Differential Regulation of p90 Ribosomal S6 Kinase and Big Mitogen–Activated Protein Kinase 1 by Ischemia/Reperfusion and Oxidative Stress in Perfused Guinea Pig Hearts

Yasuchika Takeishi, Jun-ichi Abe, Jiing-Dwan Lee, Hisaaki Kawakatsu, Richard A. Walsh, Bradford C. Berk

Abstract—Reactive oxygen species (ROS) activate members of the Src kinase and mitogen-activated protein kinase superfamily, including big mitogen-activated protein kinase 1 (BMK1) and extracellular signal-regulated kinases (ERK1/2). A potentially important downstream effector of ERK1/2 is p90 ribosomal S6 kinase (p90RSK), which plays an important role in cell growth through the activation of several transcription factors, as well as the Na+/H+ exchanger. Previously, we showed that Src regulates BMK1 via a redox-sensitive signaling pathway. Because ROS are generated during ischemia and reperfusion after ischemia, we assessed the effects of these stimuli (H2O2, ischemia, and reperfusion) in the activation of ERK1/2, p90RSK, Src, and BMK1 in perfused guinea pig hearts. H2O2 (100 μmol/L) significantly activated all kinases. Ischemia alone stimulated p90RSK, Src, and BMK1 but not ERK1/2. These results suggest that p90RSK activation through ischemia occurs via a pathway other than ERK1/2. A role of Src in ischemia-mediated BMK1 activation was demonstrated through inhibition with the Src inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine. Reperfusion after ischemia stimulated both p90RSK and ERK1/2. In contrast, although ROS increase during reperfusion after ischemia, the activities of both BMK1 and its upstream regulator, Src, were markedly attenuated by reperfusion after ischemia. The activation of C-terminal Src kinase during ischemia but not during reperfusion suggests that the attenuation of Src and BMK1 activity by reperfusion was not regulated by C-terminal Src kinase activity. The antioxidant N-2-mercaptopropionylglycine completely inhibited ERK1/2 and p90RSK activation by reperfusion but only partially inhibited ischemia-induced Src and BMK1 activation. The present study is the first to show the coregulation of Src and BMK1 by reperfusion after ischemia, which we propose to occur via a novel, ROS-independent pathway. (Circ Res. 1999;85:1164-1172.)

Key Words: transduction ■ oxidative stress ■ myocardium ■ ischemia

In ischemic myocardial disease, the heart is exposed to numerous cell stresses, including the increased production of reactive oxygen species (ROS), ionic imbalances, osmotic stress, mechanical stress, and metabolic deprivation.1,2 ROS are generated in the heart during ischemia (and reperfusion after ischemia) via several mechanisms, including mitochondrial uncoupling, xanthine oxidase, cytochrome P-450 monoxygenase, and NADH/NADPH oxidase. ROS stimulate intracellular signal events similar to those activated by growth factors and cytokines. ROS stimulate small G proteins and kinases such as Src and mitogen-activated protein kinases (MAPKs) that lead to the activation of transcription factors both in vitro and in the perfused heart.3,4 The subsequent changes in gene expression are likely to account for many of the changes in cell function induced by ROS.

Four subfamilies of MAPKs that are sensitive to ROS have been identified: extracellular signal-regulated protein kinase (ERK1/2), c-Jun NH2 terminal kinase (JNK), p38 kinase, and big MAP kinase 1 (BMK1, or ERK5).1 Each subfamily may be regulated via different signal transduction pathways and modulate specific cell functions.5 ERK1/2 is activated by an upstream kinase (MAPK kinase 1, or MEK1) via dual phosphorylation of the TEY motif, whereas JNK and p38 kinases are activated by MEK4/7 and MEK3/6 via phosphorylation of the TPY and TGY motifs, respectively. BMK1 is a recently identified MAPK family member that shares the TEY activation motif with ERK1/2 but is activated by MAPK kinase 5 (MEK5). We have shown that BMK1 is very strongly activated by ROS and that ROS-mediated BMK1 activation requires Src in cultured fibroblasts.6,7
Increased oxidative stress due to increased ROS generation, a relative deficit in the endogenous antioxidant reserve, or both can cause cardiac contractile depression. Importantly, Dhall et al reported that oxidative stress is one of the contributing factors in the transition from compensated hypertrophy to decompensated heart failure. It is established that ischemia and reperfusion produce ROS in the heart, but the signal transduction mechanisms via which ischemia and reperfusion alter cardiac function remain unclear.

