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Abstract—Recent studies suggest that p38 mitogen-activated protein kinase (MAPK) may be involved in ischemic preconditioning (PC). To further test this possibility, the regulation of MAPK-activated protein kinase 2 (MAPKAPK2), a kinase immediately downstream from p38 MAPK, and the activity of c-Jun NH_2-terminal kinase (JNK), a second MAPK, were examined in preconditioned hearts. Isolated, perfused rabbit hearts were subjected to 20 to 30 minutes of global ischemia. Ventricular biopsies before treatment and after 20 minutes of ischemia were homogenized, and the activities of MAPKAPK2 and JNK were evaluated. For the MAPKAPK2 experiments, 7 groups were studied, as follows: control hearts; preconditioned hearts; hearts treated with 500 nmol/L R(–) N^6-(2-phenylisopropyl) adenosine (PIA), an A_1-adenosine receptor agonist; preconditioned hearts pretreated with 100 μmol/L 8-(*p*-sulfophenyl) theophylline (SPT), an adenosine receptor antagonist; preconditioned hearts also treated with SB 203580, a potent inhibitor of p38 MAPK activation; hearts treated with 50 ng/mL anisomycin (a p38 MAPK/JNK activator); and hearts treated with both anisomycin (50 ng/mL) and the tyrosine kinase inhibitor genistein (50 μmol/L). MAPKAPK2 activity was not altered in control hearts after 20 minutes of global ischemia. By contrast, there was a 3.8-fold increase in activity during ischemia in preconditioned hearts. Activation of MAPKAPK2 in preconditioned hearts was blocked by both SPT and SB 203580. MAPKAPK2 activity during ischemia increased 3.5-fold and 3.3-fold in hearts pretreated with PIA or anisomycin, respectively. MAPKAPK2 activation during ischemia in hearts pretreated with anisomycin was blocked by genistein. In separate hearts, anisomycin mimicked the anti-infarct effect of PC, and that protection was abolished by genistein. JNK activity was measured in control and preconditioned hearts. There was a comparable, modest decline in activity during 30 minutes of global ischemia in both groups. As a positive control, a third group of hearts was treated with anisomycin before global ischemia, and in these, JNK activity increased by 290% above baseline. These results confirm that the p38 MAPK/MAPKAPK2 pathway is activated during ischemia only if the heart is in a preconditioned state. These data further support p38 MAPK as an important signaling component in ischemic PC. **(*Circ Res*. 2000;86:144-151.)**

Key Words: anisomycin ■ p38 MAPK ■ ischemic preconditioning ■ JNK ■ MAPKAPK2

Brief exposure of the myocardium to ischemia substantially enhances tolerance to a subsequent ischemic insult by a process known as ischemic preconditioning (PC).[1] Although ischemic PC has been observed in all species examined, the mechanism of protection is not fully understood. Activation of protein kinase C (PKC) has been proposed as an important step in the cellular pathway to PC.[2,3] However, the cellular pathways beyond activation of PKC are still not clear and are currently being sought.

Recently, several studies have examined the potential role of mitogen-activated protein kinases (MAPKs) in PC.[4–6] The MAPKs are proline-directed protein-serine/threonine kinases, and several distinct families of MAPKs have been identified in mammals, each having a unique activation pathway.[7] The major MAPKs found in cardiac tissue include the extracellular signal–regulated kinases (ERK1/ERK2), the p46 and p54 c-Jun NH_2-terminal kinase/stress-activated protein kinases (JNK/SAPKs), and the α and β isoforms of p38.[8] The ERKs are strongly activated by mitogenic stimuli such as angiotensin.[9] JNK and p38 MAPK can be activated by various stresses including heat,[10] osmotic shock,[11,12] UV light,[13,14] endotoxin,[12] cytokines,[15,16] and ischemia/reperfusion.[17,18] The activation of p38 MAPK requires phosphorylation of Thr180 and Tyr182 within a TGY motif.[12] Phosphorylation of both of these residues is carried out by dual-specificity MAPK kinases (MKKs), and MKK3 and MKK6 are the physiolog-

