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Stimulation of c-Jun Kinase and Mitogen-Activated Protein Kinase by Ischemia and Reperfusion in the Perfused Rat Heart

Richard J. Knight and Denis B. Buxton[1]

Department of Molecular and Medical Pharmacology, UCLA School of Medicine, Los Angeles, CA 90095-6948

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Ischemia and reperfusion lead to the rapid induction of proto-oncogenes in the heart and subsequent induction of genes with cardioprotective functions. The activity of the transcription factors c-Jun and ATF-2 can be stimulated by activation of c-Jun amino-terminal kinase (JNK) in response to a variety of stresses. Here we show that ischemia and reperfusion led to the activation of JNK and also of the distantly-related mitogen activated protein kinase (MAPK). Activation of JNK, but not (MAPK), was abolished by removal of calcium from the perfusate immediately prior to ischemia. In contrast, infusion of the hydrogen peroxide scavenger catalase abolished activation of MAPK in response to ischemia and reperfusion, but activation of JNK was inhibited significantly by catalase only when superoxide dismutase was also present. Hydrogen peroxide infusion activated MAPK but not JNK, supporting a role for hydrogen peroxide produced during reperfusion in MAPK activation. We conclude that while ischemia and reperfusion activate both JNK and MAPK, the mechanisms of activation are different for the 2 kinases. Activation of these kinases is likely to contribute to altered gene expression in response to ischemia and reperfusion.

Transient periods of myocardial ischemia of insufficient severity to cause cell death lead to reversible dysfunction, a phenomenon often referred to as myocardial "stunning". The reversibly injured myocardium is also characterized by alterations in gene expression; genes encoding proto-oncogenes [1, 2], heat shock proteins and [2–4] and antioxidant enzymes [2] are amongst those expressed in response to myocardial ischemia and reperfusion. Induction of gene expression is also found in response to other stresses, including hypoxia, oxidative stress and hyperthermia [5].

Induction of proto-oncogene expression represents a very early response to ischemia and reperfusion [1, 2], and the products of proto-oncogenes are likely to be involved in the regulation of transcription of other stress-inducible genes. *c-jun* and *c-fos* encode transcription factors which can bind to AP-1 regulatory sites as c-Jun homodimers or c-Jun-c-Fos heterodimers. *c-jun* transcription can be activated by a phorbol ester response element that binds c-Jun homodimers [6] or c-Jun/ATF-2 heterodimers [7]. c-Jun transcriptional activity is enhanced by phosphorylation of 2 serine residues in its transactivation domain, Ser-63 and Ser-73 [8, 9].

Recently a family of novel kinases which bind to the c-Jun transactivation domain and phosphorylate Ser-63 and Ser-73 have been identified [10, 11]. These kinases, known as c-jun NH_2-terminal kinases (JNKs) or stress-activated protein kinases (SAPKs) are activated in response to cellular stresses, including UV light [10, 11], heat shock [11] and to inflammatory cytokines [11, 13]. Activation of JNK has also been shown in response to ischemia and reperfusion in the kidney [14]. AP-1 activity can also be increased by activation of MAPK; while MAPK isoforms do not transcriptionally activate c-Jun or c-Fos directly by phosphorylation, they phosphorylate and activate the transcription factor TCF/Elk-1, leading to increased expression from the c-Fos gene [15]. The aim of this study was to investigate the activation of JNK and MAPK in response to ischemia and reperfusion in the perfused heart, and the mechanisms contributing to kinase activation.

MATERIALS AND METHODS

Materials. Myelin basic protein MAP kinase substrate peptide and cAMP-dependent protein kinase inhibitor peptide were obtained from Upstate Biotechnology (Lake Placid, NY). A pGEX-2T plasmid encoding GST-c-$jun_{(1–79)}$ provided by Dr

[1] Corresponding author. Fax: (310) 825-2843.

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R Davis, U. Mass, was expressed in E. coli, and the fusion protein purified by previously described methods [16]. Superoxide dismutase and catalase were obtained from Sigma (St Louis, MO). Phorbol 12-myristate 13-acetate (PMA) was obtained from Biomol (Plymouth Meeting, PA).

