan) (34), and H. Sabe (Kyoto University, Kyoto, Japan) (35), respectively. The constructs of all expression plasmids have been described elsewhere (32–35). All plasmid DNA was prepared by using plasmid DNA preparation kits from QIAGEN (Hilden, Germany).

Cell culture. Primary cultures of cardiac myocytes were prepared from ventricles of 1-d-old Wistar rats as described previously (36) according to the method of Simpson and Savion (37). In brief, cardiomyocytes were plated at a field density of 1×10^5 cells per cm^2 on 35-mm culture dishes with 2 ml of culture medium (DME with 10% FBS). 24 h after seeding, the culture medium was changed to serum-free DME and cells were cultured for 48 h before stimulation.

Transfection. 24 h after plating the cells on culture dishes, DNA was transfected by the calcium phosphate method as described previously (38). For each dish, 2.5 μg of HA-ERK2 plasmid was cotransfected with 7.5 μg of control vector plasmid, D.N.Ras, D.N.Raf-1, CSK, or CSK$^-$ plasmid. After 15–20 h of transfection, the culture medium was removed, and the cells were washed with PBS twice and were maintained in serum-free DME for 48–72 h before treatment with H_2O_2 or other reagents.

Kinase assay of endogenous ERK. Endogenous ERK activity was measured by MBP assay (39). Briefly, after stimulation, cardiomyocytes were lysed with lysis buffer A (25-mM Tris-HCl, pH 7.4; 25-mM NaCl; 1-mM sodium orthovanadate; 10-mM sodium pyrophosphate; 10-nM okadiac acid; 0.5-mM EGTA; and 1-mM phenylmethylsulfonyl fluoride) and the lysates were incubated with anti-ERK polyclonal antibody (31) for 1 h at 4°C. After incubation, the immunecomplex was precipitated using protein A Sepharose, washed, resuspended in 25 μl of the kinase buffer (25-mM Tris-HCl, pH 7.4; 10-mM $MgCl_2$; 1-mM DTT; 40-μM ATP; 2-μCi [γ-^{32}P]ATP; 2-μM protein kinase inhibitor; and 0.5-mM EGTA) and incubated with 25-μg MBP as a substrate at 25°C for 10 min. After incubation, the reaction was terminated by adding the Laemmli sample buffer (0.002% bromophenol blue, 10-mM sodium phosphate buffer, pH 7.0; 10% glycerol; 0.4% SDS; and 1% 2-mercaptoethanol) to the samples, and the samples were boiled for 5 min. The supernatants were subjected to SDS-PAGE and the gel was dried and subjected to autoradiography.

Kinase assay of transfected HA-tagged ERK2. After stimulation, cardiomyocytes into which HA-ERK2 was transfected were lysed with the lysis buffer A and the lysates were incubated with anti-HA polyclonal antibody for 1 h at 4°C. After incubation, the immunecomplex was precipitated using protein A Sepharose, resuspended in 25 μl of the kinase buffer and incubated with 25-μg MBP as a substrate at 25°C for 10 min. After incubation, the reaction was terminated by adding the Laemmli sample buffer to the samples. The supernatants were subjected to SDS-PAGE, and the gel was dried and subjected to autoradiography.

Western blot analysis of phosphorylated p38MAPK. Protein extracts were subjected to Western blot analysis as described previously (40) using a polyclonal phosphorylated p38MAPK–specific antibody, which recognizes only activated p38MAPK that is phosphorylated at the 182 tyrosine residue. The anti–rabbit IgG conjugated with horseradish peroxidase was used as the second antibody and immune complexes were visualized using the enhanced chemiluminescence (ECL) detection kit (Boehringer Mannheim Biochemicals, Indianapolis, IN) according to the manufacturer's directions.

Assay of p38MAPK activity. After transfection of Flag-tagged p38MAPK and stimulation with 1-mM H_2O_2 for 15 min, cardiomyocytes were lysed with the lysis buffer A and the transfected p38 MAPK was immunoprecipitated with an anti-Flag monoclonal antibody. The immunecomplex was resuspended in the kinase buffer (25-mM Tris-HCl, pH 7.4; 10-mM $MgCl_2$; 1-mM DTT; 40-μM ATP; 2-μCi [γ-^{32}P]ATP; 2-μM protein kinase inhibitor; and 0.5-mM EGTA) and incubated with MBP as a substrate at 25°C for 10 min. The sample was subjected to SDS-PAGE, and the gel was dried and exposed to X-ray film.

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL). Cardiomyocytes plated on a cover glass were fixed with 4% paraformaldehyde solution for 30 min at room temperature. After rinse with PBS, the samples were first incubated with a monoclonal antibody against myosin heavy chain (MF-20) (41) for 1 h at 37°C and next with an anti–mouse IgG conjugated with Rhodamine for 1 h at room temperature. Next, 50-μl TUNEL reaction mixture containing both terminal deoxynucleotidyl transferase, which catalyzes polymerization of nucleotides of single to free 3′-OH DNA ends and FITC-dUTP was added on to each sample for 1 h at 37°C. These samples were analyzed by fluorescence microscopy.

Agarose gel electrophoresis for DNA fragmentation. Cells (4×10^5) were lysed in 0.2 ml of lysis buffer B (10-mM Tris-HCl, pH 7.4; 10-mM EDTA; and 0.5% Triton X-100) followed by incubation with 40-μg RNase (Boehringer Mannheim Biochemicals) for 1 h at 37°C and 100-μg proteinase K (Boehringer Mannheim Biochemicals) for 1 h at 37°C and only fragmented DNA was extracted. The pellet was resuspended in TE buffer (10-mM Tris-HCl, pH 8.0; 1-mM EDTA) and treated with DNase-free RNase (Boehringer Mannheim Biochemicals) for 1 h at 37°C. DNA was ethanol precipitated and finally resuspended in distilled water. The fragmented DNA was electrophoretically fractionated on 1.5% Agarose gel and stained with ethidium bromide as described previously (42).