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ical activators of p38.[19] The MKKs themselves are activated by many upstream protein-serine/threonine kinases including the large mixed-lineage kinase family, transforming growth factor-β–activated kinase-1, and the MAPK/ERK kinase kinase family.[7] Once active, p38 MAPK then stimulates MAPKAPK2 in a phosphorylation-dependent manner, which in turn leads to the phosphorylation of the low molecular weight heat shock protein, HSP27.[10,16,20] In many cells, phosphorylation of HSP27 leads to the polymerization of actin,[21] which appears to increase the tolerance of the cytoskeleton to stress.[22]

It has been reported that p38 MAPK can be activated by adenosine[23,24] and phenylephrine, endothelin, and the PKC activator phorbol 12-myristate 13-acetate in neonatal myocytes,[25] indicating a link between PKC and p38 MAPK. Maulik et al[5] also demonstrated that MAPKAPK2 activities were increased in the preconditioned rat heart. Our studies have shown that the level of phosphorylation of the activation site of p38 MAPK is specifically increased during ischemia, but only in preconditioned hearts.[6] Moreover, pretreatment with anisomycin, an activator of the p38 MAPK/JNK pathways, mimicked cardioprotection.[6,26,27] Together, these results support a role for the p38 MAPK cascade in the path to ischemic PC.

To further test the role of p38 MAPK in PC, we first examined whether the activity of MAPKAPK2 was similarly increased during ischemia in preconditioned hearts and whether PC-mimetic drugs could duplicate this effect. We also tested whether either a pharmacological inhibitor of PC protection or a specific antagonist of p38 MAPK activation could abolish this increase. p38 MAPK is a dually regulated kinase, requiring phosphorylation of both a tyrosine and a nearby threonine residue for activity.[12] Because genistein, an inhibitor of tyrosine kinases, blocks PC protection, we hypothesized that the site of blockade might be at the tyrosine phosphorylation of p38. To test whether the phosphorylation of p38 MAPK could be the site of the blockade of genistein, we activated p38 MAPK with anisomycin and tested whether genistein could block the resulting increase in MAPKAPK2 activity. Finally, we examined whether JNK, also stimulated by anisomycin, was activated in the preconditioned heart before or during ischemia.

Materials and Methods

All procedures were performed in compliance with the *Guide for the Care and Use of Laboratory Animals* (Washington, DC: National Academic Press, 1996).

Isolated Rabbit Heart Model and MAPKAPK2 Studies

As previously described,[28] hearts were isolated from New Zealand White rabbits, mounted on a Langendorff apparatus, and perfused with Krebs buffer. For the infarct studies only, a snare was passed around a coronary artery branch. As shown in Figure 1, the following groups were studied: control (n=4); PC (n=3); PC+the adenosine receptor antagonist 8-(*p*-sulfophenyl) theophylline (SPT, 100 μmol/L) (n=3); PC+the selective antagonist of p38 MAPK activation SB 203580 (SB, 10 μmol/L) (n=3); R(–) N^6-(2-phenylisopropyl) adenosine (PIA), an A_1-adenosine receptor agonist (500 nmol/L) (n=4); anisomycin, a p38 MAPK/JNK activator (50 ng/mL) (n=3); and combined anisomycin+genistein, a tyrosine kinase inhibitor (50 μmol/L) (n=4). Transmural biopsies were obtained just before global ischemia in the control group or before treatment in all other groups as indicated by the arrows in Figure 1. A second biopsy was taken in all hearts after 20 minutes of global ischemia.

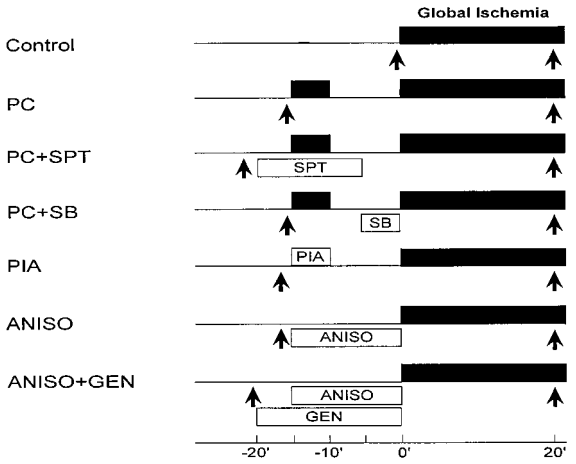

Figure 1. Experimental protocols for the MAPKAPK2 study. Timing of interventions is shown in relation to the 20-minute period of global ischemia. Arrows indicate timing of biopsies of the left ventricle. Open areas represent continuous infusion of drugs; solid areas, global ischemia. PC indicates ischemic PC; ANISO, anisomycin; and GEN, genistein.

