PKC-dependent activation of p44/p42 MAPKs during myocardial ischemia-reperfusion in conscious rabbits

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Ping, Peipei, Jun Zhang, Xinan Cao, Richard C. X. Li, Deying Kong, Xian-Liang Tang, Yumin Qiu, Srinivas Manchikalapudi, John A. Auchampach, Richard G. Black, and Roberto Bolli. PKC-dependent activation of p44/p42 MAPKs during myocardial ischemia-reperfusion in conscious rabbits. *Am. J. Physiol.* 276 (*Heart Circ. Physiol.* 45): H1468–H1481, 1999.—Using conscious rabbits, we examined the effect of ischemic preconditioning (PC) on p44 and p42 mitogen-activated protein kinases (MAPKs). We found that both isoforms contribute significantly to total MAPK activity in the heart (in-gel kinase assay: p44, 59 ± 1%; p42, 41 ± 1%). Ischemic PC (6 cycles of 4-min occlusion/4-min reperfusion) elicited a pronounced increase in total cellular MAPK activity (+89%). This increase, which occurred exclusively in the nuclear fraction, was contributed by both isoforms (in-gel kinase assay: p44, +97%; p42, +210%) and was accompanied by migration of the two proteins from the cytosolic to the nuclear compartment. In control rabbits, MAPK kinase (MEK)1 and MEK2, direct activators of p44 and p42 MAPKs, were located almost exclusively in the cytosolic fraction. Ischemic PC induced a marked increase in cytosolic MEK activity (+164%), whereas nuclear MEK activity did not change, indicating that MEK-induced activation of MAPKs occurred in the cytosolic compartment. Activation of MAPKs after ischemic PC was completely blocked by the protein kinase C (PKC) inhibitor chelerythrine. Selective overexpression of PKC-ε in adult rabbit cardiomyocytes induced activation of both p44 and p42 MAPKs and reduced lactate dehydrogenase release during simulated ischemia-reperfusion, which was abolished by the MEK inhibitor PD-98059. The results demonstrate that *1*) ischemic PC induces a rapid activation of p44 and p42 MAPKs in hearts of conscious rabbits; *2*) the mechanism of this phenomenon involves activation of p44 and p42 MAPKs in the cytosol and their subsequent translocation to the nucleus; and *3*) it occurs via a PKC-mediated signaling pathway. The in vitro data implicate PKC-ε as the specific isoform responsible for PKC-induced MAPK activation and suggest that p44/p42 MAPKs contribute to PKC-ε-mediated protection against simulated ischemia. The results are compatible with the hypothesis that p44 and p42 MAPKs may play a role in myocardial adaptations to ischemic stress.

extracellular signal-regulated kinases 1 and 2; mitogen-activated protein kinase kinases 1 and 2; protein kinase C-ε isoform; nuclear translocation

ISCHEMIC PRECONDITIONING (PC), i.e., the phenomenon whereby brief episodes of ischemia render the heart more resistant to subsequent ischemic insults, induces both an early and a late phase ("second window") of protection (3, 8, 9, 14, 15, 17, 19, 26, 31, 32, 36, 42, 43, 45). Mounting evidence indicates that protein kinase C (PKC) plays a key role in the signaling pathways underlying both phases of ischemic PC (2, 14, 17, 30, 35, 41, 49). In the conscious rabbit, brief episodes of ischemia-reperfusion induce selective translocation of the ε-isoform of PKC to the particulate fraction (38); furthermore, both the ischemic PC-induced activation of PKC-ε and the subsequent cardioprotection are completely abolished by the PKC inhibitor chelerythrine (39), suggesting a key role of the ε-isoform in the development of ischemic PC. Nevertheless, the identity of the downstream effector(s) and/or mediator(s) that are activated by PKC-ε during PC remains elusive and represents a major unresolved problem.

A plausible target for PKC-mediated signaling events is the family of mitogen-activated protein kinases (MAPKs), which has been shown to be involved in a number of growth hormone- and stress-activated cellular responses (6, 7, 16, 17a, 20). Recent evidence implicates PKC in the activation of two members of this kinase family, i.e., the p44 and p42 MAPKs, also known as extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2, respectively) (11, 27). Accordingly, in the present investigation we tested the following two hypotheses: *1*) regional ischemia-reperfusion activates the p44 and p42 MAPKs through a PKC-dependent signaling pathway in vivo; and *2*) selective activation of the ε-isoform of PKC [analogous to that elicited by ischemic PC in vivo (38)] leads to the activation of the p44 and p42 MAPKs in isolated cardiac myocytes.

A number of other issues were also addressed in this investigation. Previous studies (5, 25, 33) showed that total MAPK activity (the sum of p44/p42 and p38 activities) increases after reperfusion in isolated rat hearts subjected to global ischemia. These investigations (5, 25, 33), which focused on either the p38 MAPK (5, 33) or the cytosolic fraction of the p44/42 MAPKs (5, 25), have provided important evidence for an involvement of MAPKs in the signaling events associated with myocardial ischemia-reperfusion. However, the subcellular redistribution of p44/p42 MAPKs and the cellular mechanism underlying ischemia-reperfusion-induced activation of these kinases have not been previously investigated. Because it is well established that in noncardiac cells the p44/p42 MAPKs exert their biologi-

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cal function in specific subcellular compartments (11, 18, 27), we investigated whether ischemia-reperfusion causes increased activity and protein expression of p44/p42 MAPKs in the cytosolic, membrane, or nuclear compartment. Because it is unknown whether the effects of an ischemic stimulus are restricted to the p44/p42 kinases or involve more broadly the MAPK cascade, we also determined whether ischemia-reperfusion causes activation of MAPK kinases 1 and 2 (MEK1 and MEK2), the direct activators of p44/p42 MAPKs (16, 17a), and whether the activation of p44/p42 MAPKs correlates with the activation of MEK1 and MEK2 in a subcellular compartment-specific manner.

The above hypotheses were examined in two consecutive phases using two different but complementary experimental settings, an in vivo setting (a well-characterized conscious rabbit model of ischemic PC) and an in vitro setting (isolated adult rabbit cardiac myocytes). In the in vivo setting, an ischemic PC protocol that was previously shown to induce late PC (9, 10, 31, 39, 40, 44) was examined to correlate the changes in MAPKs with the development of cardioprotection. To determine whether ischemic PC-induced activation of MAPKs is mediated by PKC, the specific PKC inhibitor chelerythrine was used. Because MAPKs are activated by cellular stress (16, 37) and because the response of these enzymes to the stress of anesthesia, surgical trauma, elevated catecholamines, fluctuations in temperature, release of cytokines, exaggerated generation of reactive oxygen species (29, 46), and other factors associated with open-chest preparations is unknown, these studies were conducted in conscious animals. To identify the subcellular compartment in which the enhanced activity of p44/p42 MAPKs occurs, the subcellular distribution of these kinases after the ischemia-reperfusion protocol was examined. To further elucidate the mechanism of activation of the p44 and p42 MAPKs, the analysis was expanded to include the subcellular protein distribution and kinase activity of MEK1 and MEK2. In the in vitro setting, recombinant adenoviruses expressing wild-type and dominant negative mutant cDNAs of PKC-ε were used to selectively manipulate the activity of this isoform of PKC and to specifically interrogate the role of the PKC-ε isozyme in p44/p42 MAPK activation in cardiac myocytes. The in vitro studies made it possible to examine the signaling pathways leading to MAPK activation in a specific cell type, i.e., in adult cardiac myocytes.

MATERIALS AND 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 the *Guide for the Care and Use of Laboratory Animals* (DHHS Publication No. [NIH] 86–23).

Studies in Conscious Rabbits (Phase I)

Experimental preparation. The conscious rabbit model of ischemic PC has been described in detail previously (1, 9, 10, 31, 38, 40, 44). Briefly, male New Zealand White 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. 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 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. Rabbits were assigned to four groups (see Fig. 1). *Group I* (control) did not undergo coronary occlusion. At 10–14 days after surgery (time corresponding to interval between instrumentation 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 (38)]. 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 p44 and p42 MAPKs 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 late PC in this conscious rabbit model (39). 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 administration of chelerythrine (time interval corresponding to interval between treatment and euthanasia in *group IV*). In all groups, the samples were frozen within 60–90 s of the bolus of KCl.

Tissue sample preparation. Tissue samples were processed for the determination of protein expression and phosphorylation activity of p44 MAPK, p42 MAPK, MEK1, and MEK2. 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 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 1 μM Microcystin LR (an inhibitor of protein phosphatase), and 0.3% β-mercaptoethanol.

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 to 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 (HDAC) 1 (23).

Protein concentration was determined using the method of Bradford (Biorad). 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 MEK 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 Western immunoblotting and activity assay was performed in at least five of the eight control rabbits (*group I*) and in all five rabbits in the other groups.

