The signal transduction pathways that mediate activation of trans acting factors controlling an organ's response to ischemia are unknown. The stress-activated protein kinases (SAPKs), a subfamily of the extracellular signal-regulated kinases (ERKs), phosphorylate c-Jun within the amino-terminal transactivation domain and are activated in response to a variety of cellular stresses. We determined whether SAPKs are activated in response to ischemia, an extreme, albeit common, pathophysiologic stress. Rats underwent 40 min of renal ischemia followed by reperfusion for 0, 5, 20, or 90 min. SAPKs were immunoprecipitated from kidney lysates and kinase activity assayed with recombinant GST-c-Jun(1-135) containing the amino-terminal transactivation domain of c-Jun as substrate. SAPKs were not activated by ischemia alone, but reperfusion for as little as 5 min was associated with a 4.6-fold increase in kinase activity. Kinase activity was increased 7.6-fold at 20 min following reperfusion and remained elevated at 90 min of reperfusion (4.9-fold). In contrast, activity of the related ERK-1 and -2 was increased only 1.3-fold and only at the 5-min reperfusion time point. When SAPKs were immunodepleted from kidney extracts prior to incubation of the extracts with agarose-coupled GST-c-Jun(1-135), it was found that SAPKs accounted for the majority of the amino-terminal c-Jun kinase activity of kidney at 5 min following reperfusion. In Madin-Darby canine kidney epithelial cells, ATP depletion induced by chemical anoxia, was associated with a 9-15-fold activation of SAPKs with a similar time course of activation to that seen in the kidney after ischemia and reperfusion. In conclusion, the SAPKs are markedly activated very early after reperfusion of ischemic kidney and following ATP depletion of anoxic cells in culture. We propose that this activation of SAPKs may trigger part of the kidney's early genetic response to ischemia, possibly by enhancing trans acting activity of c-Jun.

Reperfusion of ischemic tissue can lead to a variety of different fates for the affected cells, depending upon the genetic programs that are activated. In the kidney, tubular epithelial cells are particularly vulnerable to ischemia. If the insult to the cell is severe enough, the cell will die. If the cell survives while others near it have died, then the cell dedifferentiates and enters the cell cycle to replace irreversibly injured cells (1, 2). The dedifferentiation and proliferative responses, which ultimately lead to tissue repair, depend on a poorly defined genetic program involving cis- and trans acting factors. The trans acting factors controlling these responses and the signal transduction pathways modulating their activity are largely unknown.

In general, there are many similarities between the gene expression induced by ischemic renal damage and that following growth factor stimulation of cultured cells. Induction of jun and fos has been reported in response to ischemia and reperfusion in the kidney (2-5), heart (6), and brain (7, 8) and in cardiac myocytes made chemically hypoxic (9, 10). c-Jun transcription is controlled by a 12-O-tetradecanoyl-phorbol 13-acetate response element in the c-Jun promoter that binds AP-1, a c-Jun homodimer or a heterodimer of transcription factors of the Jun and Fos families. Trans acting activity of c-Jun is likely positively regulated by phosphorylation of 2 serine residues in the amino terminus (11-14). Phosphorylation of these sites in vitro is induced by phorbol esters, growth factors, several oncogenes, cytokines, UV radiation, and heat shock (11, 12, 15-19). The stress-activated protein kinases (SAPKs)' (18, 21-), are a subfamily of the extracellular signal-regulated kinase (ERK) family of serine/threonine kinases, but, unlike the related ERK-1 and ERK-2, are weakly activated by growth factors. Instead, the SAPKs are markedly activated in response to the inflammatory cytokine tumor necrosis factor-α, UV irradiation, and cellular stresses including heat shock and inhibitors of protein synthesis (18, 22). The SAPKs and, to a lesser extent, ERK-1 and -2 stoichiometrically phosphorylate c-Jun at the putative regulatory amino-terminal serine residues in vitro (12).

Transcription of c-fos is under control of the serum-response element, which is bound by the transcription factors p65 and p52 in a ternary complex. p65 and the related Elk-1, are phosphorylated by ERK-1 and -2 and this phosphorylation enhances ternary complex formation (23, 24).