Western blot analysis of CPP32. Protein extracts were subjected

to Western blot analysis using a monoclonal anti-CPP32 antibody as described previously (40). This antibody reacts with both p32 proenzyme and p17 active subunit of CPP32. The anti–mouse IgG conjugated with horseradish peroxidase was used as the second antibody and immune complexes were visualized using ECL according to the manufacturer's directions.

Statistics. Statistical comparison of the control group with treated groups was carried out using the paired-sample *t* test with *P* values corrected by the Bonferroni method. The accepted level of significance was $P < 0.05$.

Results

H_2O_2 activates ERKs in cardiac myocytes. To determine the effects of oxidative stress on cardiac myocytes, we first examined whether H_2O_2 activates ERKs in cultured cardiac myocytes of neonatal rats. When cardiac myocytes were exposed to 1-mM H_2O_2 for 10 min, ERKs were markedly activated (Fig. 1). When cardiomyocytes were pretreated for 20 min with 0.5% DMSO, a potent hydroxyl radical scavenging agent (43), activation of ERKs by H_2O_2 was strongly suppressed (Fig. 1). These results suggest that hydroxyl radicals derived from H_2O_2 are involved in activation of ERKs. H_2O_2 rapidly and transiently activated ERKs in cardiac myocytes (Fig. 2). The activity of ERKs increased from 6 min, peaked at 9 min and returned to the basal levels at 60 min after addition of 1-mM H_2O_2. Activation of ERKs by H_2O_2 was concentration dependent at the range of 10-μM to 1 mM (Fig. 3). A significant increase in ERK activities was detected from 10 μM of H_2O_2 and maximum activation was obtained by 1-mM H_2O_2.

H_2O_2–induced ERK activation is independent of PKC, protein kinase A (PKA) and Ca^{2+} in cardiac myocytes. We have reported recently that activation of PKC, PKA, or an increase in Ca^{2+} activate ERKs in cultured cardiac myocytes (15, 20, 38, 44). To elucidate how hydroxyl radicals activate ERKs, we examined the role of PKC, PKA, and Ca^{2+} in H_2O_2–induced ERK activation using specific inhibitors and chelators (Table I). When cardiac myocytes were pretreated with 10^{-7}-M TPA for 24 h or with a PKC-specific inhibitor, Cal.C (10^{-6} M) (20) for 60 min, 10^{-7} M TPA did not activate ERKs in cardiac myocytes (44, data not shown). However, either treatment did not affect H_2O_2–induced ERK activation (Fig. 4), suggesting that PKC is not involved in H_2O_2–induced ERK activation. Inhibition of PKA with 10^{-4}-M RpcAMP(45) for 10 min or chelation of intracellular or extracellular Ca^{2+} with 4×10^{-5} M BAPTA

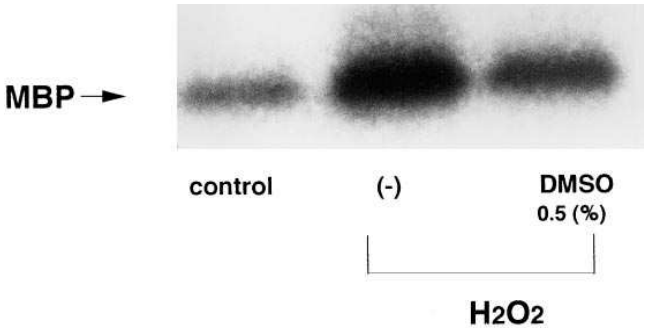

Figure 1. H_2O_2–induced ERK activation in cardiac myocytes. Cultured cardiac myocytes of neonatal rats were stimulated with 1-mM H_2O_2 for 10 min and ERK activities were determined by the immune complex kinase assay. ERKs were immunoprecipitated using a polyclonal anti-ERK antibody and kinase activities were assayed using bovine brain MBP as a substrate. After electrophoresis on SDS-polyacrylamide gels, the gel was dried and subjected to autoradiography. DMSO (0.5%) was added to cultured cardiomyocytes 20 min before the stimulation with H_2O_2.

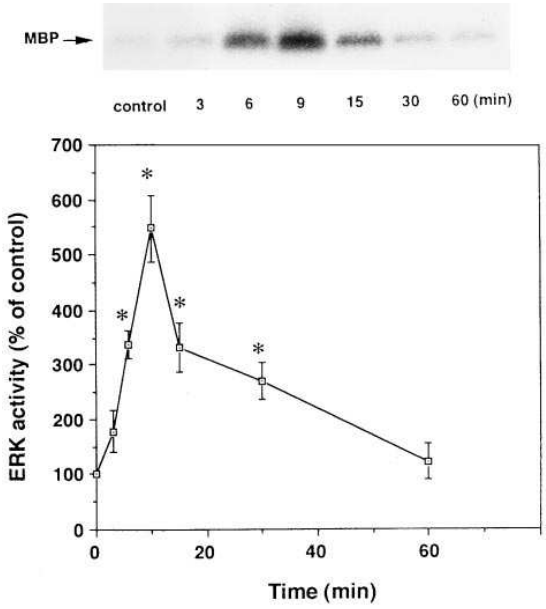

Figure 2. Time course of ERK activation by H_2O_2. Cardiac myocytes were treated with 1-mM H_2O_2 for 3–60 min. ERKs were immunoprecipitated from cell lysates using a polyclonal anti-ERK antibody, and kinase activities were assayed using MBP as a substrate. Intensities of the 28-kD MBP band in the autoradiogram were measured by densitometric scanning. The data represent the average percentage of the controls (100%) from three independent experiments (mean±SEM). $*P < 0.05$ versus control.