Western immunoblotting analysis of subcellular distribution of p44 MAPK, p42 MAPK, MEK1, and MEK2. Fifty micrograms of proteins derived from either the nuclear or the cytosolic fraction of homogenates were electrophoresed on a 10% SDS-PAGE gel and blotted onto a nitrocellulose membrane (Amersham). As previously detailed (38), gel transfer efficiency was carefully recorded by making photocopies of membranes dyed with reversible Ponceau staining, and gel retention was determined by Coomassie Blue staining. Adequate background blocking was accomplished by incubating the nitrocellulose membranes with 5% nonfat dry milk in Tris-buffered saline. The nitrocellulose membrane was incubated with the proper antibodies and stained with a chemiluminescence system (ECL kit, Amersham). Monoclonal antibodies against p44 MAPK, p42 MAPK, MEK1, and MEK2 were obtained from Upstate Biotechnology, Transduction Laboratories, Biolab, and Santa Cruz Biotechnology. The MAPK and MEK signals and the corresponding records of Ponceau stains were quantitated with an image-scanning densitometer (Personal PI, Molecular Dynamics). The MAPK or MEK protein in either the cytosolic or the nuclear fraction was expressed as a percentage of total myocardial MAPK or MEK protein.

To assure consistency in the data analysis, the cytosolic or the nuclear fractions of all five tissue samples in each group were run on the same gel (see Fig. 2, *A* and *B*). Each immunoblotting experiment was repeated twice, and the results were averaged. Particular care was taken to normalize MAPK expression to the sample protein content as accurately as possible, so as to enable valid comparisons among samples. In addition to loading equal amounts of proteins in all lanes of the gel, we achieved internal control of each MAPK signal by normalizing this signal to the corresponding Ponceau stain signal (used as a measure of "housekeeping" proteins) determined by densitometric analysis of the Ponceau stain record, as detailed previously (38).

MAPK activity assays. The phosphorylation activity of the p44 and p42 MAPKs was determined using both an enzyme assay in solution and 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. In each assay, pilot experiments were carried out at 25, 30, and 37°C; the reaction temperature chosen was that at which the optimal sensitivity for the enzyme was achieved. Autophosphorylation 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.

PHOSPHORYLATION ASSAY IN SOLUTION. The p44/42 MAPK activity in the cytosolic and nuclear fractions was quantitated using a modified assay system from Upstate Biotechnology. Briefly, 15 μg of myocardial tissue protein were incubated with 10 μCi of [γ-^{32}P]ATP, 0.1125 mM ATP, 16.875 mM $MgCl_2$, 20 mM MOPS (pH 7.2), 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol (DTT), and 20 μg of myelin basic peptide (MBP) at 30°C for 15 min. Particular care was taken to avoid nonspecific substrate phosphorylation by other kinases such as PKC, protein kinase A (PKA), calmodulin (CaM) kinase, and p38 MAPK, which would confound the results of the assay. Accordingly, to measure p44/42 MAPK-selective phosphorylation of the MBP, 5 μM PKC inhibitor peptide, 0.5 μM PKI (a PKA inhibitor), and 5 μM CaM kinase inhibitor (compound R-24571) were included in the final reaction. Because MBP contains a phosphorylation domain for p38 MAPK (22), 60 μM SB-203580 (a potent p38 MAPK inhibitor) was also added to the reaction. Each sample was assayed in triplicate. MAPK activity was expressed as picomoles of phosphate incorporated into MBP per minute per milligram of sample proteins.

IN-GEL KINASE ASSAY. The isoform-specific activity of the p44 and p42 MAPKs was determined by an in-gel kinase assay using the method described by Sugden and colleagues (5, 6). Denatured cytosolic and nuclear proteins were fractionated on a 10% polyacrylamide gel containing 0.5 mg/ml of MBP. 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 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 for 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 DTT, and 10 mM $MgCl_2$ (pH 8.0) for 1 h at RT. In-gel phosphorylation of the substrate was then carried out in 40 mM HEPES, 10 mM $MgCl_2$, 0.5 mM EGTA, 2 μM PKI, 60 μM SB-203580, and 40 μM [γ-^{32}P]ATP (5 μCi/ml or 40 μCi per gel; pH 8.0) at 30°C for 1 h. The phosphorylated gel was washed in 5% (vol/vol) trichloroacetic acid and 1% (vol/vol) sodium pyrophosphate to remove the unincorporated free [γ-^{32}P]ATP and then dried and autoradiographed. Each sample was assayed in duplicate.

To verify the phosphorylation signal of the p42 and p44 MAPKs, in *phase I* we purified the myocardial p44 and p42 MAPKs in *groups I–IV* via immunoprecipitation using monoclonal antibodies against the p44 and p42 MAPK proteins (Santa Cruz Biotechnology). The immunoprecipitates were then subjected to phosphorylation assays as described in PHOSPHORYLATION ASSAY IN SOLUTION. When expressed as a percentage of control (*group I*), the measurements of both p44 and p42 MAPK phosphorylation activity obtained after immunoprecipitation in *groups II–IV* confirmed the measurements obtained without immunoprecipitation (data not shown).

MEK activity assay. Total MEK activity in the cytosolic and nuclear fractions was quantitated using a modified assay system from Upstate Biotechnology. The assay consisted of two steps. Briefly, in *step 1*, 15 μg of myocardial tissue protein were incubated with (in mM) 0.1125 ATP, 16.875 $MgCl_2$, 20 MOPS (pH 7.2), 25 β-glycerol phosphate, 5 EGTA, 1 sodium orthovanadate, and 1 DTT and 1.4 μg of glutathione *S*-transferase (GST)-p42 MAPK at 30°C for 30 min. To measure MEK-selective phosphorylation of p42 MAPK, 5 μM PKC inhibitor peptide, 0.5 μM PKI, 5 μM CaM kinase inhibitor (compound R-24571), and 60 μM SB-203580 of p38 MAPK inhibitor were included in the final reaction. In *step 2*, 5 μl of the final reaction mixture from *step 1* were incubated with 10 μCi of [γ-^{32}P]ATP, 0.1125 mM ATP, 16.875 mM $MgCl_2$, 20 mM MOPS (pH 7.2), 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM DTT, and 1.4 μg of MBP at 30°C for 10 min. Endogenous phosphorylation of the p42 MAPK was determined by omitting GST-p42 MAPK from *step 1* of the reaction. The specific activity was obtained by subtracting the endogenous activity from the total activity. Each sample was assayed in triplicate. MEK activity was expressed as counts per min per microgram of sample proteins.

Studies in Isolated Cardiac Myocytes (Phase II)

Isolation of adult rabbit cardiac myocytes. Rabbit cardiac myocytes were isolated using collagenase (type II, Worthington Biochemical Corporation; Ref. 21). Cardiac myocytes were plated at subconfluence (0.5×10^6 cells/well of a 6-well plate) and cultured in 2% fetal bovine serum-M199 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 (Clonetech) 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 wild-type full-length PKC-ε cDNA (PKC-FL) and a dominant negative mutant PKC-ε cDNA (PKC-DN) were constructed through site-directed mutagenesis. PKC-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). 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 (34). Positive recombinant adenoviruses were isolated by plaque purification and propagated in H293 cells that had been transformed with E1 genes (34). 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. Ten plaque-forming units per cell of recombinant adenovirus were transfected in cardiac cells. Four experimental groups were studied. The control group (*group I*) received recombinant adenovirus expressing no cDNA insert. *Group II* received recombinant adenovirus expressing PKC-FL. *Group III* received recombinant adenovirus expressing PKC-DN. *Group IV* received recombinant adenovirus expressing PKC-FL in conjunction with a PKC inhibitor, Ro-31–8220 (100 nM Ro-31–8220 was added to cells during last 60 min of 24-h incubation with adenovirus). This concentration of Ro-31–8220 was chosen on the basis of its IC_{50} for PKC-ε (48). A higher concentration of Ro-31–8220 was avoided to elude its nonspecific actions on other kinases (4). Each group included four to nine experiments, each from a different rabbit heart. All cells were harvested 24 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 p44/p42 MAPK activity. PKC-ε transgene protein expression was determined by Western immunoblotting using HA antibodies, and the signal was confirmed by PKC-ε antibodies. PKC-ε isoform activity was selectively measured by immunoprecipitating the cardiac cell lysates with PKC-ε isoform antibodies (Upstate) followed by a phosphorylation assay using a PKC-ε-selective substrate (ERMRPRKRQGSVRRRV). The optimal substrate concentration, 1 nM, was determined by the dose-response (substrate vs. phosphorylation activity) curve in our pilot experiments. The phosphorylation activity of the p44 and p42 MAPKs was determined by immunoprecipitation of total cell lysates followed by phosphorylation assay of these kinases. In separate experiments, recombinant adenovirus expressing green fluorescence peptide was used to determine the transfection efficiency (see Fig. 8*A*).