There is a growing body of evidence for a key role of the plasma membrane Na+/H+ exchanger isoform 1 (NHE-1) in the pathophysiology of cardiac ischemia and reperfusion. Several investigators have shown that H2O2 stimulates both ERK1/2 and NHE-1 in neonatal cardiac myocytes. Because phosphorylation regulates NHE-1 exchanger activity and the inhibition of ERK1/2 decreases NHE-1 activity, it appears likely that the ERK1/2 pathway regulates NHE-1. Recently, we showed that p90 ribosomal S6 kinase (p90RSK), one of the downstream regulators of ERK1/2, is a serum-stimulated NHE-1 kinase. However, we have recently found that p90RSK is strongly activated by ROS in an ERK1/2-independent manner in cultured fibroblasts and Jurkat T cells, suggesting that other MAPKs may be required, such as BMK1. In fact, we demonstrated that ROS-mediated BMK1 activation requires Src. Thus, to define the relative roles of BMK1 and p90RSK in ischemia and ischemia/reperfusion, we compared the effects of H2O2 and ischemia/reperfusion on BMK1 and p90RSK activity in the perfused heart. We found that ischemia activates p90RSK and BMK1 but, surprisingly, not ERK1/2. On reperfusion after ischemia, both p90RSK and ERK1/2 are activated, but BMK1 was inhibited by ischemia/reperfusion. The antioxidant N-2-mercaptopyrrolidinyl glycine (MPG) completely inhibited ERK1/2 and p90RSK activation by reperfusion but only partially inhibited ischemia-induced Src and BMK1 activation. These results demonstrate a new ischemia-sensitive mechanism responsible for the activation of Src and BMK1 that is not mediated by ROS.

Materials and Methods

Heart Perfusion

Adult male Charles River guinea pigs were anesthetized with 54 mg/kg ketamine IP, 1.8 mg/kg acepromazine IP, and 10.9 mg/kg xylazine IP and heparinized. Hearts were quickly excised and perfused with Krebs-Henseleit buffer (50 mmol/L NaH2PO4, 50 mmol/L NaCl, 5 mmol/L EDTA, 5 mmol/L Na2HPO4, 100 mmol/L NaCl, 1 mmol/L MgSO4, 1.1 mmol/L KH2PO4, 0.12 mmol/L NaHCO3, 23.6 mmol/L CaCl2, 2.5 mmol/L mannitol, and glucose 11.0, as previously reported. The solution was saturated with 95% O2/5% CO2 (pH 7.4) at 37°C. A saline-filled latex balloon attached to a 3F micromanometer catheter (Millar Instruments) was inserted into the left ventricle through the mitral valve for pressure measurements. The balloon was inflated to achieve 10 mm Hg initial minimum diastolic pressure and was kept isovolumic during the perfusion. The coronary flow rate was adjusted to 10 mL·min⁻¹·g net heart wt⁻¹ and was kept constant throughout the experiment. Atrial pacing was carried out at 200 bpm, except for the period of global ischemia and the first 3 minutes of reperfusion thereafter.