These data suggest that members of the ERK family, including the SAPKs, may play a role in activating transcription in response to growth factors, oncogenes, and cellular stress. Given the similarities between the gene expression induced by these stimuli and by the extreme cellular stress of ischemia, we postulated that the SAPKs might be activated in response to ischemia and reperfusion, and that this activation might modulate...
late the genetic response. Our data suggest the SAPKs are major c-Jun amino-terminal kinases activated early after ischemia and reperfusion, and may transduce an important ischemia-induced signal to the nucleus.

**EXPERIMENTAL PROCEDURES**

**Animal Protocol**—Male Sprague-Dawley rats weighing 200–300 g (Charles River Breeding Laboratories) were anesthetized with sodium pentobarbital (65 mg/kg) and were administered 10 ml of 0.9% NaCl intraperitoneally prior to surgery. Unilateral renal ischemia was induced by clamping the left renal vein and artery with a microaneurysm clamp (2, 25). After 40 min, the clamp was removed. Ischemic and contralateral kidneys were removed at 0, 5, 20, 40, 60 or 90 min following reperfusion and were homogenized at 4°C in homogenization buffer (20 mM HEPEs pH 7.4; 50 mM β-glycerophosphate; 2 mM EGTA; 1 mM DTT; 250 μM sucrose; 400 μM phenylmethylsulfonyl fluoride; 2 μM leupeptin; 10 μM/ml Trasylol). The lysates were centrifuged at 100,000 x g for 1 h at 4°C. Sodium orthovanadate (to 1 mM) and Triton X-100 (to 0.1%) were added to the supernatants.

**ATP Depletion Experiments**—Madin-Darby canine kidney (MDCK) tubular epithelial cells were grown to confluence in Eagle’s minimum essential medium supplemented with 20% fetal calf serum. Chemical anoxia was induced as described (26). Briefly, cell monolayers (two 10-cm dishes/condition) were incubated in a Krebs-Henseleit buffer (115 mM NaCl, 3.6 mM KCl, 1.3 mM KH₂PO₄, 25 mM NaHCO₃, 1 mM CaCl₂, 1 mM MgCl₂, pH 7.2) with or without 5 mM cycloheximide (in the absence of dextrose). After 40 min, cells were washed once and incubated for 0, 5, 20, or 90 min with Krebs-Henseleit buffer containing 10 mM dextrose. All incubations were performed at 37°C in a 95% air, 5% CO₂ incubator. Cells were washed with cold Tris-buffered saline and then lysed in lysis buffer (20 mM HEPEs pH 7.4; 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1% Triton X-100; 10% glycerol, 1 mM EGTA, 1 mM DTT, 400 μM phenylmethylsulfonyl fluoride, 2 μM leupeptin, 2 μM pepstatin, 10 units/ml Trasylol). With this protocol, during the cytoside/2-deoxyglucose treatment, ATP content of cells is reduced to less than 10% of control (26).

**Immunodepletion of SAPKs and Immune Complex Kinase Assays**—Supernatants from kidney lysates or MDCK cell lysates were matched for protein concentration (Bio-Rad) prior to immunoprecipitation with a polyclonal antiserum raised against the β1 isoform of SAPK expressed as a glutathione S-transferase fusion protein in E. coli (18). Immune complexes were collected with protein G-Sepharose beads. The supernatants were then incubated with GST-c-Jun(1-135) beads were washed once and incubated with 0, 5, 20, or 90 min with Krebs-Henseleit buffer containing 10 mM dextrose. All incubations were performed at 37°C in a 95% air, 5% CO₂ incubator. Cells were washed with cold Tris-buffered saline and then lysed in lysis buffer (20 mM HEPEs pH 7.4; 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1% Triton X-100; 10% glycerol, 1 mM EGTA, 1 mM DTT, 400 μM phenylmethylsulfonyl fluoride, 2 μM leupeptin, 2 μM pepstatin, 10 units/ml Trasylol). With this protocol, during the cytoside/2-deoxyglucose treatment, ATP content of cells is reduced to less than 10% of control (26).

