Inhibition of Extracellular Signal–Regulated Kinase Enhances Ischemia/Reoxygenation–Induced Apoptosis in Cultured Cardiac Myocytes and Exaggerates Reperfusion Injury in Isolated Perfused Heart

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Abstract—Three major mammalian mitogen-activated protein kinases, extracellular signal–regulated kinase (ERK), p38, and c-Jun NH$_2$-terminal protein kinase (JNK), have been identified in the cardiomyocyte, but their respective roles in the heart are not well understood. The present study explored their functions and cross talk in ischemia/reoxygenation (I/R)–induced cardiac apoptosis. Exposing rat neonatal cardiomyocytes to ischemia resulted in a rapid and transient activation of ERK, p38, and JNK. On reoxygenation, further activation of all 3 mitogen-activated protein kinases was noted; peak activities increased (fold) by 5.5, 5.2, and 6.2, respectively. Visual inspection of myocytes exposed to I/R identified 18.6% of the cells as showing morphological features of apoptosis, which was further confirmed by DNA ladder and terminal deoxyribonucleotide transferase–mediated dUTP nick end labeling (TUNEL). Myocytes treated with PD98059, a MAPK/ERK kinase (MEK1/MEK2) inhibitor, displayed a suppression of I/R-induced ERK activation, whereas p38 and JNK activities were increased by 70.3% and 55.0%, respectively. In addition, the number of apoptotic cells was increased to 33.4%. With pretreatment of cells with SB242719, a selective p38 inhibitor, or SB203580, a p38 and JNK2 inhibitor, I/R+PD98059–induced apoptotic cells were reduced by 42.8% and 63.3%, respectively. Hearts isolated from rats treated with PD98059 and subjected to global ischemia (30 minutes)/reoxygenation (1 hour) showed a diminished functional recovery compared with the vehicle group. Coadministration of SB203580 attenuated the detrimental effects of PD98059 and significantly improved cardiac functional recovery. The data taken together suggest that ERK plays a protective role, whereas p38 and JNK mediate apoptosis in cardiomyocytes subjected to I/R, and the dynamic balance of their activities is critical in determining cardiomyocyte fate subsequent to reperfusional injury. (Circ Res. 2000;86:692-699.)

Key Words: cardiomyocyte ■ ischemic injury ■ apoptosis ■ mitogen-activated protein kinase

The mitogen-activated protein kinases (MAPKs) are a family of serine-threonine protein kinases that are activated in response to a variety of extracellular stimuli.1,2 Three major MAPK signaling pathways, including extracellular signal–regulated protein kinase (ERK), p38 MAPK (p38), and c-Jun NH$_2$-terminal protein kinase (JNK), have been identified in mammalian cells. These 3 MAPKs have been shown to play a pivotal role in transmission of signals from cell surface receptors and various environmental cues to the transcriptional machinery in the nucleus and are involved in cell growth, differentiation, and transformation.3-5 Recent studies have suggested that MAPKs may be implicated in programmed cell death (apoptosis), but the precise roles of these 3 major MAPK signaling pathways in regulation of apoptotic cell death are still not clear and may be cell type specific.6,7

The activation of MAPKs in heart, especially in the cardiomyocyte, has been demonstrated in vitro as well as in vivo.8-10 However, the role of each pathway in cardiac myocyte apoptosis remains controversial. A recent report showed that only p38 among the 3 major MAPK pathways was activated in neonatal rat cardiomyocytes subjected to ischemia and that inhibition of p38 reduced myocyte apoptosis.11 In contrast, Zechner et al12 reported that overexpression of MAP kinase kinase 6 (MKK6), an upstream activator of p38, resulted in protection of cardiac myocytes from apopto-
sis induced by either anisomycin or MEK kinase 1 (MEKK1), an upstream activator of JNK pathway. Adding to the complexity of the matter, Wang et al.\textsuperscript{13} reported that activation of JNK alone by transfection of cultured rat neonatal cardiomyocytes with MKK7, an upstream activator of JNK, induced hypertrophy rather than apoptosis, and coactivation of both JNK and p38 led to apoptosis. Further study by this group has demonstrated that p38α is implicated in apoptosis, whereas p38β is involved in myocyte hypertrophy.\textsuperscript{14} With regard to the function of the ERK signaling pathway in the heart, a recent study reported that ERK was activated transiently in cultured rat neonatal cardiomyocytes treated with hydrogen peroxide, and inhibition of its activation resulted in an increase in the number of apoptotic myocytes.\textsuperscript{15} However, the roles of p38 and JNK were not studied. Therefore, it is not clear whether the effect of the ERK is linked to the other 2 pathways.