Biopsies were homogenized with a Polytron in ice-cold buffer containing EGTA+protease and phosphatase inhibitors. An aliquot of the 13 000*g* supernatant (1 mg protein) was applied to a Hi-trap SP fast protein liquid chromatography (FPLC) column and was eluted using a linear NaCl gradient from 0 to 0.3 mol/L NaCl at a flow rate of 1 mL/min after slight modification of previously reported elution protocols.[10,25] Fractions (1 mL) were collected and assayed immediately for their ability to phosphorylate a substrate peptide (KKLNRTLSVA) derived from the N terminus of human glycogen synthase.[29] The kinase assay mixture also contained PKI (a cAMP-dependent protein kinase inhibitor), chelerythrine (a PKC inhibitor), H-7 (a broad-spectrum kinase inhibitor), KN-62 (a calcium/calmodulin-dependent kinase [CaMK]–II inhibitor), and okadaic acid (a phosphatase inhibitor). Some fractions were also subjected to SDS-PAGE electrophoresis[30] and probed with anti-MAPKAPK2 antibodies.[31]

JNK Studies

Biopsies from 5 isolated rabbit hearts were obtained immediately before the 5-minute PC ischemia (basal); just before onset of the 30-minute global ischemia (0 minutes); and thereafter at 5, 10, 20, and 30 minutes. The basal biopsy was obviously omitted from the 5 control hearts. In anisomycin-treated hearts (n=3), 50 ng/mL of the drug was added to the perfusate for 15 minutes before ischemia. The first biopsy was obtained before anisomycin, and thereafter, timing of biopsies was identical to that noted above.

JNK activity was determined with a commercially available assay. Biopsy homogenate was incubated with amino acids 1 to 89 of a c-Jun fusion protein attached to beads so that JNK would bind to the peptide on the beads. Washed beads were then incubated with kinase buffer containing ATP to allow JNK to phosphorylate the peptide. The beads then underwent conventional 10% SDS-PAGE electrophoresis, and substrate phosphorylation was determined with c-Jun phosphospecific antibody. Activity was expressed as a function of baseline activity.

Infarct Size Studies

Control isolated hearts (n=6) were subjected to 30 minutes of regional ischemia and 2 hours of reperfusion. The anisomycin group (n=6) received anisomycin (50 ng/mL) over 45 minutes, starting 15

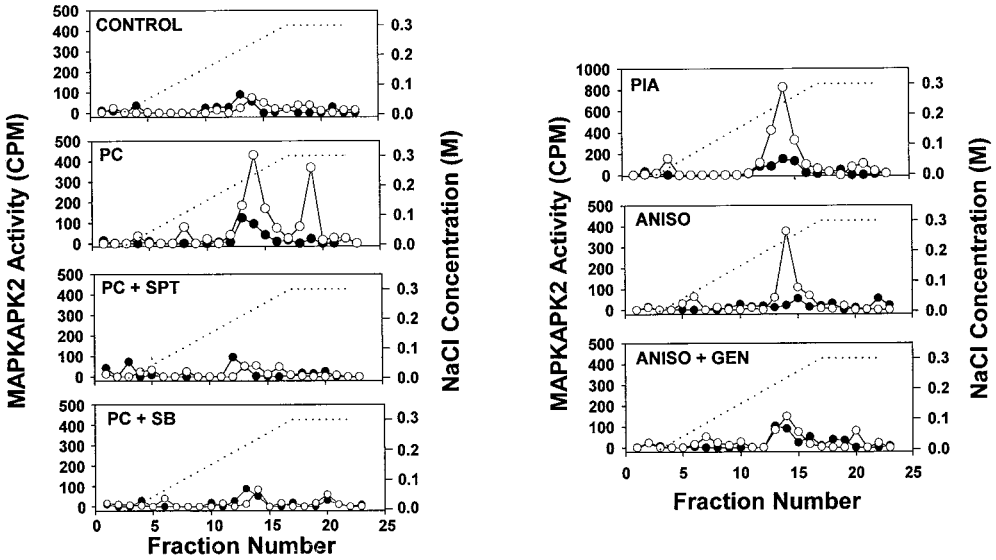

Figure 2. Representative elution profiles for myocardial samples obtained before (●) and after (○) 20 minutes of global ischemia in each of the following 7 groups: untreated control, preconditioned (PC), PC+SPT, PC+SB, PIA, anisomycin (ANISO), and ANISO+genistein (GEN). Homogenate was passed over a Hi-trap SP column using 0.3 mol/L NaCl gradient, and each column fraction was assayed for MAPKAPK2-like activity. Note that PC causes 2 peaks of activity, whereas PIA and anisomycin activate only a single peak. Dotted lines indicate NaCl gradients.