Heart perfusion. Male Sprague-Dawley rats, 200–300g, were allowed standard laboratory chow and water *ad libitum.* Rats were anesthetized with sodium pentobarbital, 10 mg/kg, and the hearts removed rapidly and placed in cold Krebs-Henseleit bicarbonate buffer [17]. Langendorff perfusion with Krebs-Henseleit bicarbonate buffer at a constant flow of 10 ml/min was performed as described previously [18]. The perfusion buffer contained glucose, 10mM and bovine serum albumin, 0.2% (w/v). Calcium was infused via syringe pump to give a final concentration of 2.5mM. Global ischemia was initiated by switching off the perfusion pump; during ischemia the heart was maintained at 37°C by bathing in warmed buffer. Reperfusion was initiated by restarting the pump.

At the end of the perfusion period, hearts were frozen rapidly by clamping between aluminium tongs cooled in liquid N_2. The frozen tissue was pulverized with a porcelain pestle and mortar cooled in dry ice, and the powder stored at −80°C. Homogenates were prepared by weighing ~0.2g of tissue into a Potter homogenizer cooled to −80°C, and homogenizing with 1ml homogenization buffer (HEPES 20mM pH 7.5; β-glycerophosphate 20mM; sodium pyrophosphate 20mM; sodium orthovanadate 0.2mM; EDTA 2mM; sodium fluoride 20mM; benzamidine 10mM; DTT 1mM; leupeptin 25μg/ml; PMSF 50μg/ml) using a teflon pestle. Homogenates were then clarified by centrifugation (10,000g, 5 min), and the supernatants assayed for protein content using the Biorad DC protein assay system.

JNKassay. JNK activity was assayed by solid phase assay, in which GST-c-jun$_{(1–79)}$ bound to glutathione agarose is used as both ligand and substrate for JNK, essentially as described by Hibi et al [10]. Homogenates (100μl) were incubated with shaking at 4°C for 3 h with 10μl of glutathione-agarose to which 2.5μg GST-c-jun$_{(1–79)}$ was pre-bound. Subsequent washes, kinase assay and SDS-PAGE were performed exactly as described [10].

MAPK assay. Homogenates were fractionated using DEAE-Sephacel as described by Mitchell et al [19], using batch elution with 0.5 M NaCl. The eluate was assayed by incorporation of ^{32}P into a myelin basic protein peptide containing residues 95–98 at 25°C for 30 min by the method of Gardner et al [20].

Presentation of results. Results are expressed as means ± standard errors. A unit of kinase activity is defined as the incorporation of 1 pmol phosphate from ATP per minute into the respective substrate, and results are expressed per mg homogenate supernatant protein. Statistical comparisons were made by ANOVA followed by t-test using the Bonferroni method to correct for multiple comparisons.

RESULTS AND DISCUSSION

Activation of JNK and MAPK in response to ischemia and reperfusion. The effects of ischemia and reperfusion on cardiac JNK and MAPK activity are shown in Figure 1. Hearts were perfused and freeze-clamped prior to ischemia, at the end of 15 min ischemia, or after increasing periods of post-ischemic reperfusion. Ischemia alone had no effect on JNK activity but after 5 min of reperfusion, JNK activity was increased 2.7 fold, and at 15 min a 5.1 fold activation was found. After 60 min of reperfusion, activity of JNK had returned to baseline. Activation of JNK in the heart thus follows a similar pattern to that found in the kidney, where a 5-fold activation was found

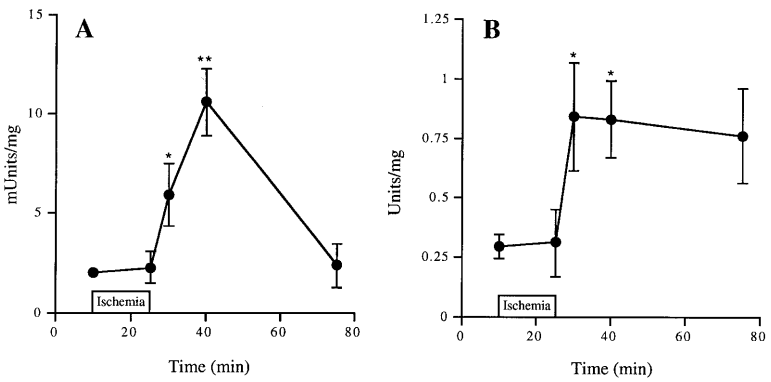

FIG. 1. Time course of JNK and MAPK activation in response to ischemia and reperfusion. Following 10 min of perfusion to stabilize function, hearts were freeze clamped immediately, after 15 min global normothermic ischemia, or after increasing periods of reperfusion. Cardiac homogenates were then assayed for JNK (A) and MAPK (B) as described in the Materials and Methods section. Results are means ± SE for 3–6 hearts at each time point. *, $p < 0.05$ vs pre-ischemia; **, $p < 0.001$ vs pre-ischemia.