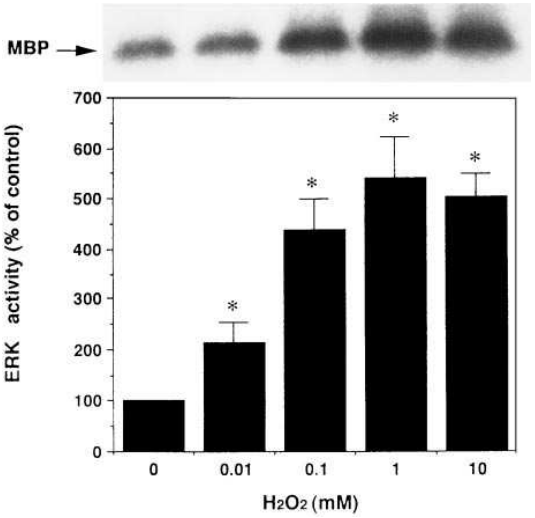

Figure 3. Dose-response of ERK activation by H_2O_2. Cardiac myocytes were exposed to various concentrations of H_2O_2 (0.01–10 mM) for 10 min. ERK activities were assessed as described in the Fig. 1 legend. Intensities of the 28-kD MBP band in the autoradiogram were measured by densitometric scanning. The data represent the average percentage of the controls (100%) from three independent experiments (mean±SEM). $*P < 0.05$ versus control.

Table I. Concentrations and Target Molecules of Various Inhibitory Agents Used in this Study

Drug	Concentration	Target molecule	Reference
EGTA	1 mM	Extracelluiar Ca^{2+}	(45)
BAPTA	10 μM	Intracellular Ca^{2+}	(46)
Rp-cAMPs	300 μM	PKA	(45)
Calphostin C	1 μM	PKC	(20)
TPA	100 nM	PKC	(44)
Genistein	2×10^{-5} M	Tyrosine kinases	(20)
Suramin	0.3 mM	Growth factors	(47)
PD98059	50 μM	MEK1/2	(48)

Cardiac myocytes were pretreated with 10^{-7}-M TPA for 24 h, with 10^{-6}-M Cal.C, 20-μM PD98059 for 60 min, with 1-mM suramin for 45 min, with 4×10^{-5}–M BAPTA, 5×10^{-3}–M EGTA, 2×10^{-5}–M genistein for 30 min, with 10^{-4}-M RpcAMP for 10 min, and then stimulated with H_2O_2 for 10 min. Activation of ERKs was determined by the immunecomplex kinase assay using MBP as a substrate. The molecules of which functions might be affected by the drugs are shown (*Target molecule*). PKC, protein kinase C; Rp-cAMPs, Rp-adenosine 3′, 5′-cyclic phosphorothioate; *MEK*, MAPK/ERK kinase.

(46) or 5×10^{-3} M EGTA (45), respectively, for 30 min did not also have any effects on H_2O_2–induced ERK activation (Fig. 4). These results suggest that H_2O_2 induces activation of ERKs not through PKC-, PKA-, or Ca^{2+}-dependent pathways in cardiac myocytes.

Tyrosine kinases are critical for H_2O_2–induced ERK activation. Many tyrosine kinases including receptor and nonreceptor type tyrosine kinases have been reported to activate ERKs in many cell types (12–14). To elucidate whether H_2O_2 activates ERKs in cardiomyocytes through tyrosine kinase-dependent pathways, we pretreated cardiomyocytes with genistein (2×10^{-5} M for 30 min), a specific inhibitor of tyrosine kinases (20). The activation of ERKs by H_2O_2 was completely inhibited by the genistein pretreatment (Fig. 5), suggesting that H_2O_2 activates ERKs through tyrosine kinase-dependent pathways. We next examined the involvement of growth factor receptors in the activation of ERKs using suramin. Suramin is known to block ligand-receptor interactions and can inhibit

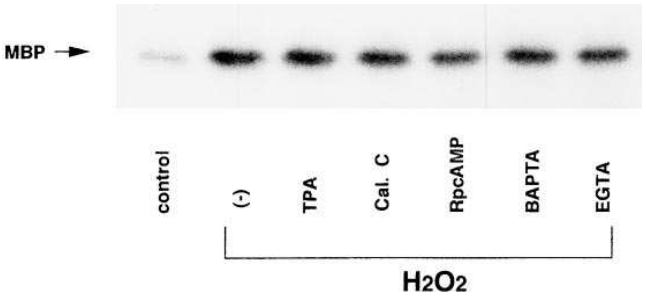

Figure 4. Role of PKC, PKA, and Ca^{2+} in H_2O_2–induced ERK activation. Cardiac myocytes were preincubated with 10^{-6} M Cal.C for 60 min, 10^{-4}-M RpcAMP for 10 min, 4×10^{-5} M BAPTA-AM or 5×10^{-3} M EGTA for 30 min or with 10^{-7} M TPA for 24 h. After stimulation with 1-mM H_2O_2 for 10 min, ERK activities were assayed as described in the Fig. 1 legend.