The current study was therefore designed to investigate the following: (1) whether all 3 major MAPK signaling pathways are activated in cardiomyocytes undergoing apoptosis, (2) the role of each pathway in this process of cell death, and (3) the possible interplay and cross talk among the 3 MAPK pathways. We have developed an apoptotic model in cultured neonatal rat cardiomyocytes exposed to ischemia/reoxygenation (I/R), the most pathologically relevant form of cardiac stress in vivo. In this model, ERK, JNK, and p38 pathways are all activated during I/R. To dissect the role of each pathway, the present study used an experimental design that does not require overexpression of the proteins involved but rather inhibition of the endogenous kinases by the following 3 types of inhibitors: PD98059, a selective inhibitor of MAP/ERK kinase 1 and 2 (MEK1/MEK2), the upstream activator of the ERK1/ERK2,\textsuperscript{16,17} SB242719, a JNK-sparing p38 inhibitor; and SB203580, an inhibitor of both p38 and JNK2.\textsuperscript{18} To further confirm the results observed in cultured cardiomyocytes, an I/R model in isolated perfused rat heart was used to study the role of MAPKs in I/R-induced injury. In this model, activation of MAPKs has been demonstrated.\textsuperscript{19,20} The data presented in this report demonstrate that ERK plays a role in protecting cardiomyocytes, whereas p38 and JNK mediate cell apoptosis after I/R injury. Our results suggest that inhibition of ERK pathway may shift the balance between cell death and survival toward cell death; in contrast, inhibition of p38 and JNK pathways shifts the balance toward cell survival.

**Materials and Methods**

**Primary Neonatal Rat Cardiomyocyte Cultures**

Primary cultures of neonatal rat cardiomyocytes from 1- to 2-day-old Sprague-Dawley rats were prepared by the method reported previously.\textsuperscript{21} Ischemia was induced by replacing medium with a serum-/glucose-free DMEM and exposing cells to 1% O\textsubscript{2} at 37°C for an indicated time period; the medium was then replaced with the maintenance medium, and the cells were exposed to normoxic atmosphere (reoxygenation).

**Morphological Assessment and Quantification of Apoptotic Myocytes**

To quantify apoptotic myocytes, cell monolayers were fixed and stained with Hoechst 33324. The morphological features of apoptotic (cell shrinkage, chromatin condensation, and fragmentation) were monitored by fluorescence microscopy. At least 400 cells from 12 randomly selected fields per dish were counted, and each treatment was performed in triplicate.\textsuperscript{21}

**DNA Ladder**

Myocytes were lysed in lysis buffer and electrophoresed on 2% agarose gel. The gel was stained with ethidium bromide, and DNA fragments were visualized under ultraviolet light.\textsuperscript{22}

**In Situ End Labeling for Detection of Apoptotic Myocytes**

In situ detection of apoptotic myocytes was performed by using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) with an in situ cell death detection kit (Boehringer Mannheim).\textsuperscript{21}

**MAPK Activity Assays**

MAPK activities were assayed as described previously.\textsuperscript{22,23} The cell lysates were immunoprecipitated with antibodies specific for ERK1/ERK2, p38, JNK1/JNK2, or MAPK-activated protein kinase-2 (MAPAPK2) and were assayed by using myelin basic protein (MBP) for ERK, glutathione-S-transferase activating transcription factor-2 (GST-ATF2) for p38, GST-c-Jun(1–81) for JNK and heat shock protein 27 for MAPAPK2 as the substrate, respectively.