Studies of simulated ischemia in cardiac myocytes. To simulate the conditions encountered during ischemia, myocytes were incubated for 6 h at 37°C in 1.5 ml of a glucose-free modified Krebs buffer (pH 6.5) containing (in mM) 120 NaCl, 12 KCl, 1 $MgSO_4$, 1 $CaCl_2$, 20 sodium lactate, and 25 HEPES under hypoxic conditions in an anaerobic chamber (Plas Labs, Lansing, MI) containing 85% nitrogen, 10% hydrogen, and 5% CO_2. The oxygen content in the chamber [as measured by an oxygen meter (YSI, Columbus, OH)] was <0.1 mmHg during the entire incubation period. After 6 h of simulated ischemia, the cells were removed from the anaerobic chamber and the supernatant was collected. The cells were reoxygenated by adding 1.5 ml of culture medium to each plate and by placing the plates in a cell culture incubator (95% air-5% CO_2) for 1 h. The culture medium was then removed and pooled with the supernatant collected after simulated ischemia. The myocytes left on the plates were scraped into hypotonic lysis buffer (10 mM Na^+-HEPES, 2 mM EDTA, pH 7.4). LDH activity was measured in the supernatants and lysates using a standard assay kit (Sigma). The extent of cellular injury was expressed as the percentage of total LDH that was released into the supernatants during simulated ischemia and reoxygenation. The basal release of LDH (background, defined as the amount of LDH present in the supernatant of cells not subjected to simulated ischemia) was subtracted from all measurements in the various treatment groups. Twenty-four hours before simulated ischemia, myocytes were

transfected with a recombinant adenovirus, expressing either a null vector (sham control) or PKC-FL, or cotransfected with two types of adenovirus expressing both PKC-FL and PKC-DN of PKC-ϵ (at a FL-to-DN ratio of 1:3). The inhibitor of the p44/p42 MAPK pathway, PD-98059 (1 μM), was added to the culture medium at the time of the adenovirus transfection.

Statistical Analysis

Data are reported as means ± SE. To facilitate comparisons, measurements of MAPK activity by in-gel assays and of protein expression in each individual rabbit were expressed as a percentage of the average value for the control group. Differences among the four experimental groups in the in vivo studies and among the various 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 (47).

RESULTS

Exclusions

A total of 23 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). All rabbits in *groups I–IV* successfully completed the protocol. A total of 42 rabbits were used for the in vitro experiments in *phase II*. In five rabbits, we were unable to obtain viable cardiac cells. In the remaining 37 rabbits, each isolation procedure yielded 20–30 × 10^6 cardiac myocytes per heart.

Phase I: PKC-Dependent Activation of p44 and p42 MAPKs During Ischemic PC in Conscious Rabbits

In *phase I* of the study, we first determined whether ischemic PC has an effect on the phosphorylation activity of the p44 and p42 MAPKs in vivo and whether ischemic PC-induced activation of these MAPKs is mediated by PKC. We next investigated possible mechanisms underlying activation of the p44/p42 MAPKs by examining the subcellular distribution of these kinases. Finally, we characterized the effects of ischemia-reperfusion on the activity of MEK1 and MEK2, which are proximal elements of this cascade and direct activators of p44/42 MAPKs.

Expression of p44 and p42 MAPKs in rabbit heart. Our results show that, unlike other mammalian cells (6, 11, 27), the adult rabbit heart expresses both the p44 and the p42 MAPK. After immunoprecipitation with the general MAPK antibody (which recognizes both the p44 and the p42 isoforms), rabbit heart tissue lysates exhibited two sharp signals at 44 and 42 kDa as detected by Western immunoblotting (Fig. 2*A*). These signals were further confirmed by the isoform-specific p44 MAPK and p42 MAPK antibodies. Analysis of subcellular distribution revealed that 94 ± 2% of the p44 MAPK resides in the cytosolic fraction and 6 ± 2% in the nuclear fraction and that 94 ± 2% of the p42 MAPK is located in the cytosolic fraction and 6 ± 2% in the nuclear fraction. No expression of p44 MAPK or p42 MAPK protein was detectable in the membrane fraction using currently available antibodies. Using in-gel kinase assays, we identified phosphorylated (i.e., activated) p44 MAPK and p42 MAPK signals both in the cytosolic fraction, where p44 contributed 59 ± 1% and p42 contributed 41 ± 1% of the MAPK activity, and in the nuclear fraction, where p44 contributed 65 ± 2% and p42 contributed 35 ± 2% of the MAPK activity (Fig. 2*B*). The results of the in-gel kinase assays indicate that MAPKs are active in the heart of control conscious rabbits, which implies that beside responding to extracellular stimulation, the p44 and p42 MAPKs may be important in maintaining cardiac function under basal conditions.

Effect of ischemic PC on MAPK activity. The ischemic PC protocol examined (6 cycles of 4-min coronary occlusion/4-min reperfusion) was shown previously to induce late PC against myocardial stunning (9, 10, 31, 39) and infarction (40, 44). Compared with control rabbits (*group I*), the total MAPK activity determined by the phosphorylation assay (Fig. 3*A*) increased by 89% ($P < 0.05$) after six cycles of 4-min occlusion/4-min

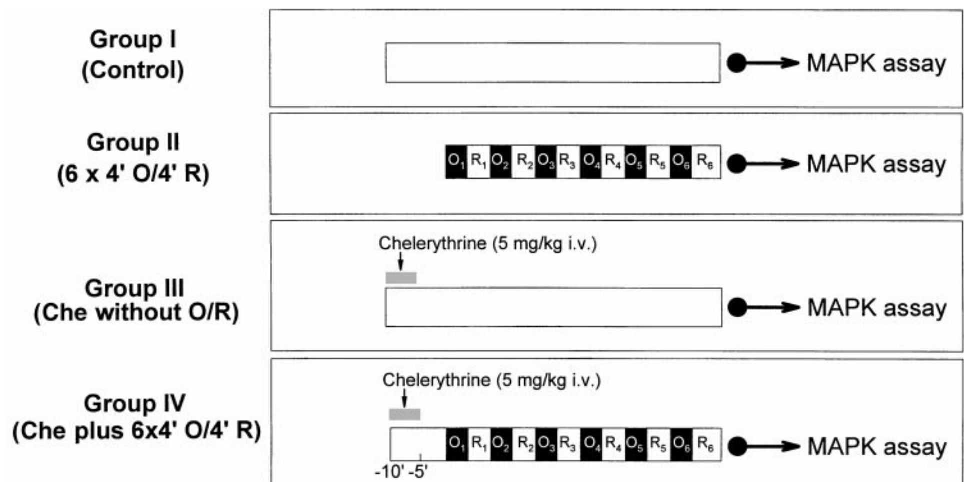


Fig. 1. Diagram of experimental protocols. Mitogen-activated protein kinase (MAPK) assays were performed immediately after collection of tissue samples. O, coronary occlusion; R, coronary reperfusion; Che, chelerythrine. 6 × 4′ O/4′ R, 6 cycles of 4-min occlusion/4-min reperfusion.

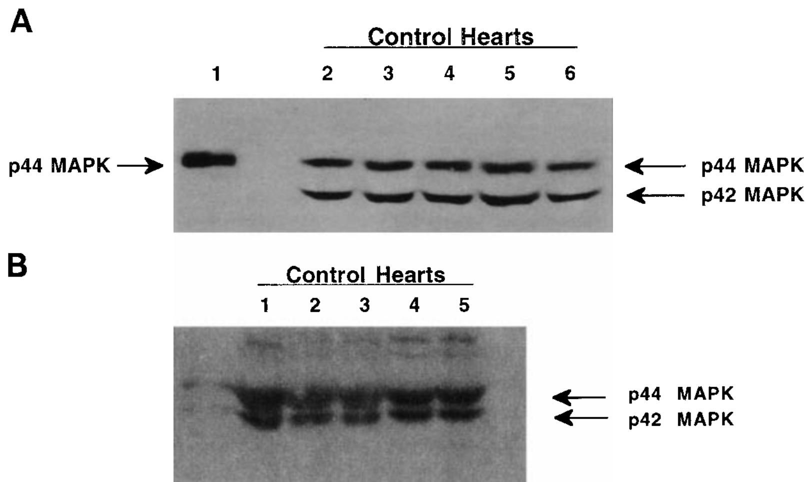


Fig. 2. *A*: Western blot performed to identify p44 and p42 MAPK isoform expression in 5 control rabbits that did not undergo coronary occlusion (*group I*). *Lane 1*, positive control of p44 MAPK obtained from human recombinant p44 MAPK protein (Upstate Biotechnology). *Lanes 2–6*, cytosolic fractions of p44 and p42 MAPKs. *B*: in-gel kinase assay for determination of isoform-specific p44 and p42 phosphorylation activity. *Lanes 1–5* are nuclear fractions of MAPKs.

reperfusion (*group II*). In-gel kinase assays demonstrated that the increase in total MAPK activity in *group II* was accounted for by a rise in the p44/p42 MAPK activity in the nuclear fraction, with no appreciable changes in the cytosolic fraction (Fig. 3, *B* and *C*); the nuclear fraction of the p44 MAPK activity increased by 97% ($P < 0.05$) (Fig. 4*A*) and the nuclear fraction of the p42 MAPK activity by 210% ($P < 0.05$) (Fig. 4*B*). The cytosolic fraction of the p44 and p42 MAPK activity was not significantly altered by this PC protocol (data not shown). Thus ischemic PC induced activation of p44/p42 MAPKs, and this activation was restricted to the nuclear fraction.

