Role of F-actin organization in p38 MAP kinase-mediated apoptosis and necrosis in neonatal rat cardiomyocytes subjected to simulated ischemia and reoxygenation

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Okada, Takayuki, Hajime Otani, Yue Wu, Shiori Kyo, Chiharu Enoki, Hiroyoshi Fujisawa, Tomohiko Sumida, Reiji Hattori, and Hiroji Imamura. Role of F-actin organization in p38 MAP kinase-mediated apoptosis and necrosis in neonatal rat cardiomyocytes subjected to simulated ischemia and reoxygenation. Am J Physiol Heart Circ Physiol 289: H2310–H2318, 2005. First published July 22, 2005; doi:10.1152/ajpheart.00462.2005.—Activation of p38 mitogen-activated protein (MAP) kinase (MAPK) has been implicated in the mechanism of cardiomyocyte (CMC) protection and injury. The p38 MAPK controversy may be related to differential effects of this kinase on apoptosis and necrosis. We have hypothesized that p38 MAPK-mediated F-actin reorganization promotes apoptotic cell death, whereas it protects from osmotic stress-induced necrotic cell death. Cultured neonatal rat CMCs were subjected to 2 h of simulated ischemia followed by reoxygenation. p38 MAPK activity measured by phosphorylation of MAP kinase-activated protein (MAPKAP) kinase 2 was increased during simulated ischemia and reoxygenation. This was associated with translocation of heat shock protein 27 (HSP27) from the cytosolic to the cytoskeletal fraction and F-actin reorganization. Cytochrome c release from mitochondria, caspase-3 activation, and DNA fragmentation were increased during reoxygenation. Robust lactate dehydrogenase (LDH) release was observed under hyposmotic (140 mosM) reoxygenation. The p38 MAPK inhibitor SB-203580 abrogated activation of p38 MAPK, translocation of HSP27, and F-actin reorganization and prevented cytochrome c release, caspase-3 activation, and DNA fragmentation. Conversely, SB-203580 enhanced LDH release during hyposmotic reoxygenation. The F-actin disrupting agent cytochalasin D inhibited F-actin reorganization and prevented cytochrome c release, caspase-3 activation, and DNA fragmentation, whereas it enhanced LDH release during hyposmotic reoxygenation. When CMCs were incubated under the isoosmotic condition for the first 15 min of reoxygenation, SB-203580 and cytochalasin D increased ATP content of CMCs and prevented LDH release after the conversion to the hypoxic condition. These results suggest that F-actin reorganization mediated by activation of p38 MAPK plays a differential role in apoptosis and protection against osmotic stress-induced necrosis during reoxygenation in neonatal rat CMCs; however, the sarcolemmal fragility caused by p38 MAPK inhibition can be reversed during temporary blockade of physical stress during reoxygenation.

mitogen-activated protein kinase; osmotic stress; apoptotic and necrotic cell death

P38 MITOGEN-ACTIVATED PROTEIN (MAP) KINASE (MAPK) EXERTS A PLEIOTROPIC EFFECT ON CARDIOMYOCYTES (CMCS) DURING ISCHEMIA AND REPERFUSION (1, 39). IT HAS BEEN DEMONSTRATED THAT P38 MAPK ACTIVATION AGGRAVATES ISCHEMIA-REPERFUSION INJURY (8, 30, 32, 38, 43, 47), WHEREAS IT IS NECESSARY TO MEDIATE CARDIOPROTECTION OF ISCHEMIC PRECONDITIONING (34–36). THE P38 MAPK CONTROVERSY MAY BE RELATED TO DIFFERENTIAL EFFECTS OF THIS KINASE ON APOPTOSIS AND NECROSIS, BOTH OF WHICH ARE KNOWN TO CONTRIBUTE TO THE DEVELOPMENT OF MYOCARDIAL INFARCTION AFTER CORONARY ARTERY OCCLUSION AND REPERFUSION (23). IT HAS BEEN SHOWN THAT P38 MAPK ACTIVATION TRIGGERS APOPTOSIS BY ACTIVATING THE MITOCHONDRIAL CELL DEATH PATHWAY, INCLUDING PERMEABILITY TRANSITION PORE OPENING AND CYTOCHROME C RELEASE FROM THE INTERMEMBRANE SPACE (10, 16, 54). IN CONTRAST TO GENERAL AGREEMENT THAT P38 MAPK PLAYS A ROLE IN CMC APOPTOSIS DURING HYPOXIA OR ISCHEMIA-REPERFUSION (11, 30, 31), THE ROLE OF P38 MAPK IN CMC NECROSIS IS CONTROVERSIAL (29, 30, 43, 46) AND THE MECHANISM OF AN ANTI-NECROTIC EFFECT OF P38 MAPK HAS NOT BEEN ELUCIDATED.

