Nuclear Mitogen-activated Protein Kinase Activation by Protein Kinase Cζ during Reoxygenation after Ischemic Hypoxia*

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We examined the upstream kinases for mitogen-activated protein kinase (MAPK) activation during ischemic hypoxia and reoxygenation using H9c2 cells derived from rat cardiomyocytes. Protein kinase C (PKCζ), an atypical PKC isoform mainly expressed in rat heart, has been shown to act as an upstream kinase of MAPK during ischemic hypoxia and reoxygenation by analyses with PKC inhibitors, antisense DNA, a dominant negative kinase defective mutant, and constitutively active mutants of PKCζ. Immunocytochemical observations show PKCζ staining in the nucleus during ischemic hypoxia and reoxygenation when phosphorylated MAPK is also detected in the nucleus. This nuclear localization of PKCζ is inhibited by treatment with wortmannin, a phosphoinositide 3-kinase inhibitor that also inhibits MAPK activation in a dose-dependent manner. This is supported by the inhibition of MAPK phosphorylation by another blocker of phosphoinositide 3-kinase, LY294002. An upstream kinase of MAPK, MEK1/2, is significantly phosphorylated 15 min after reoxygenation and observed mainly in the nucleus, whereas it is present in the cytoplasm in serum stimulation. The phosphorylation of MEK is blocked by PKC inhibitors and phosphoinositide 3-kinase inhibitors, as observed in the case of MAPK phosphorylation. These observations indicate that PKCζ, which is activated by phosphoinositide 3-kinase, induces MAPK activation through MEK in the nucleus during reoxygenation after ischemic hypoxia.

Various pathways in mammalian cells are induced in response to ischemia and reperfusion leading to cell death and organ dysfunction. In the heart, prolonged ischemia causes necrosis and contractile dysfunction, but the heart can recover from injury following brief ischemia. Many genes are expressed in the heart during recovery from ischemia. In particular, the expression of immediate early genes (e.g. c-fos, c-jun, and Egr-1) is rapidly up-regulated during post-ischemic reperfusion (1–4). Among transcription factors, the induction of c-fos is regulated by a serum response element in the promoter. Transcription factors p67SF and p62TCF bind to the serum response element, the activity of which is regulated by the phosphorylation of p62TCF by mitogen-activated protein kinase (MAPK) (5). This indicates that MAPKs serves as an important regulator of nuclear transcriptional activity. MAPK is a serine/threonine protein kinase whose activity is up-regulated by MAPK/extracellular signal-regulated kinase (MEK)-catalyzed phosphorylation on tyrosine and threonine residues (6–9). MEKs are substrates of Raf-1 (10, 11), which has been reported to be activated through protein kinase C (PKC) isoform-dependent or -independent pathways (12–20). PKC seems to play an important role in the heart during ischemia. It has been reported that ischemia-reperfusion injures the myocardium by Ca2+ influx through a Na+/H+ -exchanger whose activity appears to be regulated by PKC (21–23). Furthermore, cycles of brief ischemia and reperfusion (designated preconditioning) protect the myocardium against infarction caused by subsequent sustained ischemia. This preconditioning effect might be mediated by PKC isoforms (24–28). In preceding studies, we demonstrated that PKCζ, δ, ε, and ζ isoforms translocate to distinct fractions during ischemia (29–31). In particularly, PKCζ, which is expressed mainly in rat heart, translocates from the cytoplasm to the nucleus during ischemia (30). In the nucleus, the nuclear proteins nucleolin and heterogeneous nuclear ribonucleoprotein are PKCζ substrates, suggesting that PKCζ may play a role in nuclear signal transmission (32, 33). However, the role of PKCζ in the ischemic heart is presently unknown.