minutes before and continuing until the end of ischemia. In the genistein group (n=6), genistein (50 μmol/L) was infused for 50 minutes, starting 20 minutes before the 30-minute regional ischemia. The 2 drug protocols were then combined in the anisomycin+genistein group (n=5). As previously detailed,[28] at the end of reperfusion, the risk zone was determined with fluorescent particles and infarct size with triphenyltetrazolium chloride. Infarction is expressed as a percentage of the risk region.

Statistics

One-way ANOVA with the Scheffé post hoc test was used to test for differences in baseline hemodynamics and infarct size between groups. ANOVA with repeated measures was used to test for differences in hemodynamics within any given group.

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

Results

MAPKAPK2 Studies

Hemodynamic parameters were evaluated in all experimental groups. There were no significant differences at baseline in heart rate (range 201 to 224 bpm), developed pressure (range 90 to 118 mm Hg), or coronary flow (range 48 to 69 mL/min) among the groups. Representative MAPKAPK2 activity profiles are illustrated in Figure 2. In the untreated control heart, MAPKAPK2 activity was unchanged before (closed circles) and 20 minutes after (open circles) the onset of global ischemia. In contrast, 2 peaks of kinase activity were observed in preconditioned hearts after 20 minutes of global ischemia, whereas the basal activity in the same heart was negligible. This increase in kinase activity was completely blocked by the adenosine receptor antagonist SPT, as well as the potent antagonist of p38 MAPK activation, SB 203580. Other treatments known to mimic ischemic PC also increased MAPKAPK2 activity after 20 minutes of global ischemia. Both the A_1-adenosine receptor agonist PIA and the p38 MAPK/JNK activator anisomycin elicited a single peak of MAPKAPK2 activity after 20 minutes of ischemia compared with baseline. Interestingly, the anisomycin-induced kinase activity was greatly attenuated by pretreatment with the protein-tyrosine kinase inhibitor genistein.

The observation that MAPKAPK2 activity was detected in 2 peaks after FPLC separation was unexpected. Furthermore, because ischemic PC activated 2 peaks whereas pharmacological PC activated only a single peak, it became important to identify which, if either, peak was MAPKAPK2. This issue was addressed by Western blotting analysis of the fractions using an anti-MAPKAPK2 antibody, which demonstrated that the first peak corresponded with the MAPKAPK2 protein (Figure 3). Both known isoforms of MAPKAPK2 (≈50 and

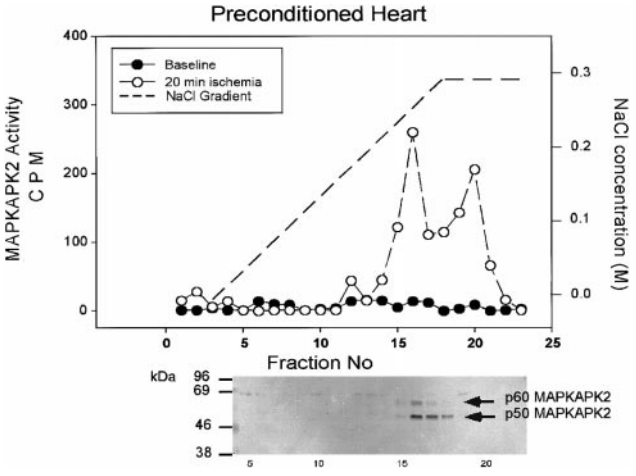

Figure 3. Elution profile of a myocardial biopsy from 1 preconditioned heart after 20 minutes of global ischemia. Note that 2 peaks of MAPKAPK2-like activity are seen. Western blotting of the samples reveals that the 2 isoforms of MAPKAPK2 are found only in the first peak. The identity of the second peak is unknown.

