

PKC-dependent activation of p46/p54 JNKs during ischemic preconditioning in conscious rabbits

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Ping, Peipei, Jun Zhang, Shuang Huang, Xinan Cao, Xian-Liang Tang, Richard C. X. Li, Yu-Ting Zheng, Yumin Qiu, Angela Clerk, Peter Sugden, Jiahuai Han, and Roberto Bolli. PKC-dependent activation of p46/p54 JNKs during ischemic preconditioning in conscious rabbits. *Am. J. Physiol.* 277 (Heart Circ. Physiol. 46): H1771–H1785, 1999.—A conscious rabbit model was used to study the effect of ischemic preconditioning (PC) on stress-activated kinases [c-Jun NH₂-terminal kinases (JNKs) and p38 mitogen-activated protein kinase (MAPK)] in an environment free of surgical trauma and attending external stress. Ischemic PC (6 cycles of 4-min ischemia/4-min reperfusion) induced significant activation of protein kinase C (PKC)- ϵ in the particulate fraction, which was associated with activation of p46 JNK in the nuclear fraction and p54 JNK in the cytosolic fraction; all of these changes were completely abolished by the PKC inhibitor chelerythrine. Selective enhancement of PKC- ϵ activity in adult rabbit cardiac myocytes resulted in enhanced activity of p46/p54 JNKs, providing direct in vitro evidence that PKC- ϵ is coupled to both kinases. Studies in rabbits showed that the activation of p46 JNK occurred during ischemia, whereas that of p54 JNK occurred after reperfusion. A single 4-min period of ischemia induced a robust activation of the p38 MAPK cascade, which, however, was attenuated after 5 min of reperfusion and disappeared after six cycles of 4-min ischemia/reperfusion. Overexpression of PKC- ϵ in cardiac myocytes failed to increase the p38 MAPK activity. These results demonstrate that ischemic PC activates p46 and p54 JNKs via a PKC- ϵ -dependent signaling pathway and that there are important differences between p46 and p54 JNKs with respect to the subcellular compartment (cytosolic vs. nuclear) and the mechanism (ischemia vs. reperfusion) of their activation after ischemic PC.

stress-activated protein kinases; p38 mitogen-activated protein kinases; c-Jun NH₂-terminal kinases; ischemia-reperfusion; protein kinase C

RECENTLY, MUCH INTEREST has focused on the late phase of ischemic preconditioning (PC), that is, the phenomenon whereby exposure of the heart to a brief ischemic insult confers increased tolerance to a subsequent ischemic insult 24–96 h later (1, 2, 7–9, 26, 30, 48). Protein kinase C (PKC) has been identified as an important element of the intracellular signaling transduction cascade that underlies the genesis of late PC (2,

40, 42). Specifically, studies in conscious rabbits have shown that ischemic PC is associated with isoform-selective translocation of the ϵ -isozyme of PKC (40) and that inhibition of PKC- ϵ translocation results in inhibition of the late PC effect (42). Considerable evidence indicates that PKC is intimately involved not only in the late phase but also in the early phase of ischemic PC (14, 19, 29, 34, 46, 53) as well as in various forms of pharmacologically induced PC (see review, Ref. 14). However, the downstream signaling pathways that are activated by PKC in the setting of myocardial ischemia-reperfusion remain poorly characterized.

In the present study, we tested the hypothesis that two subgroups of the mitogen-activated protein kinase (MAPK) family, the p38 MAPK and the p46 and p54 c-Jun NH₂-terminal kinases (JNKs), are downstream targets of PKC during ischemic PC. The p38 MAPK and the p46/p54 JNKs are known to play an important role in a multitude of cellular functions in response to stress (e.g., regulation of transcription, phosphorylation of small heat shock proteins) (3, 11, 16, 47). In noncardiac cells, activation of the p38 MAPK and JNKs appears to be coupled to PKC (15, 17, 18, 21, 22, 36), and several studies suggest that these kinases may participate in PKC-triggered signal transduction events (21, 22, 31, 36, 47). Although myocardial ischemia-reperfusion has been shown to induce activation of the p38 MAPK and the p46/p54 JNKs in isolated perfused hearts (4, 14, 25, 32, 35, 52), it is unclear whether this phenomenon occurs in vivo and whether it may be related to activation of these kinases by the stress associated with the in vitro conditions. Furthermore, it is unknown whether activation of the p38 MAPK, the p46 JNK, and the p54 JNK during myocardial ischemia-reperfusion is PKC dependent. Finally, no information is available regarding whether these kinases are coupled to the ϵ -isoform of PKC (which appears to be an essential element in the signal transduction pathway that underlies ischemic PC) in cardiac myocytes (19, 40, 42).

A study consisting of three consecutive phases was designed to address these issues. In *phase I* of the study, a well-established conscious rabbit model of late PC (7, 9, 40, 42, 43, 49) was used to examine the effect of ischemic PC on the phosphorylation activity of the p38 MAPK and the p46/p54 JNKs. Ten different PKC isoforms are expressed in the adult rabbit heart (40). Because previous studies have suggested that PKC- ϵ is the isozyme responsible for PKC signaling during the

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development of late PC (40, 42), we tested the hypothesis that ischemic PC-induced activation of the p38 MAPK and JNK cascades is part of the downstream signaling events triggered by activation of the ϵ -isoform of PKC. Isoform-selective measurements of PKC- ϵ phosphorylation activity were performed in the absence and presence of the PKC inhibitor chelerythrine, and the results were correlated with the activity of the p38 MAPK and the p46/p54 JNKs. We used an ischemic PC protocol consisting of six cycles of 4-min coronary occlusion/4-min reperfusion, which has previously been shown to induce late PC against myocardial stunning (7, 9, 42) and infarction (43, 49) as well as translocation of PKC- ϵ to the particulate fraction (40). A conscious animal model was employed to obviate any possible activation of MAPKs by surgical trauma and attending external stress. In *phase II* of this investigation, we determined whether selective activation of the PKC ϵ -isoform is sufficient to induce activation of the p38 MAPK and the p46/p54 JNKs in cardiac myocytes *in vitro*. By overexpressing PKC- ϵ in these cells to mimic the intracellular signaling events that occur *in vivo* during ischemic PC, we directly tested whether a molecular coupling exists between the ϵ -isozyme and the p38 MAPK or the p46/p54 JNKs. Because the results of *phase I* showed that the phosphorylation activity of the p38 MAPK was unchanged after six cycles of 4-min occlusion/4-min reperfusion, in *phase III* of the study we investigated whether this is due to a nonsustained activation of this kinase during repetitive cycles of ischemia-reperfusion or to an inherent lack of responsiveness of the p38 MAPK cascade to myocardial ischemia-reperfusion in this model. The results demonstrate, for the first time, that ischemic PC induces activation of the p46/p54 JNKs via a PKC-dependent pathway *in vivo*, that the response of the p38 MAPK to ischemic PC differs from that of the p46 and p54 JNKs with respect to its time course and

subcellular distribution, and that p46/p54 JNKs are coupled to the ϵ -isoform of PKC in cardiac myocytes.

METHODS

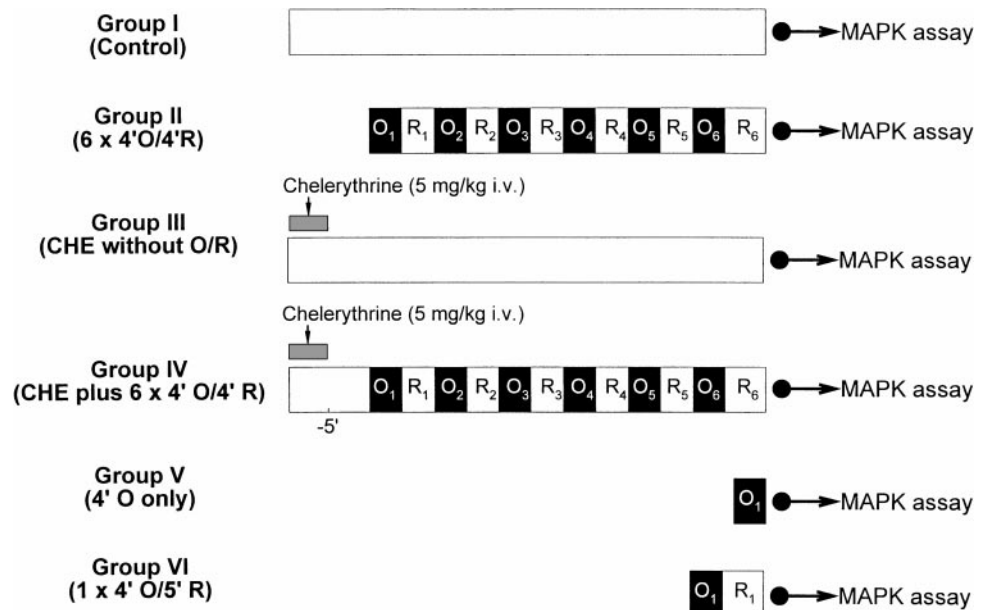
The present study was performed in accordance with the guidelines of the Animal Care and Use Committee of the University of Louisville School of Medicine and with the *Guide for the Care and Use of Laboratory Animals* [DHHS Publication No. (NIH) 86-23].

Studies in Conscious Rabbits (Phases I and III)

Experimental preparation. The conscious rabbit model of ischemic PC has been described in detail previously (7, 9, 40, 42, 43, 49). Briefly, New Zealand White male rabbits (2.0–2.5 kg) were instrumented under sterile conditions with a balloon occluder around a major branch of the left coronary artery, a 10-MHz pulsed ultrasonic crystal in the region to be rendered ischemic, and electrocardiogram (ECG) leads on the chest wall. The chest wound was closed in layers, and a small tube was left in the thorax for 3 days to aspirate air and fluids. Gentamicin was administered before surgery and on the first and second postoperative days (0.7 mg/kg im each day). The animals were allowed to recover for a minimum of 10 days after surgery. Throughout the experiments, the rabbits were kept in a cage in a quiet, dimly lit room. Left ventricular (LV) systolic wall thickening, range gate depth, and ECG were continuously recorded on a thermal array chart recorder (Gould TA6000). Coronary artery occlusion was produced by inflating the balloon occluder. The performance of successful occlusions was verified by observing the appearance of S-T segment elevation and the widening of the QRS complex on the ECG and by the development of paradoxical systolic wall thinning on the ultrasonic crystal recordings. Successful reperfusion was documented by the normalization of the ECG and by the resumption of active systolic wall thickening. No sedative or antiarrhythmic agents were given at any time.