Effect of chelerythrine on ischemic PC-induced MAPK activation. To determine whether activation of p44 and p42 MAPKs during ischemic PC is dependent on PKC activation, we measured p44 and p42 MAPK activity in rabbits undergoing six cycles of 4-min coronary occlusion/4-min reperfusion after pretreatment with 5 mg/kg of chelerythrine (*group IV*). Previous studies demonstrated that this dose of chelerythrine completely blocks the translocation of PKC-ϵ and the development of late PC in this conscious rabbit model (39) and that chelerythrine alone does not have any significant effect on the subcellular distribution of PKC-ϵ (39). In the present study, we found that chelerythrine completely blocked the activation of p44 and p42 MAPKs in the nuclear fraction after six cycles of 4-min occlusion/4-min reperfusion (*group IV*) (Fig. 3, *A–C*). In the absence of ischemic PC (*group III*), chelerythrine significantly attenuated total MAPK activity (Fig. 3*A*) and cytosolic MAPK activity (Fig. 3*B*) but had no effect on either the p44 or the p42 MAPK activity in the nuclear fraction (Figs. 3*C*, 4*A*, and 4*B*). These results indicate that the activation of p44 and p42 MAPKs during ischemic PC occurs via a PKC-dependent pathway.

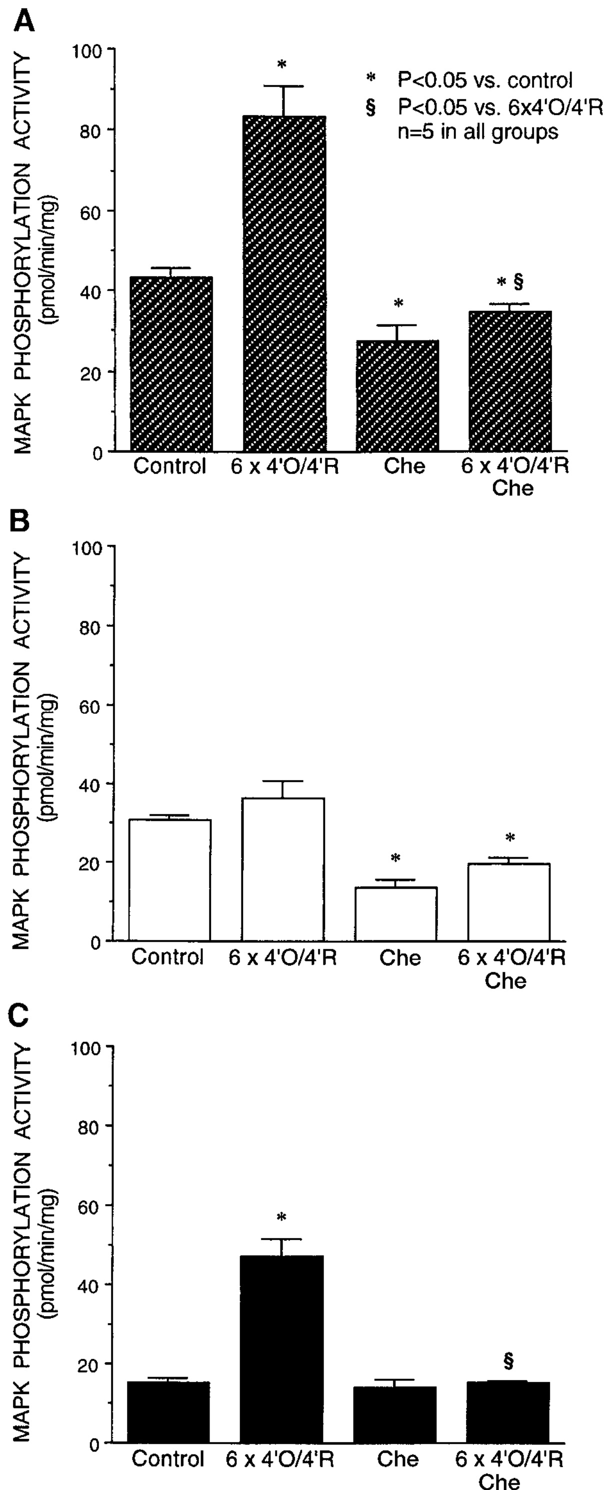
Effect of repetitive ischemia-reperfusion on subcellular distribution of MAPK. The finding that repetitive ischemia enhances the activity of MAPKs in the nuclear (rather than the cytosolic) fraction represents a novel observation. Additional studies were performed to elucidate the mechanism of this phenomenon. In theory, it could be caused by activation of p44/p42 proteins already present in the nucleus or by a shift of new proteins from the cytosolic to the nuclear compartment. To test the hypothesis that the increased activity was the result of a nuclear translocation of p44 and/or p42 MAPK, we correlated the changes in the subcellular distribution of MAPK phosphorylation activity (Fig. 4, *A* and *B*) with the corresponding changes in the subcellular distribution of MAPK protein expression after six cycles of 4-min ischemia/4-min reperfusion (Fig. 5, *A* and *B*). We reasoned that if activation of MAPKs in the nucleus was caused by translocation, the increases in activity should parallel the increases in protein content.

As detailed above, both the phosphorylation assay and the MBP in-gel kinase assay demonstrated that ischemia-reperfusion increased p44 and p42 MAPK phosphorylation activity in the nuclear fraction after six cycles of 4-min occlusion/4-min reperfusion (Figs. 3*C*, 4*A*, and 4*B*). Western immunoblotting demonstrated that, compared with *group I* (controls), protein expression of p44 and p42 MAPKs in the nuclear fraction rose by 202 and 211%, respectively, after six 4-min occlusion/4-min reperfusion cycles (*group II*) (Figs. 5*A* and 6; $P < 0.05$). When calculated as percentages of control, the changes in nuclear p44 and p42 protein expression (Fig. 5*A*) paralleled the corresponding changes in nuclear phosphorylation activity (Fig. 4, *A* and *B*). This correlation was very close for both isoforms (Figs. 4 and 5), supporting the concept that the increased nuclear activity of p44 and p42 MAPKs was the result of the migration of activated proteins from the cytosol.

As expected from the results obtained with the nuclear fraction, MAPK protein expression in the cytosolic fraction decreased significantly after six cycles of 4-min occlusion/4-min reperfusion. Compared with the control group (*group I*), the cytosolic p44 and p42 MAPK proteins decreased by 12 and 14%, respectively, in *group II* (Fig. 5*B*; $P < 0.05$). The decreases in protein content in the cytosolic fraction mirrored the increases in the nuclear fraction (Fig. 5*A*). The finding that MAPK activity in the cytosolic fraction did not decrease (Fig. 3*B*) despite a decrease in cytosolic MAPK protein

expression (Fig. 5*B*) is not surprising. Although it is generally accepted that essentially all of the MAPKs present in the nucleus are in the active form (12, 16, 20, 50, 51), not all of the cytosolic MAPKs are active. Consequently, changes in cytosolic MAPK protein content are not necessarily paralleled by changes in cytosolic MAPK activity.