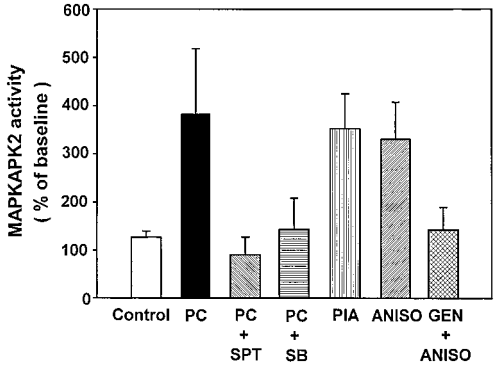

Figure 4. Mean phosphotransferase or MAPKAPK2-like activity expressed as a percentage of the baseline activity (μmol/L ATP/mg protein · min^{-1}) after 20 minutes of global ischemia for the 7 experimental groups.

60 kDa) were confined to the first peak.[32] The kinase present in the second peak has yet to be identified. Interestingly, activation of p38 MAPK indirectly with PIA and more directly with anisomycin produced only 1 peak, but SB 203580 treatment of ischemically preconditioned hearts eliminated both peaks.

Figure 4 presents the mean data from these studies. Phosphotransferase or MAPKAPK2-like activity after 20 minutes of ischemia is expressed as a percentage of basal activity (μmol/L ATP/mg protein/min) in each experimental group. There was no change in the MAPKAPK2-like activity after global ischemia alone (control). However, ischemic PC elicited a 3.8-fold increase in MAPKAPK2 activity (first peak) that was completely abolished by pretreatment with SPT. Similar abolition of activity was observed in preconditioned hearts treated with SB 203580. Treatment with the adenosine agonist PIA, in lieu of PC, increased MAPKAPK2 activity 3.5-fold, whereas pretreatment with anisomycin increased the enzyme activity 3.3-fold. The effect of the latter was reversed by genistein, indicating that the dual-specificity kinase that phosphorylates p38 may be inhibited by genistein.

JNK Studies

There were no significant differences in hemodynamics between the 3 experimental groups. Western blots from representative control and preconditioned hearts are presented in Figure 5. As determined from the amount of phosphorylated c-Jun at each time point, there was a small decrease in JNK activity during the initial 10 minutes of ischemia compared with the preischemic activity in both control and

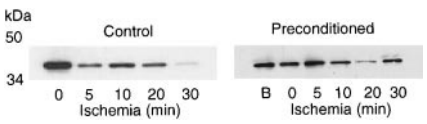

Figure 5. JNK activity of control and preconditioned rabbit hearts before ischemia (0 minutes in control and B [baseline] in preconditioned hearts); after 5 minutes of ischemia and 10 minutes of reperfusion in preconditioned hearts (0 minutes); and after 5, 10, 20, and 30 minutes of global ischemia in the 2 groups. Extracts of heart tissue were analyzed for JNK by immunodetection of the ≈37-kDa phospho-c-Jun substrate. In both control and preconditioned hearts, JNK activity decreased during ischemia.

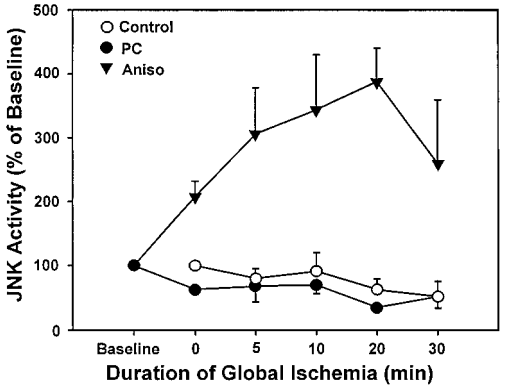

Figure 6. Average JNK activity presented as a percentage of preischemic activity in control, ischemically preconditioned, and anisomycin-treated hearts at the time points described in Figure 5. During ischemia, there was a modest decline in JNK activity in control and preconditioned hearts, and no differences between the 2 groups were observed. In contrast, JNK activity significantly increased in hearts exposed to anisomycin.

preconditioned hearts. There was an additional, more substantial decrease in the final 20 minutes of ischemia. Again, the change seemed to be comparable in the 2 hearts. The group data in Figure 6 confirm a modest and equivalent decrease in JNK activity in both control and preconditioned hearts throughout 30 minutes of global ischemia. As a positive control, 3 hearts were pretreated with anisomycin before the period of global ischemia. As is evident from Figure 6, there was a dramatic increase in JNK activity, with a peak 290% above baseline after 20 minutes of ischemia. Therefore, our assay system was clearly capable of detecting increases in JNK activity.

