MEK1-ERK2 Signaling Pathway Protects Myocardium From Ischemic Injury In Vivo

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Background—Myocardial infarction causes a rapid and largely irreversible loss of cardiac myocytes that can lead to sudden death, ventricular dilation, and heart failure. Members of the mitogen-activated protein kinase (MAPK) signaling cascade have been implicated as important effectors of cardiac myocyte cell death in response to diverse stimuli, including ischemia-reperfusion injury. Specifically, activation of the extracellular signal–regulated kinases 1/2 (ERK1/2) has been associated with cardioprotection, likely through antagonism of apoptotic regulatory pathways.

Methods and Results—To establish a causal relationship between ERK1/2 signaling and cardioprotection, we analyzed *Erk1* nullizygous gene-targeted mice, *Erk2* heterozygous gene-targeted mice, and transgenic mice with activated MEK1-ERK1/2 signaling in the heart. Although MEK1 transgenic mice were largely resistant to ischemia-reperfusion injury, $Erk2^{+/-}$ gene-targeted mice showed enhanced infarction areas, DNA laddering, and terminal deoxynucleotidyl transferase–mediated dUTP biotin nick-end labeling (TUNEL) compared with littermate controls. In contrast, enhanced MEK1-ERK1/2 signaling protected hearts from DNA laddering, TUNEL, and preserved hemodynamic function assessed by pressure-volume loop recordings after ischemia-reperfusion injury.

Conclusions—These data are the first to demonstrate that ERK2 signaling is required to protect the myocardium from ischemia-reperfusion injury in vivo. (***Circulation*. 2004;109:1938-1941.**)

Key Words: ischemia ■ cardiac output ■ mitogen-activated protein kinases ■ infarction ■ signal transduction

The mitogen-activated protein kinases (MAPK) constitute an essential signal transduction cascade that occupies a central position in cell growth, differentiation, apoptosis, and transformation.[1] Although it is somewhat oversimplified, the MAPK signaling pathway consists of a sequence of successively acting kinases that ultimately result in the dual phosphorylation and activation of the terminal kinases p38, c-Jun N-terminal kinase (JNKs), and extracellular signal–regulated kinase (ERKs).[2] The MAPK signaling cascade is initiated in cardiac myocytes by G-protein–coupled receptors, receptor tyrosine kinases, cardiotrophin-1 (gp130 receptor), and stress stimuli.[3] The major upstream activators of ERK1/2 are 2 MAP kinase kinases (MAPKK), MEK1 and MEK2, which directly phosphorylate the dual site on ERK kinases (Thr-Glu-Tyr).[2]

Within the heart, members of the MAPK cascade have been implicated in regulating myocyte survival after ischemia-reperfusion injury, oxidative stress, and anthracycline exposure. Indeed, a number of studies support the hypothesis that the MEK1-ERK1/2 branch of the MAPK pathway is cardioprotective by directly antagonizing myocyte apoptosis.[4] Although MEK1-ERK1/2 signaling is thought to protect the myocardium from apoptotic insults, definitive genetic data demonstrating a necessary function for this pathway in vivo have not been reported.