Experimental protocol. In *phase I* of the study, rabbits were assigned to four groups (Fig. 1). *Group I* (control) did not undergo coronary occlusion. At 10–14 days after surgery (time corresponding to the interval between instrumentation

Fig. 1. Diagram of experimental protocols. Mitogen-activated protein kinase (MAPK) assays were performed immediately after the tissue samples were collected. O, coronary occlusion; R, coronary reperfusion; CHE, chelerythrine.



and euthanasia in the other groups), the rabbits were given heparin (1,000 U iv), after which they were anesthetized with pentobarbital sodium (50 mg/kg iv) and euthanized with a bolus of KCl. The heart was immediately excised, and myocardial samples (~0.5 g) were rapidly removed from the anterior LV wall and stored in liquid nitrogen until used. *Group II* underwent an ischemic PC protocol consisting of six cycles of 4 min of coronary occlusion separated by 4 min of reperfusion. The rabbits were euthanized 5 min after the last reperfusion [a time point at which marked activation of PKC was found previously in this model (40)]. Myocardial samples were rapidly removed from the ischemic-reperfused region (whose boundaries had been marked with sutures at the time of instrumentation) and stored in liquid nitrogen. To determine whether activation of the p46/p54 JNKs during ischemic PC is mediated by PKC, *group III* received the PKC inhibitor chelerythrine (5 mg/kg iv) without ischemia-reperfusion, whereas *group IV* received chelerythrine (5 mg/kg iv 5 min before 1st occlusion) and then underwent the sequence of six cycles of 4-min occlusion/4-min reperfusion. This dose of chelerythrine was shown previously to effectively block translocation of PKC- ϵ and the protection of late PC in this conscious rabbit model (42). In *group IV*, the rabbits were euthanized 5 min after the last reperfusion and tissue samples were obtained as described above. In *group III*, the rabbits were euthanized 54 min after the administration of chelerythrine (time interval corresponding to the interval between treatment and euthanasia in *group IV*).

In *phase III* of the study, two additional groups of rabbits were used to determine whether the activation of p38 MAPK was affected by repetitive cycles of ischemia-reperfusion (Fig. 1). *Group V* underwent one 4-min coronary occlusion without reperfusion; the rabbits were euthanized at 4 min of coronary occlusion, and the heart was immediately excised. *Group VI* underwent one cycle of 4-min coronary occlusion followed by reperfusion; the rabbits were euthanized 5 min after reperfusion, and myocardial samples were obtained as described above. In all groups, the samples were frozen within 60–90 s of the administration of the bolus of KCl.

Tissue sample preparation. Tissue samples were processed for the determination of protein expression and phosphorylation activity of p46/p54 JNKs, MAPK/extracellular signal-regulated kinase (ERK) kinases 3 and 6 (MEK3 and MEK6), p38 MAPK, and MAPK-activated protein kinase 2 (MAPKAPK-2). Frozen myocardial tissue samples were powdered in a prechilled stainless steel mortar and pestle. Total cellular proteins were obtained by glass-glass homogenization of the powdered tissue in sample buffer containing 50 mM Tris·HCl (pH 7.5), 5 mM EDTA, 10 mM EGTA, 10 mM benzamidine, 50 μ g/ml phenylmethylsulfonyl fluoride (PMSF), 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin A, 1 μ M Microcystin LR (an inhibitor of protein phosphatase), and 0.3% β -mercaptoethanol. The nuclear, cytosolic, and membrane fractions were prepared as previously described (12).

PREPARATION OF NUCLEAR FRACTION. The homogenate was loaded onto a sucrose gradient, which contained 2 ml of 1 M sucrose in sample buffer, and was centrifuged at 1,600 g for 10 min to pellet the nuclear fraction. The pellet from the 1,600- g centrifugation was resuspended in tissue sample buffer containing 0.5% NP-40, 0.1% deoxycholate, and 0.1% Brij 35, incubated on ice for 60 min, and recentrifuged at 10,000 rpm for 5 min. The supernatant became the nuclear fraction.

PREPARATION OF CYTOSOLIC FRACTION. The supernatant from the 1,600- g centrifugation was loaded onto a second 1 M sucrose cushion and centrifuged at 150,000 g for 60 min. The supernatant became the cytosolic fraction.

PREPARATION OF MEMBRANE FRACTION. The pellet from the 150,000- g centrifugation was resuspended in tissue sample buffer containing 0.5% NP-40, 0.1% deoxycholate, and 0.1% Brij 35, incubated on ice for 60 min, and recentrifuged at 10,000 rpm for 5 min. The supernatant became the membrane fraction. Careful preliminary experiments were conducted to ensure that powdering the frozen tissue did not fractionate the nuclei of the myocardial samples. The purity of the particulate fractions was examined using lactate dehydrogenase (LDH) as a cytosolic marker, and it was found that <2% of total myocardial tissue LDH was present in nuclear and membrane fractions. The purity of the nuclear preparation was further confirmed by staining the myocardial nuclear extracts for the nuclear histone deacetylase 1 (24).

Protein concentration was determined using the method of Bradford (Bio-Rad). The yields of total cellular proteins, nuclear proteins, and cytosolic proteins were carefully recorded for each tissue sample tested. Total myocardial proteins were calculated as the sum of the proteins from the cytosolic, nuclear, and membrane fractions. The proteins in the cytosolic fraction averaged $67 \pm 1\%$ of total myocardial proteins, those in the nuclear fraction $22 \pm 1\%$, and those in the membrane fraction $11 \pm 1\%$. To ensure the most accurate assessment of MAPK and JNK protein expression and to avoid any decay in the kinase phosphorylation activity, samples were processed by either Western immunoblotting or phosphorylation assays immediately after tissue sample preparation. Each phosphorylation activity assay was performed in at least five of the eight control rabbits (*group I*) and in all five rabbits in the other groups.

PKC ϵ -isoform-selective phosphorylation activity assay. To determine the phosphorylation activity of the ϵ -isoform of PKC, 50 μ g of proteins from either the cytosolic or the particulate fraction (the latter fraction includes both membrane and nuclear fractions) were immunoprecipitated overnight with PKC ϵ -isoform monoclonal antibodies (Transduction Laboratories) and protein A/G agarose beads (Santa Cruz Biotechnology) in buffer containing 150 mM NaCl, 50 mM Tris (pH 7.4), 1% NP-40, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM PMSF, 16 μ g/ml benzamidine-HCl, 10 μ g/ml phenanthroline, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 10 μ g/ml pepstatin A. The cytosolic and particulate fractions of the tissue samples were prepared as previously described (38–40). The immunoprecipitation-enriched and -purified tissue PKC- ϵ enzyme was then subjected to a phosphorylation assay in a reaction mixture containing 2.3 μ g/ml phorbol 12-myristate 13-acetate (PMA), 28.8 μ g/ml L- α -phosphatidyl-L-serine, and 1 nM PKC ϵ -isoform-selective substrate (ERM₁PRKRQGSVRRRV) (38–40).

JNK activity assays. The phosphorylation activity of the p46/p54 JNKs was determined by immunoprecipitation followed by an in-gel kinase assay. The amount of proteins applied in each assay was chosen on the basis of the optimal sensitivity of the enzyme, which was derived from the sample protein and enzymatic activity dose-response curves. Auto-phosphorylation of the enzyme was determined by omitting the substrate peptide from the reaction. Specific enzymatic activity was calculated by subtracting the nonspecific activity (autophosphorylation and basal background activity) from the total activity.

IMMUNOPRECIPITATION OF p46/p54 JNKs. Briefly, 60 μ g of myocardial tissue protein were immunoprecipitated overnight with 0.5 μ g of either the p46 JNK or p54 JNK monoclonal antibodies and protein A/G agarose beads (Santa Cruz).

IN-GEL KINASE ASSAY. The isoform-specific activity of the p46 and p54 JNKs was further determined by an in-gel kinase

assay using the method described by Sugden and colleagues (5, 6). The immunoprecipitates were fractionated on a 10% polyacrylamide gel containing 0.5 mg/ml of c-Jun fusion protein. The gel was washed with 20% (vol/vol) isopropyl alcohol in 50 mM Tris·HCl (pH 8.0) three times for 1 h at room temperature (RT) and then washed again with 5 mM β -mercaptoethanol and 50 mM Tris·HCl three times for 1 h at RT. Proteins were further denatured by washing the gel in 6 M guanidine-HCl and 50 mM Tris·HCl buffer three times at RT. Proteins were renatured by incubation in 0.04% Tween 40 (vol/vol), 5 mM β -mercaptoethanol, and 50 mM Tris·HCl (pH 8.0) at 4°C overnight. The gel was then equilibrated in a preincubation buffer containing 40 mM HEPES, 2 mM dithiothreitol (DTT), and 10 mM MgCl₂ (pH 8.0) for 1 h at RT. In-gel phosphorylation of the substrate was then carried out in 40 mM HEPES, 10 mM MgCl₂, 0.5 mM EGTA, 2 μ M PKI (a protein kinase A inhibitor), and 40 μ M [γ -³²P]ATP (5 μ Ci/ml or 40 μ Ci per gel; pH 8.0) at 30°C for 1 h. The phosphorylated gel was washed in 5% (wt/vol) trichloroacetic acid and 1% (wt/vol) sodium pyrophosphate to remove the unincorporated free [γ -³²P]ATP and was then dried and autoradiographed. Each sample was assayed in duplicate. Pilot experiments confirmed equal loading of proteins in the in-gel kinase assays.

p38 MAPK cascade activity assays. The phosphorylation activity of the kinases in the p38 MAPK cascade was determined with an assay system developed by Upstate Biotechnology.