We have demonstrated that ischemia induces the nuclear translocation of MAPK and c-Jun N-terminal kinase 1, both of which are activated during post-ischemic reperfusion, suggesting that a MAPK activation pathway may be present in nuclei in ischemic heart (4, 34, 35). However, the upstream pathway for MAPK activation during ischemia and reperfusion remain unknown. The present study uses an ischemia and reperfusion cell model to examine the upstream kinases of MAPK activation. The results obtained here indicate that during reoxygenation after ischemic hypoxia, an atypical PKC, PKCζ, that is

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activated by phosphoinositide 3-kinase (PI3K) participates in the activation of MAPK through MEK in the nucleus.

EXPERIMENTAL PROCEDURES

Materials and Plasmids—Anti-phospho-MAPK antibody (Tyr-204), anti-phospho-Erk-1 (Ser-383) antibody, Erk-1 fusion protein, anti-phospho-MEK1/2 antibody (Ser-217/Ser-221), and PD98059 (MEK inhibitor) were bought from New England Biolabs, Inc. (Beverly, MA). Anti-MAPK antibody (for p42MAPK and p44MAPK, K-25), anti-MAPK antibody (for p42MAPK, C-161 antibody, and anti-gal IgG antibody coupled to peroxidase were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-PKCs, δ, ε, λ, and ζ antibodies were from Transduction Laboratory, Inc. (Lexington, KY). Anti-rabbit and anti-mouse IgG antibodies coupled to peroxidase were obtained from Promega Co. (Madison, WI). Anti-mouse IgG-linked Cy3 and anti-rabbit IgG-Cy2 were from Amersham Pharmacia Biotech Ltd. (Buckinghamshire, UK). The enhanced chemiluminescence reaction assay kit was from Pierce (Rockford, IL). For the construction of constitutively active PKCζ1 A119E and kinase-deficient dominant negative PKCζ K275W mutants, site-directed mutagenesis of a full-length PKCζ cDNA derived from mouse brain (donated by H. Mischaik, Munich, Germany) was performed as described previously (19, 36, 37). The orientation of insertion was determined by analysis and sequencing. All cDNAs for PKCζ mutants were subcloned into the pEFneo expression vector. All other chemicals were commercially available.

Cell Culture and Ischemic Hypoxia and Reoxygenation—An embryonic rat heart-derived cell line, H9c2 cells, was plated at a density of 5–105 cells per dish in 100-mm culture dishes. After incubation in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum for 72 h, the cells were cultured in serum-free DMEM for 60–72 h. Simulated ischemia was achieved as described previously (38). Briefly, the cells were incubated in slightly hypotonic Hanks’ balanced saline solution (1.3 mM CaCl2, 5 mM KCl, 0.3 mM KH2PO4, 0.5 mM MgSO4, 69 mM NaCl, 4 mM NaHCO3, and 0.3 mM Na2HPO4) without glucose or serum for 2 h at 37 °C. Hypoxia was achieved using an air-tight incubator from which oxygen was removed by replacement with nitrogen concentation increased to 1%. After incubation under the conditions of ischemic hypoxia, the cells were incubated in DMEM without serum under normoxic conditions (2% O2, 5% CO2) at 37 °C for the indicated times.

Electrophoresis and Immunoblotting—Cells were washed with cold phosphate-buffered saline (PBS) and lysed with lysis buffer (1% Triton X-100, 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM Na3VO4, 10 mM NaF, 1 mM sodium orthovanadate, 1 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride). The cell extracts and molecular mass standards were electrophoresed in 10% (w/v) polyacrylamide gels in the presence of SDS and transferred to polyvinylidene difluoride membranes (0.45 μm, Millipore Corp., Bedford, MA) in the case of phospho-MAPK and phospho-Erk-1, or transferred to nitrocellulose membranes in the case of other proteins. The blots were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.05% (w/v) Tween 20, and incubated with antibody. The blots were washed with PBS, and incubated for an additional hour at room temperature with Cy3-conjugated anti-mouse IgG or/and Cy2-conjugated anti-rabbit IgG at 1:800 dilution in PBS containing 3% bovine serum albumin. The blots were incubated for 15 min at room temperature with antibody against PKCζ, PKCδ, PKCε, PKCλ, or PKCζ and then incubated in serum-free medium for 60–72 h. For the transfection of expression vectors, subconfluent cell cultures were transfected with the indicated plasmids using Tfx-50 (Promega) as described above. To obtain H9c2 cells that stably expressed PKCζ mutants, clones of stable transfectants were isolated by their ability to grow in the presence of G418 (750 μg/ml, Promega). Untransfected cells could not proliferate up to 7 days in the presence of G418 (750 μg/ml). The expression of PKCζ was confirmed by immunoblotting using anti-PKCζ antibody. After incubation under the conditions of ischemic hypoxia and reoxygenation, the cells were used for biochemical assay.