Signal transduction pathways downstream of p38 MAPK involve activation of MAP kinase-activated protein (MAPKAP) kinase 2 (MAPKAPK2) (35) that phosphorylates several members of the small heat shock protein family such as heat shock protein 27 (HSP27) and aB crystallin (21, 24). Translocation of these small heat shock proteins from the cytosol to the cytoskeleton occurs along with their phosphorylation. Accumulating evidence suggests that phosphorylation of these small heat shock proteins is cytoprotective against a wide variety of cellular stress. It has been demonstrated that overexpression of a nonphosphorylatable form of HSP27 was much less effective in mediating protection in Chinese hamster CCL-39 cells (18). Furthermore, HSP27 phosphorylation-mediated cytoprotection is mediated by actin polymerization and physical reinforcement of plasma membrane actin cytoskeleton in the murine fibrosarcoma cell lines (21), suggesting that HSP27 phosphorylation and subsequent reorganization of actin cytoskeleton may be a crucial step for cytoprotection. On the other hand, transgenic overexpression of wild-type HSP27 or nonphosphorylatable HSP27 provided equal protection against ischemia-reperfusion injury in the mouse heart (19), suggesting that either form of HSP27 plays a cardioprotective role, although the mechanism of cardioprotection may be different.

With regard to the role of HSP27 phosphorylation in cytoprotection against physical stress under the cytotoxic environment, it is assumed that sarcolemmal membrane fragility may be prevented by p38 MAPK activation and HSP27 phosphorylation during ischemia and reperfusion. Sarcolemmal fragility

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is a characteristic feature of reperfusion injury in the heart (4, 15). Studies using the contractile blocker 2,3-butanedione monoxide have demonstrated that temporary blockade of contractile activity during reperfusion prevents myocardial necrosis (45, 50). The importance to block mechanical stress at the time of reperfusion was further supported by the observations (25) that cardioprotection mediated by ischemic preconditioning was potentiated by temporary blockade of contractile activity during reperfusion. Moreover, our recent study (46) demonstrated that temporary blockade of contractility during reperfusion converted the p38 MAPK inhibitor SB-203580 from the pronecrotic to the antinecrotic agent. This antinecrotic effect mediated by p38 MAPK inhibition was thought to be due to the enhancement of the reparative process against sarcolemmal fragility by improving mitochondrial function. These previous studies have prompted us to hypothesize that reorganization of actin cytoskeleton exerts a protective effect against necrosis when the sarcolemmal membrane is weakened by preceding ischemia.

Actin cytoskeleton is not only involved in maintaining the integrity of cell architecture but is also involved in signal transduction regulating cell growth, motility, survival, and death (9). Thus reorganization of actin cytoskeleton mediated by activation of p38 MAPK may modulate the apoptotic signal transduction. It has been shown that actin cytoskeleton provides an essential linkage in mediating signal transduction between the intracellular and extracellular compartments (53). Indeed, the mitochondria-mediated cell death pathway induced by activation of p38 MAPK proceeds through actin cytoskeletal reorganization and subsequent downregulation of the antiapoptotic signal transduction pathway and translocation of proapoptotic Bcl-2 family proteins (16, 27).

In the present study, we focused on the role of reorganization of actin cytoskeleton mediated by p38 MAPK activation during simulated ischemia-reoxygenation in necrosis and apoptosis in cultured neonatal rat CMCs. The results of the present study suggest that reorganization of actin cytoskeleton mediated by p38 MAPK differentially regulates apoptotic and necrotic death of CMCs during reoxygenation.

MATERIALS AND METHODS

Isolation and cultivation of neonatal rat CMCs. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996) and approved by the Animal Care Committee of Kansai Medical University. Neonatal rat CMCs were isolated and cultured as described previously (48). Briefly, neonatal rats were euthanized by decapitation, and their hearts were rapidly removed and placed into ice-cold Dulbecco’s modified Eagle’s medium (DMEM, GIBCO-BRL, Rockville, MD) containing penicillin G (300 U/ml), streptomycin (300 µg/ml), and amphotericin B (0.75 µg/ml, GIBCO-BRL). The ventricles were minced and incubated at 37°C for 10 min in Joklik’s modified Eagle’s minimal essential medium (DMEM) with 10% fetal calf serum (FCS). The precipitate was removed and incubated in buffer with 0.01% collagenase at 37°C for 10 min. These procedures were repeated four to five times. The supernatants were centrifuged at 600 g and resuspended in DMEM with 10% FCS. Cells were plated for 30 min to reduce the number of contaminating non-CMCs. CMCs were pooled, counted, and incubated in DMEM with serum. Bromodeoxyuridine (100 µM) was included in the incubation medium to inhibit proliferation of non-CMCs. CMCs (2 × 10⁶ cells) were plated on a 60-mm culture dish and incubated for 48 h at 37°C under 5% CO₂ in a tissue culture incubator.