MAPKAPK-2 ACTIVITY ASSAY. Protein (15 μ g, which was found to be the optimal sample dose for assessment of MAPKAPK-2 activity) was incubated with 10 μ Ci of [γ -³²P]ATP, 0.1125 mM ATP, 16.9 mM MgCl₂, 5 mM calmodulin kinase inhibitor (compound R-24571, Sigma), 12.5 mM β -glycerol phosphate, pH 7.0, 25 mM EDTA, 2.5 mM magnesium acetate, and 250 mM substrate peptide (KKLNRTLVA) in 20 mM MOPS, pH 7.2, 25 mM β -glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, and 1 mM DTT for 15 min at 30°C. The reaction was terminated by transferring the phosphorylated substrates to P81 binding papers (Upstate Biotechnology) prewet with 0.75% phosphoric acid. P81 binding papers were washed three times in 0.75% phosphoric acid and once in acetone, and radioactivity was measured using a beta scintillation counter. MAPKAPK-2 activity was calculated from the specific counts (total counts minus nonspecific counts). Nonspecific counts were determined by performing parallel assays in the absence of the substrate peptide. One unit of MAPKAPK-2 was defined as the amount that catalyzed the incorporation of 1 pmol of phosphate into MAPKAPK-2 substrate peptide per minute per milligram of protein.

p38 MAPK ACTIVITY ASSAY. According to the cascade reaction from the Upstate Biotechnology protocol, the p38 MAPK activity assay consists of two sequential steps. *Step 1* measures the phosphorylation of glutathione *S*-transferase (GST)-MAPKAPK-2 by p38 MAPK. After activation by p38 MAPK, the phosphorylated MAPKAPK-2 transfers the γ -phosphate of [γ -³²P]ATP to a specific peptide substrate (*step 2*). In *step 1*, 15 μ g of protein were incubated with 15 mM MgCl₂, 0.1 mM ATP, 60 μ M H-7, and 200 ng of GST-MAPKAPK-2 in a final volume of 25 μ l of assay dilution buffer (20 mM MOPS, 25 mM β -glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, and 1 mM DTT) for 20 min at 30°C with gentle agitation. *Step 2* was initiated by adding a cocktail buffer containing 10 μ Ci of [γ -³²P]ATP and 122.86 mM substrate peptide in dilution buffer (total reaction volume 70 μ l) and incubating for 15 min at 30°C with agitation. The reaction was stopped by lowering the temperature to 4°C with ice, and the phosphorylated substrates were transferred to the P81 binding papers. The papers were washed three times in 0.75%

phosphoric acid and once in acetone, and radioactivity was measured using a beta scintillation counter. p38 MAPK activity was calculated from the specific counts (total counts minus nonspecific counts). Nonspecific counts were determined by performing parallel assays in the presence of the substrate peptide but in the absence of the GST-MAPKAPK-2 substrate. One unit of p38 MAPK was defined as the amount that catalyzed the incorporation of 1 pmol of phosphate into MAPKAPK-2 substrate peptide per minute per milligram of protein.

MEK3/6 ACTIVITY ASSAY. MEK3 and MEK6 activity was determined with a cascade reaction consisting of three steps, according to the protocol from Upstate Biotechnology. In *step 1*, 15 μ g of protein was incubated in a total volume of 40 μ l of a mixture containing 5 mM MgCl₂, 0.1 mM ATP, and 350 ng of inactive p38 MAPK (Upstate) at 30°C for 30 min. The reaction was stopped with the addition of 10 μ l of ice-cold dilution buffer. *Step 2* was started by aliquoting 11 μ l of the mixture from *step 1* into 9 μ l of reaction cocktail, which contained 300 ng of GST-MAPKAPK-2, 15 mM MgCl₂, and 0.1 mM ATP. The mixture was incubated at 30°C for 15 min with gentle agitation. *Step 3* was begun by adding 20 μ l of buffer containing 10 μ Ci of [γ -³²P]ATP, 0.1125 mM ATP, 16.9 mM MgCl₂, 5 mM compound R-24571, 12.5 mM β -glycerol phosphate, pH 7.0, 25 mM EDTA, 2.5 mM magnesium acetate, and 250 mM MAPKAPK-2 substrate peptide to the reaction mixture from *step 2*. The reaction was carried out for 10 min at 30°C with agitation. The final reaction was stopped by lowering the temperature to 4°C with ice, and the phosphorylated substrates were transferred to the P81 binding papers. The papers were washed three times in 0.75% phosphoric acid and once in acetone, and radioactivity was measured using a beta scintillation counter. MEK3/6 activity was calculated from the specific counts (total counts minus the nonspecific counts). Nonspecific counts were determined by performing parallel assays in the presence of the substrate peptide and the GST-MAPKAPK-2 substrate but in the absence of the inactive p38 MAPK. MEK3/6 activity was defined as the amount that catalyzed the incorporation of 1 pmol of phosphate into MEK3/6 substrate peptide per minute per milligram of protein and was expressed as a percentage of the control.

Studies in Isolated Cardiac Myocytes (Phase II)

Isolation of adult rabbit cardiac myocytes. Rabbit cardiac myocytes were isolated using collagenase (type II, Worthington Biochemical) (20). This method yielded 80–85% rod-shaped cardiac cells, which generated an average total of 20–30 million cells per rabbit heart. Cardiac myocytes were plated at subconfluency (0.5×10^6 cells/well of a 6-well plate) and cultured in 2% fetal bovine serum-medium 199 for 48 h before gene transfection.

Construction of recombinant adenovirus expressing rabbit PKC- ϵ cDNAs. The full-length rabbit heart PKC- ϵ cDNA (~2.3 kb) was cloned from a rabbit heart cDNA library (Clontech) using a cDNA probe kindly provided by Dr. Shigeo Ohno (Yokohama City University, Yokohama, Japan). A human hemagglutinin (HA) epitope tag was attached to the 5' end of the rabbit PKC- ϵ cDNA through site-directed mutagenesis. The expression of this HA epitope enabled us to differentiate the expression of the transfected PKC- ϵ from the endogenously expressed rabbit PKC- ϵ . The rabbit HA-PKC- ϵ cDNA was sequenced and characterized. Preliminary studies demonstrated that the HA epitope, consisting of a nine-amino acid sequence, did not affect the protein expression or the enzymatic activity of the rabbit PKC- ϵ isoform. To alter PKC- ϵ isoform activity in cardiac myocytes, a full-length wild-type

PKC- ϵ cDNA (PKC- ϵ -FL) and a dominant negative mutant PKC- ϵ cDNA (PKC- ϵ -DN) were constructed through site-directed mutagenesis. PKC- ϵ -DN was generated through a double mutation by converting K to R (amino acid 436) and A to E (amino acid 159). This double mutation permanently impairs the ATP-binding site of the enzyme but still allows the enzyme to compete for substrates, thereby effectively attenuating the activity of the ϵ -isoform (28, 39). Recombinant adenoviruses expressing the wild-type and the dominant negative mutant of the rabbit PKC- ϵ gene were generated by cloning HA-PKC- ϵ cDNAs into the E1 region of human adenoviral type 5 genomic DNA (33). Positive recombinant adenoviruses were isolated by plaque purification and propagated in H293 cells that had been transformed with E1 genes (33). The recombinant viral cell lysates were purified by double CsCl gradient. The integrity of the PKC- ϵ transgene structure was confirmed by both PCR and Southern blotting.

PKC- ϵ gene transfer into cardiac myocytes. To elucidate the role of PKC- ϵ in the activation of the p46 and p54 JNKs in cardiac cells, four experimental groups were studied. Ten plaque-forming units (pfu) per cell of recombinant adenovirus were transfected. The control group (*group I*) received recombinant adenovirus expressing no cDNA insert. *Group II* received recombinant adenovirus expressing PKC- ϵ -FL. *Group III* received recombinant adenovirus expressing PKC- ϵ -DN. *Group IV* received recombinant adenovirus expressing PKC- ϵ -FL (10 pfu/cell) in conjunction with PKC- ϵ -DN (30 pfu/cell). Each group included four to nine experiments, each from a different rabbit heart. All cells were harvested 18 h after recombinant adenovirus transfection. Cells from three wells were pooled together, and total cardiac cell lysates were used to determine PKC- ϵ protein expression, PKC- ϵ protein activity, and p46/p54 JNK activity. PKC- ϵ transgene protein expression was determined by Western immunoblotting using HA antibodies, and the signal was confirmed by PKC- ϵ antibodies. The isoform-selective phosphorylation activity of PKC- ϵ was measured as described above. The phosphorylation activity of the p46 and p54 JNKs was determined by immunoprecipitating the total cell lysates, followed by phosphorylation assay of these kinases. In separate experiments, the transfection efficiency was determined using recombinant adenovirus expressing green fluorescence peptide (39).

To elucidate the role of PKC- ϵ in activation of the p38 MAPK signaling pathway, we transfected cardiac cells with recombinant adenoviruses expressing the null vector, the PKC- ϵ -FL, the constitutively active MEK3 (MEK3-KE), or the constitutively active MEK6 (MEK6-KE). Both MEK3 and MEK6 are direct activators of the p38 MAPK. Their corresponding mutants, MEK3-KE and MEK6-KE, were generated as described previously (23). Ten plaque-forming units per cell of recombinant adenovirus were used for transfection. Eighteen hours after transfection, total cell lysates were collected and immunoprecipitated and p38 MAPK assays were performed.

Statistical Analysis

Data are reported as means \pm SE. To facilitate comparisons, measurements of kinase activity and protein expression in each individual rabbit were expressed as a percentage of the average value for the control group. Differences among the six experimental groups in the *in vivo* studies and among the four groups in the *in vitro* studies were analyzed using a one-way ANOVA. If the ANOVA showed an overall difference, post hoc contrasts were performed with Student's *t*-tests for unpaired data using the Bonferroni correction (50).

RESULTS

Exclusions

A total of 33 conscious rabbits were instrumented for the *in vivo* experiments. In *phase I*, eight rabbits were assigned to *group I* (control group), five to *group II* (6 cycles of 4-min occlusion/4-min reperfusion), five to *group III* (chelerythrine without occlusion-reperfusion), and five to *group IV* (chelerythrine followed by 6 cycles of 4-min occlusion/4-min reperfusion) (Fig. 1). In *phase III*, five rabbits were assigned to *group V* (4-min occlusion only) and five to *group VI* (4-min occlusion/5-min reperfusion). All rabbits in *groups I-VI* successfully completed the protocol.