Infarct Size Studies

The above results confirm that genistein can block activation of MAPKAPK2 by anisomycin. In light of that result, we tested whether genistein could also reverse the anti-infarct effect of anisomycin. Baseline hemodynamics were not different in any group. Anisomycin did not affect any of the hemodynamic parameters. Genistein induced a small but significant reduction in developed pressure (114±3 to 104±4 mm Hg, $P<0.05$) and increase in coronary flow (68±3 to 77±1 mL/min; $P<0.05$). Figure 7 reveals that anisomycin reduced infarction from 33.0±3% of the risk zone to 7.5±1.6% ($P<0.05$), similar to that seen with ischemic PC. However, protection was completely blocked by inhibition of tyrosine kinase with genistein (37.4±3.8% infarction), which itself did not affect infarct size (33.9±2.6% infarction). These data further support the hypothesis that PC protects by activating the p38 MAPK pathway during ischemia and that genistein blocks protection from ischemic PC by preventing the tyrosine phosphorylation of p38 MAPK.

Discussion

We have previously shown that the level of phosphorylation of Tyr182 (one of the activation sites) of p38 MAPK is elevated during the sustained ischemia in preconditioned hearts, whereas no changes are seen in nonpreconditioned

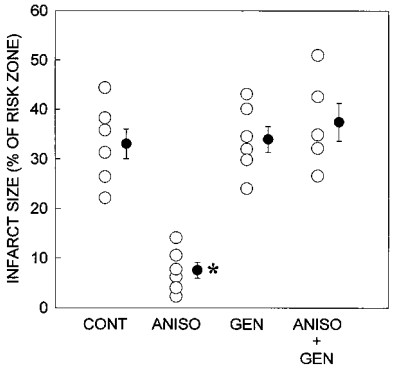

Figure 7. Infarct size as a percentage of the risk (ischemic) zone for control (CONT), anisomycin (ANISO)-, and genistein (GEN)-treated hearts. Shown are data from individual experiments (○) and mean±SEM for each group (●). Note that the protection by anisomycin was blocked by the tyrosine kinase inhibitor genistein (ANISO+GEN). *$P<0.05$ vs CONT.

hearts.[6] Furthermore, peak phosphorylation was observed after 20 minutes of global ischemia. Although we concluded that ischemic PC activated p38 MAPK, in fact we proved only that PC increased phosphorylation of the Tyr182 residue of that kinase. Because activation of p38 MAPK requires dual phosphorylation of both Tyr182 and Thr180 residues,[12] increased phosphorylation of only the tyrosine residue does not necessarily imply activation of the kinase. However, the present study extends the prior findings by demonstrating that MAPKAPK2-like activity increased nearly 4-fold during the sustained ischemic period in preconditioned hearts. As MAPKAPK2 is the immediate downstream substrate for p38 MAPK,[10,20] an increase in the activity of the former should be an index of p38 activation. This activation appears to be specific in that there was no discernible change in MAPKAPK2 activity during ischemia in hearts that did not experience PC ischemia.

Pretreatment with the nonselective adenosine receptor antagonist SPT, which blocks infarct size reduction from ischemic PC,[33,34] also prevented the increase in MAPKAPK2 activity in preconditioned hearts. Moreover, we found that pharmacological agents that mimic ischemic PC in whole heart infarct models, including the A_1-selective adenosine receptor agonist PIA,[35] had a similar effect on MAPKAPK2 activity. The bacterial product anisomycin activates MKK3, -4, -6, and -7[36,37] and, therefore, will strongly activate p38 MAPK and JNK, but not ERK. Hence, not surprisingly, perfusion of the whole heart with anisomycin before prolonged ischemia also elevated MAPKAPK2 activity and protected the ischemic heart with a potency equivalent to that of ischemic PC. Finally, the potent inhibitor of p38 MAPK activation SB 203580[38] blocked the increased activity previously observed in preconditioned hearts. All of these observations are consistent with a pathway that includes p38 MAPK and MAPKAPK2 playing a causative role in the cardioprotection provided by ischemic PC.