Other Assays—MAPK activity was measured by immunoprecipitation kinase assay using Elk-1 as a substrate as described previously (34, 41).

RESULTS

PKCζ Is Involved in MAPK Activation during Ischemic Hypoxia and Reoxygenation—We used the cells incubated for 60–72 h in serum-free DMEM since MAPK activity was detected for up to 48 h in untreated cells after the removal of serum. We first examined the time course of MAPK activation during ischemic hypoxia and reoxygenation by immunoblotting using an anti-phospho-MAPK antibody that recognizes the tyrosine phosphorylation of MAPK (Tyr-204) necessary for activation. MAPK phosphorylation was increased approximately 10-fold after 15 min of reoxygenation following ischemic hypoxia (Fig. 1, A and C), and p42MAPK was preferentially activated as compared with p44MAPK. The amount of MAPK remained almost the same during ischemic hypoxia and reoxygenation (Fig. 1B). To confirm the amount of MAPK protein, two MAPK antibodies (PKC-23, antibody for p42MAPK and p44MAPK, and C-16, antibody for p42MAPK) were used. These antibodies produced essentially similar results in all experiments (data not shown). MAPK phosphorylation was consistent with MAPK activity measured by immunoprecipitation kinase assay using Elk-1 as a substrate (Fig. 1D). To identify the upstream kinase(s) for MAPK activation, we examined the phosphorylation of MAPK in the presence of various inhibitors after 15 min of reoxygenation following 2 h of ischemic hypoxia. As shown in Fig. 2, A–C, both GF109203X (3 μM) and chelerythrine (5 μM), inhibitors of the ATP-binding site of PKC (42, 43), blocked MAPK phosphorylation to the control level, while calphostin C (1 μM), an inhibitor that interacts with the phorbol ester-binding site of PKC (44), suppressed MAPK phosphorylation only weakly. Phorbol 12-myristate 13-acetate (20 nM) significantly increased the phosphorylation of MAPK and MEK1/2 at 15 min after stimulation; this phosphorylation was largely inhibited by 1 μM calphostin C (data not shown). The results indicate that calphostin C works as an effective inhibitor of phorbol 12-myristate 13-acetate-sensitive PKC. These observations suggest that an atypical PKC isoform lacking a phorbol ester-binding site, may be involved in MAPK activation during reoxygenation. Since PKCζ, among PKC isoforms expressed mainly in the heart, has no phorbol ester-binding site we transfected an antisense oligonucleotide against PKCζ into cells using a liposome method. The antisense DNA inhibited PKCζ expression by 75% compared with the sense DNA (data not shown). These DNAs had no effect on the expression of other PKC isoforms (data not shown). The expression of PKCζ isoforms was almost the same in three independent experiments. Another member of the atypical PKC isoform, PKCζ/δ, was not detected by immunoblotting using the anti-PKCζ antibody (for the amino acid sequence of human PKCζ) under the conditions used in this study. Cells in which the PKCζ protein was depleted by the antisense DNA showed a significant inhibition of MAPK phosphorylation after 15 min of reoxygenation, while...
sense DNA-treated cells showed significantly more MAPK phosphorylation the same amount as in untreated cells (Fig. 3, A-C). The antisense DNA for PKCa caused a significant inhibition of the expression of PKCa, whereas it had no effect on the expression of PKCζ. Treatment with an antisense DNA for PKCa resulted in the induction of MAPK phosphorylation after 15 min of reoxygenation to almost the same extent as in the mock control (Fig. 5). The biochemical and biological properties of the mutants have been described previously by Überall et al. (37). MAPK phosphorylation by 10% fetal calf serum was significantly increased in the mock control, whereas it had no effect on phospho-MAPK (p42MAPK and p44MAPK) were determined from the immunoblots by densitometric analysis (mean ± S.E., n = 5; *p < 0.05 versus control; #, p < 0.05 versus ischemic hypoxia and reoxygenation) (C). Chl, chelerythrine; GF, GF109203X; Cal C, calphostin C.