A total of 27 rabbits were used for the *in vitro* experiments in *phase II*. In seven rabbits, we were unable to obtain viable cardiac cells. In the remaining 20 rabbits, each isolation procedure yielded 20–30 \times 10⁶ cardiac myocytes per heart.

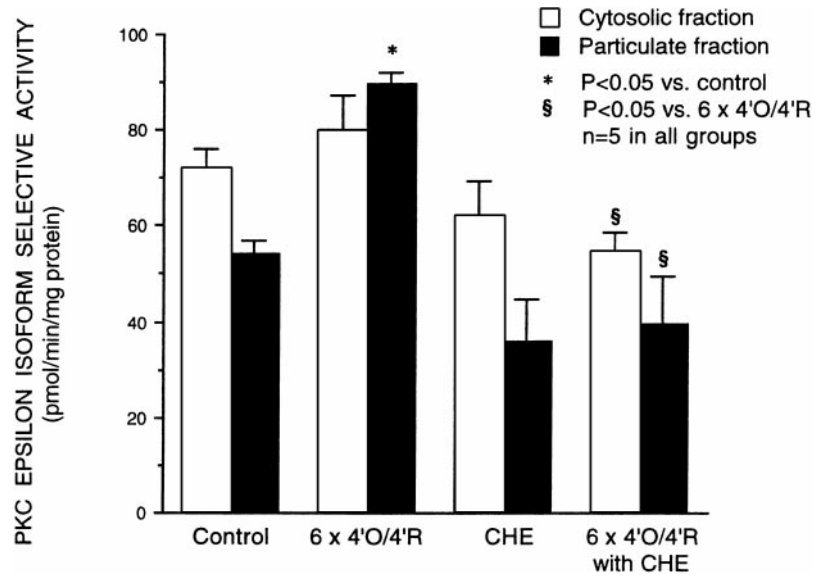
Phase Ia: Isoform-Selective Activation of PKC- ϵ by Ischemic PC in Conscious Rabbits

Previous studies in conscious rabbits have demonstrated that ischemic PC causes translocation of PKC- ϵ from the cytosolic to the particulate fraction (40). However, it remained uncertain whether this redistribution of PKC- ϵ protein was associated with increased enzymatic activity. In the present study we used immunoprecipitation to purify PKC- ϵ enzymes from the tissue samples. Using this technique, we found that ischemic PC significantly enhanced the isoform-selective phosphorylation activity of PKC- ϵ (Fig. 2). Analysis of the subcellular compartments revealed that the enhanced PKC- ϵ activity was caused by a robust rise in the particulate fraction from 54.3 ± 2.8 pmol \cdot min⁻¹ \cdot mg protein⁻¹ in control (*group I*) to 89.7 ± 2.4 pmol \cdot min⁻¹ \cdot mg protein⁻¹ after ischemic PC (*group II*) ($P < 0.05$) (Fig. 2). In contrast, the cytosolic activity did not change significantly (Fig. 2). Chelerythrine completely blocked the ischemic PC-induced activation of the ϵ -isoform in the particulate fraction (*group IV*) (Fig. 2). These data expand our previous findings (40) by demonstrating that translocation of PKC- ϵ is accompanied by enhanced phosphorylation activity in the particulate fraction. Thus ischemic PC induces not only translocation but also isoform-selective activation of the ϵ -isoform of PKC.

Phase Ib: PKC-Dependent Activation of p46/p54 JNKs During Ischemic PC in Conscious Rabbits

Expression of p46 and p54 JNKs in the rabbit heart. We found that the adult rabbit heart expresses both the p46 and p54 JNKs (Fig. 3, A and B). Analysis of subcellular distribution revealed that $84.8 \pm 2.1\%$ of the p46 JNK resides in the cytosolic fraction and $15.2 \pm 2.1\%$ in the nuclear fraction and that $90.6 \pm 2.0\%$ of the p54 JNK is located in the cytosolic fraction and $9.4 \pm 2.0\%$ in the nuclear fraction. No expression of p46 or p54 JNK protein was detected in the membrane fraction using currently available antibodies. Using immu-

Fig. 2. Protein kinase C (PKC) ε-isoform-selective phosphorylation activity, measured after immunoprecipitation, in 4 experimental groups (*n* = 5 rabbits in each group). Compared with the control group (*group I*), the phosphorylation activity of PKC-ε in the particulate fraction was significantly increased after 6 cycles of 4-min occlusion/4-min reperfusion (6 × 4'O/4'R; *group II*). In the absence of ischemia (*group III*), chelerythrine had no significant effect on PKC-ε phosphorylation activity. In rabbits subjected to ischemic PC after receiving chelerythrine (6 × 4'O/4'R with CHE; *group IV*), chelerythrine completely abolished the increase in PKC-ε phosphorylation activity induced by the 6 O/R cycles. Data are means ± SE.



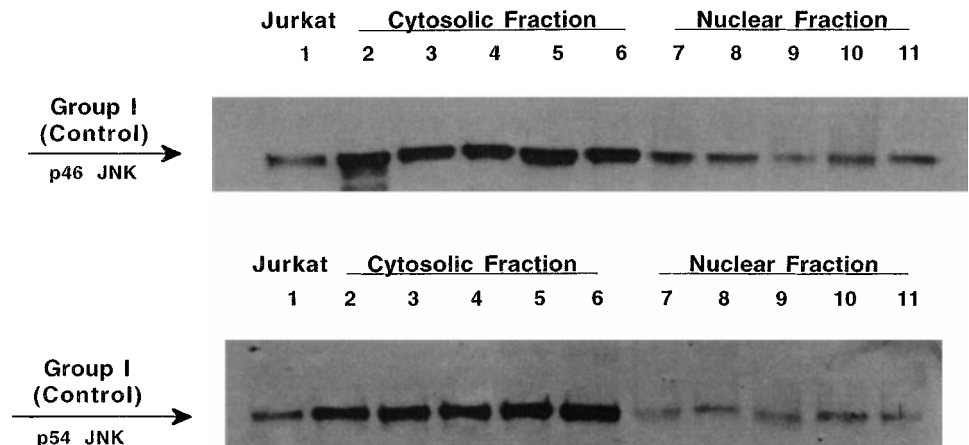
noprecipitation followed by in-gel kinase assay, we observed basal p46 and p54 JNK activity in both the cytosolic and the nuclear fraction (Figs. 4 and 5). An example of an in-gel kinase assay is shown in Fig. 5. These data indicate that JNKs are active in the heart of conscious rabbits under control conditions, which implies that besides responding to extracellular stimulation, these kinases may be important in maintaining cardiac function under basal conditions.

Effect of ischemic PC on JNK activity. To examine the effect of ischemic PC on the p46/p54 JNKs, we used a protocol (6 cycles of 4-min coronary occlusion/4-min reperfusion) that was shown previously to induce late PC against myocardial stunning (7, 9, 42) and infarction (43, 49). The ischemic PC protocol did not affect the protein expression of p46 and p54 JNKs, as determined by Western immunoblotting (data not shown). However, both the p46 and p54 JNK activities (determined by in-gel kinase assay) were significantly increased after the ischemic PC protocol (*group II*) compared with control rabbits (*group I*) (Fig. 4, A and B). The increase in the p46 JNK activity was accounted for exclusively by a rise in the nuclear fraction (Fig. 4A), whereas the cytosolic p46 JNK activity was unchanged (Fig. 4A). In

contrast, the increase in the p54 JNK activity was accounted for solely by a rise in the cytosolic fraction (Figs. 4B and 5), whereas the nuclear p54 JNK activity was unaffected (Fig. 4B). Thus ischemic PC induced activation of the p46 JNK in the nuclear fraction and the p54 JNK in the cytosolic fraction, suggesting that these JNKs are targeted at proteins in different subcellular compartments of the heart.

Effect of chelerythrine on ischemic PC-induced JNK activation. To determine whether ischemic PC-induced activation of the p46/p54 JNKs is dependent on PKC activation, we measured JNK activity in rabbits undergoing the ischemic PC protocol after pretreatment with 5 mg/kg chelerythrine (*group IV*). Previous studies in this conscious rabbit model have documented that this dose of chelerythrine blocks both the ischemic PC-induced translocation of PKC-ε and the cardioprotective effects of late PC against stunning and infarction (40, 42). Chelerythrine completely blocked the activation of the p46/p54 JNKs induced by ischemic PC (*group IV*) (Figs. 4, A and B, and 5). These results indicate that in the adult rabbit heart p46 and p54 JNKs are located downstream of PKC and that their

Fig. 3. *Top:* Western blot performed to identify p46 c-Jun NH₂-terminal kinase (JNK) expression in 5 control rabbits that did not undergo coronary occlusion (*group I*). *Lane 1:* positive control for p46 JNK obtained from human Jurkat cell lysates [American Type Culture Collection (ATCC)]. *Lanes 2–6:* cytosolic fractions of p46 JNK. *Lanes 7–11:* nuclear fractions of p46 JNK. *Bottom:* Western blot performed to identify p54 JNK expression in 5 control rabbits that did not undergo coronary occlusion (*group I*). *Lane 1:* positive control for p54 JNK obtained from human Jurkat cell lysates (ATCC). *Lanes 2–6:* cytosolic fractions of p54 JNK. *Lanes 7–11:* nuclear fractions of p54 JNK.