20 min post-reperfusion [14]. Cardiac MAPK was also not affected by ischemia alone, but increased 2.9-fold after 5 min reperfusion and remained elevated. The changes in cardiac MAPK activity differ markedly from those obtained in the kidney, where MAPK activation in response to ischemia and reperfusion was much smaller (1.3 fold at 5 min) and more transient, returning to baseline by 20 min [14].

For comparison, hearts were also perfused with the phorbol ester PMA, which is known to activate MAPK in the perfused heart [21]. Infusion of PMA, 1μM, for 5 min led to an 18-fold increase in MAPK activity, from 0.29 ± 0.05 ($n = 6$) to 5.3 ± 1.0 U/mg protein ($n = 2$; $p < 0.001$). In contrast, no significant change in JNK activity was found (control 2.0 ± 0.4 vs PMA 1.9 ± 0.03 mU/mg protein). In control experiments, no change in JNK or MAPK activity was found with increasing time of perfusion (results not shown).

The effect of the duration of ischemia on subsequent activation of JNK and MAPK in response to reperfusion are shown in Figure 2. Short periods of ischemia, 2–10 min, did not lead to significant activation of either MAPK or JNK following 15 min reperfusion. Increasing the ischemic period to 15 min led to pronounced activation of both JNK and MAPK, and extending the ischemic period further to 30 min led to no further change in enzyme activation. Global normothermic ischemia for periods greater than 15–20 min leads to progressive increases in irreversible cell damage, associated with uncontrolled increases in tissue calcium content [22].

Involvement of free radicals in kinase activation. Reperfusion of ischemic myocardium leads to the production of oxygen free radicals, including hydrogen peroxide and superoxide, which are believed to contribute to the pathophysiological effects of ischemia and reperfusion on myocardial function [23]. There is increasing evidence that free radicals may participate in signal transduction pathways. H_2O_2 has been suggested to play a role in the activation of the transcription factor NF-κB [24], and signalling by platelet derived growth factor (PDGF) in rat vascular smooth muscle cells has been shown to require H_2O_2 generation [25]; PDGF signalling, including phosphorylation and activation of MAPK, was inhibited by catalase. Free radical scavengers were therefore employed to assess the potential role of free radicals in activation of the JNK and MAPK pathways during cardiac ischemia and reperfusion. Figure 3 shows that infusion of superoxide dismutase, which dismutes superoxide to O_2 and H_2O_2, had little effect on MAP kinase activation in response to ischemia and reperfusion. In contrast, the inclusion of catalase, which reduces H_2O_2 to O_2 and H_2O, abolished activation of MAPK by ischemia and reperfusion. For JNK, a different pattern was found; neither superoxide dismutase nor catalase alone had significant effects on JNK activation,

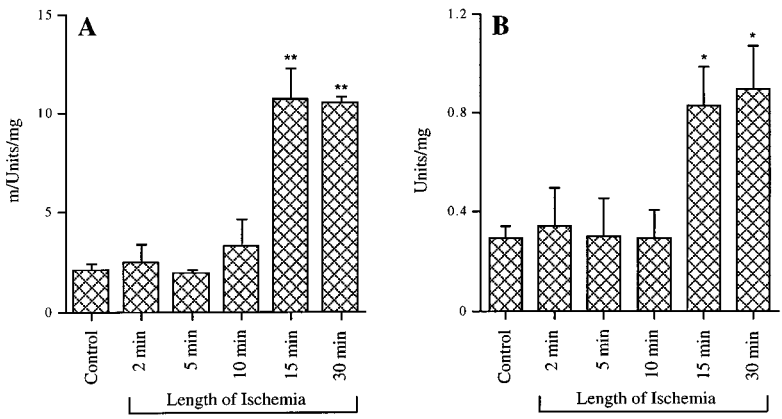

FIG. 2. Effect of length of ischemia on activation of JNK (A) and MAPK (B). Hearts were made globally ischemic for the indicated period, and then reperfused for 15 min. Results are means ± SE for 3–6 hearts. *, $p < 0.025$ vs control; **, $p < 0.001$ vs control.