The JNK family consists of at least 2 isoforms, the 46-kDa JNK1 and the 54-kDa JNK2, both of which are present in the heart.[18] Clerk et al[18] have reported that both are strongly activated on reperfusion but are not affected by ischemia alone, although a recent preliminary report suggests that JNK1 can also be activated by simple coronary occlusion.[39] Furthermore, stimulation of G_q-coupled receptors and subsequently PKC[40] can also activate JNK. Ping et al[41] have recently demonstrated that transfection of rabbit cardiomyocytes with the wild-type cDNA of PKC-ε induced activation of p46/p54 JNK, whereas the activation of JNK by coronary occlusion and reperfusion in rabbit hearts was abolished by chelerythrine.[39] Barancik et al[42] have reported significant increases in JNK activity in preconditioned pig hearts, and Htun et al[43] have correlated a reduction in infarction with increased JNK activity in hearts exposed to anisomycin. Because of these observations, we also investigated whether ischemic PC in the rabbit heart would increase JNK activity. There was no increase in either nonpreconditioned or preconditioned hearts before or during ischemia, nor did we see any differences between the groups. On the other hand, JNK activity did increase substantially after treatment with anisomycin. It is possible that species variability accounts for the different conclusions. Nonetheless, our data suggest that JNK is not part of the ischemic PC signal transduction cascade in rabbits. Of course, one cannot exclude the possibility that selective activation of the JNK pathway might in some way produce cardioprotection.

After FPLC fractionation, we observed 2 peaks of MAPKAPK2 activity in ischemically preconditioned hearts. This was an unexpected finding. However, Western blotting indicated that only the first peak contains the 2 isoforms of MAPKAPK2. Furthermore, PIA and anisomycin activated the enzymes eluting in only the first peak. An intriguing issue for further study is the identity of the second peak of activity. The amino acid sequence of the substrate peptide is reportedly selective for MAPKAPK2.[44] However, $p90^{s6k}$ kinase and CaMK-II have been reported to also phosphorylate this peptide.[20,29] To eliminate possible interference from these kinases, the kinase reaction mixture contained H-7, a potent inhibitor of several kinases, including $p90^{s6k}$ kinase,[45] and KN-62, a potent CaMK-II inhibitor.[46] The second peak may represent the recently described PRAK (p38-regulated/-activated kinase), which is also regulated by p38 MAPK.[47] Because the sequence homology between MAPKAPK2 and PRAK is only ≈30%, they are not considered isoforms. Yet both can phosphorylate HSP27. However, if the second peak were indeed PRAK, then anisomycin should have also induced a second peak of activity, given that both PRAK and MAPKAPK2 are thought to be under the control of p38 MAPK. It is more probable that the second peak reflects a kinase in a parallel pathway that is activated with PC but the activation of which is unrelated to p38 MAPK. The second peak could even be part of a redundant parallel pathway capable of causing protection independent of PKC. Multiple cycles of PC reportedly can overcome PKC blockade and restore protection.[48,49] It is possible that the second peak reflects this bypass pathway. Unexpectedly, the potent p38 MAPK inhibitor SB 203580 also abolished the second peak. It is unclear why SB 203580 can prevent appearance of the second peak, whereas anisomycin cannot induce it. Further investigative studies are clearly required to identify it.

In our preliminary experiments, we found direct assay of tissue homogenate to be unreliable, and it was only after

resorting to FPLC to purify the samples that we obtained clear, reproducible results. Despite the relatively small number of replications, all groups included at least 3 hearts, and all replications yielded consistent data. The complexity of the assay and time required to process the samples from each heart limited the number of hearts that could be studied. We measured MAPKAPK2 activity at only 1 time point during ischemia in these hearts. The 20-minute time point was chosen, because that was when the peak of p38 MAPK phosphorylation was observed in the study by Weinbrenner et al.[6] It is interesting to note that the pattern of MAPKAPK2 activity was identical to that of p38 phosphorylation, ie, there is only activation during ischemia if the heart is in a preconditioned state. This pattern is consistent with previous pharmacological studies using kinase inhibitors. The protein-tyrosine kinase inhibitors genistein and lavendustin A[26] and the PKC inhibitors staurosporine and chelerythrine[14,50] had no effect on infarction in nonpreconditioned hearts, but each abolished protection in preconditioned hearts. Furthermore, protection was prevented only if the inhibitor was present during the prolonged ischemic insult and not the brief PC ischemia.[26,28] Blockade of adenosine receptors at the beginning of the prolonged ischemic period also prevents protection.[51] Together with the present study, these data would suggest that PC elicits a coupling between adenosine and p38 MAPK that normally does not exist in a nonpreconditioned heart.