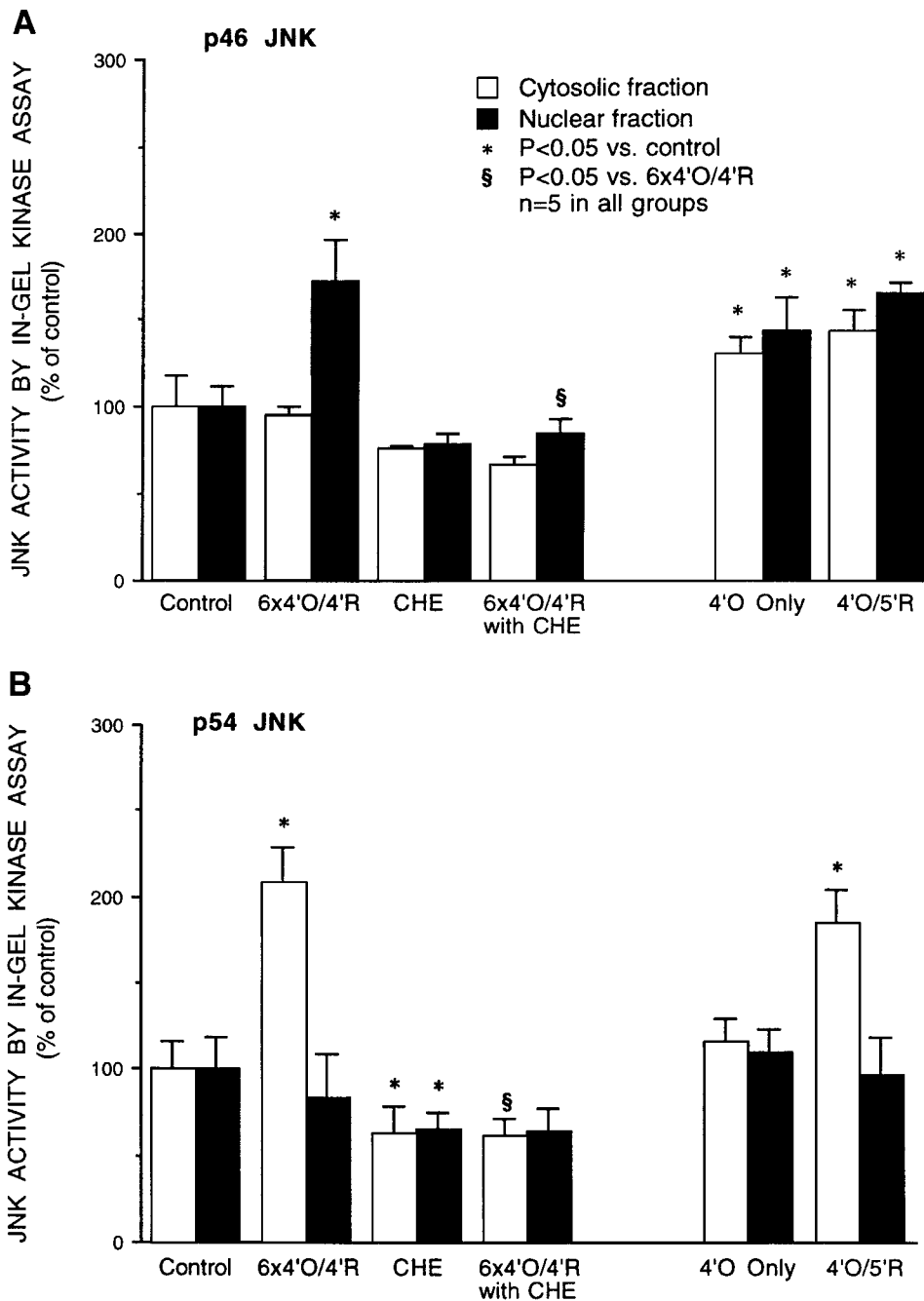


Fig. 4. Nuclear and cytosolic phosphorylation activity of the p46 and p54 JNKs, as determined by in-gel kinase assays following immunoprecipitation, in the 6 experimental groups ($n = 5$ rabbits in each group). Compared with the control group (*group I*), the nuclear p46 (*A*) and the cytosolic p54 (*B*) JNK activities were significantly increased after 6 cycles of 4-min occlusion/4-min reperfusion ($6 \times 4'O/4'R$; *group II*). In the absence of ischemia (*group III*), chelerythrine had no significant effect on p46 JNK activity but attenuated p54 JNK activity. Chelerythrine blocked the ischemic PC-induced increase in the nuclear p46 (*A*) and the cytosolic p54 (*B*) JNK activity. A single episode of 4-min ischemia (4'O only; *group V*) induced significant activation of the p46 JNK but did not affect the p54 JNK. A 5-min period of reperfusion following the 4-min ischemia (4'O/5'R; *group VI*) induced significant activation of the p54 JNK in the cytosolic fraction. Activation of the p46 JNK was evident in both the nuclear and cytosolic fractions in *groups V* and *VI* (4'O/5'R). Data are means \pm SE.

activation during ischemic PC occurs via a PKC-dependent pathway.

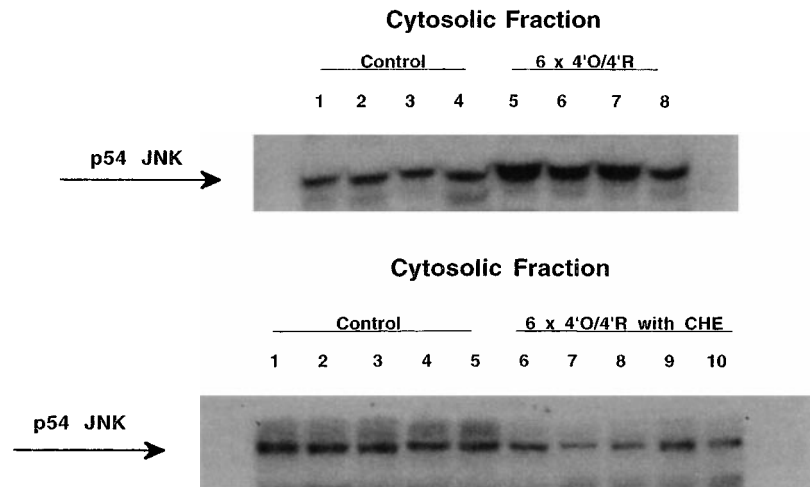
Phase Ic: Phosphorylation Activity of the p38 MAPK Cascade During Ischemic PC in Conscious Rabbits

At least four isoforms of p38 MAPK have been identified (47). Using monoclonal antibodies, we found that the rabbit myocardium expresses at least two isoforms of the p38 MAPK family of enzymes: the α - and β -isoforms. Most of the α -isoform of the p38 MAPK protein was found in the cytosolic fraction, as shown in Fig. 6. We detected weak expression of the β -isoform of the p38 MAPK and were unable to detect the γ -isoform (data not shown). Because antibodies for the δ -isoform

are not currently available, it is not possible to determine the phosphorylation activity of each individual isoform of the p38 MAPK family. Consequently, we measured the total phosphorylation activity for the entire p38 MAPK family.

Figure 7 shows the subcellular distribution of the phosphorylation activity of the three elements of the p38 MAPK signaling cascade: MEK3/6, p38 MAPK, and MAPKAPK-2 (Fig. 7, *A*, *B*, and *C*, respectively). Surprisingly, the same ischemic PC protocol (6 cycles of 4-min coronary occlusion/4-min reperfusion) that induced activation of the p46/p54 JNKs (Fig. 4, *A* and *B*) did not exert a discernible effect on the phosphorylation activity of the p38 MAPK (Fig. 7*B*). Chelerythrine did not

Fig. 5. Cytosolic p54 JNK activity after ischemic PC, as determined by in-gel kinase assay following immunoprecipitation. *Top*: cytosolic phosphorylation activity of p54 JNK was significantly increased after 6 cycles of 4-min occlusion/4-min reperfusion ($6 \times 4'O/4'R$; *group II*) compared with control (*group I*). *Bottom*: cytosolic phosphorylation activity of p54 JNK was decreased after 6 cycles of 4-min occlusion/4-min reperfusion with chelerythrine ($6 \times 4'O/4'R$ with CHE; *group IV*) compared with control.



affect the p38 MAPK activity in either the absence (*group III*) or presence (*group IV*) of ischemic PC (data not shown). The phosphorylation activity of MEK3/6, the direct activators of the p38 MAPK, was only marginally increased after six cycles of occlusion-reperfusion [compared with the striking increase noted after a 4-min occlusion only (*group V*)] (Fig. 7A). The phosphorylation activity of MAPKAPK-2, the substrate of p38 MAPK, was unaffected (Fig. 7C). Thus, despite robust activation of the PKC ϵ -isoform (Fig. 2), the phosphorylation activity of the p38 MAPK cascade was essentially unchanged after an ischemic PC protocol consisting of six cycles of 4-min occlusion/4-min reperfusion.

Phase II: PKC- ϵ -Dependent Activation of p46/p54 JNKs and p38 MAPK in Isolated Cardiac Myocytes

Having established that ischemia-reperfusion causes activation of the p46/p54 JNKs via a PKC-dependent pathway in vivo, we next examined whether increased PKC- ϵ activity could reproduce such activation in isolated cardiac myocytes in vitro. Ten plaque-forming units per cell of recombinant adenovirus produced consistently high transfection efficiency (>85% of cells transfected) in adult rabbit cardiac myocytes. Overexpressing full-length wild-type PKC- ϵ significantly increased the isoform-selective PKC- ϵ activity (Fig. 8A) and caused a marked elevation of both the p46 and p54 JNK activities (Fig. 8B). Expressing the dominant negative mutant of PKC- ϵ attenuated the basal PKC- ϵ activity in cardiac cells (Fig. 8A) but had no significant

effect on the basal activity of either the p46 or p54 JNK (Fig. 8B). Coexpressing the full-length wild-type PKC- ϵ in conjunction with the dominant negative mutant of PKC- ϵ inhibited the activation of PKC- ϵ (Fig. 8A) and abolished the increased activity of p46 and p54 JNK (Fig. 8B). These data demonstrate that selective activation of the PKC ϵ -isoform enhances the activity of p46/p54 JNKs in adult cardiac myocytes, indicating that PKC- ϵ is coupled to the p46/p54 JNK signaling cascade.

Figure 8C demonstrates that overexpression of PKC- ϵ in cardiac myocytes had no effect on the total phosphorylation activity of the p38 MAPK. To determine whether this lack of response of p38 MAPK to PKC- ϵ activation was due to an inherent limitation in the extent to which p38 MAPK activity can be enhanced in this system, we overexpressed MEK3 and MEK6, which are the direct activators of the p38 MAPK. In contrast to PKC- ϵ , both MEK3 and MEK6 produced marked increases in the p38 MAPK activity of cardiac myocytes (+252% of control for MEK3; +589% of control for MEK6; $P < 0.05$ for both) (Fig. 8C). These data suggest that, in contrast to p46/p54 JNKs, the ϵ -isoform of PKC is not coupled to the p38 MAPK cascade in adult rabbit cardiac myocytes.