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**Immunodepletion of SAPKs**—For immunodepletion experiments, supernatants from kidney lysates or MDCK cell lysates were incubated with a 1:100 dilution of either anti-SAPK antiserum or preimmune serum for 2 h at 4°C, followed by the addition of a second 1:100 dilution of anti-pan versus preimmune serum for 2 h. Immune complexes were collected with protein G-Sepharose beads. The supernatants were then incubated with GST-c-Jun(1-135), which had been adsorbed onto glutathione-agarose beads (27). After 40 min, the GST-c-Jun(1-135) beads were washed once with reaction buffer, and the 1:1 mixture was resuspended 1:1 in assay buffer. Kinase reactions were started by the addition of MgCl₂ (to 10 mM) and (γ-32P)ATP (to 100 μM). After 15 min at 30°C, the reactions were stopped, and the samples were processed for SDS-polyacrylamide gel electrophoresis as above.

**ERK-1 and -2 Activity**—ERK-1 and -2 activity was assayed as described (28) with some modifications. The supernatants from kidney lysates or MDCK lysates were matched for protein and loaded onto a Mono Q anion exchange column (Pharmacia), which had been pre-equilibrated with 50 mM β-glycerophosphate, pH 7.3, 1 mM EGTA, 1 mM EDTA, 0.1% sodium orthovanadate, 400 μM phenylmethylsulfonyl fluoride, 2 μM leupeptin, 2 μM pepstatin, and 1 mM dithiothreitol (Trasylol). Proteins were eluted with an 18-mL linear gradient of NaCl (0–500 mM) and 1-mL fractions were collected. Ten microliters of the column fractions or appropriate controls were added to 6 μl of assay buffer (150 mM Tris, pH 7.4, 5 mM DTT, 45 μM heat stable inhibitor of protein kinase A) containing 1 mg/ml of myelin basic protein, which is not a substrate for the SAPKs, and the reaction was started by the addition of MgCl₂ and (γ-32P)ATP as above. After 20 min at 30°C, 15 μl of the assay mixture was spotted on phosphocellulose squares and immersed in 10% trichlo-

**RESULTS**

**Rerfusion of Ischemic Kidney Activates SAPKs**—Rats were subjected to unilateral renal ischemia for 40 min followed by reperfusion for 0, 5, 20, 40, 60, or 90 min (all n = 2 except 40 and 60 min, n = 1) as described under “Experimental Procedures.” This duration of ischemia followed by reperfusion typically results in renal tubular necrosis, particularly in the S3 segment of the proximal tubule, followed by a regenerative response (2). After 40 min of ischemia alone without reperfusion, SAPK activity was reduced to 32% of sham-operated control. Reperfusion was associated with activation of the SAPKs in all animals (p < 0.001 versus sham control or contralateral kidney control). After 5 min of reperfusion, kinase activity increased 14-fold compared with the pre-reperfusion value and was increased 4.6-fold over sham-operated control (Fig. 1A). SAPK activity increased further with longer durations of reperfusion to a peak at 20 min (7.5-fold versus sham control), and remained elevated at 90 min of reperfusion (4.9-fold; p < 0.01 for all reperfusion time points versus sham-operated control or contralateral kidney control). In the nonischemic contralateral kidney, SAPK activity increased to a maximum of 1.5-fold over the sham control at the 20-min time point (data not shown).

**Activity of the related ERK-1 and -2 also decreased during ischemia (Fig. 1B). In contrast to the SAPKs, ERK-1 and -2 were only minimally (and not statistically significantly) activated following reperfusion. Total ERK-1 and -2 kinase activity was increased 1.3-fold after 5 min of reperfusion. After 20 min of reperfusion, ERK-1 and -2 activity was not different from control. Thus ischemia and reperfusion markedly activated the SAPKs, and this activation is not due to a nonspecific activation of protein kinases in posts ischemic tissue.