Experimental protocols. Experimental protocols are shown in Fig. 1. CMCs were cultured overnight in Krebs-Henseleit bicarbonate (KHB) buffer solution containing (in mM) 118 NaCl, 4.7 KCl, 1.7 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, 25 NaHCO₃, and 10 glucose, pH 7.4, when incubated in 5% CO₂. CMCs were then treated with the vehicle (0.05% dimethyl sulfoxide, control), 10 µM SB-203580 (Alexis), or 1 µM cytochalasin D (Sigma) and incubated for 165 min under a normoxic and isosmotic condition or under a hyposmotic condition (140 mosM with reduced NaCl) to produce physical stress for the last 30 min of normoxic incubation. Simulated ischemia was created in GasPak (Becton Dickinson) in modified KHB buffer solution in which 10 mM glucose was replaced by 2-deoxy-d-glucose. Reoxygenation was performed with the isosmotic KHB or the hypoxic modified KHB for 30 min or the isosmotic KHB for 15 min followed by the hyposmotic KHB for 15 min. In the simulated ischemia-reoxygenation protocol, the vehicle (control), SB-203580, or cytochalasin D was administered for 15 min before and during 2 h of simulated ischemia and during reoxygenation.

Immunoblot analysis. After 2 h of normoxic incubation or simulated ischemia and 15 min after reoxygenation, CMCs were treated with lysis buffer containing (in mM) 150 NaCl, 10 Tris (pH 7.5), 1 EDTA, 1 EGTA, 1 β-glycerophosphate, 0.5 phenylmethylsulfonyl fluoride, 1 Na₃VO₄, and 50 NaF, and a protease inhibitor cocktail (Complete, Roche). Cells were then scraped off the dishes and drawn into a 3-ml syringe fitted with a 27-gauge needle to further lyse the cells. The lysate solution was then centrifuged at 14,000 g for 15 min at 4°C, and the supernatant was transferred into a new tube and stored at −80°C until needed. Because SB-203580 does not block the phosphorylation of p38 MAPK but binds reversibly to its catalytic site, it can be washed away during the p38 MAPK activity assay (55). Therefore, we determined phosphorylation of MAPKAPK2 to evaluate the effect of SB-203580 on p38 MAPK activity. Immunoblot analyses for phospho-MAPKAPK2 and MAPKAPK2 were performed with the use of antibodies specific for phospho-MAPKAPK2 and MAPKAPK2 (Cell cytotechnology, San Diego, CA).

Fig. 1. Experimental protocol. Cultured neonatal rat cardiomyocytes (CMCs) were incubated for 165 min under a normoxic and isosmotic condition (solid bar) or under a hyposmotic condition (open bar) for the last 30 min of normoxic incubation. CMCs were subjected to 2 h of simulated ischemia (SI, shaded bar) followed by reoxygenation for 30 min under an isosmotic or hyposmotic condition for the last 30 min of reoxygenation. SB-203580 (SB) and cytochalasin D (CD) were administered for 15 min before and during SI and during reoxygenation.
Signaling), respectively, and the ratio of immunoreactivity for phospho-MAPKAPK2 and MAPKAPK2 was quantified by densitometric analysis with the use of the image analyzing software system Win Roof (Mitani).

For the immunoblot assay of HSP27 and phospho-HSP27, CMCs were treated with Tris-Triton extraction buffer containing 100 mM Tris·HCl, pH 7.4, 2% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, and a protease inhibitor cocktail. Cells were scraped off the dishes and drawn into a syringe as described above. The lysate solution was centrifuged at 15,000 g for 5 min at 4°C. The Triton X-100-insoluble pellet was designated as the cytoskeletal fraction, and the supernatant was designated as the soluble fraction. The Triton X-100-soluble and -insoluble fractions were analyzed for nonphosphorylated and phosphorylated HSP27 by immunoblot analysis with the use of rabbit polyclonal antibodies against HSP27 and phospho-HSP27 (Santa Cruz Biotechnology).