FIG. 2. Effect of PKC inhibitors on MAPK phosphorylation during reoxygenation after ischemic hypoxia. Cell extracts (40 μg of protein) were prepared from H9c2 cells reoxygenated for 15 min after ischemic hypoxia for 2 h with or without PKC inhibitors, and subjected to immunoblotting with anti-phospho-MAPK antibody (A) or anti-MAPK antibody (B). The figure shows representative immunoblots obtained from five independent experiments. The levels of MAPK phosphorylation (sum of the phosphorylation levels of p42MAPK and p44MAPK) were determined from the immunoblots by densitometric analysis (mean ± S.E., n = 5; *p < 0.05 versus control; #, p < 0.05 versus ischemic hypoxia and reoxygenation) (C). Chl, chelerythrine; GF, GF109203X; Cal C, calphostin C.

The PKCζ mutant (data not shown) as detected by immunoblotting using anti-PKCζ antibody. In agreement with the data obtained with the PKCζ antisense DNA, the increased phosphorylation of MAPK observed after 15 min of reoxygenation following ischemic hypoxia was strongly inhibited in cells expressing the DN mutant of PKCζ, in which an alanine in the pseudosubstrate domain of PKCζ is replaced by glutamate. The transfection of the CA PKCζ mutant into cells increased the expression of the PKCζ mutant (data not shown). The expression of the CA PKCζ mutant in cells led to a significant phosphorylation of MAPK after reoxygenation as compared with the mock control (Fig. 5). The biochemical and biological properties of the mutants have been described previously by Überall et al. (37). MAPK phosphorylation by 10% fetal calf serum was significantly increased in the mock control, an increase similar to that in cells expressing DN and CA PKCζ (data not shown). These findings show that PKCζ mainly participates in the activation of MAPK during reoxygenation after ischemic hypoxia. In unstimulated cells, the expression of CA PKCζ mutants did not affect MAPK activity, suggesting that the localization of PKCζ may play a role in MAPK activity.

Nuclear Localization and Activation of PKCζ MAPK Pathway during Ischemic Hypoxia and Reoxygenation—We examined the localization of PKCζ and phosphorylated MAPK during ischemic hypoxia and reoxygenation by immunocytochemical analysis.

FIG. 1. Time course of tyrosine phosphorylation (Tyr192) of MAPK and MAPK activity during ischemic hypoxia and reoxygenation. Cell extracts (40 μg of protein) were prepared from H9c2 cells exposed to ischemic hypoxia and reoxygenation for the indicated times, and subjected to immunoblotting with anti-phospho-MAPK antibody (A) or anti-MAPK antibody (B). The figure shows representative immunoblots obtained from three independent experiments.

The PKCζ mutant (data not shown) as detected by immunoblotting using anti-PKCζ antibody. In agreement with the data obtained with the PKCζ antisense DNA, the increased phosphorylation of MAPK observed after 15 min of reoxygenation following ischemic hypoxia was strongly inhibited in cells expressing the DN mutant of PKCζ, in which an alanine in the pseudosubstrate domain of PKCζ is replaced by glutamate. The transfection of the CA PKCζ mutant into cells increased the expression of the PKCζ mutant (data not shown). The expression of the CA PKCζ mutant in cells led to a significant phosphorylation of MAPK after reoxygenation as compared with the mock control (Fig. 5). The biochemical and biological properties of the mutants have been described previously by Überall et al. (37). MAPK phosphorylation by 10% fetal calf serum was significantly increased in the mock control, an increase similar to that in cells expressing DN and CA PKCζ (data not shown). These findings show that PKCζ mainly participates in the activation of MAPK during reoxygenation after ischemic hypoxia. In unstimulated cells, the expression of CA PKCζ mutants did not affect MAPK activity, suggesting that the localization of PKCζ may play a role in MAPK activity.