Methods

Murine In Vivo Ischemia-Reperfusion and Pressure-Volume Acquisition

Adult mice (aged 8 to 10 weeks) were anesthetized, placed on a heating plate, and ventilated (250 μL; 200 strokes per minute). A left thoracotomy was performed, and the left anterior descending artery was ligated with 8-0 Prolene with a slipknot, which was subsequently removed 60 or 90 minutes later to allow reperfusion for 4 hours, 24 hours, or 7 days. To assess physiological function, a 1.4F conductance micromanometer was inserted through the right common carotid artery and into the left ventricle in mice 7 days after
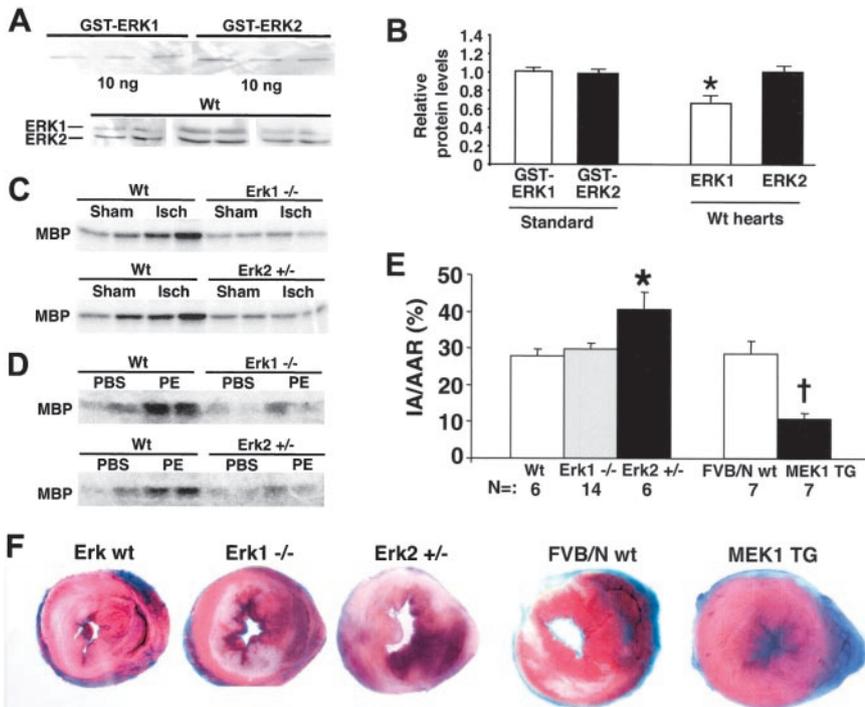
Figure 1. A, Western blot with bacterium-generated ERK1 and ERK2 GST fusion protein standards to verify antibody equivalence (top) and 6 independent wild-type (Wt) heart extracts (bottom). B, Western blot quantitation of ERK1 and ERK2 fusion protein standards and the content of endogenous ERK1 and ERK2 in the heart. C, Total ERK immune kinase activity against myelin basic protein (MBP) from hearts of wild-type, $Erk1^{-/-}$, and $Erk2^{+/-}$ mice after 30 minutes of ischemia (Isch) in vivo or after sham operation. D, ERK immune kinase activity after 30 minutes of phenylephrine (PE) stimulation in vivo for the indicated groups. Similar results were observed in 2 independent experiments. E, Infarct area (IA) was normalized to the area at risk (AAR). F, Representative TTC- and Evans blue–stained heart cross sections from each of the cohorts. *$P<0.05$ vs C57Bl/6 or Bl/6sv129 wild-type; †$P<0.05$ vs FVB/N wild-type control. TG indicates transgenic.

ischemic injury. Infarct size and area at risk were determined 24 hours after ischemic injury by religating the left anterior descending artery and infusing 300 μL of 2% Evans blue dye, followed by staining heart cross sections in 2% 2,3,5-triphenyltetrazodium chloride (TTC) solution at 37°C.

Biochemical and Molecular Analyses
Wild-type, $Erk1^{-/-}$, and $Erk2^{+/-}$ mice were either injected subcutaneously with 10 mg/kg phenylephrine or PBS vehicle or subjected to 30 minutes of ischemia. Hearts were later harvested for Western blotting or ERK immunoprecipitation kinase assay as previously described with a rabbit polyclonal ERK1/2 antibody (cell signaling No. 9102).[5,6] Conditions for generating cultured neonatal cardiac myocytes and recombinant adenovirus were described previously.[6,7] DNA laddering and terminal deoxynucleotidyl transferase–mediated dUTP biotin nick-end labeling (TUNEL) from ischemia-reperfusion–injured hearts or from cultured cardiomyocytes were performed as described previously.[6,7]

Statistical Analysis
A Student t test or 1-way ANOVA was used when multiple groups were compared. Data are reported as mean±SEM. Probability values ≤0.05 were considered significant.

Results

Characterization of ERK1 and ERK2 Protein and Activity in Gene-Targeted Mice
A gene-targeting approach was used to evaluate the necessary functions of ERK1 and ERK2 gene products as regulators of cardiac homeostasis and stress responsiveness. *Erk1* and *Erk2* gene-targeted mice were each described previously.[8,9] *Erk1*-null mice are fully viable as adults, but *Erk2* gene-targeted mice were analyzed in the heterozygous state because nulls die early during embryonic development.[9] Western blotting with bacterium-generated ERK1 and ERK2 protein standards (GST fusion proteins) demonstrated equivalent antibody reactivity, from which a standard curve was generated to quantify endogenous ERK1 and ERK2 levels in the heart,

demonstrating higher ERK2 protein content (Figure 1A, 1B). Cardiac ERK activation was assessed by immunoprecipitation kinase assay from hearts of wild-type, $Erk1^{-/-}$, and $Erk2^{+/-}$ mice after an acute 30 minutes of ischemia (Figure 1C) or acute phenylephrine injection (Figure 1D). The immune kinase assays showed a consistent profile of inhibited total ERK activity from either $Erk1^{-/-}$ or $Erk2^{+/-}$ hearts by either stimulation. For example, 30 minutes of ischemia induced a 2-fold increase in ERK activity in wild-type hearts but essentially no increase in $Erk1^{-/-}$ or $Erk2^{+/-}$ hearts ($P<0.05$) (Figure 1C). In response to phenylephrine stimulation, a similar percentage of reduction in ERK kinase activity was observed in both $Erk1^{-/-}$ and $Erk2^{+/-}$ hearts ($P<0.05$). These results indicate that both $Erk1^{-/-}$ and $Erk2^{+/-}$ mice show a similar defect in ERK kinase activation in the heart (see Discussion).