To quantify cytochrome c release, immunoblot analyses of cytochrome c in the mitochondria and the cytosol fractions were performed as described previously (48). Briefly, at designated time points after exposure to experimental treatments, CMCs (2 × 10⁶ cells) were trypsinized and then washed with ice-cold buffer A (in mM: 250 sucrose, 20 HEPES-KOH, 1 EDTA, and 1 EGTA, and the protease cocktail, pH 7.4). Cells were resuspended in 200 μl of buffer A and carefully homogenized with the use of a Teflon homogenizer. The homogenate was separated into the cytosol and the mitochondrial fractions by differential centrifugation. Mitochondrial (5 μg) and cytosolic (25 μg) proteins were subjected to immunoblot analysis with the use of cytochrome c antibodies (Santa Cruz Biotechnology), and the immunoreactive band was quantified by densitometric analysis.

Confocal fluorescence microscopy. To visualize F-actin, CMCs were plated on fibronectin-coated slides. They were then fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. F-actin was detected with the use of tetramethylrhodamine isothiocyanate-conjugated phalloidin (Molecular Probes). Cells were examined by confocal laser microscopy (Olympus Fluov View).

To stain mitochondria and cytochrome c, CMCs were treated with 0.5 μM FITC-conjugated MitoTracker (Molecular Probes) for 30 min, washed with PBS, fixed with 4% paraformaldehyde. After permeabilization with 0.1% Triton X-100 in PBS, the slides were incubated with anti-cytochrome c antibodies (Santa Cruz Biotechnology) and tetramethylrhodamine isothiocyanate-conjugated secondary antibodies for 2 h incubation each. Saponin (0.2%, wt/vol) was added with the immunostaining reagents to enhance antibody accessibility. Cells were examined by confocal laser microscopy.

Caspase-3 activity assay. Caspase-3 activity in CMCs was evaluated by a colorimetric assay method as described previously (48). Briefly, CMCs (2 × 10⁶ cells) were suspended in 50 μl of cold cell lysis buffer, incubated on ice for 10 min, centrifuged for 1 min at 10,000 g, diluted to 50 μg of protein, added to 50 μl of reaction buffer and 5 μl of the 4 mM Asp-Glu-Val-Asp-p-nitroanilide (DEVD-pNA) substrate (200 μM of final concentration), and incubated at 37°C for 1 h. The pNA fluorescence was analyzed with the use of a microtiter plate reader (Bio-Rad) at an emission wavelength of 405 nm.

DNA fragmentation assay. DNA fragmentation in CMCs was quantitatively measured with a Cell Death Detection ELISA kit (Roche) as described previously (48). Briefly, CMCs (2 × 10⁶ cells) were centrifuged for 10 min at 200 g. The supernatant of the medium was stored at 4°C. The pellet was incubated in the cell lysis buffer for 30 min and centrifuged for 10 min at 200 g. The immunoreagent (biotin-labeled mouse monoclonal antibodies against histone and peroxidase-conjugated mouse monoclonal antibodies against DNA) were added to 20 μl of the cell lysate or the supernatant and incubated for 2 h. The 2.2′-azino-di(3-ethylbenzthiazolin-sulfonate) substrate was then added, and the fluorescence was analyzed with the use of a microtiter plate reader (Bio-Rad) at an emission wavelength of 405 nm.

Lactate dehydrogenase release. CMC necrosis was determined by lactate dehydrogenase (LDH) release in the culture medium. LDH was measured with the use of an LDH assay kit (Sigma).

Measurement of ATP content of CMCs. ATP content of CMCs was measured as described previously (48). Briefly, CMCs were treated with 500 μl of 0.6 N ice-cold perchloric acid and centrifuged at 3,000 g for 5 min at 4°C. The supernatants were neutralized with KOH and the extracts were centrifuged at 13,000 g for 5 min at 4°C to remove the KClO4 precipitates. The supernatant was assayed for ATP with the use of high-performance liquid chromatography (Tosho Techno System).

Statistical analysis. All numerical data are expressed as means ± SE. Statistical analysis was performed by one-way ANOVA followed by the Bonferroni post hoc test. The differences were considered significant at a P value of <0.05.

RESULTS

Simulated ischemia and reoxygenation activates p38 MAPK. p38 MAPK was significantly activated 2 h after simulated ischemia and during reoxygenation as evaluated by phosphorylation of MAPKAPK2 (Fig. 2). p38 MAPK activation induced by simulated ischemia and reoxygenation was abrogated by treatment with SB-203580.