Phase III: Effect of Ischemia and Subsequent Reperfusion on Activity of the p38 MAPK Cascade and p46/p54 JNKs in Conscious Rabbits

The observation that ischemic PC had a marginal effect on the activity of the p38 MAPK cascade was

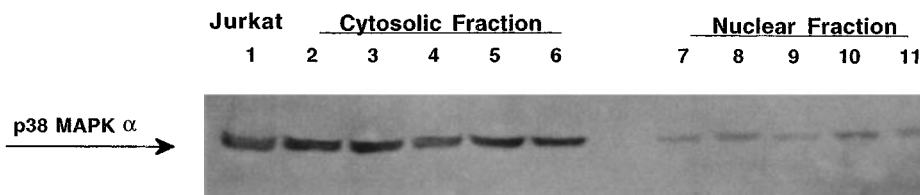


Fig. 6. Western blot performed to identify p38 MAPK α -isoform expression in 5 control rabbits that did not undergo coronary occlusion (*group I*). Fifty micrograms of either cytosolic or nuclear proteins were loaded in each lane. *Lane 1*: positive control for the α -isoform of p38 MAPK obtained from human Jurkat cell lysates (ATCC). *Lanes 2-6*: cytosolic fractions of the α -isoform of p38 MAPK. *Lanes 7-11*: nuclear fractions of the α -isoform of p38 MAPK. Note that most of the α -isoform of p38 MAPK resides in the cytosolic fraction.

unexpected. We therefore conducted further studies to address this issue. The lack of a response of p38 MAPK to the six cycles of occlusion-reperfusion may be due to the fact that ischemia-reperfusion has no effect on p38 MAPK in our model or, alternatively, that ischemia

induces a transient activation of the p38 MAPK cascade that is diminished by the reperfusion process so that the enhanced p38 MAPK activity returns to control values after six cycles of 4-min occlusion/4-min reperfusion. To discern between these two possibilities, in *phase III* of the present investigation we studied two additional groups of rabbits: *group V* underwent only 4 min of ischemia, whereas *group VI* underwent 4 min of ischemia followed by 5 min of reperfusion. Because it was unclear from the results of *phase I* whether the activation of the p46/p54 JNKs requires ischemia, reperfusion, or both, p46/p54 JNK activity was also measured in these two groups of rabbits.

Phosphorylation activity of the p38 MAPK cascade. A single episode of 4 min of ischemia (*group V*) induced a pronounced increase in the phosphorylation activity of all components of the p38 MAPK cascade. The increase occurred exclusively in the cytosolic fraction (Fig. 7, A–C). The cytosolic MEK3/6 activity increased to $3,185 \pm 310\%$ of control ($P < 0.05$; Fig. 7A), the cytosolic p38 MAPK activity increased to $539 \pm 102\%$ of control ($P < 0.05$; Fig. 7B), and the cytosolic MAPKAPK-2 activity increased to $269 \pm 34\%$ of control ($P < 0.05$; Fig. 7C). After a 5-min period of reperfusion (*group VI*), the cytosolic MEK3/6 activity decreased to $1,779 \pm 213\%$ of control ($P < 0.05$ vs. *group V*; Fig. 7A) and the cytosolic p38 MAPK activity decreased to $237 \pm 31\%$ of control ($P < 0.05$ vs. *group V*; Fig. 7B). The cytosolic MAPKAPK-2 activity did not change appreciably (Fig. 7C). The nuclear activities of MEK3/6, p38 MAPK, and MAPKAPK-2 remained unaltered in both *groups V* and *VI* (Fig. 7, A–C).

In summary, a single 4-min period of ischemia induced a marked increase in the cytosolic phosphorylation activity of the entire p38 MAPK cascade. This activation, however, was significantly attenuated during the subsequent 5-min period of reperfusion (except for MAPKAPK-2) and disappeared completely after six cycles of 4-min ischemia/4-min reperfusion.

Phosphorylation activities of p46/p54 JNKs. Compared with control rabbits (*group I*), the phosphorylation activity of the p46 JNK increased significantly ($P < 0.05$) after 4 min of ischemia (*group V*; Fig. 4A). The

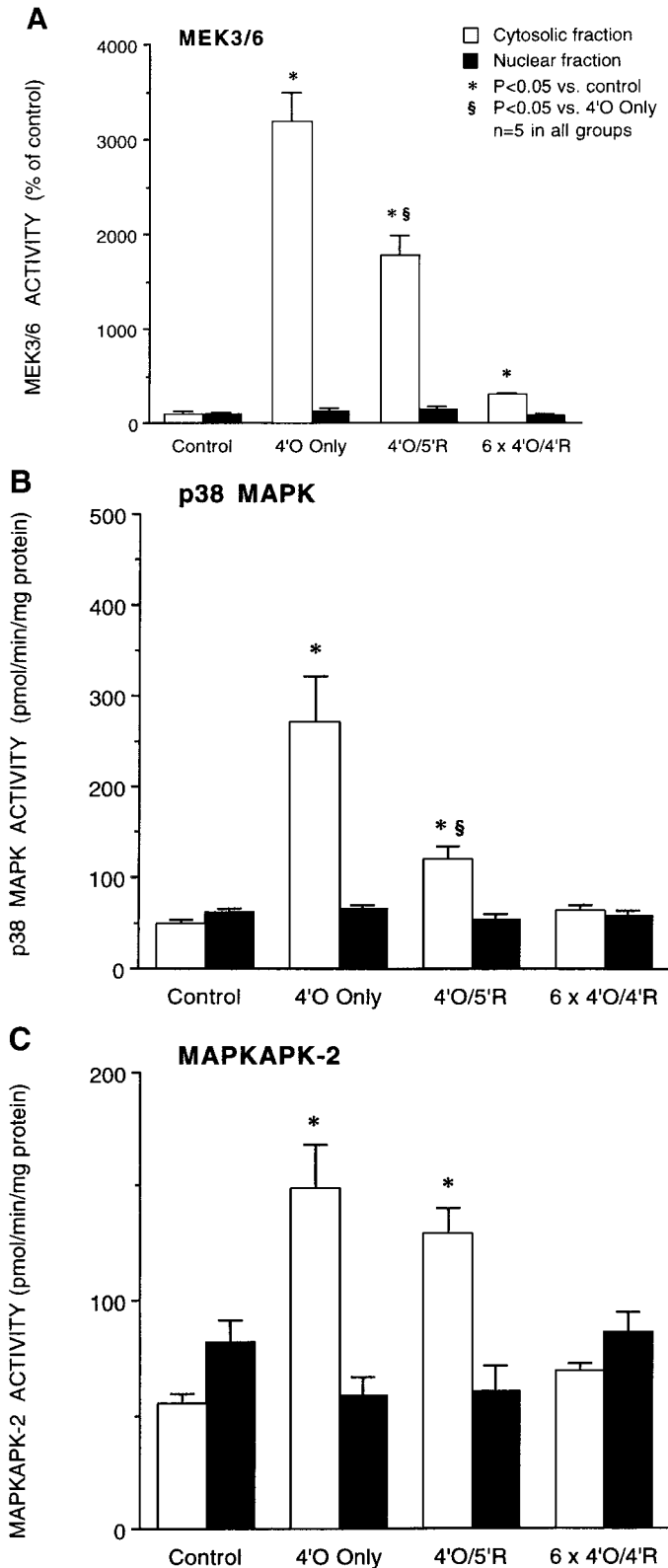


Fig. 7. Phosphorylation activity of the p38 MAPK cascade during ischemic PC. A: MAPK/extracellular signal-regulated kinase 3/6 (MEK3/6) activity. Compared with control (*group I*), the cytosolic MEK3/6 activity increased markedly (31-fold) after 4 min of ischemia (4'O only; *group V*) but decreased after 5 min of reperfusion (4'O/5'R; *group VI*) and decreased even further after 6 cycles of 4-min occlusion/4-min reperfusion (6 × 4'O/4'R; *group II*). B: p38 MAPK activity. Compared with control (*group I*), the cytosolic p38 MAPK activity increased significantly after 4 min of ischemia (4'O only; *group V*) but decreased after 5 min of reperfusion (4'O/5'R; *group VI*) and returned to control levels after 6 cycles of 4-min occlusion/4-min reperfusion (6 × 4'O/4'R; *group II*). C: MAPK-activated protein kinase 2 (MAPKAPK-2) activity. Compared with control (*group I*), the cytosolic MAPKAPK-2 activity increased significantly after 4 min of ischemia (4'O only; *group V*) and after 5 min of reperfusion (4'O/5'R; *group VI*) but returned to control levels after 6 cycles of 4-min occlusion/4-min reperfusion (6 × 4'O/4'R; *group II*). Ischemia-reperfusion did not have a significant effect on the phosphorylation activity of MEK3/6, p38 MAPK, or MAPKAPK-2 in the nuclear fraction. Data are means \pm SE; n = no. of rabbits.

enhanced activity occurred in both the cytosolic and nuclear fractions and persisted after the subsequent 5-min period of reperfusion (*group VI*; Fig. 4A). In contrast, a 4-min period of ischemia was not sufficient to affect the phosphorylation activity of the p54 JNK (Fig. 4B). Activation of the p54 JNK required the subsequent reperfusion stimulus (Fig. 4B). The en-

hanced activity of p54 JNK occurred only in the cytosolic fraction (Fig. 4B). These results indicate that the pattern of activation of JNKs during ischemic PC differs: activation of the p46 JNK occurs during ischemia, whereas activation of the p54 JNK occurs after reperfusion.