**Western Blot Analysis of Total and Phosphorylated (Active) MAPKs**

Cardiomyocytes either untreated or subjected to ischemia or I/R were extracted. Total protein from each sample (100 μg) was resolved in SDS-PAGE, transferred to a nitrocellulose membrane, blocked with nonfat milk, and then incubated with the primary antibodies that recognize ERK (p44/42), phosphospecific ERK (p-p44/42), p38, phosphospecific p38, JNK1 and JNK2, and phosphospecific JNK1 and JNK2 at 4°C overnight. After washing, the membrane was incubated with the secondary antibody conjugated to horseradish peroxidase.

**Perfused Rat Hearts Subjected to Ischemia/Reperfusion**

Forty-five hearts were isolated from adult male Sprague-Dawley rats and subjected to 30 minutes of global ischemia and 60 minutes of reperfusion, as described in our previous study.\textsuperscript{24} The I/R hearts were randomly assigned to 1 of the following 3 groups: I/R + vehicle, I/R + PD98059 (2.5 mg/kg IP; 30 minutes before heart excision), and I/R + PD98059 + SB203580 (10 μmol/L; treatment was initiated 15 minutes before ischemia and remained during the entire period of reperfusion). Cardiac function was evaluated from the postischemic recoveries (% preischemic values) of left ventricular developed pressure (LVDP), dP/dt\textsubscript{max}, heart rate, and pressure-rate product (PRP) (=heart rate × LVDP).\textsuperscript{24}

**Statistical Analysis**

Statistical evaluation was performed by using 1-way ANOVA with subsequent post hoc paired comparisons. Probabilities of \( P<0.05 \) were considered statistically significant.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

**Results**

**Activation of ERK, p38, and JNK in Cultured Rat Neonatal Cardiomyocytes Subjected to I/R**

The temporal profile of the activation of the 3 MAPK pathways is shown in Figure 1. A moderate but significant increase in activities of JNK, p38, and ERK was detected 5 minutes after ischemia, reached a maximal level (fold increase, 3.50±0.65, 2.90±0.57 and 3.33±0.68, respectively) at 10 minutes, and...
returned to basal level within 1 to 1.5 hours. No further activation was observed when ischemia continued up to 6 hours (data not shown). On reoxygenation, JNK, p38, and ERK were rapidly reactivated, and the peak increase (fold) at 10 minutes was 6.21 ± 0.71, 5.24 ± 0.82, and 5.50 ± 0.65, respectively, and returned to the basal level after 1 to 2 hours. Figure 1, lower panel, is a representative autoradiogram.

The activation of ERK, p38, and JNK in cardiomyocytes subjected to I/R was further demonstrated by Western blot analysis using antibodies that recognize total ERK1/ERK2, p38, or JNK1/JNK2, respectively, or the corresponding phosphorylated (activated) forms. As shown in Figure 2, ERK, p38, and JNK were present in all samples. There was no difference in total protein levels between ERK1 and ERK2, but a higher level of JNK2 than JNK1 in the cardiac myocytes was detected. When the blot was reprobed using the antibodies that recognize only the dual phosphorylated active forms, it was observed that the activities of the 3 kinases were low in control myocytes but rapidly increased when the cells were subjected to ischemia and reactivated on reoxygenation. As shown in Figure 2, the major activated forms of ERK and JNK in the cardiomyocytes were ERK1 and JNK2.

I/R Induces Apoptosis in Cardiomyocytes

When cardiomyocytes were exposed to ischemia (2 hours) followed by reoxygenation (24 hours), a considerable fraction of myocytes showed morphological features of apoptosis such as shrinkage, blebbing, and cytoplasm condensation (Figure 3-I). Cells stained with Hoechst 33342 and assessed by fluorescence microscopy showed condensed chromatin and fragmented nuclei (Figure 3-II). The characteristic degradation of DNA into oligonucleosome-length fragmentation (DNA ladder) was only observed when the cells were exposed to ischemia followed by reoxygenation (Figure 3-III, lane 3). DNA fragments in situ were further visualized by the TUNEL assay (Figure 3-IV). When myocytes were exposed to ischemia for a longer period of time (4 to 6 hours), the number of necrotic cells increased by 5% to 10%.