Genetic Alterations in ERK Activity Correlate With Susceptibility to Ischemic Damage
Although most studies using cell culture–based models have suggested an antiapoptotic role for ERK signaling, such conclusions have yet to be definitively extended to an intact animal. In the present study, $Erk1^{-/-}$ and $Erk2^{+/-}$ gene-targeted mice underwent 60 minutes of left ventricular cardiac ischemia followed by 24 hours of reperfusion to induce myocardial infarctions and cell death in vivo. Remarkably, $Erk2^{+/-}$ mice (C57Bl/6) showed a significant increase in total left ventricular infarct area (normalized to area at risk) compared with strain-matched wild-type controls (C57Bl/6) ($P<0.05$)(Figure 1E, 1F). No significant differences were observed in the area at risk normalized to left ventricular area (data not shown). Loss of *Erk1* did not significantly enhance myocardial infarction susceptibility (see Discussion). To extend the mechanistic implications of these observations,
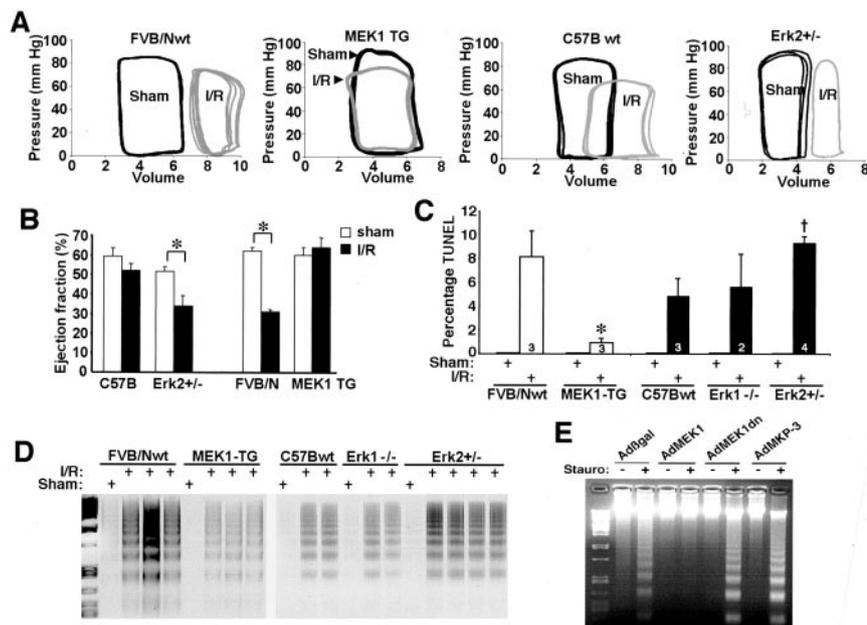
Figure 2. A, Representative pressure-volume loops measured from wild-type (wt), MEK1 transgenic (TG), or $Erk2^{+/-}$ mice 7 days after sham operation or ischemia-reperfusion (I/R) injury. B, Invasive hemodynamic assessment of ejection fraction in the indicated mice 7 days after sham operation or ischemia-reperfusion injury (*$P<0.05$ vs sham control). C, D, TUNEL and DNA laddering assessment of apoptosis from the affected region of the left ventricle in the indicated groups 24 hours after ischemic injury. E, Representative DNA ladders from staurosporine (Stauro)-treated or -untreated neonatal cardiomyocytes infected with the indicated adenoviruses.

transgenic mice expressing an activated MEK1 mutant protein in the heart were analyzed (gain of function for ERK1/2 signaling).[6] MEK1 transgenic mice were significantly protected from ischemia-reperfusion injury (Figure 1E, 1F).