DISCUSSION

A conscious animal model was utilized in this study in an effort to avoid potential activation of JNKs and p38 MAPK by the stress and the manipulations associated with open-chest preparations and isolated hearts. There are several new findings in this study. First, in the heart of conscious rabbits, ischemic PC significantly increased the phosphorylation activity of the p46 and p54 JNKs. This increase was associated with an increase in PKC- ϵ phosphorylation activity and was completely blocked by chelerythrine, demonstrating that p46/p54 JNKs are downstream of PKC and that ischemic PC activates p46/p54 JNKs via a PKC-dependent signaling pathway. Second, selective activation of the ϵ -isoform of PKC in the absence of ischemia mimicked the ischemic PC-induced activation of the p46/p54 JNKs in isolated cardiac myocytes, indicating that activation of PKC- ϵ is sufficient to enhance the phosphorylation activity of JNKs in this specific cell type in vitro. This is the first demonstration of the existence of a signal transduction pathway linking the ϵ -isoform of PKC to the p46/p54 JNKs in cardiac myocytes. Third, activation of the p46 JNK occurred during ischemia, whereas activation of the p54 JNK required the reperfusion process, indicating that PKC-mediated activation of JNKs involves at least two distinct molecular mechanisms. Finally, the phosphorylation activity of the p38 MAPK cascade was increased by a brief ischemic stimulus, but this activation was not sustained. After repetitive cycles of ischemia-reperfusion,

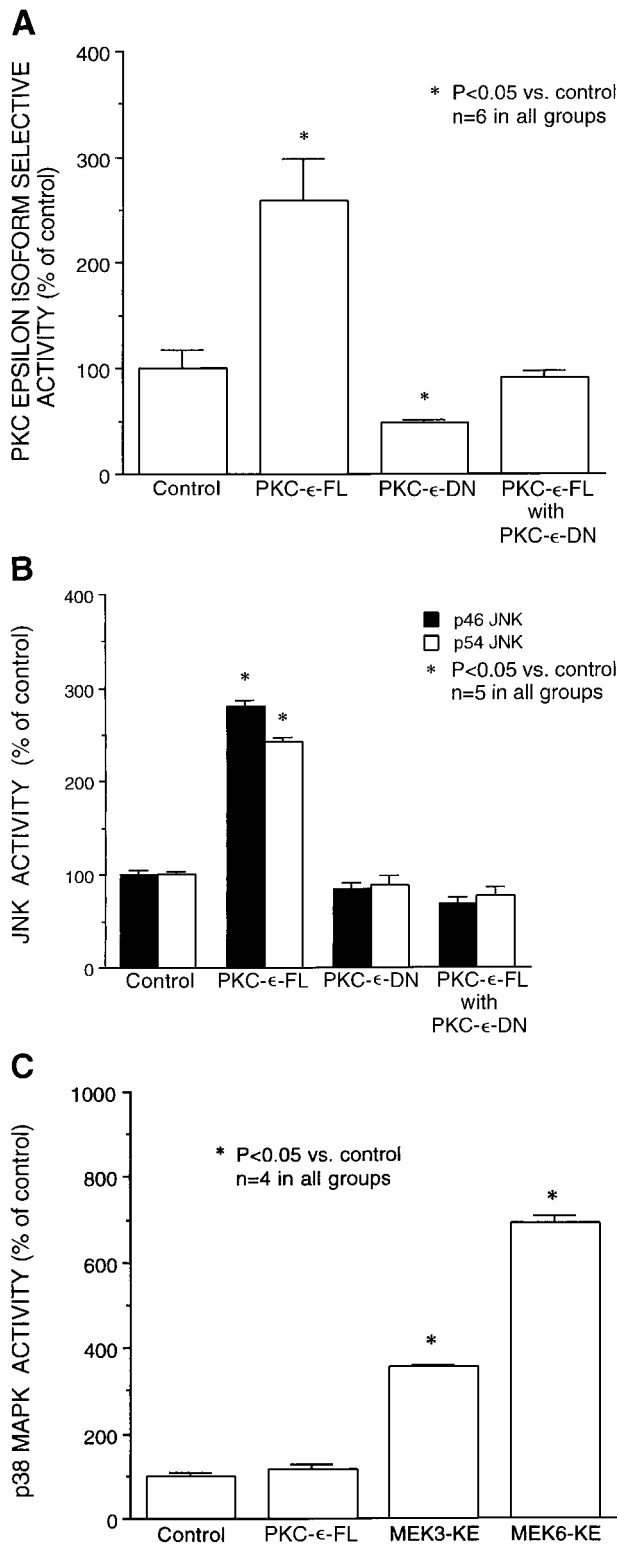


Fig. 8. *A*: PKC ϵ -isoform-selective phosphorylation activity, as determined after immunoprecipitation, in adult rabbit cardiac myocytes transfected with null recombinant adenovirus (control), cardiac cells overexpressing the wild-type full-length PKC- ϵ (PKC- ϵ -FL), myocytes expressing the dominant negative mutant of PKC- ϵ (PKC- ϵ -DN), and myocytes coexpressing both the full-length and dominant negative mutants of PKC- ϵ (PKC- ϵ -FL with PKC- ϵ -DN). The total activity of the PKC ϵ -isoform was significantly increased in cells overexpressing PKC- ϵ -FL. This enhanced PKC ϵ -isoform activity was abolished in cells coexpressing both PKC- ϵ -FL and PKC- ϵ -DN. *B*: p46 and p54 JNK phosphorylation activities, as determined after immunoprecipitation, in myocytes transfected with null recombinant adenovirus (control), myocytes overexpressing PKC- ϵ -FL, myocytes expressing PKC- ϵ -DN, and myocytes coexpressing both PKC- ϵ -FL and PKC- ϵ -DN. The total activity of the p46 and p54 JNKs was significantly increased in cells overexpressing PKC- ϵ -FL. The enhanced p46 JNK and p54 JNK activity was abolished in cells coexpressing both PKC- ϵ -FL and PKC- ϵ -DN. *C*: p38 MAPK phosphorylation activity in adult rabbit cardiac myocytes transfected with null recombinant adenovirus (control) and in myocytes transfected with PKC- ϵ -FL. Myocytes transfected with PKC- ϵ -FL did not exhibit a discernible change in the phosphorylation activity of p38 MAPK compared with control cells. In contrast, myocytes transfected with either constitutively active MEK3 (MEK3-KE) or constitutively active MEK6 (MEK6-KE), both of which are direct activators of p38 MAPK, demonstrated a significant increase in the p38 MAPK phosphorylation activity. Data are means \pm SE; *n* = no. of rabbits.

the p38 MAPK phosphorylation activity declined while the activation of the PKC ϵ -isoform persisted, suggesting that activation of the p38 MAPK was not coupled to that of the ϵ -isozyme of PKC during the development of the late phase of ischemic PC. This conclusion is directly supported by the finding that selective activation of PKC- ϵ in isolated cardiac myocytes failed to enhance p38 MAPK activity.

Subcellular Redistribution of PKC- ϵ Phosphorylation Activity During Ischemic PC

Traditionally, activation of PKC has been inferred from the subcellular redistribution of the protein (37). Using the same conscious rabbit model and the same protocol (6 cycles of 4-min occlusion/4-min reperfusion) employed in this study, we found in previous studies that ischemic PC induces a significant translocation of PKC- ϵ protein to the particulate fraction (40) and that chelerythrine, at doses that block the late PC effect, also blocks the translocation of PKC- ϵ (42). However, although the demonstration of PKC translocation illustrates the mobilization of the enzyme and strongly supports its activation, it does not provide, in itself, any information regarding the kinetic state of the enzyme. Accordingly, the criticism has been raised (10, 41) that translocation of PKC does not necessarily signify activation, so the changes in the subcellular distribution of the ϵ -protein reported previously (40, 42) may not be indicative of increased phosphorylation activity of this specific isoform.

Before concluding that PKC- ϵ mediates ischemic PC, it is undoubtedly important to document not only its translocation but also its activation. To address this concern, in the present study we directly measured the ϵ -isoform-selective phosphorylation activity using immunoprecipitation and a PKC- ϵ -selective substrate. Our results demonstrate that the ischemic PC protocol consisting of six cycles of 4-min occlusion/4-min reperfusion significantly increased the phosphorylation activity of the ϵ -isoform in the particulate fraction (Fig. 2). This increase in activity (+65%) was quantitatively similar to the increase in ϵ -protein in the particulate fraction observed in our previous studies (+88%) (40). Furthermore, the increase in PKC- ϵ phosphorylation activity was blocked by the same dose of chelerythrine (Fig. 2) that was previously shown to block the ischemic PC-induced translocation of the PKC- ϵ protein to the particulate fraction (42). Taken together, these results demonstrate that ischemic PC-induced ϵ -protein translocation is tightly coupled to the activation of the isozyme. The enhanced phosphorylation activity of PKC- ϵ further supports the role of this isozyme as an important element in ischemic PC and removes one of the major limitations of the PKC- ϵ hypothesis of late PC.

Previous Studies of the Effect of Ischemia-Reperfusion on the p46/p54 JNKs and the p38 MAPK Cascade

Previous studies of the effect of ischemia-reperfusion on stress-activated kinases (JNKs and p38 MAPK) were conducted primarily in isolated cardiac myocytes (27, 44) or in isolated hearts (4, 13, 25, 32, 35, 52). We discuss the JNKs and the p38 MAPK separately.

p46/p54 JNKs. The results of previous studies of p46/p54 JNKs are conflicting. Most investigations in isolated perfused rat hearts (4, 14, 25, 35, 52) or in isolated cardiac myocytes undergoing simulated ischemia-reperfusion (27, 44) have concluded that activation of p46 and p54 JNKs requires reperfusion-reoxygenation. In one study (35), ischemia was found to induce nuclear translocation but not activation of the p46 JNK; activation of the p46 JNK required reperfusion. However, a recent *in vivo* investigation (45) has concluded that ischemia alone (without reperfusion) activates both p54 and p46 JNKs. Furthermore, some reports indicate that both p46 and p54 JNKs are activated by reperfusion-reoxygenation (4, 13, 25, 35), whereas others have concluded that only p54 JNK is activated (52).