PD98059 Enhances I/R-Induced Apoptosis in Cardiomyocytes

In the presence of the MEK1/MEK2 inhibitor PD98059 (50 μmol/L), I/R-induced apoptosis in cardiomyocytes was increased from 18.6 ± 2.5% to 33.4 ± 3.5% (P<0.01 versus I/R alone, n=14) (Figure 4-I). PD98059 had no effect on the...
basal level of apoptotic cell death (data not shown) but enhanced apoptosis in myocytes subjected to ischemia for 2 hours (8.4±1.6 versus 3.6±1.2, P<0.05, n=8). PD98059 at 1 µmol/L did not increase apoptotic cell death induced either by ischemia (2 hours) alone or by ischemia (2 hours)/reoxygenation (24 hours). The enhancement by PD98059 (50 µmol/L) of I/R-induced apoptosis in myocytes was also demonstrated by DNA ladder (Figure 4-II, lane 4) and TUNEL assay (Figure 4-III B, more intense stain).

Effects of PD98059 on I/R-Induced Activation of ERK, p38, and JNK

As shown in Figure 5, PD98059 at 50 µmol/L essentially inhibited ERK activation (Figure 5A), whereas it increased activities of p38 and JNK by 70.3% and 54.9%, respectively (both P<0.01) (Figures 5B and 5C). In the presence of 50 µmol/L of PD98059, ischemia alone–induced activation of p38 and JNK in the cardiomyocyte was also increased significantly (both P<0.05). Figure 5, lower panel, is a representative autoradiogram.

Effects of SB203580 and SB242719 on I/R-Induced Apoptosis and Activation of p38 and JNK

MAPKAPK2 is a specific downstream target of p38, and its activity is commonly used as an indication of p38 activation. In the presence of 10 µmol/L SB242719, a selective p38 inhibitor, I/R+PD98059-induced activation of p38 was inhibited completely (Figure 6B), whereas the number of apoptotic cardiomyocytes was reduced by 42.8% (P<0.01, n=6) (Figure 6A). Meanwhile, activation of JNK in the
myocytes was not affected (Figure 6C). Besides complete inhibition of p38, SB203580 at 10 μmol/L also reduced JNK activity by 34.9 ± 1.2% (P < 0.05 versus I/R + PD98059, n=6) (Figure 6C). In addition, this inhibitor reduced the number of...
apoptotic myocytes to a greater extent than that of SB242719 (63.3%, P<0.05 versus SB242719, n=6) (Figure 6D).

SB242719 and SB203580 at 10 μmol/L also reduced I/R alone–induced cardiomyocyte apoptosis by 44.6% (P<0.01 versus vehicle) and 72.2% (P<0.01 versus SB242719), respectively. Both SB242719 and SB203580 had no effect on I/R-induced activation of ERK, as shown in Figure 6D.

Effect of ERK Inhibition on Postischemic Myocardial Function Recovery and Its Reversal by SB203580

As shown in Figures 7A and 7B, ERK was activated in the isolated hearts exposed to I/R. The maximal increase, at 15 minutes of reperfusion, was 3.8-fold over the basal level (n=5). Meanwhile, the activation of p38 and JNK was also demonstrated (Figure 7B). Pretreatment of the animals with PD98059 (2.5 mg/kg IP and 30 minutes before heart excision) reduced I/R-induced activation of ERK by 70.7% (P<0.05 versus vehicle). When SB203580 (10 μmol/L) was coadministered (15 minutes before ischemia and remaining during the entire period of reoxygenation), I/R-induced activation of p38 (measured by MAPKAPK2) was suppressed (Figure 7B). However, inhibition of JNK activity in the hearts by SB203580 was variable, ranging from 4% to 31% (19±8%, n=6).