Experimental Protocols

All hearts were allowed to equilibrate for ≥20 minutes before the protocols were begun. The animals were assigned to protocols as summarized in Figure 1. Three guinea pigs perfused with Krebs-Henseleit buffer alone were used as control animals. The guinea pig hearts were subjected to 10, 20, 30, or 40 minutes of ischemia alone or to 10 or 20 minutes of reperfusion after 20 minutes of ischemia (n=3 for each group). Ischemia was induced through the suspension of circulation of the perfusion pump. The guinea pig heart is very sensitive to ischemia, and ischemia for >30 minutes prevented recovery of the heart from ischemia. Therefore, we used a 20-minute ischemic period for the reperfusion studies. Three guinea pig hearts were perfused with 100 μmol/L H2O2 for 20 minutes. After the equilibration period, 300 μmol/L MPG was added to the perfusate and infused for 20 minutes before ischemia (n=3) and ischemia/reperfusion (n=3). The dose and perfusion time with MPG were chosen on the basis of previous reports. A dose of 10 μmol/L 4-amino-5-(4-chlorophenyl)-7-((3-buty1)pyrazolo[3,4-d]pyrimidine (PP2; Calbiochem), a selective inhibitor of the Src family of tyrosine kinases, was infused for 20 minutes before ischemia (n=3).

Immunoprecipitation and Western Blot Analysis

After the completion of perfusion, the left ventricle was quickly freeze-clamped with a liquid nitrogen– precooled Wollenberger clamp, powdered in liquid nitrogen, and stored at −80°C. After treatment, heart powders were homogenized with 4 vol of lysis buffer (50 mmol/L sodium pyrophosphate, 50 mmol/L NaF, 50 mmol/L NaCl, 5 mmol/L EDTA, 5 mmol/L EGTA, 100 μmol/L Na3VO4, 10 mmol/L HEPES, pH 7.4, 1% Triton X-100, 0.1% SDS, 500 μmol/L PMSF, and 10 μg/mL leupeptin). The heart homogenates were centrifuged at 14 000g for 30 minutes, and protein concentration was determined with the use of the Bradford protein assay (Bio-Rad). For immunoprecipitation, cell lysates were incubated with rabbit anti-BMK1 or C-terminal Src kinase (Csk) (Santa Cruz Biotechnology) antibody for 12 hours at 4°C and then incubated with 20 μL of protein A-Sepharose CL-4B (Pharmacia Biotech) for 1 hour on a roller system at 4°C. The beads were washed 2 times with 1 mL lysis buffer, 2 times with 1 mL LiCl wash buffer (500 mmol/L LiCl, 100 mmol/L Tris-Cl, pH 7.6, 0.1% Triton X-100, and 1 mL LiCl DTT), and 2 times with 1 mL wash buffer (20 mmol/L HEPES, pH 7.2, 2 mmol/L EGTA, 10 mmol/L MgCl2, 1 mmol/L DTT, and 0.1% Triton X-100). For Western blot analysis, cell lysates or immunoprecipitates were subjected to SDS-PAGE and proteins were transferred to nitrocellulose membranes (Hybond-ECL; Amer sham) as previously described. The membrane was blocked for 1 hour at room temperature with a commercial blocking buffer from GIBCO BRL. The blots were then incubated for 4 hours at room temperature with anti-BMK1, anti-Src (Santa Cruz), anti-Csk (Santa Cruz), or activated-Src antibody clone 28, followed by incubation for 1 hour with secondary antibodies (horseradish peroxidase conjugated). For ERK1/2 activation, the blots were incubated for 12 hours with anti-phosphospecific ERK1/2 (New England Biolabs) or nonspecific ERK1 and ERK2 antibodies (Santa Cruz). Immunore-
active bands were visualized with the use of enhanced chemiluminescence (ECL Kit; Amersham International).