In previous studies, it has been observed that the protein-tyrosine kinase inhibitor genistein could block protection from ischemic PC in both isolated rabbit[26] and rat[5,52] hearts. More recently, it was reported that genistein could block activation of p38 MAPK in preconditioned hearts.[53] These observations led us to speculate that the protein-tyrosine kinase in question could be phosphorylating the Tyr182 residue of p38 MAPK. This, in addition to phosphorylation of Thr180, is required for activation of p38, and the dual-specificity kinases MKK3 and MKK6 accomplish phosphorylation of both of these residues.[12] Because genistein inhibits protein-tyrosine kinases by interacting with the ATP-binding site,[54] it could potentially do the same to the catalytic domain of MKK3/MKK6. However, as of yet, there are no studies addressing this issue. When we directly activated MKKs with anisomycin, genistein blocked both the resulting reduction of infarct size (Figure 7) and activation of MAPKAPK2 (Figures 2 and 4). Because p38 MAPK and MAPKAPK2 are protein-serine/threonine and not tyrosine kinases, the dual-specificity MKKs most likely represent the genistein-sensitive step in the signaling pathway of PC. However, it should be noted that it has been suggested that anisomycin may stimulate the p38 MAPK and JNK pathways by the ribotoxic stress response rather than through a direct effect on the MKK protein itself.[55] If that were the case, then a genistein-sensitive protein-tyrosine kinase could possibly exist somewhere between the ribosome and the MKK and not necessarily at the MKK.

The present study shows only that MAPKAPK2 activity is highly correlated with the protection of ischemic PC but does not actually prove a role for this kinase, because there is no way to block MAPKAPK2 in these hearts. There are several inhibitors of p38 MAPK, including SB 203580,[38] which can effectively block protection in our cell model of ischemic PC without having an effect on nonpreconditioned cells.[6] However, Armstrong et al[4] reported that SB 203580 promoted injury in nonpreconditioned cells, whereas another group reported that SB 203580 actually protected cardiac myocytes.[27] Most recently, SB 203580 has been found to selectively block the anti-infarct effect of PC in isolated rat hearts,[53] and this blockade occurred only when SB 203580 was present during the prolonged ischemic period (Derek M. Yellon, personal communication, September 1999).

It was impossible to measure infarct size in those hearts that were being biopsied. However, it has been demonstrated repeatedly that pretreatment with a 5-minute period of ischemia, 500 nmol/L PIA, or 50 ng/mL anisomycin can precondition the isolated rabbit heart against infarction.[26,35,56] Furthermore, 100 μmol/L SPT completely abolishes the anti-infarct effect of ischemic PC in the rabbit heart.[33,34]

Among other effects, MAPKAPK2 phosphorylates the small heat shock protein HSP27,[16] an important regulator of actin dynamics. Overexpression of HSP27 confers protection against ischemia in rat neonatal myocytes.[57] Furthermore, activation of this pathway prevents oxidative stress- and cytochalasin D–induced fragmentation of actin filaments and preserves cell viability.[21,22] As prolonged ischemia is known to cause cytoskeletal disruption,[58] activation of p38 MAPK and MAPKAPK2 could contribute to the protective action of ischemic PC by maintaining the integrity of the actin cytoskeleton. Of course, the identity of the end-effector of protection is actively being pursued, and the K_{ATP} channel is a strong candidate.[59] There may be a connection between the actin cytoskeleton and K_{ATP} channels.[60]

In summary, the present study reveals that both ischemic and pharmacological PC induce strong activation of MAPKAPK2, but not JNK, during the prolonged ischemic period. Furthermore, that activation of MAPKAPK2 by ischemic PC could be blocked with an adenosine receptor antagonist. These data indicate that activation of MAPKAPK2, which is immediately downstream of p38 MAPK, is highly correlated with PC's protection.

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