Nuclear Localization and Activation of PKCζ MAPK Pathway during Ischemic Hypoxia and Reoxygenation—We examined the localization of PKCζ and phosphorylated MAPK during ischemic hypoxia and reoxygenation by immunocytochemical analysis.
PKCζ translocated from the cytoplasm to the nucleus during 2 h of ischemic hypoxia, and was present predominantly in the nucleus after 15 min reoxygenation (Fig. 6, A-C), when phosphorylated MAPK was also detected in the nucleus (Fig. 6F). The nuclear localization of PKCζ was inhibited by 100 nM wortmannin, an inhibitor of PI3K (Fig. 6, D and E). Consistent with the localization of PKCζ in the cytoplasm, wortmannin also blocked the phosphorylation of MAPK in a dose-dependent manner (1–100 nM) (Fig. 7, A-C). No PKCζ staining was observed by a secondary antibody alone (data not shown). Phosphorylated MAPK staining could not be detected in untreated cells or wortmannin-treated cells (data not shown). The expression of PKCζ as detected by immunoblotting in wortmannin-treated cells was similar to that in untreated cells using anti-PKCζ antibody (data not shown). Another inhibitor of PI3K, LY294002 (30 μM), also largely inhibited MAPK phosphorylation 15 min after reoxygenation (Fig. 7, D-F). These findings suggest that PKCζ activated by PI3K translocates to the nucleus, which leads to the activation of nuclear MAPK. We then examined the activation and localization of MEKs, upstream kinases of MAPK, using anti-phospho-MEK1/2 antibody that recognizes the phosphorylation site (Ser217/Ser222) necessary for the activation. The phosphorylation of MEK1/2 increased significantly with a peak at 15 min after reoxygenation (Fig. 8, A-D), and a time course similar to that for MAPK phosphorylation. The phosphorylation was inhibited by PI3K inhibitors, wortmannin (Fig. 8, E-H) and LY294002 (Fig. 8, I-L), and PKC inhibitors, chelerythrine and GF109203X (data not shown). A MEK inhibitor, PD98059 (50 μM), blocked MAPK activation during reoxygenation in a dose-dependent manner, and had no effect on JNK phosphorylation (data not shown). These findings suggest that the PI3K/PKCζ pathway acts as an upstream kinase of MEK, which participates in MAPK activation during reoxygenation after ischemic hypoxia. Active phosphorylated MEK1/2 was detected mainly in the nucleus during reoxygenation (Fig. 9B), whereas it was retained in the cytoplasm during serum stimulation (Fig. 9C). Phosphorylated MEK1/2 was undetectable in unstimulated cells (Fig. 9A). These findings indicate that nuclear MEK activates MAPK during reoxygenation.

**DISCUSSION**

p42MAPK was preferentially activated during reoxygenation after ischemic hypoxia as compared with p44MAPK, implying that the activating pathway of MAPK may be different than that induced by serum or PMA stimulation since both p42MAPK and p44MAPK are activated by treatment with serum and PMA. The inhibition of PKCζ expression and the activity significantly suppresses the phosphorylation of MAPK during reoxygenation, demonstrating that PKCζ is involved in MAPK activation. In cells expressing the CA mutant of PKCζ, MAPK phosphorylation was undetectable in unstimulated cells. The activation of MAPK may require that PKCζ translocates from the cytosol to other organelle where the MAPK pathway is present during ischemic hypoxia. This activation pathway might exist in the nucleus based on the following reasons.
First, during ischemic hypoxia and reoxygenation, PKCζ localizes mainly in the nucleus where phosphorylated MAPK is also detected. Second, the nuclear localization of PKCζ is blocked during ischemic hypoxia and reoxygenation in wortmannin-treated cells. Consistent with the localization of PKCζ in the cytoplasm, the phosphorylation of MAPK during reoxygenation is also suppressed by wortmannin. We also observed the nuclear translocation of MEK, an upstream kinase for MAPK, during ischemic hypoxia, and reoxygenation for 15 min after ischemic hypoxia led to phosphorylation of MEK only in the nucleus although the active form of MEK remained in the cytoplasm for 15 min after serum stimulation. The phosphorylation of MEK during reoxygenation was blocked by PKC inhibitors and PI3K inhibitors as shown in this study. These observations indicate that nuclear PKCζ activates the MEK/MAPK pathway.