**p90RSK and BMK1 Kinase Assays**

p90RSK kinase activity was measured through the use of GST-NHE-1 phosphorylation and BMK1 kinase activity was measured through the use of autophosphorylation as described previously with slight modifications. For analysis of whole cell extracts, heart powders were homogenized with 3 vol of lysis buffer and centrifuged at 14 000g (4°C for 30 minutes), and protein concentrations were determined. p90RSK or BMK1 were immunoprecipitated through the incubation of 1000 µg protein from each sample with 3 µL of the rabbit polyclonal anti-p90RSK (Santa Cruz) antibody and anti-BMK1 antibody for 3 hours, the addition of 40 µL of a 1:1 slurry of protein A/Sepharose (Pharmacia Biotech) beads to the extract/antibody mixture, and then incubation for 1 hour at 4°C. The beads were washed 2 times with 1 mL lysis buffer, 2 times with 1 mL LiCl wash buffer, and 2 times in 1 mL modified buffer A (20 mmol/L HEPES, pH 7.2, 2 mmol/L EGTA, 10 mmol/L MgCl2, 1 mmol/L DTT, and 0.1% Triton X-100). Immunoprecipitated p90RSK and BMK1 were resuspended in 25 mmol/L HEPES, pH 7.4, 10 mmol/L MgCl2, and 10 mmol/L MnCl2, and the kinase reaction was initiated by the addition of 3 µCi of [γ-32P]ATP. After the reaction proceeded for 20 minutes at 30°C, it was terminated by the addition of Laemmli’s sample buffer. BMK1 kinase activity of the immunoprecipitate was measured at 30°C for 20 minutes in a reaction mixture (40 µL) containing 15 µmol/L ATP, 10 mmol/L MgCl2, 10 mmol/L MnCl2, and 3 µCi of [γ-32P]ATP. Proteins were analyzed with 10% SDS-PAGE, followed by autoradiography. NHE-1 phosphorylation and BMK1 autophosphorylation were determined through densitometry of bands at the correct molecular weights in the linear range of film exposure with the use of a scanner and NIH Image 1.54.

**Csk Activity Assay**

Immunoprecipitated Csk kinase activity was measured through the phosphorylation of poly(E4Y) with acid precipitation onto filter paper. The phosphorylation reactions were performed in a volume of 50 µL at 30°C for 30 minutes. The standard phosphorylation reaction contained 3 µCi of [γ-32P]ATP, 1 mg/mL poly(E4Y), 6 mmol/L MgCl2, 75 mmol/L HEPES-NaOH (pH 8.0), 5% glycerol, 0.005% Triton X-100, and 0.05% 2-mercaptoethanol. At the end of the reaction time, 35 µL of the reaction mixture was spotted onto p81 Whatman filter paper, which was washed in 5% TCA at 65°C (3 times for 10 minutes each). The radioactivity incorporated into poly(E4Y) was determined through liquid scintillation counting.

**Materials**

All materials were obtained from Sigma Chemical Co, except where indicated. H2O2 was obtained from Fisher Scientific.

**Statistical Analysis**

Data are reported as mean±SD. Statistical analysis was performed with the StatView 4.0 package (Abacus Concepts). Differences were analyzed with 1- or 2-way repeated measures ANOVA as appropriate, followed by Scheffe’s correction.

**Results**

**Activation of ERK1/2 and p90RSK by Ischemia and Ischemia/Reperfusion**

To determine whether ERK1/2 and p90RSK are activated in the perfused heart in response to ischemia and ischemia/reperfusion, we performed Western blotting with antiphosphospecific ERK1/2 and an immune complex kinase assay with GST-NHE-1 as substrate, respectively. ERK1/2 was not activated by ischemia alone, as shown by other investigators, even at early time points. Figures 2A and 2C. There was a small but significant activation of ERK1/2 by ischemia/reperfusion (2.5±1.1-fold increase with 20-minute ischemia and 10-minute reperfusion; Figures 2B and 2C). In contrast, p90RSK was activated rapidly and transiently by ischemia alone (4.3±1.3-fold increase), and activity rapidly declined within 20 minutes (Figures 3A and 3C). Reperfusion after 20-minute ischemia also activated p90RSK (5.6±1.5-fold increase with 20-minute ischemia and 10-minute reperfusion) and was sustained for 20 minutes after reperfusion (Figures 3B and 3C). Although reperfusion after ischemia activated both ERK1/2 and p90RSK in the perfused heart, only p90RSK was activated by ischemia alone (Figures 2 and 3). These results suggest that in addition to ERK1/2, there is an alternative pathway for the activation of p90RSK.
Regulation of BMK1 and Src by Ischemia and Ischemia/Reperfusion