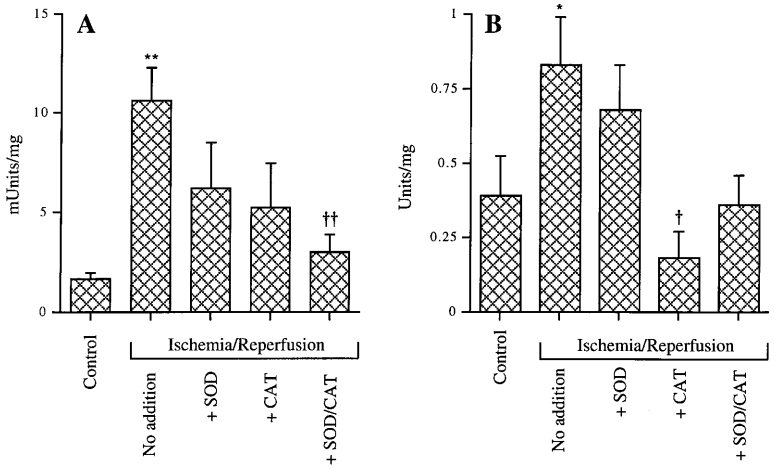

FIG. 3. Effect of infusion of free radical scavengers on activation of JNK (A) and MAPK (B) by ischemia and reperfusion. Infusion of superoxide dismutase, 100U/ml (SOD), catalase, 25U/ml (CAT) or superoxide dismutase + catalase was initiated 5 min prior to ischemia. Infusion was stopped during ischemia, and restarted at the time of reperfusion. Hearts were reperfused for 15 min prior to freeze-clamping. Results are means ± SE for 3–5 hearts. *p < 0.05 vs control; **, p < 0.005 vs control; †, p < 0.05 vs ischemia/reperfusion without scavengers; ‡, p < 0.02 vs ischemia/reperfusion without scavengers.

but in combination the free radical scavengers inhibited JNK activation in response to ischemia/reperfusion.

The experiments with free radical scavengers suggested that formation of H_2O_2 during reperfusion might be of particular importance for MAPK activation, and so the effects of H_2O_2 infusion on kinase activation were determined. Figure 4 shows that infusion of H_2O_2 led to a dose-dependent activation of MAPK. In contrast, no significant effect of H_2O_2 infusion on JNK activity was observed. Taken together, these results suggest that H_2O_2 formed during reperfusion, or other reactive species derived from H_2O_2, may play a significant role in activation of MAPK, but appears to be less important in JNK activation. Activation of MAPK but not JNK by H_2O_2 has also been demonstrated in NIH 3T3 cells [11]. It is noteworthy that AP-1 is poorly activated by H_2O_2 in HeLa cells under conditions where NF-κB is strongly activated by the free radical [26].

Calcium depletion inhibits activation of JNK but not MAPK. Ischemia leads to an increase in

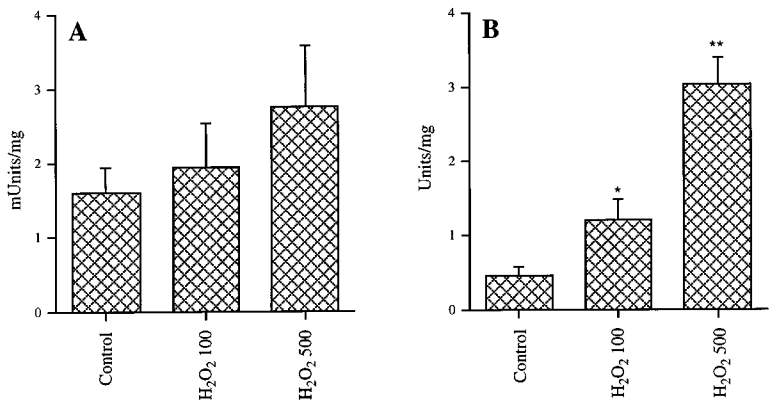

FIG. 4. Hydrogen peroxide activates MAPK but not JNK in the perfused heart. Hearts were perfused for 10 min, and hydrogen peroxide (0, 100 or 500μM) was then infused for 15 min before freeze-clamping the heart. Results are means ± SE for 3 4 hearts. *, p < 0.05 vs OμM H_2O_2; **, p < 0.001 vs OμM H_2O_2.

cytosolic calcium within 10–15 min, which after short periods of ischemia causing only reversible injury returns towards baseline levels by 10 min post-reperfusion. Time-averaged calcium measurements have shown that increased Ca^{2+} levels persist during systole but not diastole. To assess the potential role of calcium uptake in activation of kinase activity, calcium-free perfusion was initiated 1 min prior to the start of ischemia, and continued throughout the reperfusion period. Figure 5 demonstrates that activation of JNK by ischemia and reperfusion was completely abolished by calcium-free perfusion. In contrast, MAPK activation was enhanced by in the absence of calcium. A modest activation of MAPK (but not JNK) activity was also found in control hearts which were perfused in the absence of calcium for 15 min; this could reflect the increase in wall stress in the calcium-free perfused heart [27], since mechanical stretch has been shown to activate MAPK in neonatal rat myocytes [28].