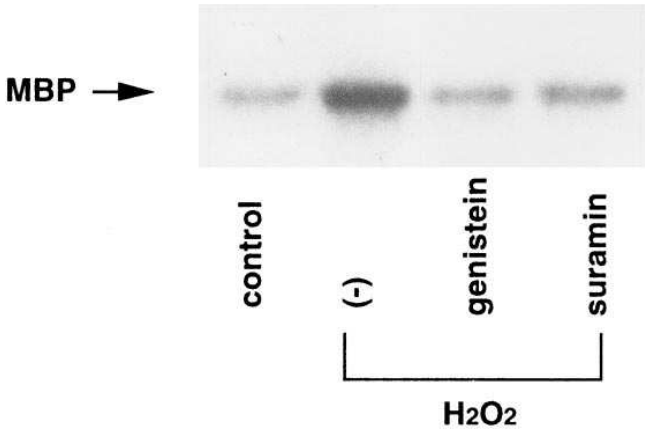

Figure 5. Role of tyrosine kinases and growth factors in H_2O_2–induced ERK activation. After pretreatment with 2×10^{-5}-M genistein for 30 min or 1×10^{-3}-M suramin for 60 min, cardiac myocytes were exposed to 1-mM H_2O_2 for 10 min. ERK activities were assayed as described in the Fig. 1 legend.

ERK activation by growth factors such as epidermal growth factor (47). Pretreatment of cardiac myocytes with 1-mM suramin for 45 min blocked H_2O_2–induced activation of ERKs (Fig. 5). These results suggest that some growth factors may be involved in H_2O_2–induced activation of ERKs by binding and activating receptor tyrosine kinases in cardiac myocytes.

Src family tyrosine kinases are involved in H_2O_2–induced ERK activation. Since among nonreceptor tyrosine kinases, Src family tyrosine kinases have been reported to activate ERKs (12–14), we examined the role of Src family tyrosine kinases in H_2O_2–evoked ERK activation using a negative regulator, CSK. CSK has been reported to phosphorylate the carboxyl tyrosine residue of Src family tyrosine kinases and inhibit their functions (35). We cotransfected CSK with HA-ERK2 into cultured cardiac myocytes and examined the activity of transfected ERK2 after incubation with 1-mM H_2O_2 for 10 min. Although CSK^- had no significant effects, ~ 70% of H_2O_2–induced increase in the ERK2 activity was suppressed by overexpression

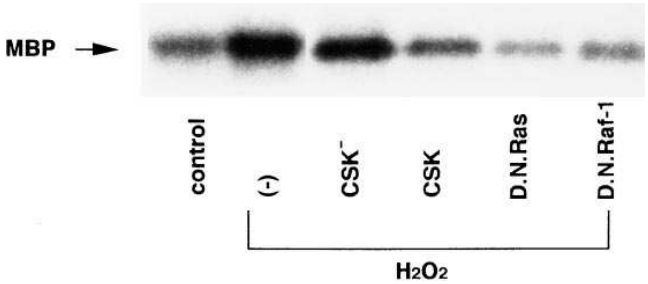

Figure 6. CSK, D.N.Ras, and D.N.Raf-1 suppress HA-ERK2 activation by H_2O_2. 12 h after transfection of HA-ERK2 with either CSK^-, CSK, D.N.Ras, or D.N.Raf-1, cardiac myocytes were washed with PBS twice and maintained in serum-free media for 2 d, and then treated with 1-mM H_2O_2 for 10 min. HA-ERK2 was immunoprecipitated from cell lysates using an polyclonal antibody against HA, and kinase activities were assayed using MBP as a substrate as described in the Fig. 1 legend.

of CSK (Fig. 6), suggesting that Src family tyrosine kinases are involved in H_2O_2–induced ERK activation.

H_2O_2–induced ERK activation is dependent on Ras and Raf-1 in cardiac myocytes. Many lines of evidence have implicated that Ras is a key signaling molecule in a variety of cell functions (13, 17) and that tyrosine kinases often activate ERKs through Ras (13, 18, 19). To determine whether Ras is involved in H_2O_2–induced ERK activation in cardiac myocytes, D.N.Ras was cotransfected into cultured cardiac myocytes with HA-ERK2. Overexpression of D.N.Ras completely blocked ERK2 activation by H_2O_2 (Fig. 6), suggesting that Ras is required for H_2O_2–induced activation of ERK2. ERKs are activated by the dual serine/threonine protein kinase, MEK, and MEK is in turn activated by serine/threonine kinases including Raf-1 (17). We thus examined whether Raf-1 is required for H_2O_2–induced ERK activation. Activation of the transfected ERK2 by H_2O_2 was suppressed completely by overexpression of D.N.Raf-1 in cardiac myocytes, suggesting that the activation of Raf-1 is a critical step to stimulate ERKs by H_2O_2.

H_2O_2 activates p38MAPK in cardiac myocytes. p38MAPK is another member of MAPK family and is activated by phosphorylation in response to the proinflammatory cytokine tumor necrosis factor-α and various cellular stresses such as ultraviolet irradiation and osmotic stress (21–24). We examined whether oxidative stress activates p38MAPK in cardiac myocytes. Cultured cardiac myocytes were treated with 1-mM H_2O_2 for 5–30 min and the activation of p38MAPK was assessed by Western blot analysis using a phosphorylated p38MAPK–specific antibody. Levels of phosphorylated p38MAPK increased from 5 min and peaked at 15 min after exposure to H_2O_2. Increased levels of phosphorylated p38MAPK remained over 30 min and returned to the basal levels at 60 min after exposure to H_2O_2 (Fig. 7 *A*). The activity of p38MAPK was directly measured using MBP as a substrate. Flag-tagged p38MAPK was transfected into cultured cardiac myocytes and immunoprecipitated using an anti-Flag monoclonal antibody after incubation for 15 min with 1-mM H_2O_2. The treatment with H_2O_2 for 15 min definitely activated p38MAPK (Fig. 7 *B*). Another member of MAPK family, JNK, was also activated by H_2O_2 in cultured cardiac myocytes (data not shown).