We showed previously that ROS activated BMK1 in vascular smooth muscle cells, endothelial cells, and fibroblasts. Because there is significant release of ROS on reperfusion after ischemia, we determined the activation of BMK1 in response to ischemia/reperfusion in the perfused heart. Ischemia alone stimulated BMK1, which was maximal at 30 minutes (5.8 ± 1.5-fold increase) and sustained for 40 minutes after ischemia (Figures 4A and 4C). However, in contrast to ERK1/2 and p90RSK, BMK1 activity was significantly inhibited by reperfusion after ischemia (Figures 4B and 4C). No difference in the amount of BMK1 was observed in BMK1 immunoprecipitated from ischemia- and ischemia/reperfusion–treated heart samples on Western blot analysis with anti-BMK1 antibody (Figures 4A and 4B, bottom).

To determine the role of Src in the BMK1 signaling pathway activated by ischemia and ischemia/reperfusion, we investigated the effect of ischemia/reperfusion on Src activation in the perfused heart. Src has been shown to be activated by ROS in Jurkat T cells, fibroblasts, and endothelial cells. In addition, we demonstrated previously that the activation of BMK1 by H₂O₂ in fibroblasts is dependent on Src. Src activity was measured through Western blotting with Src antibody clone 28, which recognizes the activated form of Src. This assay was validated in a comparison of clone 28 immunoreactivity with Src activity measured on the basis of ²⁵P incorporation into soluble enolase. There was a good correlation between the 2 techniques as previously described. Ischemia alone stimulated Src activation, which was maximal at 30 minutes (6.7 ± 1.8-fold increase) and sustained for 40 minutes after ischemia (Figures 5A and 5C). Similar to BMK1 activity, Src activity was markedly inhibited by ischemia/reperfusion (Figures 5B and 5C). No difference in the amount of Src was observed in lysates from ischemia- and ischemia/reperfusion–treated heart samples on Western blot analysis with anti-Src (Figures 5A and 5B, bottom). These results suggest that Src and BMK1 are coregulated by ischemia and reperfusion in the perfused heart.

To confirm the role of Src as an upstream signaling mediator of BMK1 activation by ischemia, we studied the...
effect of the Src-specific inhibitor PP2 on ischemia-induced BMK1 activity (Figure 6). PP2 interacts specifically with Src family kinases and is a competitive inhibitor of ATP.26 As shown in Figure 6, 10 μmol/L PP2 completely inhibited ischemia-induced BMK1 activation. These results support an important role for Src in ischemia-induced activation of BMK1.

Role of ROS in Activation of ERK1/2, p90RSK, Src, and BMK1 in Perfused Hearts

We4,6,7 and others27–31 have shown that cellular stresses, including ROS, activate Src, ERK1/2, p90RSK, and BMK1 kinases in several cell lines. All 4 kinases showed significant activation when the hearts were perfused with H2O2 (100 μmol/L) for 20 minutes (ERK1/2 4.2-fold increase, p90RSK 3.4-fold increase, Src 3.8-fold increase, BMK1 3.1-fold increase) (Figure 7). H2O2 decreased left ventricular developed pressure and increased left ventricular end-diastolic pressure in the heart as described previously (Table).32,33 These results suggested that these kinases could also be activated by endogenously generated ROS in hearts. Importantly, the control activities of these kinases were not inhibited by H2O2 stimulation, which is in contrast to the inhibition of Src and BMK1 by reperfusion in the perfused heart.