Assessment of Physiological Function

Each cohort was analyzed 7 days after ischemic injury by invasive hemodynamics of left ventricular pressure-volume loops collected in the anesthetized mouse. After 90 minutes of ischemic injury, wild-type FVB/N mice demonstrated a significant decrease in cardiac output and ejection fraction and deterioration in the pressure-volume relationship ($P<0.05$) (Figure 2A, 2B). In dramatic contrast, MEK1 transgenic mice (FVB/N) did not show a significant loss in functional performance or the rightward shift in the pressure-volume relationship during either diastole or systole (Figure 2A, 2B). $Erk1^{-/-}$ and $Erk2^{+/-}$ gene-targeted mice were also analyzed after ischemia-reperfusion injury and compared with Bl/6sv129 or C57Bl/6 strain-matched controls, respectively. In this series of experiments, slightly less injury was instituted to permit evaluation of enhanced myocardial infarction from a smaller baseline level of damage (60 minutes of ischemia). Under these conditions, $Erk1^{-/-}$ mice showed no difference in function after ischemia-reperfusion injury compared with strain-matched controls (data not show). In contrast, $Erk2^{+/-}$-null mice showed a greater decrease in ejection fraction and dP/dt$_{max}$ compared with strain-matched controls ($P<0.05$) (Figure 2B; data not shown). Analysis of individual pressure-volume loops showed a greater rightward shift in $Erk2^{+/-}$ mice after ischemia-reperfusion damage compared with sham-operated mice (Figure 2A).

ERK Signaling Influences Cardioprotection Through Regulation of Apoptosis

The observation of enhanced ischemia-reperfusion injury and decreased functional performance in $Erk2^{+/-}$ mice suggested a role in regulating myocardial cell apoptosis. To examine

apoptosis levels, TUNEL and ligation-mediated polymerase chain reaction laddering for DNA fragmentation were performed on the infarct susceptibility region of the left ventricle after ischemia and 24 hours of reperfusion. The data demonstrate significantly reduced TUNEL and DNA laddering in MEK1 transgenic hearts compared with strain-matched controls ($P<0.05$) and significantly higher TUNEL and DNA laddering in $Erk2^{+/-}$ hearts compared with strain-matched controls ($P<0.05$) (Figure 2C, 2D). These results were extended through the use of neonatal myocytes infected with recombinant adenoviruses expressing various MEK1-ERK1/2 effectors, which were subsequently treated with staurosporine (500 nmol/L) for 18 hours to induce apoptosis. Staurosporine induced significant DNA laddering in control Adβgal-infected myocytes, which was qualitatively antagonized by expression of activated MEK1 (Figure 2E). More importantly, inhibition of endogenous ERK1/2 activity with recombinant adenoviruses expressing either dominant negative MEK1 or the ERK-specific dual-specificity phosphatase MKP-3 noticeably enhanced staurosporine-induced DNA laddering (Figure 2E).

Discussion

Although previous studies have suggested the hypothesis that ERK activation can partially antagonize apoptosis, some disagreement persists in the literature concerning the role of ERK signaling in regulating apoptosis. Furthermore, most previous studies relied solely on cultured cardiomyocytes to mechanistically assess the importance of ERK signaling in regulating cardiomyocyte viability after an apoptotic insult.[4] In the present study, we used genetically altered mouse models with both enhanced and depressed ERK signaling in vivo to establish a direct relationship between this signaling pathway and susceptibility to ischemia-reperfusion injury.

An interesting aspect of the present study is that $Erk2$ heterozygote gene-targeted mice showed enhanced myocardial injury after ischemia-reperfusion stimulation, whereas

Erk1-null mice showed a level of injury equivalent to that in wild-type mice. This differential susceptibility to ischemia-reperfusion injury documented between *Erk1*-null and *Erk2* heterozygote gene-targeted mice suggests 2 possible mechanisms. Quantitative Western blotting indicated that ERK2 protein levels are higher than ERK1, as shown in certain other cell types.[10] However, *Erk2* heterozygote mice showed somewhat similar reduction in total kinase activity in the heart after acute ischemia or phenylephrine injection compared with *Erk1*-null mice (Figure 1C and 1D). Thus, the loss of 1 allele of *Erk2* appears to alter the net amount of ERK1/2 activity in the heart, similar to the result with loss of both *Erk1* alleles. Another more plausible mechanism is that ERK1 and ERK2 have specific functions, as suggested by the lack of compensation by *Erk1* in *Erk2*-null mice, the latter of which die during embryonic development.[9]

A number of downstream ERK effectors have been identified that could explain, in part, their antiapoptotic regulatory function.[10] For example, ERK1/2 can directly phosphorylate and activate p90rsk, which in turn phosphorylates the proapoptotic factor Bad in the mitochondria, resulting in cellular protection.[11] ERK factors were also shown recently to interact with PKCε within the mitochondria, facilitating cellular protection within the context of the intact heart.[12] Although numerous downstream mechanisms are likely involved,[4] the present report is the first to genetically demonstrate that MEK1-ERK2 signaling protects the heart from apoptotic insults in vivo. A future challenge will be to develop strategies that exploit this function of the ERK1/2 signaling pathway in the heart by tightly controlling activation to potentially benefit the myocardium during stress.

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