ERK activation protects cardiac myocytes from apoptosis following H_2O_2 exposure. Finally, to elucidate the role of ERKs in mediating cellular responses to oxidative stress, we examined apoptotic death of cardiac myocytes following H_2O_2 exposure in the presence or absence of a MEK inhibitor, PD98059. PD98059 has been reported to specifically inhibit MEK1 and MEK2, specific activators for ERKs but not for p38MAPK or JNK (48). When cardiac myocytes were treated with PD98059 for 60 min, H_2O_2–induced ERK activation was suppressed in cardiac myocytes (Fig. 8 *A*), while H_2O_2–induced p38MAPK activation was not affected by PD98059 (Fig. 8 *B*). PD98059 itself did not affect activities of ERKs or p38 MAPK (Fig. 8, *A* and *B*). When nuclei of cardiac myocytes were stained by the TUNEL method, few cardiac myocytes (< 5%) were positive in untreated cultures (Fig. 9, *A* and *B*, and Fig. 10). After incubation with 100-μM H_2O_2 for 48 h, the number of TUNEL-positive cardiac myocytes was increased (~ 23%)

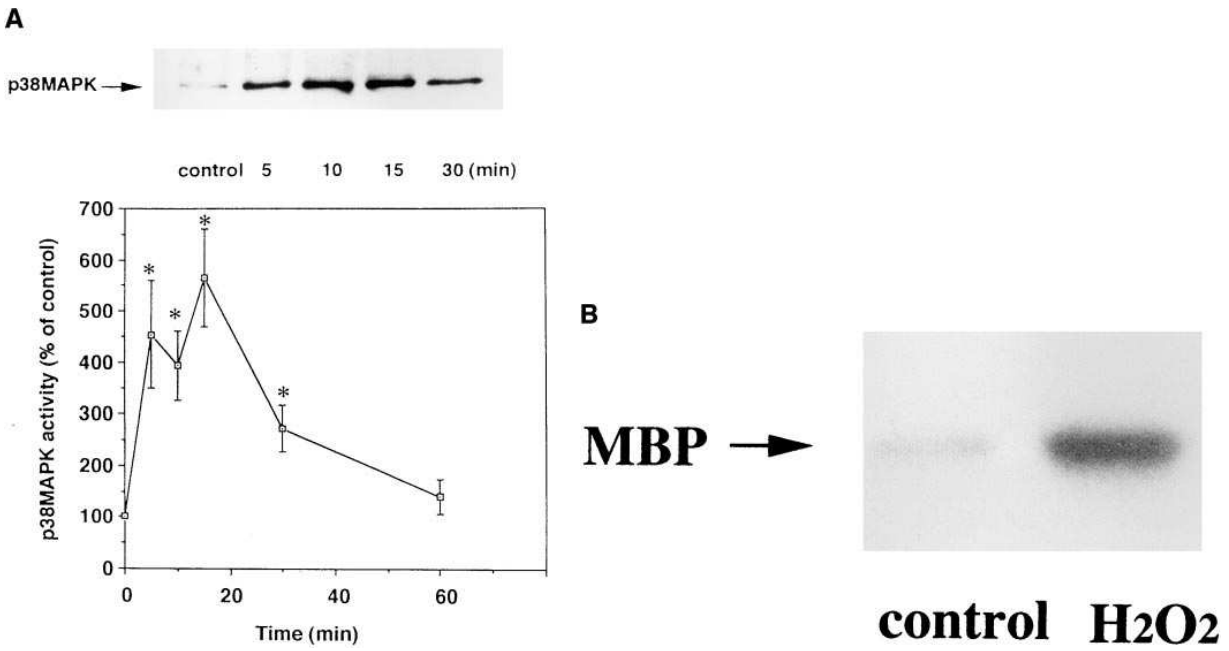

Figure 7. Time course of p38MAPK phosphorylation by H_2O_2. (*A*) Cardiomyocytes were incubated with 1-mM H_2O_2 for various periods of time. Cell lysates were subjected to SDS-PAGE and Western blot analysis was performed using phosphorylated p38MAPK–specific antibodies. The blot was developed by ECL as described in Methods. Intensities of the 38-kD bands in the autoradiogram were measured by densitometric scanning. The data represent the average percentage of the controls (100%) from three independent experiments (mean±SEM). $*P < 0.05$ versus control. (*B*) 12 h after transfection of Flag-tagged p38MAPK, cardiac myocytes were washed with PBS twice and maintained in serum-free media for 2 d, and then treated with 1-mM H_2O_2 for 15 min. Transfected p38MAPK was immunoprecipitated using an anti-Flag monoclonal antibody and the activity of p38MAPK was measured by using MBP as a substrate. Three independent experiments revealed similar results and a representative autoradiogram is shown.