To define the role of ROS in BMK1 and p90RSK activation, we pretreated the hearts with the antioxidant MPG, as described previously,18,19 and determined the effect of MPG on ERK1/2 and p90RSK activation during reperfusion and on Src and BMK1 activation during ischemia. We found that MPG completely inhibited ischemia/reperfusion–induced ERK1/2 and p90RSK activation (Figures 8A and 8B) but only partially inhibited Src and BMK1 activation during

Figure 5. Activation of Src in hearts exposed to ischemia and ischemia/reperfusion. Perfusion protocols and data analysis were performed as described in legend for Figure 2. A and B, Src kinase activity in whole extracts was measured through Western blot analysis with Src antibody clone 28, which recognizes activated form of Src (top). No difference in amount of Src was observed in lysates from any of heart samples on Western blot analysis with anti-Src antibody (Santa Cruz) (bottom). C, Densitometric analysis of Src activation (values are mean±SD, n=3; *P<0.05). IB indicates immunoblotting.

Figure 6. Src-specific inhibitor PP2 inhibits BMK1 activation during ischemia. Hearts were perfused with 10 μmol/L PP2 or vehicle for 20 minutes and then perfused under control conditions or subjected to ischemia (20 minutes). BMK1 activity was analyzed through autophosphorylation in an immune complex kinase assay. BMK1 protein level was assayed through Western blot analysis with anti-BMK1 antibody. A, Representative autoradiogram showing BMK1 kinase activity (top) and Western blot analysis showing BMK1 protein levels (bottom). B, Densitometric analysis of BMK1 kinase activity (values are mean±SD, n=3; **P<0.01). IB indicates immunoblotting.

Figure 7. Activation of ERK1/2, p90RSK, Src, and BMK1 in hearts exposed to oxidative stress. Hearts were perfused under control conditions (−) or perfused with H2O2 (100 μmol/L for 20 minutes). A, ERK1/2 activity in whole extracts was measured through Western blot analysis with a phosphospecific ERK1/2 antibody. No difference in amount of ERK1/2 was observed in lysates from any of heart samples on Western blot analysis with anti-ERK1/2 (data not shown). B, p90RSK activity was measured through use of an in vitro kinase assay with GST-NHE-1 (625-747) as substrate. C, Src kinase activity in whole extracts was measured through Western blot analysis with Src antibody clone 28. No difference in amount of Src was observed in lysates from any of heart samples with anti-Src antibody (Santa Cruz) (data not shown). D, Representative autoradiogram showing BMK1 kinase activity. No difference in amount of BMK1 was observed in immunoprecipitates from any of heart samples with anti-BMK1 antibody (data not shown). Data shown are representative of experiments repeated 2 times with different heart samples. IB indicates immunoblotting.
ischemia (Figures 8C and 8D). These data support a major role for ROS in the stimulation of ERK1/2 and p90RSK after ischemia/reperfusion but only a partial role in ischemia-induced Src and BMK1 activation. These results suggest that there is an alternative pathway to ROS for Src and BMK1 activation by ischemia.

**Regulation of Csk by Ischemia and Ischemia/Reperfusion**

The protein tyrosine kinase Csk phosphorylates Src family kinases on a tyrosine residue located near the carboxyl terminus.34 This phosphorylation downregulates Src kinase, suggesting that Csk may inhibit Src during reperfusion (Figure 5). Therefore, we determined Csk activity during ischemia and reperfusion through the use of an in vitro kinase assay with poly(E4 Y) as a substrate.22 Ischemia rapidly activated Csk activity, and the maximal activation occurred at 10 minutes (7.4 ± 1.6) (Figure 9). Unexpectedly, reperfusion activated Csk activity, and the maximal activation occurred at 106 ± 10, 7 ± 1, 97 ± 12, 1966 ± 259, 1711 ± 189, 52 ± 18*, 27 ± 13#, 25 ± 14*, 451 ± 79*, −358 ± 51* minutes, respectively.