**Chemical Anoxia of MDCK Cells in Culture Activates the SAPKs**—Ischemia results in a decrease in cellular ATP levels. To explore the role of ATP depletion in SAPK activation, we depleted MDCK cells of ATP by exposing them to cyanide plus 2-deoxyglucose for 40 min (26). ATP depletion was then accomplished by reexposure of cells to dextrose. With this protocol, SAPKs were markedly activated during ATP repletion with a time course of activation paralleling that seen with ischemia and reperfusion in the whole kidney. With ATP depletion alone, SAPK activity fell to 13% of control, but after only 5 min of ATP repletion, SAPK activity was increased 9-fold over pre-ATP depletion values and 66-fold over post-ATP depletion values (both p < 0.01; Fig. 2A). SAPK activity peaked after 20 min of ATP repletion (15-fold increase over control; p < 0.01) and then declined to control values at 90 min of reperfusion. This degree of activation of SAPKs was significantly greater than that seen following heat shock (42°C for 40 min; 4-fold activation) and...
Jun. In addition, we reasoned that if activation of the SAPKs proteins associating with the c-Jun amino terminus (ERK-1 and -2) were then activated by ischemia and reperfusion, the SAPKs should account for a significant percent of the total c-Jun amino-terminal bound kinase activity in reperfused kidney. To explore this possibility, we depleted reperfused kidney extracts of SAPKs by incubation with anti-SAPK serum (18) or exposed extracts to preimmune serum. Immune complexes were then collected with protein G beads and washed prior to kinase assay. Ischemia and reperfusion led to a marked increase in total c-Jun amino-terminal bound kinase activity (Fig. 3A). Five minutes following reperfusion, 54% of the total c-Jun amino-terminal kinase activity was removed by preincubation of the extracts with anti-SAPK serum (Fig. 3, A and B). Despite the further increase in total SAPK activity at 20 min of reperfusion (Fig. 1A), the percent of c-Jun amino-terminal kinase activity accounted for by SAPKs decreased, indicating activation of other c-Jun amino-terminal kinases or a change in the immunoreactivity of the SAPKs at the later time points. SAPKs accounted for approximately 31% of total c-Jun amino-terminal-bound kinase activity at the 20-min time point. Since the antiserum used in these experiments immunoprecipitates the β isoforms more efficiently than the α and γ isoforms, we believe the estimates of the percent of total c-Jun amino-terminal kinase activity for by SAPKs noted above may underestimate the true percentage.

Tryptic phosphopeptide mapping of the GST-c-Jun(1–135) from these experiments confirmed that the GST-c-Jun(1–135) was phosphorylated almost exclusively on peptides corresponding to ERK-1 and ERK-2. ERK activity of ischemia-reperfused kidneys was assayed using GST-c-Jun(1–135) as substrate as described under "Experimental Procedures." Kinase activity in the two peaks corresponding to ERK-1 and ERK-2 were added to give total ERK activity (ERK1/2). Value at each time point is from two experiments. The small activation of ERK-1 and -2 relative to the activation of SAPKs was similar to the pattern seen with ischemia and reperfusion in the kidney.

SAPKs Are Predominant c-Jun Amino-terminal Kinases Activated Early after Ischemic Kidney Injury—Stimuli that increase phosphorylation of serines 63 and 73 within the c-Jun transactivation domain increase binding of activated c-Jun amino-terminal kinases to the amino terminus of immobilized c-Jun (27). We wanted to determine whether ischemia and reperfusion increased binding of these activated kinases to c-Jun. In addition, we reasoned that if activation of the SAPKs was an important early response of the kidney to ischemia and reperfusion, the SAPKs should account for a significant percent of the total c-Jun amino-terminal bound kinase activity in reperfused kidney. To explore this possibility, we depleted reperfused kidney extracts of SAPKs by incubation with anti-SAPK serum (18) or exposed extracts to preimmune serum. Immune complexes were then collected with protein G beads and discarded, and the supernatants were incubated with immobilized GST-c-Jun(1–135). The GST-c-Jun beads (and proteins associated with the c-Jun amino terminus) were then washed prior to kinase assay. Ischemia and reperfusion led to a marked increase in total c-Jun amino-terminal bound kinase activity (Fig. 3A). Five minutes following reperfusion, 54% of the total c-Jun amino-terminal kinase activity was removed by preincubation of the extracts with anti-SAPK serum (Fig. 3, A and B). Despite the further increase in total SAPK activity at 20 min of reperfusion (Fig. 1A), the percent of c-Jun amino-terminal kinase activity accounted for by SAPKs decreased, indicating activation of other c-Jun amino-terminal kinases or a change in the immunoreactivity of the SAPKs at the later time points. SAPKs accounted for approximately 31% of total c-Jun amino-terminal-bound kinase activity at the 20-min time point. Since the antiserum used in these experiments immunoprecipitates the β isoforms more efficiently than the α and γ isoforms, we believe the estimates of the percent of total c-Jun amino-terminal kinase activity for by SAPKs noted above may underestimate the true percentage.