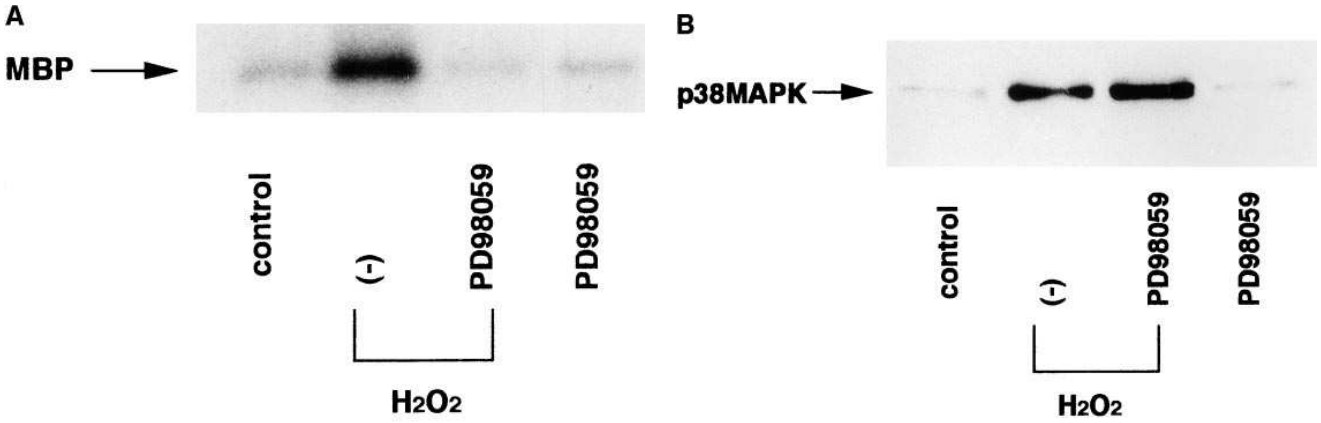

Figure 8. Effects of PD98059 on H_2O_2–induced activation of ERK and p38MAPK. Cultured cardiac myocytes were preincubated for 60 min with 20-μM PD 98059 and stimulated by 1-mM H_2O_2 for 10 min. Cells were lysed in 100 μl of lysis buffer A and 80 μl of cell lysates were used to measure ERK activities as described in the Fig. 1 legend (*A*) and 20 μl of lysates were subjected to Western blot analysis using the phosphorylated p38MAPK–specific antibodies (*B*).

(Fig. 9, *C* and *D*, and Fig. 10). Some of these TUNEL-positive nuclei were condensed and fragmented, suggesting that H_2O_2–induced apoptosis in cardiac myocytes. When ERKs but not p38MAPK were inhibited by the pretreatment with 20-μM PD98059, the number of H_2O_2–induced TUNEL-positive cardiac myocytes was increased by ~ twofold (~ 44%) (Fig. 9, *E* and *F*, and Fig. 10). Treatment with PD98059 significantly increased TUNEL-positive cells even in the absence of H_2O_2 (Fig. 10). We next examined DNA fragmentation by Agarose gel electrophoresis (42). Untreated cardiac myocytes that were cultured in serum-free medium for 48 h, showed a faint DNA ladder (Fig. 11 *D*). When cardiac myocytes were exposed to 100-μM H_2O_2 for 48 h, extracted genomic DNA showed a prominent DNA ladder characteristic of apoptosis (Fig. 11

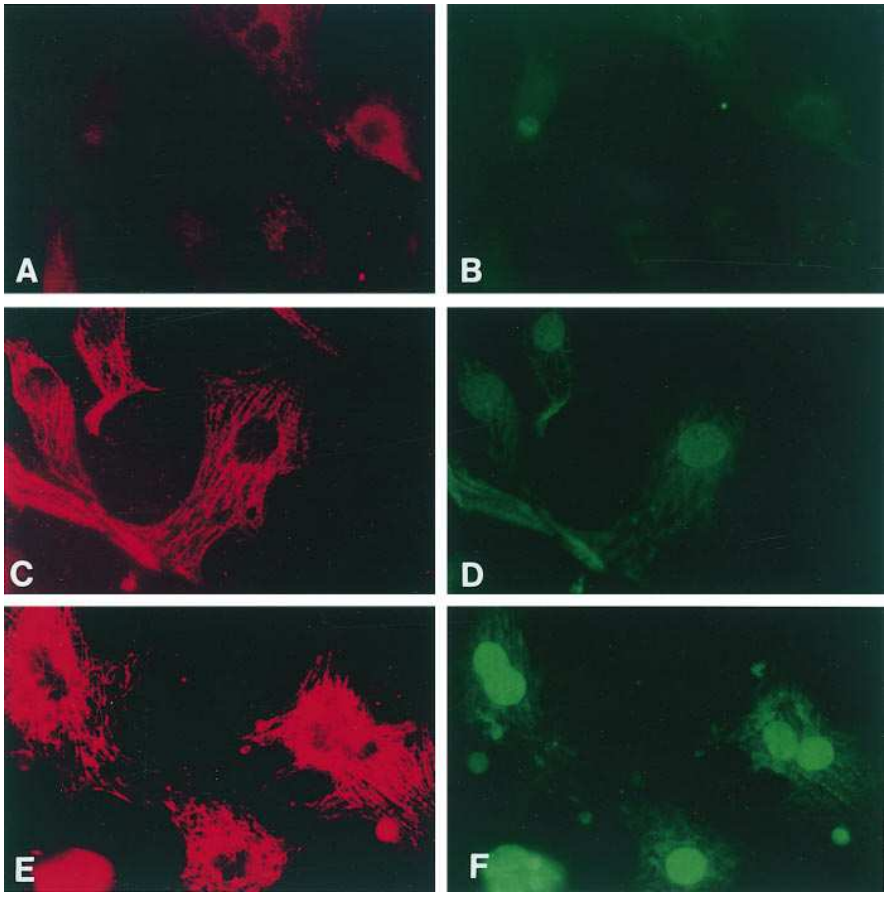

Figure 9. H_2O_2–induced apoptosis in cardiac myocytes. TUNEL was performed as described in Methods. After stimulation, cardiomyocytes were marked by staining with a monoclonal antimyosin heavy chain antibody (MF-20) followed by incubation with anti–mouse IgG conjugated with TRITC (*A*, *C*, *E*). Next, TUNEL staining was performed using FITC-conjugated dUTP (*B*, *D*, *F*). *A* and *B*, unstimulated cardiomyocytes. *C* and *D*, cardiomyocytes incubated with 100-μM H_2O_2 for 48 h. *E* and *F,* cardiomyocytes incubated with 100-μM H_2O_2 for 48 h after pretreatment with 20-μM PD98059 for 60 min. ×400.