Effect of ischemia-reperfusion on MEK activity. To determine whether the effects of ischemia-reperfusion



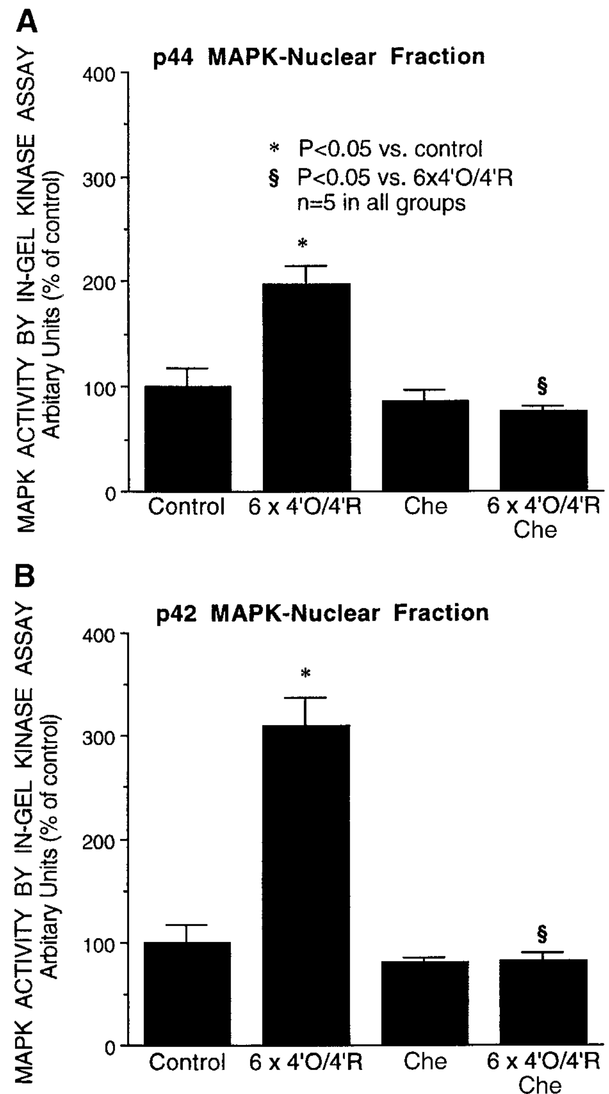


Fig. 4. Nuclear activity of p44 and p42 MAPK isoforms, as determined by in-gel kinase assay in 4 experimental groups. Compared with control group (*group I*), both nuclear p44 (*A*) and p42 (*B*) MAPK activity were significantly increased after 6 × 4′O/4′R (*group II*). In absence of ischemia (*group III*), Che had no effect on nuclear p44 or p42 MAPK activity. Che blocked ischemic PC-induced increase in p44 and p42 MAPK activity in nuclear fraction. Data are means ± SE.

Fig. 3. MAPK activity in 4 experimental groups. *A*: total MAPK activity. Compared with control group (*group I*), total MAPK activity was significantly increased after 6 cycles of 4-min occlusion/4-min reperfusion (6 × 4′O/4′R; *group II*). In absence of ischemia (*group III*), Che decreased total MAPK activity. In rabbits subjected to ischemic preconditioning (PC) after receiving Che (6 × 4′O/4′R + Che; *group IV*), Che completely abolished increase in total MAPK activity induced by the 6 O/R cycles. *B*: cytosolic MAPK activity. Cytosolic MAPK activity was not altered by ischemic PC. Che decreased cytosolic MAPK activity in absence and presence of ischemic PC. *C*: nuclear MAPK activity. Compared with control group (*group I*), nuclear MAPK activity was significantly increased after 6 × 4′O/4′R (*group II*), and increases in nuclear MAPK activity exhibited a pattern similar to that of increases in total MAPK activity (*A*). Che completely abolished ischemic PC-induced increase in MAPK activity in the nuclear fraction. Data are means ± SE.

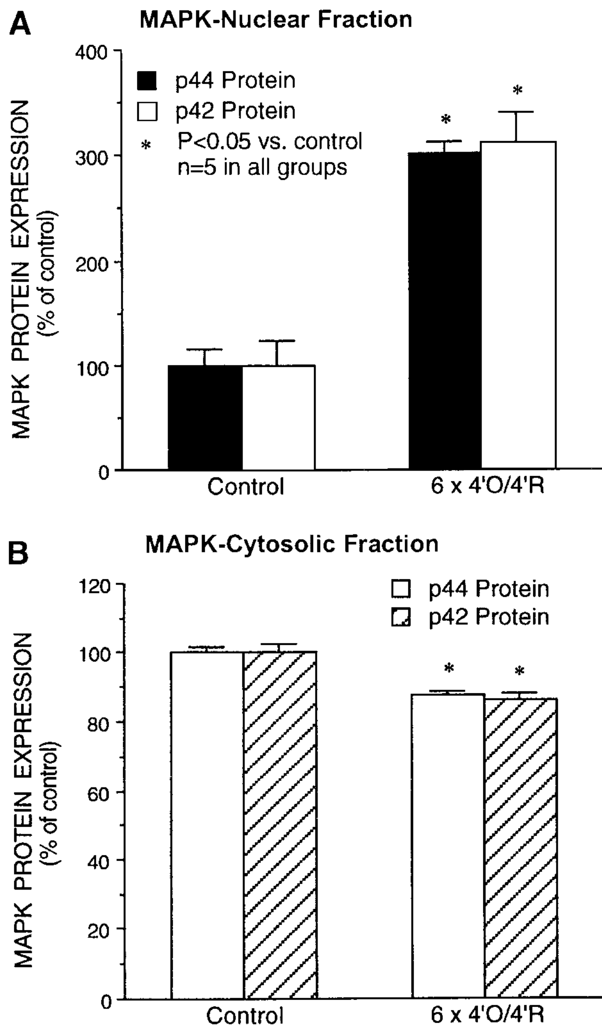


Fig. 5. Nuclear translocation of p44 and p42 MAPKs after ischemic PC. *A*: significant increase in nuclear protein expression of p44 MAPK and p42 MAPK after 6 × 4′O/4′R (*group II*). *B*: reduced expression of cytosolic p44 and p42 MAPK proteins. Note that changes in nuclear p44 and p42 protein expression mirror changes in cytosolic p44 and p42 protein expression. Data are means ± SE.

involve the p44/p42 MAPK signaling cascade or are limited to the p44/p42 MAPK elements of that cascade and whether the activation of MEK1/2 correlates with that of p44/p42 MAPKs, we examined the subcellular distribution and activity of the direct intracellular activators of MAPKs, MEK1 and MEK2. Western immunoblotting analysis showed that MEK1 is expressed only in the cytosolic fraction; no MEK1 expression was detected in either the nuclear or the membrane fraction in any of the groups examined (Fig. 7*A*). In *group I* (controls), ~98% of total MEK2 was found in the cytosol and <2% in the nuclei (Fig. 7*B*). The subcellular distribution of MEK1 and MEK2 expression was not affected by the six cycles of 4-min occlusion/4-min reperfusion (*group II*) (data not shown). Consistent with the protein expression in control rabbits (*group I*), 95% of total MEK phosphorylation activity was found in the cytosol and <5% in the nuclei (Fig. 7*C*). The ischemia-reperfusion protocol examined (6 cycles of 4-min occlusion/4-min reperfusion) markedly increased the MEK activity in the cytosolic fraction (Fig. 7*C*). In contrast, the MEK2 activity in the nuclear fraction remained unaltered (Fig. 7*C*). These data indicate that ischemia-reperfusion affects the p44/p42 MAPK signaling cascade rather than the p44/p42 MAPKs alone. Furthermore, these data support the concept that ischemia-reperfusion induces activation of the p44 and p42 MAPKs in the cytosol and that the activated MAPKs are subsequently translocated to the nucleus.

Phase II: PKC-Dependent Activation of p44/p42 MAPKs in Isolated Cardiac Myocytes

Effect of PKC-ϵ activation on p44/p42 MAPK activity. Having established that ischemia-reperfusion causes activation of the p44/p42 MAPK cascade via a PKC-dependent pathway in vivo, we next examined whether increased PKC-ϵ activity can 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 rabbit adult cardiac myocytes (Fig. 8*A*). Expressing wild-type PKC-ϵ significantly increased isoform-selective PKC-ϵ activity (Fig. 8*B*)

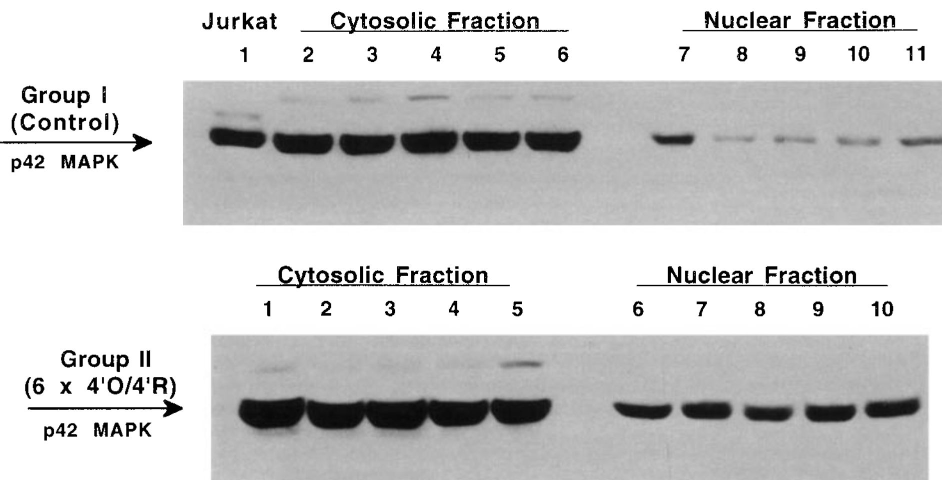


Fig. 6. Western blot performed to identify subcellular distribution of p42 MAPK protein in 5 rabbits in *group I* (control) and in 5 rabbits in *group II* (6 × 4′O/4′R). Note that in *group I*, very little p42 MAPK protein is present in nuclear fraction, whereas in *group II*, expression of p42 MAPK in nuclear fraction is increased compared with that in *group I* ($P < 0.05$).