Ischemia/reperfusion at 10 minutes

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<th>Baseline</th>
<th>End of Perfusion</th>
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<td>LVSP</td>
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<tr>
<td>Ischemia at 10, 20, 30, and 40 minutes (n=12)</td>
<td>106 ± 10</td>
<td>8 ± 1</td>
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<tr>
<td>Ischemia/reperfusion at 10 minutes (n=3)</td>
<td>104 ± 10</td>
<td>7 ± 1</td>
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<tr>
<td>Ischemia/reperfusion at 20 minutes (n=3)</td>
<td>115 ± 12</td>
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<td>H2O2 (n=3)</td>
<td>110 ± 8</td>
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LVSP indicates left ventricular peak systolic pressure; LVEDP, left ventricular end-diastolic pressure; DP, developed pressure; and dP/dt max and dP/dt min, maximal and minimum rates of left ventricular pressure development, respectively.

Distribution

The major finding of this study was that Src and BMK1 are coregulated by ischemia and ischemia/reperfusion in the perfused heart, at least in part, via a pathway other than H2O2 (Figures 7 and 10). Data to support this conclusion include the findings that ERK1/2 and p90RSK, as well as Src and BMK1, were activated when hearts were perfused by H2O2 (Figure 7). In contrast, global ischemia stimulated p90RSK, but not ERK1/2, in the perfused heart (Figures 2 and 3). On reperfusion of ischemic hearts, both ERK1/2 and p90RSK activities were increased (Figures 2 and 3). These results indicate that in addition to ERK1/2, there is an alternative pathway via which ischemia activates p90RSK. Global ischemia also stimulates Src and BMK1 activities, but the activities of Src and BMK1, in contrast to those of ERK1/2 and p90RSK, are markedly attenuated by ischemia/reperfusion (Figures 4 and 5). Furthermore, we found that the antioxidant MPG completely inhibited ischemia/reperfusion–induced ERK1/2/p90RSK activation but only partially inhibited Src and BMK1 activation during ischemia. These findings demonstrate important differences in MAPK signal transduction pathways activated via ischemia and ischemia/reperfusion in the heart.

We summarize the data for ischemia- and ischemia/reperfusion–mediated signal transduction leading to the activation of BMK1 and p90RSK in the heart based on the present study (Figure 10): the key features of these findings are (1) differential activation of BMK1 and ERK1/2 by ischemia and (2) opposing effects of reperfusion on the Src and BMK1 pathway versus the ERK1/2 and p90RSK pathway. Both Src and BMK1 are redox-sensitive kinases, and Src is required for BMK1 activation by H2O2 in fibroblasts.1 We also have shown through the use of cultured fibroblasts and Jurkat T cells that p90RSK, an important downstream effector of ERK1/2, is activated by ROS in an ERK1/2-independent manner.16 In the present study, we show for the first time that H2O2 activates p90RSK, Src, and BMK1 in the perfused heart (Figure 7). The fact that ischemia alone activates p90RSK but not ERK1/2 (Figure 10) in the present study suggests that besides ERK1/2, there is an alternative pathway by which to activate p90RSK. A candidate for this pathway is Fyn, which we have shown to regulate H2O2-mediated p90RSK activation in fibroblasts.16 Because we do not have a specific Fyn antibody for the analysis of cardiac myocytes, we could not include Fyn activation data in the present study.

We have shown that p90RSK phosphorylates Ser203 of NHE-1, and this phosphorylation is required for growth factor stimulation of Na+/H+ exchange.14 Bugge and Ytrehus35 and others36,37 suggested that the inhibition of NHE-1 by HOE642 provided cardioprotective and antiarrhythmic effects in hearts exposed to ischemia and reperfusion. The ultimate biological effects of activation of p90RSK may depend on the duration and extent of this kinase activation. Future studies will be required to determine the role of p90RSK in ischemia and reperfusion–mediated signal transduction (especially NHE-1 activity) in ischemic heart.