Tryptic phosphopeptide mapping of the GST-c-Jun(1–135) from these experiments confirmed that the GST-c-Jun(1–135) was phosphorylated almost exclusively on peptides X and Y at each point in the time course (Fig. 4). These data confirm that early after ischemia and reperfusion, the predominant c-Jun X/Y kinases are the SAPKs.

**DISCUSSION**

The molecular response of tissue to ischemia and reperfusion is a complex and poorly understood process. In the kidney, reversibly injured tubular epithelial cells can dedifferentiate.

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*J. M. Kyriakis and J. R. Woodgett, unpublished observations.*
and enter the cell cycle to replace irreversibly injured cells (1, 2). The genetic response to ischemia and reperfusion clearly includes transcription of a number of genes, including the immediate early genes, c-jun, c-fos, Egr-1, and, possibly, c-myc (2–10, 30, 31). However, the signal transduction pathways activated by the ischemic insult that might, in turn, activate critical \\textit{trans} acting factors are unknown.

Efforts have been made to identify protein kinases that are activated by ischemia since protein kinases appear to play such a critical role in the control of transcription in response to other stimuli such as growth factors and oncogenes (13, 14). Since cytosolic free [Ca\(^{2+}\)] increases during ischemia, changes in activity of the calcium/calmodulin-dependent kinase II (CaM kinase II) have been examined in ischemic and reperfused tissue. While CaM kinase II appears to undergo redistribution from a cytosolic to a particulate fraction following ischemia, there is no activation of the kinase. In most reports, CaM kinase II is rapidly inactivated following ischemia, and activity does not return to control levels for many hours following reperfusion (32–34).

Protein kinase C does appear to play a role in the response to ischemia, although the precise nature of that role is not clear. The protein kinase C inhibitor, calphostin C, inhibits the preconditioning effect in myocardium, which is the protection afforded by a short period of ischemia prior to a more sustained period (35, 36). This indicates that protein kinase C activation may be critical to the preconditioning effect. In contrast, down-regulation of protein kinase C or the use of nonselective protein kinase C inhibitors protect hippocampal neurons from anoxia (37, 38), indicating that protein kinase C may contribute to ischemic neuronal death. The mechanism of these effects are unclear since although ischemia induces translocation of protein kinase C isoforms to a particulate fraction, activity of the kinase in those fractions declines with ischemia (39).

The AMP-activated protein kinase is activated by ATP depletion induced by exposure of hepatocytes to fructose (40, 41). Although it is a candidate modulator of the response to ischemia, its activation by ischemia and reperfusion has not been examined.

A 43-kDa mitogen-activated protein kinase was reported to be activated by reperfusion of ischemic hippocampus, but the magnitude of activation was modest (2.8-fold) and the duration of activation was very brief (less than 5 min) (42). Our data on activation of ERK-1 and -2 are in agreement with these findings. We found a small activation of ERK-1 and -2 in kidneys reperfused for 5 min and in previously anoxic MDCK cells exposed to dextrose for 5 min. By 20 min of reperfusion or of dextrose reexposure, ERK-1 and -2 activity was no longer elevated. We cannot exclude an important role for ERK-1 and -2 (or for other related myelin basic protein kinases that co-elute with ERK-1 and -2) in the response to ischemia. We examined whole kidney in this experiment, and the increase in kinase activity might have been greater had we examined particular subpopulations of cells within the kidney such as S3 segment proximal tubular cells, where much of the kidney's proliferative response to ischemia is seen (1, 2). The very modest and transient activation of ERK-1 and -2 in MDCK renal tubular epi-
thelial cells subjected to chemical anoxia suggests, however, that these kinases do not play a major role in the response to the ATP depletion which occurs during ischemia.

The SAPKα1 isoform was first purified from rat liver and was markedly activated by cycloheximide (21). It shared several characteristics with ERK-1 and -2, including the requirement of both tyrosine and threonine phosphorylation for activation (20) and proline-directed substrate specificity and was initially called p54 mitogen-activated protein kinase. Although the SAPKs are 40–45% identical to ERK-1 and -2, they are activated by distinct signalling pathways. In contrast to ERK-1 and -2, mitogens and phorbol esters activate the SAPKs weakly, or not at all. Cellular stresses, such as heat shock and exposure to agents that inhibit protein synthesis or induce protein misfolding or exposure to the inflammatory cytokine tumor necrosis factor-α, dramatically activate SAPKs but have comparatively little effect on ERK-1 and -2. Accordingly, SAPKs are on a response pathway activated by cellular/ pathophysiologic stresses.