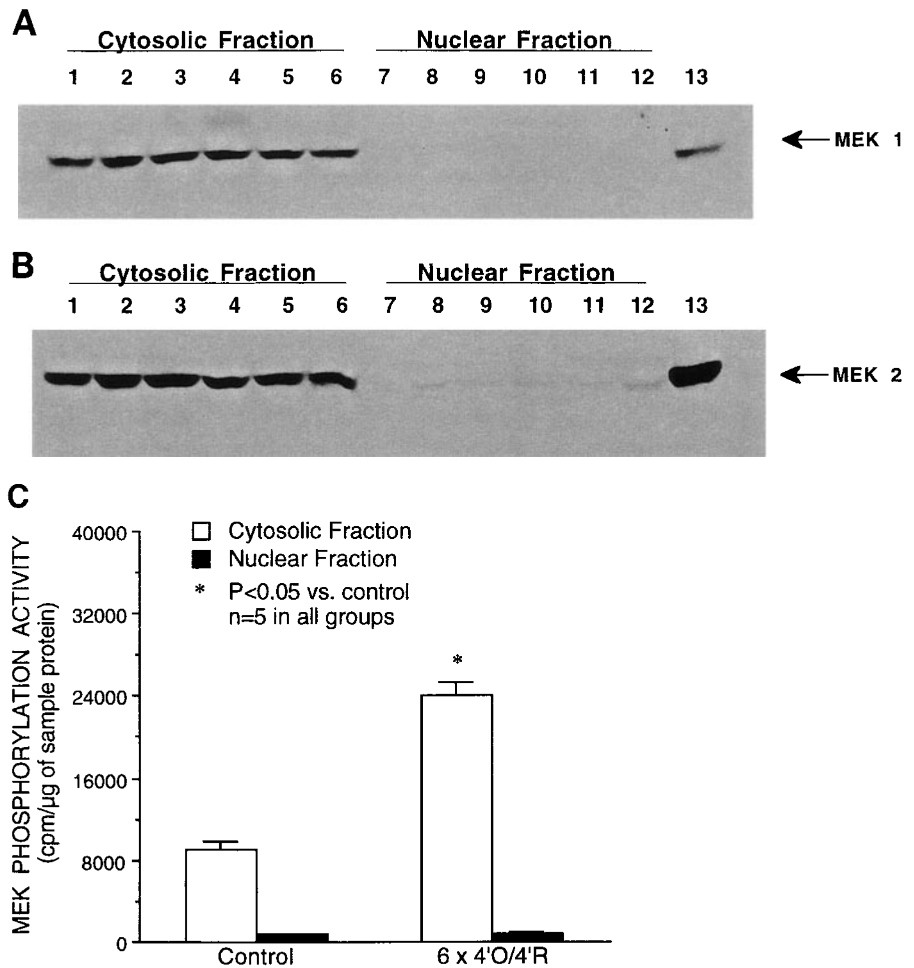


Fig. 7. Subcellular distribution of MEK1/2 protein and total MAPK (MEK) activity. *A*: *lanes 1–6* are cytosolic fractions of MEK1, whereas *lanes 7–12* are nuclear fractions of MEK1. *Lane 13* is recombinant MEK1 protein. MEK1 protein expression was detected only in cytosolic fraction. *B*: *Lanes 1–6* are cytosolic fractions of MEK2, whereas *lanes 7–12* are nuclear fractions of MEK2. *Lane 13* is recombinant MEK2 protein. More than 98% of total myocardial MEK2 was found in cytosolic fraction and <2% in nuclear fraction. *C*: total MEK activity in control and ischemic PC groups. Cytosolic MEK activity increased significantly after 6 × 4′O/4′R (*group II*). Nuclear MEK activity represented <5% of total MEK activity under control conditions and was not affected by 6 occlusion-reperfusion cycles. Note that changes in cytosolic MEK activity paralleled those in nuclear p44/p42 MAPK activity (Figs. 3*C* and 4, *A* and *B*). Data are means ± SE. cpm, Counts/min.

and induced a marked elevation of p44 MAPK activity (Figs. 8*C*). The effect of PKC-ε expression on p42 MAPK activity was less pronounced but still statistically significant (Fig. 8*C*). The PKC inhibitor Ro-31–8220 abolished both the elevated PKC-ε activity and the increased phosphorylation activity of the p44 and p42 MAPKs (Fig. 8, *B* and *C*). Expressing the dominant negative mutant of PKC-ε attenuated the basal PKC-ε activity in cardiac cells (Fig. 8*B*) but had no significant effect on the basal activity of either the p44 or the p42 MAPK (Fig. 8*C*). These data demonstrate, for the first time, that selective activation of the PKC-ε isoform induces activation of p44 and p42 MAPKs in adult cardiac myocytes, implying that PKC-ε is coupled to the p44 and p42 MAPK signaling cascade.

Effect of p44/p42 MAPK inhibition on PKC-ε-induced protection during simulated ischemia. We next used an in vitro model of simulated ischemia to determine whether activation of PKC-ε protects adult cardiac myocytes and whether activation of p44/p42 MAPKs plays a role in this phenomenon (Fig. 9). Cells were cultured in 60-mm plates. In each rabbit, 8–20 individual plates were used for each treatment. LDH release was determined in each plate, and the average LDH release for all plates in a given treatment was used as the final result of that experiment. A total of 16 rabbits were used, 4 in each treatment group. As shown in Fig. 9, 6 h of simulated ischemia caused release of 55.3 ± 5.7% of total LDH in cells that did not receive adenovirus and 54.9 ± 4.9% of total LDH in cells that received adenoviruses expressing the null vector. In cells transfected with PKC-FL, however, LDH release was significantly reduced ($P < 0.05$) to 38.8 ± 3.4% (Fig. 9), indicating that selective activation of PKC-ε protected myocytes against simulated ischemia. This protective effect was specifically caused by increased PKC-ε activity, because cotransfecting cells with PKC-DN blocked PKC-ε activation (data not shown) and abrogated the protection against simulated ischemia (Fig. 9). This protective effect was also abolished by treating cells with the p44/p42 MAPK inhibitor PD-98059 (Fig. 9), indicating that activation of PKC-ε protects cardiac myocytes from simulated ischemia via a p44/p42 MAPK dependent pathway. PD-98059 in itself had no effect (Fig. 9).

DISCUSSION

There are several major findings in this study. First, ischemic PC is associated with activation of p44 and p42 MAPKs in the heart of conscious rabbits. Second, the ischemic PC-induced activation of p44 and p42 MAPKs is completely abolished by the PKC inhibitor chelerythrine, indicating that the activation of p44 and