Simulated ischemia and reoxygenation promotes translocation of HSP27 to cytoskeletal fraction. HSP27 is an immediate downstream substrate of MAPKAPK2 (18). Therefore, we measured phosphorylation of HSP27 and its redistribution during reoxygenation. In the control CMCs, the majority of HSP27 was localized in the cytosolic fraction (Fig. 3). However, HSP27 was increased in the cytoskeletal fraction during simulated ischemia and reoxygenation associated with a reciprocal decrease in HSP27 in the cytosolic fraction. Phospho-HSP27 was markedly increased in the cytoskeletal fraction during simulated ischemia and reoxygenation. Treatment with SB-203580 during simulated ischemia and reoxygenation abolished translocation of HSP27 from the cytosolic to the cytoskeletal fraction and an increase in phospho-HSP27 in the cytoskeletal fraction. Because SB-203580 is also a potent...
inhibitor of c-Jun NH₂-terminal kinase (JNK) (13), it is possible that the inhibitory effect of SB-203580 on translocation of HSP27 was due to the inhibition of JNK. However, a JNK-specific inhibitor, SP-600125, at a concentration that successfully inhibited JNK activation after reoxygenation, did not inhibit translocation of HSP27 from the cytosolic to the cytoskeletal fraction (data not shown).

**SB-203580 and cytochalasin D inhibit F-actin reorganization during reoxygenation.** p38 MAPK activation is known to induce F-actin reorganization (21). Therefore, we studied the effect of hypoxia and reoxygenation on actin cytoskeleton. Actin fibrils stained with rhodamine phalloidin demonstrated a striated pattern of sarcomeric actin distribution in the normoxic CMCs (Fig. 4A). No noticeable effect of SB-203580 on organized sarcomeric appearance was observed under the normoxic condition (Fig. 4B). Cytochalasin D, which is known to disrupt actin cytoskeleton primarily by retarding the polymerization rate of active actin filaments with a modest effect on a more stabilized pool of actin filaments (12, 41), disrupted a sarcomeric pattern of actin filaments (Fig. 4C). F-actin formed thick bundles over the sarcomeric actin fibers after simulated ischemia followed by 10 min of reoxygenation when p38 MAPK was maximally activated (Fig. 4D). Treatment with SB-203580 during simulated ischemia and reoxygenation abolished F-actin reorganization (Fig. 4E). F-actin was observed as only punctate structures in CMCs treated with cytochalasin D during simulated ischemia and reoxygenation (Fig. 4F).

**SB-203580 and cytochalasin D inhibit cytochrome c release during simulated ischemia-reoxygenation.** Confocal laser microscopy demonstrated colocalization of cytochrome c with...
mitochondria as a punctate staining pattern in the normoxic CMCs (Fig. 5A). Cytochrome c was diffusely distributed in the cytosol in addition to colocalization with mitochondria in CMCs subjected to 2 h simulated ischemia followed by 15 min reoxygenation (Fig. 5B). Immunoblot analysis demonstrated that virtually no cytochrome c was detected in the cytosol fraction in the normoxic CMCs irrespective of whether CMCs were treated with SB-203580 or cytochalasin D (Fig. 5C). However, cytochrome c was significantly increased in the cytosol fraction during simulated ischemia, and cytosolic cytochrome c was further increased during reoxygenation without a significant decrease in cytochrome c in the mitochondrial fraction. The increase in cytochrome c in the cytosolic fraction was significantly inhibited by SB-203580 and cytochalasin D.

SB-203580 and cytochalasin D prevent caspase-3 activation and DNA fragmentation during simulated ischemia and reoxygenation. Caspase-3 activity (Fig. 6A) and DNA fragmentation (Fig. 6B) were only slightly increased during simulated ischemia but were markedly increased during reoxygenation. Treatment with SB-203580 and cytochalasin D significantly inhibited caspase-3 activation and DNA fragmentation during reoxygenation.

SB-203580 and cytochalasin D increase LDH release during reoxygenation under hyposmotic condition. Necrosis is characterized by the disintegration of cell membrane that may be unmasked by reintroduction of contractile force on reperfusion in the intact heart. However, our cultured CMCs did not contract after reoxygenation and were not exposed to mechanical stress. Therefore, we evaluated LDH release in the presence or absence of osmotic stress, which imposes mechanical stress on the sarcolemma similar to that found during in situ reperfusion after prolonged coronary occlusion (3, 37). No significant increase in LDH release was observed by any treatment modalities under the hyposmotic condition when CMCs were incubated under the normoxic condition (Fig. 7). Although no significant increase in LDH release was observed during simulated ischemia under the isosmotic condition, LDH release was significantly increased during simulated ischemia under the hyposmotic condition. This increase in LDH release was augmented by treatment with SB-203580 or cytochalasin D. A small but significant increase in LDH release occurred in the vehicle-treated CMCs during reoxygenation under the isosmotic condition. Hyposmotic reoxygenation enhanced LDH release in the vehicle-treated CMCs and provoked more drastic increase in LDH release in CMCs treated with SB-203580 or cytochalasin D.