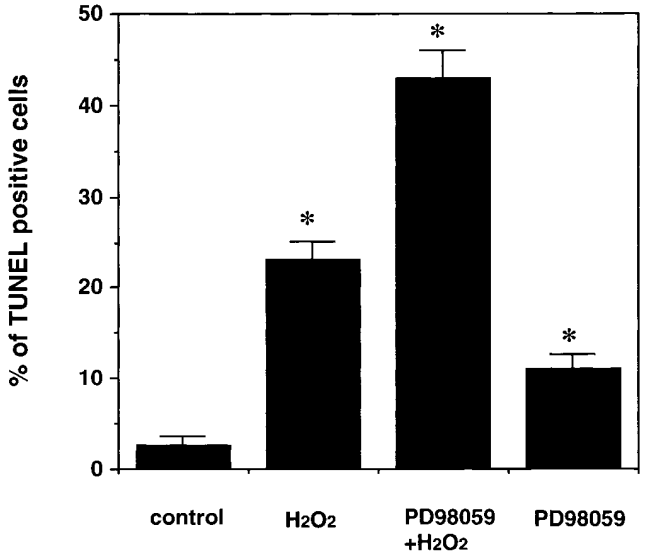

Figure 10. Number of cardiac myocytes undergoing apoptosis after exposure to H_2O_2. 100 of MF-20–positive cardiac myocytes were counted and the number of TUNEL positive cells was presented as percentage from three independent experiments (mean±SEM). $*P <$ 0.05 versus control.

B). When cultured cardiac myocytes were pretreated with PD98059 before exposure to H_2O_2, DNA fragmentation became more prominent (Fig. 11 *A*). PD98059 treatment itself slightly induced DNA ladder formation (Fig. 11 *C*). To confirm whether H_2O_2 induces apoptosis in cardiac myocytes, we also examined activation of one of ICE family, CPP32 (also called as apopine (49), Yama [50]). A growing body of evidence has suggested that CPP32 plays a critical role in the development of apoptosis, probably because CPP32 cleaves poly-ADP ribose polymerase that appears to be involved in DNA repair and the maintenance of genome integrity (48–51). Cultured cardiac myocytes were treated with 100-μM H_2O_2 for 30–90 min and the activation of CPP32 was assessed by Western blot analysis using an anti-CPP32 antibody. The amounts of active subunit (p17) increased from 30 min and peaked at 60 min after addition of 100-μM H_2O_2 (data not shown). These results suggest that H_2O_2 induces apoptosis in cardiac myocytes and that inhibition of ERKs increases H_2O_2–induced apoptotic death of cardiac myocytes.

Discussion

Much evidence has suggested that MAPK family protein kinases play critical roles in proliferation and differentiation in many cell types including cardiac myocytes (12, 16). In this study, we showed that hydrogen peroxide–derived free radicals activated two distinct MAPKs, ERKs, and p38MAPK, in cultured cardiac myocytes of neonatal rats. H_2O_2–induced ERK activation was strongly inhibited by a tyrosine kinase inhibitor and by overexpression of CSK. D.N.Ras and D.N.Raf-1 also completely blocked H_2O_2–induced activation of transfected ERK2. On the contrary, inhibition of PKC and PKA, or chelation of intra- and extracellular Ca^{2+} did not affect activation of ERKs by H_2O_2. These results suggest that tyrosine kinases including Src family tyrosine kinases, Ras and Raf-1 are important for H_2O_2–induced activation of ERKs but that PKC, PKA, or Ca^{2+} is not involved in the activation. In addition, we examined the role of ERKs in H_2O_2–induced injuries of cardiac myocytes. H_2O_2 induced the apoptotic death of cultured cardiac myocytes, and the inhibition of ERKs but not of p38MAPK enhanced apoptosis of cardiac myocytes. These results suggest that ERKs have a protective role against oxidative stress in cardiac myocytes.

H_2O_2 is often used as an experimental source of oxygen-derived free radicals. It has been reported that the iron chelator *o*-phenanthroline effectively inhibited activation of ERKs by H_2O_2 (47), suggesting that metal-dependent reactions are required for kinase activation by H_2O_2. In the presence of metal ions, H_2O_2 undergoes conversion via dismutation reactions to other oxygen-derived free radical species including hydroxyl radicals (43, 47). Indeed, DMSO, a hydroxyl radical–specific scavenger, blocked H_2O_2–mediated ERK activation in this study (Fig. 1). Therefore, oxidation by hydroxyl radical–like species may mediate H_2O_2–induced ERK activation in cardiac myocytes.