It was initially thought that MEK is always localized in the cytoplasm, but Tolwinski et al. recently demonstrated that MEK also translocates to the nucleus in mammalian cells.

Y. Mizukami, K. Mogami, N. Todoroki-Ikeda, and S. Kobayashi, unpublished observations.

**FIG. 5.** MAPK phosphorylation during reoxygenation after ischemic hypoxia in H9c2 cells expressing a constitutively active mutant of PKCζ A119E. H9c2 cells were pretreated with 1 μg of DNA for 1 h in DMEM containing liposomes (Tfx-50), and cells containing the introduced vector were selected by G418 (750 μg/ml). Cell extracts (40 μg of protein) were prepared from cells reoxygenated for 15 min after ischemic hypoxia for 2 h, and subjected to immunoblotting with anti-phospho-MAPK antibody (A) or anti-MAPK antibody (B). The figure shows representative immunoblots obtained from three independent experiments. The levels of MAPK phosphorylation (sum of phosphorylation levels of p42MAPK and p44MAPK) were determined from the immunoblots by densitometric analysis (mean ± S.E., n = 3; *, p < 0.05 versus control; #, p < 0.05 versus ischemic hypoxia and reoxygenation) (C).

**FIG. 6.** Effect of wortmannin on the localization of PKCζ during ischemic hypoxia and reoxygenation. H9c2 cells were unincubated (A) or incubated in the absence (B, C, and F) or presence of wortmannin (D and E) under the conditions of ischemic hypoxia for 2 h (B and D) or reoxygenation for 15 min (C, E, and F), fixed in cold acetone/methanol (50/50), and stained with anti-PKCζ antibody (A-E) or/and anti-phospho-MAPK antibody (F) as described under “Experimental Procedures.” The figure shows representative photographs obtained from three independent experiments. Final magnification, ×400.

**FIG. 7.** Effect of phosphoinositide 3-kinase inhibitors on MAPK phosphorylation during reoxygenation after ischemic hypoxia. Cell extracts (40 μg) were prepared from H9c2 cells reoxygenated for 15 min after ischemic hypoxia for 2 h in the presence of the indicated concentrations of wortmannin or LY294002 (30 μM), and subjected to immunoblotting with anti-phospho-MAPK antibody (A) or anti-MAPK antibody (B). The figure shows representative immunoblots obtained from three independent experiments. The levels of MAPK phosphorylation (sum of phosphorylation levels of p42MAPK and p44MAPK) were determined from the immunoblots by densitometric analysis (mean ± S.E., n = 3; *, p < 0.05 versus control; #, p < 0.05 versus ischemic hypoxia and reoxygenation) (C).
in a regulatable manner during mitosis (45). How does MEK uptake into the nucleus during ischemic hypoxia occur? One possibility is that a decrease in intracellular ATP by depletion of molecular oxygen and glucose during ischemic hypoxia may lead to the nuclear accumulation of MEK by inhibiting nuclear export via a nuclear export signal that is known to be dependent on intracellular ATP (46–48). Another possibility is that the interaction of MEK to CRM1/exportin in the nuclear membrane (49–52) may be inhibited by a protein kinase activated during ischemic hypoxia, since protein phosphorylation has been suggested to modulate a nuclear export signal by masking an adjacent protein sequence (53–55). The nuclear MEK/MAPK pathway will be able to induce gene expression rapidly under pathophysiological conditions.