Temporary isosmotic reoxygenation reverses osmotic fragility by SB-203580 and cytochalasin D. Finally, we have tested the hypothesis that p38 MAPK inhibition facilitates a repair process of osmotic fragility during reoxygenation under the isosmotic condition by increasing ATP content of CMCs. This idea comes from our recent study (46) demonstrating that the treatment with SB-203580 during reperfusion under contractile arrest improved mitochondrial function and mitigated sarcosomal fragility and prevented CMC necrosis after reintroduction of contractile activity in the isolated and perfused rat heart.
heart. To this end, SB-203580- or cytochalasin D-treated CMCs were reoxygenated for 15 min under the isosmotic condition followed by hyposmotic incubation for 15 min. ATP content was significantly decreased after simulated ischemia and reoxygenation in all the groups of CMCs (Fig. 8A). However, ATP content of CMCs treated with SB-203580 or cytochalasin D during reoxygenation under the isosmotic condition was significantly higher than the vehicle-treated CMCs. On the contrary, treatment with SB-203580 or cytochalasin D significantly decreased ATP content of CMCs compared with the vehicle-treated CMCs during hyposmotic reoxygenation. However, isosmotic incubation for the first 15 min of reoxygenation significantly increased ATP content in CMCs treated with SB-203580 or cytochalasin D compared with the vehicle-treated CMCs associated with significant inhibition of LDH release after conversion to the hyposmotic condition (Fig. 8B). In contrast to LDH release, hyposmotic reoxygenation reduced caspase-3 activity and DNA fragmentation in all groups of CMCs compared with isosmotic reoxygenation (Fig. 8, C and D). Isosmotic reoxygenation followed by hyposmotic aerobic incubation restored caspase-3 activity and DNA fragmentation to the same level as observed under isosmotic reoxygenation.

DISCUSSION

We investigated the role of p38 MAPK activation in apoptosis and necrosis of cultured neonatal CMCs subjected to simulated ischemia and reoxygenation. We found that p38 MAPK plays a differential role in apoptosis and necrosis in CMCs during reoxygenation depending on the presence or absence of osmotic stress. p38 MAPK activity was increased during simulated ischemia and reoxygenation. This was associated with phosphorylation and translocation of HSP27 from the cytosol to the cytoskeletal fraction and reorganization of F-actin. SB-203580 inhibited p38 MAPK activation, phosphorylation, and translocation of HSP27 and reorganization of F-actin, whereas cytochalasin D inhibited reorganization of F-actin induced by p38 MAPK activation. SB-203580 and cytochalasin D inhibited cytochrome c release from mitochondria during simulated ischemia and

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Fig. 7. Lactate dehydrogenase (LDH) release. Vehicle (0.05% dimethyl sulfoxide), SB (10 μM), or CD (1 μM) was administered for 15 min under normoxic condition or for 15 min before and during SI or for 15 min before and throughout SI and reoxygenation. Isosmotic (solid bar) and hyposmotic (open bar) conditions are shown. Each bar graph represents means ± SE of 5 experiments. *P < 0.05 compared with normoxia vehicle; †P < 0.05 compared with SI vehicle; #P < 0.05 compared with reoxygenation vehicle.

Fig. 8. A: ATP content. B: LDH release. C: caspase-3 activity. D: DNA fragmentation. These parameters were measured after normoxic incubation or 30 min after reoxygenation under isosmotic, hyposmotic, or 15 min of isosmotic followed by 15 min of hyposmotic condition (Iso/Hypo). Vehicle (0.05% dimethyl sulfoxide, solid bar), SB (10 μM, open bar), or CD (1 μM, shaded bar). Each bar graph represents means ± SE of 5 experiments. *P < 0.05 compared with isosmotic vehicle; †P < 0.05 compared with hyposmotic vehicle; #P < 0.05 compared with isosmotic-hyposmotic vehicle.
reoxygcnation and prevented caspase-3 activation and DNA fragmentation during reoxygcnation. Robust LDH release was not observed during reoxygcnation under the isosomotic condition. However, marked LDH release was induced by hypsomotic reoxygcnation, and this was enhanced by treatment with SB-203580 or cytochalasin D. Although care must be taken to interpret the data obtained from experiments that use these drugs with multiple nonspecific actions, these results are consistent with the hypothesis that F-actin reorganization plays a differential role in p38 MAPK-mediated apoptosis and necrosis during simulated ischemia and reoxygcnation in neonatal rat CMCs.