We postulated that the SAPKs might be important in the response to ischemia and reperfusion based on several observations. First, the SAPKs are activated by diverse agents that induce cellular stress, and ischemia and reperfusion represent an extreme pathophysiologic stress that induces ATP depletion, acidosis, and substrate depletion. Second, the pattern of gene expression, including transcription of c-jun and c-fos, was compatible with activation of ERK family members since one or more ERKs have been implicated in modulation of transcription factors controlling expression of these genes (12, 23, 24), yet activation of ERK-1 and -2 was minimal (42). Finally, other protein kinases had emerged as likely candidates to modulate the response to ischemia and reperfusion.

We found that after 5 min (data not shown) and 40 mm of ischemia, activity of the SAPKs was not increased, but after reperfusion for as little as 5 min, the SAPKs were markedly activated. Activity peaked at 20 min of reperfusion and then began to decline but remained elevated at 90 min. Given the minimal observed activation of ERK-1 and -2 and the data from studies of the CaM kinase II noted above, the activation of the SAPKs does not appear to be a nonspecific generalized activation of kinases, even of closely related ones, in response to ischemia.

The mechanism of activation of the SAPKs by ischemia and reperfusion is not clear. The ischemia-induced increase in cytosolic free [Ca2+] is likely not sufficient to activate the SAPK pathway since the calcium ionophore, ionomycin, is ineffective in cultured cells (18). Although the SAPKs require phosphorylation of both a threonine and a tyrosine residue, like ERK-1 and -2, the ERK-1 and -2-specific mitogen-activated protein kinase/ERK kinases (MEK) do not activate the SAPKs. If a unique SAPK-mitogen-activated protein kinase/ERK kinase upstream of the SAPKs, its activator(s) is also likely to be unique, since stimuli activating the Ras/Raf-1 pathway do not strongly activate SAPKs (18). Bacterial sphingomyelinase added to cells does activate the SAPKs (18), suggesting that a product of the sphingomyelinase, possibly ceramide, may activate the SAPK pathway. Following ischemia and reperfusion, or after ATP depletion, a sphingomyelinase could be activated, or the reperfusion-induced activation of lipases or free radical-mediated lipid peroxidation could generate intermediates that might act as analogs to ceramide.

As noted, transcription of c-jun, controlled by TPA response elements in the c-jun promoter that bind AP-1, increases in many tissues exposed to ischemia and reperfusion. The SAPKs phosphorylate 2 serine residues in the amino-terminal transactivation domain of c-Jun, serines 63 and 73, which are found on tryptic phosphopeptides Y and X, respectively, and this correlates with increased trans activating activity of c-Jun. ERK-1 and -2 also phosphorylate these sites, although at one-fifth to one-seventh the rate of the SAPKs (12). Stimulate that increase the phosphorylation of peptides X and Y also stimulate the binding of c-Jun kinase-2 to immobilized GST-c-Jun. We therefore determined whether ischemia increased the binding of c-Jun amino-terminal kinases to immobilized GST-c-Jun (1–135), and what proportion of the ischemia-activated c-Jun binding kinase activity might be due to the SAPKs. We found that after 5 min of reperfusion, GST-c-Jun (1–135)-binding kinase activity was markedly increased, and over 50% of that activity could be depleted by SAPK antisera. At later time points, c-Jun amino-terminal kinases other than the SAPKs (and other than ERK-1 and -2) may become more active since the SAPKs account for one-third of total activity at these later times.

In conclusion, we have found that the SAPK family of serine/threonine kinases are markedly activated very early after reperfusion of ischemic kidney and after reperfusion of cellular ATP levels following chemical anoxia. Furthermore, we have identified the SAPK family to be the major c-Jun amino-terminal bound kinases activated early after reperfusion. Given the time course and magnitude of activation, we propose that the SAPKs transduce an important early signal to the nucleus that may, in turn, trigger the complex genetic response to ischemia.

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Stress-activated Protein Kinases and Ischemia