During global ischemia (30 minutes), coronary flow was reduced to 0, and myocardial contraction was completely absent. When perfusion was restored, functional contraction resumed within 5 minutes. LVDP, dP/dtmax, and PRP all gradually recovered and reached a maximal level between 20 and 40 minutes, an observation consistent with the previous studies.25 At 60 minutes of reoxygenation, LVDP, PRP, and dP/dtmax recovered to 63±1.8%, 48±2.1%, and 46±1.1%, respectively, in vehicle-treated hearts (Figure 7C). Pretreatment with PD98059 significantly aggravated cardiac functional injury, as evidenced by diminished recovery in LVDP, dP/dtmax, and PRP compared with the corresponding recoveries in the vehicle group (P<0.01 for all parameters). In contrast, when SB203580 was added to the perfusion system at 10 μmol/L at 15 minutes before ischemia, a significant improvement in cardiac contractile function was observed. The PRP recovery was increased by 54% compared with I/R+PD98059 (P<0.01) (Figure 7C).

Discussion

The ischemic model of cultured cardiomyocytes used in this study combines the following 2 properties of ischemia: decrease in energy source and hypoxia (oxygen level at 1%). To determine the effect of ischemia and reoxygenation on cardiac MAPKs, we examined the activities of 3 major MAPK signaling pathways in a full time course. It was of interest to note that the 3 major MAPK subfamily members, PD98059+SB203580 (10 μmol/L, added to the perfusion system 15 minutes before ischemia and remaining during the entire period of reoxygenation) were subjected to 30 minutes of 0-flow global ischemia and 60 minutes of reoxygenation. Sham I/R hearts were continuously perfused with Krebs-Henseleit solution without any treatment. a, P<0.01 vs sham (bar 1); b, P<0.01 vs vehicle; c, P<0.01 vs I/R+PD98059 (n=8 to 12).

Figure 7. A, Effect of PD98059 on I/R-induced activation of ERK in isolated rat hearts. PD98059 was administered (2.5 mg/kg IP) 30 minutes before heart excision. After 30 minutes of global ischemia and 15 minutes of reoxygenation, hearts were immediately frozen for measurement of MAPK activities (n=5). *P<0.05 vs I/R alone. B, Representative autoradiogram showing activation of ERK, p38, and JNK in perfused heart subjected to I/R. C, Effects of ERK inhibition with PD98059 on postischemic myocardial functional recovery and its reversal by SB203580. Isolated rat hearts treated with vehicle, PD98059, or
ERK, p38, and JNK, were activated simultaneously in cardiomyocytes when exposed to ischemia and reoxygenation. The temporal profile as well as the extent of activation of the 3 MAPK subfamily members were similar, suggesting that their activation may be a common response of the cardiomyocytes to I/R, and all 3 MAPK pathways may play a role in the response of the cell to the noxious stimuli.

The ERK pathway has been shown to be required for survival signaling in response to growth factors in noncardiomyocytic cells.28,29 However, several recent studies have suggested that the ERK pathway is involved in the regulation of cell death.28,29 To dissect the role of ERK in the I/R-induced cardiac apoptosis, PD98059, an ERK pathway inhibitor, was used in this study. Both in vitro and intact cell studies have shown that PD98059 is highly specific for MEK1/MEK2, the upstream activator of ERK1/ERK2, with no effect on many other kinases, including p38 and JNK.16,17 Inhibition of ERK did not induce apoptosis in cardiac myocytes when the cells were exposed to normoxic conditions, suggesting that the basal level of this pathway does not play a major role in cardiac myocyte survival. However, when the cardiomyocytes were subjected to ischemia or I/R in which the ERK pathway was activated, apoptosis induced by ischemia alone or by I/R was significantly potentiated by the inhibition of ERK. Ischemia alone for 2 hours resulted in a mild increase in the number of apoptotic cells compared with the basal level (P>0.05). However, in the presence of PD98059, ischemia-triggered myocyte apoptosis was significantly enhanced, indicating clearly that ERK activation prevented the cardiomyocytes from apoptosis during ischemia when the JNK and p38 were moderately activated. ERK activation could not totally prevent reoxygenation-induced myocyte apoptosis, when the activities of p38 and JNK reached the maximal levels, but the protective effect of ERK was still demonstrated by the observation that I/R-induced apoptotic cell death was increased by 79.5% when ERK was inhibited (Figure 4-1). These data suggest that activation of ERK during ischemia or I/R plays an important role in preventing stress-induced myocyte apoptosis.