Role of F-actin reorganization in p38 MAPK-mediated apoptosis. It has been shown that p38 MAPK mediates mitochondrial dysfunction and triggers activation of the cell death pathway (10, 16, 54). However, the mechanism of p38 MAPK-mediated apoptosis has not been fully elucidated. Oxidative stress is known to play a crucial role in apoptotic signal transduction both upstream and downstream of p38 MAPK activation (14, 49), suggesting that oxidative stress promotes an amplifying loop for p38 MAPK activation and the apoptotic death pathway. Of the p38 MAPK-mediated signal transduction elements, phosphorylation of HSP27 seems to play a key role in switching the cellular redox state from antioxidation to prooxidation. It has been shown that the phosphorylation status of HSP27 is intimately related to cellular resistance against oxidative stress. Only unphosphorylated HSP27 behaves as the molecular chaperon that confers resistance against oxidative stress (33, 40). Indeed, oxidative stress after ischemia and reperfusion was prevented in nonphosphorylatable HSP27 mutant but not in the wild-type HSP27 transgenic mouse heart (19). Because phosphorylation of HSP27 is associated with the reorganization of actin cytoskeleton (18), it is suggested that p38 MAPK-mediated apoptotic signal transduction proceeds through reorganization of actin cytoskeleton. Our study demonstrating that cytochalasin D inhibited cytochrome c release, caspase-3 activation, and DNA fragmentation supports the role of actin cytoskeleton in apoptotic signal transduction. In this context, it would be intriguing to assume that cytoskeletal actin reorganization is involved in the translocation of proapoptotic Bcl-2 family proteins that has been implicated in p38 MAPK-mediated apoptosis in nitric oxide-induced neuronal cell injury (16). On the other hand, reorganization of actin cytoskeleton is involved in cardioprotective signal transduction mediated by ischemic preconditioning (7). Thus the ultimate effect of p38 MAPK activation-mediated cytoskeletal actin reorganization may be determined by the balance of signaling strength between cell survival and death.

Role of F-actin reorganization in p38 MAPK-mediated necrosis. In addition to the proapoptotic role of p38 MAPK, a growing body of evidence also indicates that activation of this kinase is cytoprotective and that the cytoprotective role of p38 MAPK is attributed to phosphorylation of HSP27 and subsequent reorganization of F-actin (2, 20, 22, 52). Because phosphorylation of HSP27 dissipates molecular chaperon activity and renders cells susceptible to oxidative stress and apoptosis, the mechanism of cytoprotection conferred by phosphorylation of HSP27 should be different from that mediated by the unphosphorylated form of HSP27. This assumption is reminiscent of the recent study (19) demonstrating that transgenic overexpression of wild-type HSP27 or nonphosphorylatable HSP27 provided equal protection against ischemia-reperfusion injury in the mouse heart. The unphosphorylated form of HSP27 acts as F-actin cap-binding proteins and inhibits actin polymerization, hence providing a mechanism to explain the in vivo observations that phosphorylation of HSP27 regulates actin polymerization and modulates filament stability (28). This reorganization of F-actin appears to be involved in resistance against actin fragmentation and cell death induced by oxidative stress (22). Similarly, the importance of the actin cytoskeletal reorganization in maintaining cell structure and function was proposed by the Aoudjit et al. (2), who demonstrated that phosphorylation of HSP27 was protective against complement-induced disruption of the actin cytoskeleton in glomerular epithelial cells. Our study also points to the conclusion that physical reinforcement of actin cytoskeleton by p38 MAPK-mediated F-actin reorganization confers resistance against osmotic fragility during reoxygcnation in cultured neonatal CMCs. We demonstrated that SB-203580 and cytochalasin D aggravated necrosis under the hypsomotic condition. Although the mechanism of osmotic fragility during lethal ischemia and reperfusion has not been completely understood, recent studies (5, 6, 25), including our own, suggest that loss of dystrophin and β-dystroglycan, both of which play a crucial role in stabilizing sarcolemma, during ischemia and reperfusion predisposes the sarcolemmal fragility that is only unmasked by imposing physical stress. Therefore, it is suggested that cytoskeletal actin reorganization mediated by p38 MAPK activation strengthens the fragile sarcolemmal membrane and prevents sarcolemmal injury on reintroduction of mechanical force at the time of reperfusion or reoxygcnation.