The present study provides the following new findings. 1) It demonstrates, for the first time, that ischemia-reperfusion activates both p46 and p54 JNKs in intact animals in the conscious state. 2) It indicates that, after a full PC stimulus (6 cycles of 4-min occlusion/4-min reperfusion), this activation occurs in a subcellular compartment-selective manner (in the nucleus for p46 JNK and in the cytosol for p54 JNK). 3) It demonstrates that both p46 and p54 JNKs are downstream of PKC and that the activation of both of these kinases is PKC dependent. The distinct subcellular localization of activated p46 and p54 JNKs suggests different targets for these two kinases (i.e., nuclear proteins for p46 JNK and cytosolic proteins for p54 JNK) and thus may have important functional significance. Furthermore, the present study indicates that a single episode of 4-min ischemia is sufficient to increase the phosphorylation activity of the p46 JNK (Fig. 4A), whereas the activation of the p54 JNK requires reperfusion (Fig. 4B). This finding suggests that different mechanisms are operative for the two JNKs and that reperfusion-associated events (e.g., reactive oxygen species formation) are specifically necessary to trigger the activation of p54 JNK but not p46 JNK. Thus, although ischemic PC activates both p46 and p54 JNKs, our results demonstrate important differences between these two subgroups of enzymes with respect to the subcellular location (nucleus vs. cytosol) and the timing (ischemia vs. reperfusion) of their activation. To our knowledge, this is the first indication of a differential effect of ischemia-reperfusion on the p46 and p54 JNKs *in vivo*, a finding that implies not only a differential mechanism for the activation of these kinases but also, possibly, different functional roles.

p38 MAPK. One of the most intriguing findings of our study is that activation of the p38 MAPK cascade during repetitive cycles of ischemia-reperfusion *in vivo* is transient and that it is triggered by ischemia but then attenuated by reperfusion. Previous investigations *in vitro* (isolated cells or hearts) have concluded that myocardial ischemia activates p38 MAPK but have yielded conflicting results regarding the duration of this phenomenon, with some studies reporting only a transient activation (45, 52) and others reporting a sustained activation (4, 32, 44). In one study that

compared ischemia with reperfusion, activation of the p38 MAPK persisted, but was not enhanced, during the reflow phase (4). In contrast, we found that in the heart of conscious rabbits the activation of the p38 MAPK cascade triggered by 4 min of ischemia was significantly attenuated during the subsequent 5 min of reperfusion and returned to control levels after repetitive cycles of ischemia-reperfusion (Fig. 7). The reason for these apparent discrepancies is unknown.

Because of the numerous fundamental differences between regional ischemia in the blood-perfused heart *in situ* and global ischemia in the isolated buffer-perfused heart *in vitro*, it is not possible to directly compare our results with these prior studies of JNKs and p38 MAPK during ischemia-reperfusion (4, 25, 27, 32, 35, 44, 52). Aside from the differences in the experimental models, the divergence between our results and those of previous studies could also be due to other factors, including species differences (rat vs. rabbit), different durations of ischemia (4 min vs. 10 or 15 min), analysis of both cytosolic and nuclear fractions in this study versus whole tissue lysates or cytosolic fractions only in previous studies, analysis of individual p46 and p54 JNK activities in this study versus analysis of the whole JNK subgroup in previous studies. Our conclusions are supported by a recent study by Weinbrenner et al. (51), who found that ischemic PC (5 min occlusion/10 min reperfusion) did not alter phosphorylation of tyrosine 182 on the p38 MAPK in isolated rabbit hearts.

PKC- ϵ -Dependent Activation of the p46/p54 JNKs During Ischemic PC

Although previous investigations have implicated the ϵ -isoform of PKC as a critical signaling element in the genesis of the late phase of ischemic PC (40, 42), the signaling cascades downstream of PKC- ϵ remain poorly characterized. Studies in both cardiac (4) and noncardiac (15, 22, 36) cells have reported activation of the JNKs by PMA, a non-isoform-selective PKC activator. To our knowledge, the present results represent the first documentation of PKC ϵ -isoform-selective activation of these two JNKs in cardiac myocytes, providing evidence for the existence of a signaling pathway linking the ϵ -molecule to the JNKs in this cell type. This finding may have implications that transcend ischemic PC, because both PKC and JNKs play an important role in many physiological and pathological processes. The precise molecular mechanism(s) linking PKC- ϵ to p46/p54 JNKs remains to be elucidated. Our finding that these kinases are located in different subcellular compartments (PKC- ϵ in the particulate fraction and p54 JNK in the cytosolic fraction) implies the involvement of additional intermediary signaling elements. In the present study we focused on PKC- ϵ because this appears to be the isoform responsible for the development of late PC in rabbits (40, 42). Our results, however, do not exclude the possibility that other isozymes of PKC may also activate p46/p54 JNKs in cardiac myocytes. The finding that PKC- ϵ activation triggers the activation of two subfamilies of JNKs (p46

and p54) suggests 1) that the signaling events triggered by PKC- ϵ activation during ischemic PC are complex, involving the recruitment of multiple signaling pathways, and 2) that both p46 JNK- and p54 JNK-dependent cellular functions may participate in the development of ischemic PC. Evaluation of the functional significance of JNK activation during ischemic PC is not currently possible and must await the development of specific inhibitors of these kinases. Our findings, however, provide a rationale for future studies aimed at elucidating the role of both p46 and p54 JNKs in the genesis of the cardioprotective effects of PC.

Phosphorylation Activity of the p38 MAPK Cascade During Ischemic PC

To our surprise, the phosphorylation activities of the p38 MAPK cascade (MEK3/6, p38 MAPK, and MAPKAPK-2) were similar in rabbits with and without the stimulus of an ischemic PC protocol (6 cycles of 4-min occlusion/4-min reperfusion) that is known to induce late PC against both stunning (7, 9, 42) and infarction (43, 49) and that induces marked activation of all other members of the MAPK family [ERK1/2 (39) and the JNKs (Fig. 4, A and B)]. The only discernible effect of ischemic PC was a marginal increase in the activity of MEK3/6 (Fig. 7A), which, however, was minuscule compared with the increase observed after a 4-min occlusion (Fig. 7A). The divergent effects of ischemic PC on p46/p54 JNKs and p38 MAPK were unexpected, because JNKs and p38 MAPK usually respond to cellular stress in unison (47).

In an effort to gain insight into this issue, we conducted additional studies *in vivo* and *in vitro*. We first examined the possibility that the apparent lack of response of the p38 MAPK cascade to ischemic PC could be due to an inherent defect in this signaling pathway in the rabbit heart. To our knowledge, increased activity of the p38 MAPK pathway in the rabbit heart during ischemic PC or other stimuli *in vivo* has never been reported. It is theoretically possible that the coupling of the p38 MAPK to its direct activators (MEK3/6) may be impaired in this species. However, analysis of isolated rabbit myocytes (*phase II*) revealed that the p38 MAPK signaling pathway is intact in these cells, because overexpressing either the MEK3 or the MEK6, the two direct activators of p38 MAPK, induced profound activation of the p38 MAPK (Fig. 8). Therefore, the failure of the p38 MAPK to respond to ischemic PC cannot be ascribed to a lack of functional coupling between the p38 MAPK and the MEK3/6.

We therefore examined the second possibility, namely, that p38 MAPK activation may abate with recurrent ischemia-reperfusion cycles. This appears to be the case, because the results of *phase III* show that the phosphorylation activities of the three components of the p38 MAPK cascade (MEK3/6, p38 MAPK, and MAPKAPK-2) were significantly increased at the end of a single 4-min ischemic episode, but (with the exception of MAPKAPK-2) this increase was blunted after the subsequent 5 min of reperfusion (Fig. 7). The activation of MAPKAPK-2 resolved completely after six cycles of

occlusion-reperfusion (Fig. 7C). Therefore, in contrast to the persistent activation of the p46/p54 JNKs, the activation of the p38 MAPK cascade was not sustained and disappeared with recurrent episodes of ischemia-reperfusion, indicating that ischemic PC exerts differential effects on these two subfamilies of MAPKs in the rabbit heart. The fact that the activation of MAPKAPK-2 was unabated after a 5-min period of reperfusion (Fig. 7C) suggests that either the kinetics of this enzyme differ from those of MEK3/6 and p38 MAPK or, in addition to p38 MAPK, other kinases may also activate MAPKAPK-2 in the heart of conscious rabbits.

The fact that six cycles of 4-min ischemia/4-min reperfusion induced a sustained activation of PKC- ϵ (Fig. 2) but only a transient activation of the p38 MAPK cascade (Fig. 7, A–C) suggests that the mobilization of these kinases in response to ischemia-reperfusion involves distinct cellular mechanisms. The chronologic dissociation between the increased phosphorylation activity of PKC- ϵ and that of the p38 MAPK also makes it unlikely that the activation of the ϵ -isozyme represents an important signaling event for the activation of the p38 MAPK during ischemic PC. Nevertheless, it should be stressed that our results do not in any way exclude a potential role of p38 MAPK in ischemic PC. It is conceivable that even a transient activation of the p38 MAPK may be sufficient to trigger the protective effect, because the p38 MAPK may rapidly phosphorylate downstream substrates before it returns to its original nonphosphorylated state. This issue is further compounded by the recent recognition of the existence of several isoforms of p38 MAPK (α , β , γ , and δ) (47). Because monoclonal antibodies for all of the currently known isoforms are not yet available, the isoform-selective effects of ischemic PC on the p38 MAPK subfamily cannot be assessed at the present time. Just as ischemic PC activates only 2 of the 11 isoforms of PKC (ϵ and η) without increasing the total PKC phosphorylation activity (40), ischemic PC may selectively activate one individual p38 MAPK isoform without affecting the total p38 MAPK phosphorylation activity. Thus definitive evaluation of the effect of ischemic PC on the p38 MAPK must await a complete characterization of the entire p38 MAPK family and the development of methods to determine the protein expression and isoform-selective phosphorylation activity of individual p38 MAPK isoforms in the rabbit heart.

In conclusion, the present study demonstrates that ischemic PC induces activation of both the p46 and p54 JNKs via a PKC-dependent pathway in the heart of conscious rabbits. The mechanism for the increased phosphorylation activity of the p46 and p54 JNKs differs, because activation of the p46 JNK occurs during ischemia, whereas that of the p54 JNK requires reperfusion. The present study further demonstrates that activation of the ϵ -isoform of PKC in itself, in the absence of ischemia-reperfusion, is sufficient to reproduce the activation of the p46 and p54 JNKs in rabbit cardiac myocytes. In contrast to the sustained activation of PKC- ϵ and of p46 and p54 JNKs, activation of the p38 MAPK cascade during ischemic PC is tran-

sient. These results significantly expand our understanding of the signaling pathways activated by myocardial ischemia and identify potential downstream targets of PKC-dependent phosphorylation in this setting. Because the p46/p54 JNKs and the p38 MAPK are known to regulate gene transcription and to phosphorylate small heat shock proteins in response to various stimuli (3, 47), activation of these kinases may be important not only in ischemic PC but also in many other pathological processes associated with recurrent ischemic stress.

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