Simultaneous measurement of the activities of ERK, p38, and JNK in cardiomyocytes further elucidates the mechanism by which ERK inhibition enhances myocyte apoptosis and the signal integration among the 3 MAPK signaling pathways. In agreement with previous reports,16,17 PD98059 up to 50 μmol/L had no effect on the basal activities of p38 or JNK in the cardiomyocyte under normoxia (data not shown). When the myocytes were exposed to ischemia or I/R, PD98059 at 50 μmol/L significantly enhanced the activities of both p38 and JNK in the cells, along with an increase in the number of apoptotic myocytes. Moreover, neither the activities of p38 and JNK nor the number of apoptotic cells was affected by PD98059 at 1 μmol/L, which had no effect on the ERK. These results indicate a possible implication of p38 and JNK in PD98059-enhanced cell death triggered by I/R. This hypothesis was further supported by the study with p38 inhibitors. When the p38 pathway was blocked by the selective p38 inhibitor SB242719, I/R+PD98059-induced cell apoptosis was significantly reduced. As SB242719 had no effect on JNK or ERK in the stimulated myocytes (Figures 6C and 6D), the protective effect of this compound was clearly due to inhibition of p38. SB203580 at 10 μmol/L completely blocked the p38 pathway and partially inhibited the JNK pathway (34.9%), whereas additional myocytes were rescued from apoptosis (63.3% versus 42.8% reduction with SB242719, P<0.05). It is conceivable that inhibition of JNK might contribute to the increased protection of SB203580 against apoptosis in cardiomyocytes. The dose of SB203580 used could not be increased further because of concerns of possible nonspecific inhibition of other kinases. As a specific and potent inhibitor of JNK is not yet available, the real importance of JNK in I/R-induced cardiomyocyte apoptosis remains to be further determined.

The data derived from isolated rat heart study further confirm the findings in cultured cardiomyocytes; that is, inhibition of ERK pathway exacerbates cardiac injury. It has been previously demonstrated in this model that I/R activated MAPK subfamily members,19,30 resulting in apoptotic cardiomyocytes.31 In the present study, treatment of the animals with PD98059 in vivo before heart excision resulted in a 70% reduction in the peak activity of ERK in the hearts as well as a 35% reduction in the recovery of PRP compared with the vehicle group. Because of the limited solubility of PD98059, it was not possible to test the compound at a higher dose. Therefore, the maximal effect of ERK inhibition might not have been observed. Nevertheless, the detrimental effect of inhibition of this MAPK pathway in I/R-induced cardiac injury was clear. Moreover, SB203580 showed a clear protection in this model that also supported the findings in the cultured myocytes. SB203580 completely inhibited I/R-induced activation of p38 in the perfused heart. However, inhibition of JNK in the heart by SB203580 was variable. Therefore, whether the cardioprotective effect of SB203580 in the perfused heart can also be attributed to its inhibition of JNK remains to be further defined.

In summary, the present results represent the first report that studies the interplay and cross talk of the 3 major MAPKs in the cardiomyocytes subjected to I/R. Three major MAPK pathways are activated in cardiac myocytes subjected to I/R. The ERK pathway is important for survival of cells by protecting them from programmed cell death caused by stress-induced activation of JNK and p38. This interplay among the different MAPK signaling pathways may serve as part of the defense mechanism for the cardiomyocyte in response to an individual stressor and, therefore, is crucial to the coordinated responses of the cell.

References


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