Temporary inhibition of physical stress at the time of reoxygcnation confers resistance to necrosis in CMCs treated with SB-203580 and cytochalasin D. Osmotic fragility is known to be associated with loss of ATP in a variety of cell types. We have, therefore, thought that osmotic fragility can be reversed by maneuvers that increase cellular ATP content. Because SB-203580 and cytochalasin D prevented cytochrome c release during simulated ischemia and reoxygcnation under the hypsomotic condition, it is suggested that mitochondrial permeability transition pore opening, which is a central executor of mitochondria-mediated death pathway and mitochondrial dysfunction (17, 26), was inhibited by treatment with these drugs under the isomotic condition. Such improvement of mitochondrial function should be associated with an increase in ATP content in CMCs and prevention of necrosis during reoxygcnation if physical stress-induced sarcolemmal disruption is prevented at the time of reoxygcnation. When SB-203580- and cytochalasin D-treated CMCs were reoxygcnated for the first 15 min under the isomotic condition and were then switched to the hypsomotic condition, ATP content in CMCs was increased and LDH release was prevented, suggesting that temporary reoxygcnation without physical stress was indeed capable of replenishing ATP and conferring resistance to osmotic stress in CMCs treated with SB-203580 or cytochalasin D. This conversion of CMCs from injurious to cardioprotective phenotype by treatment with SB-203580 or cytochalasin D under the isomotic condition is most likely to be explained by an enhanced repair process of sarcolemmal fragility through the improvement of mitochondrial function in the absence of...
osmotic stress that otherwise induces sarcolemmal membrane dysfunction at the time of reoxygenation and aggravation of mitochondrial dysfunction by allowing unlimited entry of Ca^{2+} into mitochondria.

In contrast to LDH release, hyposmotic reoxygenation reduced caspase-3 activity and DNA fragmentation in all groups of CMCs compared with isosmotic reoxygenation. Isosmotic reoxygenation followed by hyposmotic aerobic incubation restored caspase-3 activity and DNA fragmentation to the same level as observed under isosmotic reoxygenation. The mechanism by which hyposmotic reoxygenation inhibits caspase-3 activation and DNA fragmentation is currently unclear. However, a possible explanation is that enhanced necrosis and severe ATP depletion during hyposmotic reoxygenation inhibit an apoptotic cascade, because apoptosis is an energy-dependent process in that loss of ATP converts the form of cell death from apoptosis to necrosis (44). Furthermore, the decrease of DNA fragmentation under the condition of enhanced LDH release may preclude the occurrence of necrotic DNA degradation as a predominant source of fragmented DNA in our experimental model. However, more specific methods for quantitative detection of apoptosis such as in situ oligoligation assay would be necessary to unequivocally discriminate apoptosis from necrosis.

**Study limitations.** In this study, we used SB-203580 as an inhibitor of p38 MAPK. Because SB-203580 can inhibit both the \( \alpha \)- and the \( \beta \)-isofoms, the differential effect of SB-203580 on apoptosis and necrosis may arise from the differential role of these isoforms in apoptotic signal transduction and actin cytoskeletal reorganization. The \( \alpha \)- and the \( \beta \)-isofoms are known to exert a direct effect on myocyte function and survival (51). It has been reported that neonatal rat CMCs expressing the dominant negative p38\( \alpha \) are resistant to lethal simulated ischemia (42). Thus it is possible that p38\( \alpha \) is involved in apoptotic CMC injury, whereas p38\( \beta \) is responsible for cytoskeletal reorganization through phosphorylation of HSP27. Nevertheless, this issue has not been investigated thus far and needs to be addressed in the future by using p38 MAPK gene manipulation techniques. Another problem related to the use of SB-203580 is that this compound can also inhibit JNK, which is activated by the same cellular stress as activation of p38 MAPK (13). Thus the role of JNK in apoptotic signal transduction and protection against osmotic fragility cannot be excluded at present.

In conclusion, the present study suggests that F-actin reorganization plays a differential role in p38 MAPK-mediated apoptosis and necrosis during simulated ischemia and reoxygenation in neonatal rat CMCs. F-actin cytoskeleton is involved in apoptotic signal transduction when p38 MAPK is activated during simulated ischemia and reoxygenation, whereas F-actin reorganization protects CMCs with fragile sarcolemmal membrane from osmotic stress-induced necrosis. However, p38 MAPK inhibition reverses sarcolemmal membrane fragility during reoxygenation, presumably through improvement of mitochondrial function and an increase in ATP generation when osmotic stress is temporarily eliminated. Future studies are warranted to explore how these in vitro observations are translated to the clinical setting of cardioprotection against ischemia and reperfusion injury.