PKCζ, working as an upstream kinase of MAPK during reoxygenation after ischemic hypoxia, is activated by PI3K in response to various stimuli including ischemic hypoxia as indicated in this study (20, 30, 56–58). Joyal et al. (59) show that PI3K is activated by an intracellular Ca2+-dependent effector protein, calmodulin, and that the increase in Ca2+ induced by ischemic hypoxia may be involved in the activation of PI3K. Recently, it was reported that phosphatidylinositol 3,4,5-triphosphate, a product of PI3K, directly activates PDK, which leads to the activation of PKCζ (60). The inhibition of PI3K activity can inhibit the increased cell survival induced by growth factors (61–63). One target of PI3K is c-Akt (also designated protein kinase B), which promotes survival through the phosphorylation of BAD (64). Recently, Burnet et al. (65) reported that c-Akt phosphorylates Forkhead transcription factor, which is involved in the promotion of cell survival in late phase. PI3K can also activate the MAPK pathway (66–68), and the activated MAPK produces signals capable of improving survival in PC12 cells (69). Another target of PI3K, PKCζ, may also play a role in cell survival through MAPK during ischemia and reperfusion since PKCζ can activate the MAPK pathway via Ras-dependent (19) or -independent pathways (20). Consistent with this idea, the inhibitions of the PKCζ/MAPK pathway induces apoptotic cell death during reoxygenation. Cardiomyocyte-specific proteins and functions were conserved in the H9c2 cells used in this study, and the pathway of apoptotic inhibition might also be observed in a primary cardiac cell system. In cardiac myocytes that have lost their mitogenic activity, the preservation of cell viability by inhibition of apoptotic cell death may be critical for the maintenance of normal cardiac function. Further investigations are required to eluci-

**FIG. 8.** Time course of phosphorylation of MEK1/2 (Ser\(^{217}/\text{Ser}^{222}\)) during reoxygenation after ischemic hypoxia and the effect of phosphoinositide 3-kinase inhibitors. A–D, cell extracts (40 \(\mu\)g of protein) were prepared from H9c2 cells exposed to ischemic hypoxia and reoxygenation for the indicated times, and subjected to immunoblotting with anti-phospho-MEK1/2 antibody (A), anti-MEK1 antibody (B), or anti-MEK2 antibody (C). The figure shows representative immunoblots obtained from four independent experiments. The phosphorylation level of MEK1/2 was determined from the immunoblots by densitometric analysis (mean ± S.E., \(n = 4\)) (D) as described under “Experimental Procedures.” E–L, cell extracts (40 \(\mu\)g) were prepared from H9c2 cells reoxygenated for 15 min after ischemic hypoxia for 2 h in the presence of the indicated concentrations of wortmannin (E–H) or LY294002 (30 \(\mu\)M) (I–L), and subjected to immunoblotting with anti-phospho-MEK1/2 antibody (E and I), anti-MEK1 antibody (F and J), or anti-MEK2 antibody (G and K). The figure shows representative immunoblots obtained from three independent experiments. The levels of MEK1/2 phosphorylation were determined from the immunoblots by densitometric analysis (mean ± S.E., \(n = 3\); *, \(p < 0.05\) versus control; #, \(p < 0.05\) versus ischemic hypoxia and reoxygenation) (H and L).

**FIG. 9.** Localization of phosphorylated MEK during reoxygenation after ischemic hypoxia. H9c2 cells were unincubated (A), or incubated under the conditions of reoxygenation for 15 min after ischemic hypoxia for 2 h (B), or with 10% serum for 15 min (C), fixed in cold acetone/methanol (50/50), and stained with anti-phospho-MEK1/2 antibody (A, B, and C) as described under “Experimental Procedures.” The figure shows representative photographs obtained from four independent experiments. Final magnification, ×400.
date the role of the PKCζ/MAPK pathway in pathogenic processes.

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