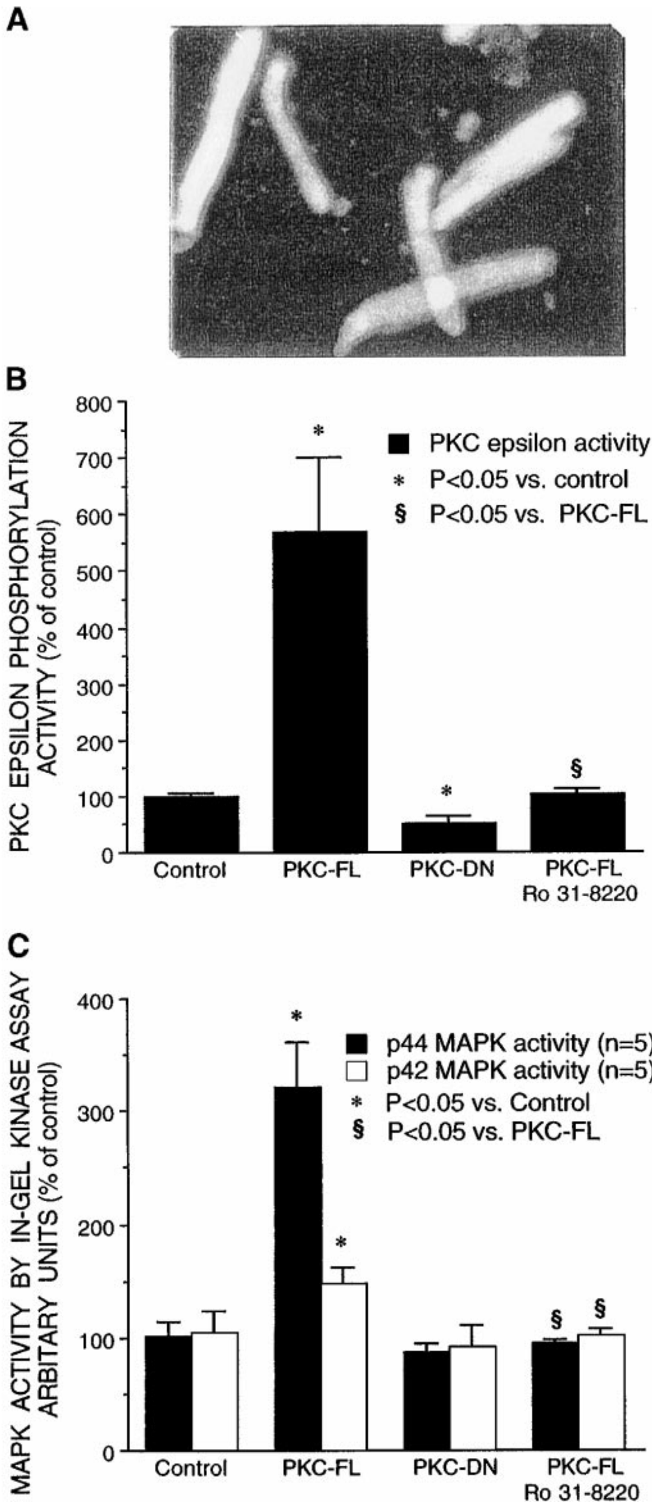


Fig. 8. *A*: adult rabbit cardiac myocytes transfected with recombinant adenoviruses expressing fluorescence peptide. *B*: PKC-ϵ-selective phosphorylation activity in cardiac cells overexpressing wild-type full-length cDNA of PKC-ϵ (PKC-FL), in cardiac cells expressing dominant negative mutant cDNA of PKC-ϵ (PKC-DN) and in cardiac cells expressing PKC-FL treated with PKC inhibitor Ro-31–8220. *C*: total cellular activity of p44 and p42 MAPKs as determined by in-gel kinase assay in cardiac cells overexpressing PKC-FL, in cardiac cells expressing PKC-DN, and in cardiac cells expressing PKC-FL treated with PKC inhibitor Ro-31–8220. Data are means ± SE.

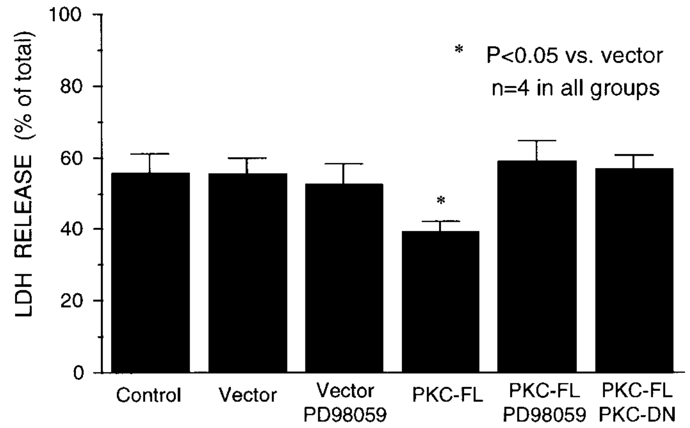


Fig. 9. Lactate dehydrogenase (LDH) release in isolated adult rabbit cardiac myocytes subjected to 6 h of simulated ischemia followed by 1 h of reoxygenation. LDH release in supernatant at end of 6 h of simulated ischemia and 1 h of reoxygenation is expressed as percentage of total LDH (LDH in supernatant after ischemia and reoxygenation + LDH left in myocytes at end of protocol). Shown is LDH release in nontransfected myocytes, myocytes transfected with a null vector, myocytes expressing PKC-FL, myocytes cotransfected with both PKC-FL and PKC-DN, myocytes transfected with null vector treated with p44/p42 MAPK pathway inhibitor PD-98059 (vector + PD-98059), and myocytes expressing PKC-FL treated with PD-98059. Data are means ± SE.

p42 MAPKs is downstream of, and dependent on, PKC activation and suggesting that p44 and p42 MAPKs may play a role in PKC-mediated ischemic PC. Third, selective elevation of the activity of a specific PKC isoform, the ϵ-isoform, in adult rabbit cardiac myocytes results in increased p44/p42 MAPK activity, providing direct evidence that this isoform of PKC [which appears to play a pivotal role in ischemic PC (38, 39)] activates the p44/p42 MAPK signaling pathway in the adult rabbit heart. Fourth, selective activation of PKC-ϵ protects rabbit cardiac myocytes against simulated ischemia, and this effect is abolished by the p44/p42 MAPK inhibitor PD-98059, indicating that activation of the p44/p42 MAPK pathway plays an important role in PKC-ϵ-induced protection in vitro. Finally, both the phosphorylation activity and the protein expression of p44 and p42 MAPKs increase in the nuclear fraction, indicating that ischemic PC causes nuclear translocation of these MAPK isoforms. The increases in p44 and p42 activity in the nuclear fraction are paralleled by (and hence can be accounted for by) the increases in the nuclear p44 and p42 protein content. This, coupled with the finding that MEK activity increases exclusively in the cytosol, indicates that ischemia activates p44 and p42 MAPKs in the cytosol, which then leads to translocation of the kinases to the nucleus.

Previous studies (5, 25, 33) reported MAPK activation in the cytosolic fraction of isolated, buffer-perfused hearts subjected to global ischemia followed by reperfusion. To our knowledge, this is the first study to demonstrate that *1*) the p44/p42 MAPKs are activated after regional myocardial ischemia in vivo; *2*) this activation is mediated by PKC; *3*) it occurs exclusively in the nuclear fraction, with no change in the cytosolic fraction; *4*) the ϵ-isoform of PKC can induce a similar

activation of p44/p42 MAPKs in cardiac myocytes; and *5*) the ϵ-isoform of PKC protects cardiac myocytes via a p44/p42 MAPK-dependent mechanism. This is also the first study to examine the effect of ischemic PC on MAPK in a conscious animal model. Because the ischemia-reperfusion protocol tested (6 cycles of 4-min occlusion/reperfusion) induces late PC against both myocardial stunning (9, 10, 31, 39) and myocardial infarction (40, 44), the present results are compatible with the hypothesis that p44/p42 MAPKs may contribute to the development of the late phase of ischemic PC. Apart from PC, however, activation of p44/p42 MAPKs after repetitive ischemia-reperfusion may also play a role in PKC-dependent signaling and attending phenotypic changes in various other pathophysiological conditions.

Methodological Considerations

In an effort to perform a comprehensive analysis of the effect of ischemic PC on the p44/p42 MAPK pathway, we measured not only total p44 and p42 MAPK activity but also the isoform-selective activity of each of these kinases. To this end, two assays of MAPK activity were used, the phosphorylation assay in solution and the in-gel kinase assay with MBP. The phosphorylation activity assay, which is based on the phosphorylation reaction in solution, provides a highly sensitive and quantitative measurement of the total MAPK activity in the tissue sample. However, it does not differentiate the isoform-dependent activity of the MAPKs. The in-gel kinase assay, which is based on the phosphorylation reaction in the gel, is less sensitive compared with the former, but enables one to selectively measure either the p44 or the p42 MAPK activity (6, 24). Therefore, these two assays complement and confirm one another. The potential nonspecific phosphorylation of MBP by the p38 MAPK was avoided by the addition of 60 μM SB-203580 in the reaction solutions. In the present study, the results obtained with the phosphorylation assay and the in-gel kinase assay were concordant, i.e., both showed increased MAPK activity after ischemia-reperfusion. The in-gel assay further showed that the increase in total MAPK activity was contributed by both the p44 and p42 isoforms, thereby providing additional useful information compared with that which would have been obtained with the phosphorylation assay alone.

Our assay of MEK activity was designed to specifically measure the MEK phosphorylation that occurred in vitro without the confounding influence of the variable degrees of endogenous phosphorylation of p42 MAPK that occurred in vivo before the assay. Because ischemia-reperfusion had a major effect on the amounts of p42 MAPK that were phosphorylated in vivo (Fig. 3*A*), inclusion of these amounts in our measurements would have led to erroneous and potentially misleading results. Accordingly, the amount of p42 MAPK phosphorylated in vivo (i.e., before the assay) was subtracted from the total amount of phosphorylated p42 MAPK present at the end of the reaction; the difference between the two amounts reflects the degree of MEK-catalyzed phosphorylation that took place in vitro.