It was recognized some years ago that there was a significant release of ROS and increase in H2O2,28 OH−,30 and O2−,39 in ischemia and in reperfusion occurring after ischemia. Therefore, the effect of ischemia/reperfusion of inhibition of Src-BMK1 activity was an unexpected finding. One of the candidate inhibitors was Csk,34,42 which has been found to phosphorylate the carboxyl-terminal tyrosine ( Tyr527) of c-Src, thereby suppressing c-Src kinase activity. However, we found that ischemia/reperfusion did not increase Csk activity,
although Csk was activated by ischemia. These results suggest 2 possible mechanisms to regulate Src activity during reperfusion: (1) there is another unknown Src Tyr527 tyrosine kinase other than Csk or (2) ischemia/reperfusion inhibits an Src phosphatase that dephosphorylates Src Tyr 527. Future studies will be required to characterize the relative roles of these mediators.

In contrast to the present study, Knight and Buxton 43 and Shimizu et al 44 reported ischemia-induced ERK1/2 activation. There are several differences in the ischemia procedures of the present study compared with those of these groups. First, Knight and Buxton 43 and Shimizu et al 44 used rats instead of guinea pig. Second, Knight and Buxton 43 reported ERK1/2 activation during ischemia, but they used fractionated homogenates and assayed via the incorporation of 32 P into myelin basic protein peptide. Therefore, it is possible that another “MBP kinase,” like p38 kinase or BMK1, may have contaminated the fractions that were used. Finally, in contrast to the Langendorff model of the present study, Shimizu et al 44 reported ERK1/2 activation in a coronary artery ligation model.

The biological consequences of Src and BMK1 activation by ischemia and inhibition by ischemia/reperfusion in the heart are poorly understood. Kato et al 45 reported that BMK1 activity is required for epidermal growth factor–mediated cell

or vehicle for 20 minutes and then perfused under control conditions, subjected to ischemia/reperfusion (20 minutes/20 minutes) (A and B), or subjected to ischemia (20 minutes) (C and D). ERK1/2, p90RSK (A and B), Src, and BMK1 (C and D) were assayed as described in Materials and Methods. B and D, Den- sitometric analysis of ERK1/2 and p90RSK (B) and Src and BMK1 kinase activity (D). Results were normalized for all experiments by arbitrarily setting radioactivity of control heart samples (time=0) at 1.0 (values are mean±SD, n=3; *P<0.05). IB indicates immunoblotting.

Figure 8. Effects of MPG on ERK1/2 and p90RSK activation through ischemia/reperfusion and Src and BMK1 activation through ischemia. Hearts were perfused with 300 μmol/L MPG
proliferation and cell cycle progression. In contrast to p38 and JNK, the activation of BMK1 is not apoptotic.\textsuperscript{45,46} BMK1 has recently been reported to phosphorylate MEF2C, which in these 2 redox-sensitive kinase pathways serve different intra-activated p90RSK but “shut off” Src and BMK1 suggests that pathway in ischemic myocardium.

In summary, we have shown that p90RSK, Src, and BMK1 are activated by ischemia. The fact that ischemia/reperfusion activated p90RSK but “shut off” Src and BMK1 suggests that these 2 redox-sensitive kinase pathways serve different intracellular functions with respect to reperfusion.

Acknowledgments

This study was supported by National Institutes of Health Grants HL-44721 and HL-49192 (to Dr Berk) and Grant HL-52318 (to Dr Walsh). The authors wish to thank Drs C. Yan, H. Ueba, M. Okuda, and H. Umemori for their invaluable assistance and critical reading of the manuscript.

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Effects of I/R on p90RSK and BMK1 Activity

Figure 10. Comparison of Src, BMK1, ERK1/2, and p90RSK activation through ischemia and ischemia/reperfusion. Perfusion protocols and data analysis were performed as described in legend for Figure 2. To more clearly show comparison, we did not include SDs.


Differential Regulation of p90 Ribosomal S6 Kinase and Big Mitogen–Activated Protein Kinase 1 by Ischemia/Reperfusion and Oxidative Stress in Perfused Guinea Pig Hearts

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*Circ Res.* 1999;85:1164-1172
doi: 10.1161/01.RES.85.12.1164

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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