Differential requirement for calcium for activation of JNK and MAPK was also found in rat 1a cells transfected with muscarinic acetycholine receptors. Chelation of intracellular calcium blocked activation of JNK but not MAPK in response to carbachol, and also blocked JNK activation in response to UV light only partially [19]. A co-stimulatory role for calcium in JNK activation has also been reported in Jurkat cells, where PMA and calcium ionophore have a synergistic effect on activation of JNK but not MAPK [29].

Significance of kinase activation in response to ischemia and reperfusion. The rapid activation of JNK and MAPK following ischemia and reperfusion is consistent with a role for the kinases in activation of proto-oncogene expression and induction of cardioprotective genes, and could be important for "preconditioning", in which exposure of the heart to a stress protects the heart against damage during subsequent ischemia [30]. Initially it was shown that exposure of the heart to a short ischemic episode led to transient protection against a longer ischemic insult, but that protection was lost after 1–2 hrs [31]. More recently, a second delayed period of protection has been demonstrated several hours after the preconditioning stimulus, and other stimuli, including heat shock, bacterial lipopolysaccharide, interleukin 1, tumor necrosis factor and catecholamines lead to delayed preconditioning [30]. Delayed preconditioning is associated with induction of proteins thought to be cardioprotective, including heat shock proteins and antioxidant enzymes, but the mechanisms by which induction of these proteins occurs is unknown. The potential role of JNK and MAPK in induction of cardioprotective genes thus merits further investigation; it is noteworthy that many of the stimuli leading to delayed preconditioning have also been shown to activate JNK, including

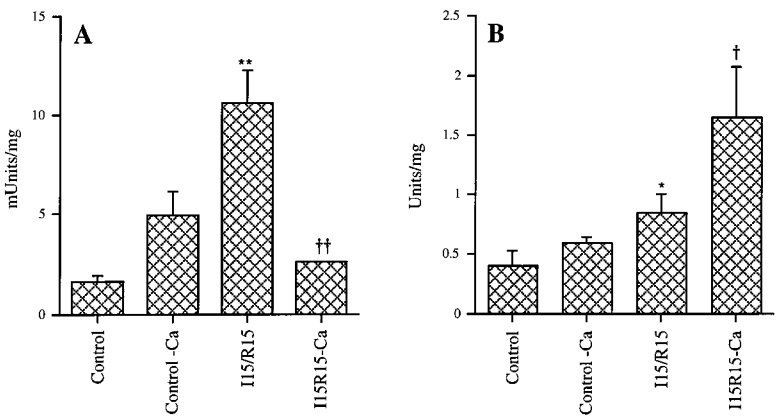

FIG. 5. Removal of perfusate calcium blocks activation of JNK but not MAPK. Control hearts were perfused normally for 40 min; ischemia/reperfusion hearts were subjected to 15 min ischemia and 15 min reperfusion (I15/R15). For I15R15-Ca^{2+}, infusion of calcium was terminated 1 min prior to initiation of ischemia; calcium was also absent during reperfusion. Control - Ca^{2+} hearts were perfused with calcium for 25min, and then calcium-free for the final 15 min of perfusion. Results are means ± SE for 2–4 hearts. *, $p < 0.05$ vs control; **, $p < 0.05$ vs control †, $p < 0.01$ vs I15R15; ‡, $p < 0.025$ vs I15R15.

heat shock, [11], tumor necrosis factor [11, 13], interleukin 1 [32] and ischemia/reperfusion [14; this study].

In summary, we have shown that reperfusion of ischemic myocardium leads to the activation of JNK and MAPK. However, the mechanisms by which the two pathways are activated differ. Activation of MAPK is blocked by the hydrogen peroxide scavenger catalase, and can be mimicked by infusion of hydrogen peroxide. In contrast, JNK was not activated significantly by hydrogen peroxide, and significant inhibition by free radical scavengers required both superoxide dismutase and catalase. Removal of perfusate Ca^{2+} prior to ischemia had no effect on subsequent activation of MAPK by ischemia and reperfusion, but blocked JNK activation.

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