Role of PKC in Activation of p44 and p42 MAPKs During Ischemic PC

One of the most important findings of this study is that the nuclear activation of p44/p42 MAPKs associated with ischemic PC is PKC dependent. Although previous studies suggested that PKC activates MAPKs in neonatal cardiac cells (6) and isolated hearts (5), virtually no information is available regarding *1*) whether PKC activates MAPKs in vivo and *2*) if so, which PKC isoform is specifically involved. It is also unknown whether, in the setting of ischemic PC, mobilization of PKC occurs in parallel to MAPK activation or is a distal event. Our finding that chelerythrine, a specific PKC inhibitor, blocked ischemia-induced MAPK activation demonstrates two important points: *1*) PKC plays an obligatory role in the stimulation of p44/p42 MAPKs during ischemic PC; and *2*) PKC activation precedes MAPK activation in the cascade that leads to PC. Because PKC activation is required for late PC to develop (2, 39), these results suggest that p44 and p42 MAPKs may be downstream phosphorylation targets of PKC and in the PKC-induced signaling pathways that mediate ischemic PC.

Having found that PKC is necessary to activate MAPKs in vivo, we performed additional studies to gain insights into which isoform of PKC is involved. The use of isolated adult rabbit myocytes enabled us to identify a specific cell type in which PKC activates MAPKs. We focused on the ϵ-isoform of PKC because our previous studies have shown that ischemic PC in the conscious rabbit selectively translocates this isoform to the particulate fraction (38) and that inhibition of such translocation abrogates the PC effect (39), implicating the ϵ-isoform as a critical mediator of ischemic PC. Our in vitro results demonstrate that a selective increase in the activity of this specific isotype of PKC results in MAPK activation (Fig. 8, *B* and *C*). The fact that the dominant negative mutant of PKC-ϵ decreased PKC-ϵ activity but had no effect on basal MAPK activity (Fig. 8, *B* and *C*) suggests that, in addition to PKC-ϵ, other stimuli account for the basal activity of MAPKs in cardiac myocytes and can compensate for the loss of activity of the ϵ-isozyme.

Because assessing the role of p44/p42 MAPK activation in the cardioprotective effects of ischemic PC in conscious rabbits would be prohibitively expensive, we utilized an in vitro model of simulated ischemia to determine whether inhibition of the p44/p42 MAPK pathway with the specific inhibitor PD-98059 blocks the protection conferred by PKC-ϵ activation. We found that activation of PKC-ϵ significantly reduced cell death during 6 h of simulated ischemia followed by 1 h of reoxygenation (Fig. 9) and that this effect was reversed by cotransfection with the dominant negative mutant of PKC-ϵ, indicating that it is specifically attributable to this isozyme (Fig. 9). To our knowledge, this is the first indication that a selective increase in PKC-ϵ activity is cytoprotective. The protection afforded by PKC-ϵ activation was completely abolished by PD-98059, supporting the notion that PKC-ϵ-

dependent activation of p44/p42 MAPKs in cardiac myocytes is not simply an epiphenomenon but rather an important signaling mechanism for the development of cardioprotection. Although data obtained in artificial in vitro systems must obviously be extrapolated to intact animals with caution, these results are compatible with the general conceptual paradigm that the p44/p42 MAPKs participate in the signaling events whereby ischemic PC triggers protection in vivo.

Previous Studies of MAPK During Ischemia-Reperfusion

Previous studies have addressed the effect of ischemia on MAPK in in vitro models of global ischemia (isolated rat hearts) and have yielded conflicting results. Maulik and colleagues (33) showed that four cycles of 5-min ischemia/10-min reperfusion caused a significant increase in total MAPK phosphorylation activity and in the activity of MAPK-activated protein kinase 2. Knight and Buxton (25) reported that a single episode of ischemia of ≤10 min followed by 15 min of reperfusion had no effect on total MAPK phosphorylation activity; a 15-min period of ischemia in itself had no effect but was associated with increased MAPK activity after 5 min of reperfusion. In contrast, using a similar isolated, perfused rat heart model, Bogoyevitch et al. (5) reported that 10 or 20 min of ischemia with or without reperfusion failed to activate p44 or p42 MAPKs. The reason for these discrepancies is unclear. Maulik et al. and Knight and Buxton determined total MAPK activity using a phosphorylation assay that measures the sum of p44/p42 and, to a certain extent, p38 activities; the activities of the p44 and p42 isoforms were not individually assessed. Thus evidence that ischemic PC activates the p44/p42 pathway (as opposed to other MAPK pathways) is still lacking. In all three studies (5, 25, 33), only the cytosolic fraction of the heart was examined. In the present study we examined p44 and p42 individually; furthermore, we analyzed both the nuclear and the cytosolic fractions of the heart. In agreement with the aforementioned investigations in vitro (5, 25, 33), we found that myocardial ischemia-reperfusion does not enhance the cytosolic MAPK phosphorylation activity in conscious rabbits (Fig. 3*B*). However, we did observe a significant elevation in the nuclear MAPK phosphorylation activity (Fig. 3*C*). Thus this study provides the new observation that brief ischemia activates MAPK in the nucleus rather than in the cytosol.

MAPK Nuclear Translocation During Ischemic PC

One of the characteristic features of all MAPKs is their ability to translocate to the nucleus, in which they phosphorylate and activate transcription factors, thereby regulating gene expression (12, 16, 17a, 18, 20, 37). For example, the p44 and p42 MAPKs have been shown to activate the expression of immediate-early genes such as c-*Jun* and c-*Fos*, as well as the transcriptional factor Elk1 (12, 16, 17a, 20, 37). Our results demonstrate that ischemic PC elicits a rapid increase in nuclear p44 and p42 MAPK phosphorylation activity (Fig. 4, *A* and *B*), which is associated with a parallel shift of these proteins from the cytosolic to the nuclear compartment (Fig. 5, *A* and *B*). Because the nuclear entry of the p44 and p42 MAPKs has been shown to be activation dependent (12, 20, 51), and because in this study the changes in activity and protein expression of p44 and p42 MAPKs in the nuclear fraction correlated closely (Figs. 4*A*, 4*B*, 5*A*, 5*B*, and 6), we propose that myocardial ischemia-reperfusion causes activation of p44 and p42 MAPKs in the cytosol, which is followed by migration of the activated isoforms into the nucleus. This hypothesis is supported by the measurements of MEK1 and MEK2, the activators of p44 and p42 MAPKs. We found that MEK1 is located exclusively in the cytosolic fraction in the adult rabbit heart, both under control conditions and after ischemia-reperfusion (Fig. 7, *A* and *C*). We also found that almost all of MEK2 is expressed in the cytosolic fraction, with the amount present in the nuclear fraction being consistently <2% under control conditions as well as after myocardial ischemia-reperfusion (Fig. 7*B*). In keeping with these protein expression measurements, we observed that the preponderance of total MEK phosphorylation activity is located in the cytosol and <5% in the nucleus (Fig. 7*C*); importantly, ischemia-reperfusion did not cause a discernible increase in nuclear MEK activity, although it did cause a marked increase in the cytosolic activity (Fig. 7*C*). Taken together, these results strongly support the concept that activation of p44 and p42 MAPKs during ischemia-reperfusion occurs in the cytosol, not in the nucleus.

In summary, the present study demonstrates that ischemic PC induces nuclear translocation and activation of both p44 and p42 MAPKs in the heart of conscious rabbits. The mechanism for the increased nuclear activity of p44/p42 appears to involve activation of these isozymes by cytosolic MEKs followed by migration of the activated p44/p42 proteins to the nucleus. The present study also demonstrates that MAPK activation during ischemic PC is downstream of, and mediated by, PKC activation and that the ε-isoform of PKC, which is selectively activated during ischemic PC (38), can account for this effect in cardiac myocytes. Furthermore, the studies with simulated ischemia in vitro provide the first indication that the ε-isoform of PKC protects cardiac myocytes and that this protection is dependent on the p44/p42 MAPK pathway. These results significantly expand our understanding of the signaling pathways activated by myocardial ischemia and identify potential downstream targets of PKC activation in this setting. The present observations provide a rationale for investigating the role of p44/p42 MAPKs in the development of protection during the early and/or late phase of ischemic PC in vivo. However, it is important to note that the implications of the present results are not limited to ischemic PC. Because p44/p42 MAPKs are known to modulate the expression of immediate-early response genes and other genes (12, 16, 17a, 20, 37), activation of these kinases in the nucleus may contribute to altered gene expression in

other pathological processes associated with recurrent ischemic stress.

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