A growing body of evidence has suggested that ERKs are key molecules in the signal transduction pathways leading to mitogenesis and differentiation originating from distinct recep-

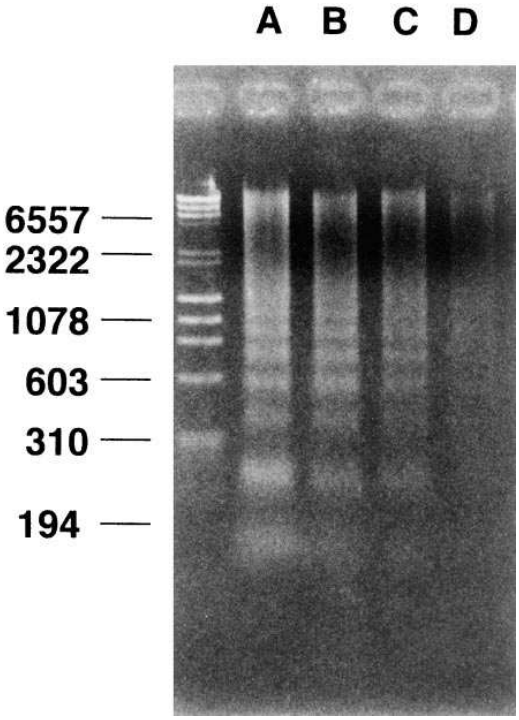

Figure 11. H_2O_2–induced DNA fragmentation in cardiomyocytes. Cultured cardiac myocytes (4×10^5) were lysed in lysis buffer B consisting of 1-M Tris-HCl, pH 7.4, 0.5-M EDTA, 10% Triton X-100, followed by incubation with RNase and finally with proteinase K. By this method, only fragmented DNA was extracted. DNA was separated by electrophoresis in 1.5% Agarose gels and stained by ethidium bromide. Cultured cardiac myocytes were incubated with 100-μM H_2O_2 for 48 h with (*A*) or without (*B*) the pretreatment by 20-μM PD98059 for 60 min. (*C*) Cardiac myocytes were incubated for 60 min with 20-μM PD98059. (*D*) Untreated cardiomyocytes. Molecular weight is shown at the left.

tors such as receptor tyrosine kinases and G protein–coupled receptors (18, 19). Activation of ERKs is also critical for the development of cardiac hypertrophy (16, 19). Stimulation of receptor tyrosine kinases often activates ERKs through the Ras/Raf-1/MEK cascade in many cell types (17, 19). In smooth muscle cells, Ang II activates ERKs through Src and Ras (18). In contrast, we have reported recently that in cardiac myocytes, Ang II–induced ERK activation was dependent on the activation of PKC, but not on Src or Ras (20). These results suggest that signal transduction pathways leading to activation of ERKs are highly divergent among cell types. It has been reported that ultraviolet irradiation activates Src and Ras (52) and phosphorylates EGF receptor via ROI (53). Ras has been reported to be necessary for ERK activation by H_2O_2 in HeLa, Rat1, NIH3T3, PC12, and smooth muscle cells (47). It has also been reported that PKC is partly involved in ERK activation by H_2O_2 in Jurkat T cells (54). In this study, we demonstrated that H_2O_2 activates ERKs through Src family tyrosine kinases and Ras, but not through PKC in cardiac myocytes. It remains to be determined how oxygen-derived free radicals activate Src family tyrosine kinases. Since pretreatment with suramin abolished H_2O_2–induced ERK activation, some growth factors may be involved in H_2O_2–induced Src activation (49). What growth factors are involved in free radical–induced ERK activation awaits future studies.

Apoptosis is one of the fundamental mechanisms of cell death in many tissues (55). The signaling pathways leading to apoptosis are beginning to be defined, and a number of proteins that either induce or prevent apoptosis have been identified (28–30, 56–58). In cardiac myocytes, it has been reported that apoptosis is induced by hypoxia/reperfusion in both in vivo (59) and in vitro (60). Mechanical stress has also been reported to induce apoptosis in cardiac myocytes (61). Although expression of Fas antigen has been reported to be increased in ischemic cardiomyocytes (60), cellular mechanisms of apoptosis in cardiac myocytes were largely unknown. We asked whether H_2O_2 induces apoptosis of cardiac myocytes by examining TUNEL staining, DNA ladder formation and activation of CPP32. Although we can not rule out the possibility that H_2O_2 induces necrotic cell death of cardiac myocytes, all these examinations suggest that at least a part of cardiomyocytes showed apoptotic cell death after exposure to H_2O_2.

Recently, Xia et al. reported that activation of ERKs promotes cell survival, whereas activation of JNK and p38MAPK induces apoptosis (28). Verheij et al. have also demonstrated that ceramide-induced activation of JNK is important for the induction of apoptosis (29). These results suggest that ERKs have a protective role against cellular stresses, while activation of p38MAPK/JNK leads to induction of apoptotic death. We examined whether this is true in oxidative stress–induced apoptosis of cardiac myocytes using a MEK inhibitor PD98059. When activation of ERKs was inhibited by the pretreatment with PD98059, the number of cardiomyocytes that showed apoptotic death after exposure to H_2O_2 was increased. Although three MAPK members such as ERKs, JNK, and p38MAPK are structurally related, they are generally activated by different extracellular stimuli and through different signaling pathways (12–14, 23, 62). Because PD98059 specifically inhibits MEK1 and MEK2, both of which are upstream kinases of ERK, but not of JNK and p38MAPK, the results in this study suggest that activation of ERKs is important to prevent cardiac myocytes from oxidative stress–induced apoptosis. Whether activation of JNK and p38MAPK is critical for promoting apoptosis in cardiac myocytes or how JNK and p38MAPK are activated by free radicals remains to be determined. In summary, this study suggests that hydroxyl radicals derived from H_2O_2 activate ERKs through the Src- and Ras-dependent pathways in cardiac myocytes, and that activation of ERKs is important to protect cardiomyocytes from apoptosis.

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