An Inhibitor of p38 Mitogen-activated Protein Kinase Protects Neonatal Cardiac Myocytes from Ischemia*

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Cellular ischemia results in activation of a number of kinases, including p38 mitogen-activated protein kinase (MAPK); however, it is not yet clear whether p38 MAPK activation plays a role in cellular damage or is part of a protective response against ischemia. We have developed a model to study ischemia in cultured neonatal rat cardiac myocytes. In this model, two distinct phases of p38 MAPK activation were observed during ischemia. The first phase began within 10 min and lasted less than 1 h, and the second began after 2 h and lasted throughout the ischemic period. Similar to previous studies using in vivo models, the nonspecific activator of p38 MAPK and c-Jun NH2-terminal kinase, anisomycin, protected cardiac myocytes from ischemic injury, decreasing the release of cytosolic lactate dehydrogenase by approximately 25%. We demonstrated, however, that a selective inhibitor of p38 MAPK, SB 203580, also protected cardiac myocytes against extended ischemia in a dose-dependent manner. The protective effect was seen even when the inhibitor was present during only the second, sustained phase of p38 MAPK activation. We found that ischemia induced apoptosis in neonatal rat cardiac myocytes and that SB 203580 reduced activation of caspase-3, a key event in apoptosis. These results suggest that p38 MAPK induces apoptosis during ischemia in cardiac myocytes and that selective inhibition of p38 MAPK could be developed as a potential therapy for ischemic heart disease.

The heart is subjected to episodes of ischemia followed by reperfusion in a number of situations, including angina, myocardial infarction, and cardiac surgery, and these stresses can result in cell injury and death. Part of the cellular response to ischemia/reperfusion is activation of several members of the mitogen-activated protein kinase (MAPK) family. In many different cell types, p38 MAPK and c-Jun NH2-terminal kinase (JNK) family members are activated predominantly by cellular stresses or inflammatory signals, e.g. hyperosmolarity, chemical or heat stress, endotoxin, and cytokines (1–4), whereas the extracellular signal-regulated kinase (ERK) family is activated by ischemia, this stress results in translocation of JNK1 to the nucleus, where it is then phosphorylated and activated on reperfusion (9). Ischemia and reperfusion also activate members of the MAPK family in kidney and liver differentially (7, 10, 11). However, it is not clear from these studies whether activation of these kinases is part of the protective response of the cell or if these signals mediate the cellular damage and death caused by ischemia or ischemia/reperfusion. Evidence suggests that myocardial ischemic cell death occurs by both apoptosis and necrosis (12, 13). From the timing of p38 MAPK activation during ischemia and initiation of apoptosis, Yin et al. (7) speculate that activation of p38 MAPK initiates the signal for apoptotic cell death. Indeed, p38 MAPK activation has been implicated in mediating apoptosis in several cell types (14–16). Recent studies in neonatal rat cardiac myocytes support a role for the α isoform of p38 MAPK in mediating apoptosis; overexpression of activated MAPK kinase 3b, which phosphorylates and activates p38 MAPK, induces apoptosis that is increased by coexpression of p38α and is decreased by expression of a dominant negative form of this isoform (17).

In contrast, a separate study demonstrates that activation of p38 MAPK can prevent apoptosis in neonatal rat cardiac myocytes (18). Furthermore, others have proposed that p38 MAPK activation mediates a phenomenon termed preconditioning, which confers cardiac protection from ischemia. Preconditioning is a highly effective method of protecting the heart from ischemic damage by subjecting it to sublethal periods of ischemia before the prolonged ischemia (6, 8, 19, 20). A protective function for p38 MAPK is supported by a recent study in which the role of p38 MAPK in preconditioning was examined in isolated rabbit cardiac myocytes (21). Pretreatment with anisomycin, an activator of p38 MAPK, protects isolated rabbit cardiac myocytes against ischemia-induced cell fragility, leading to the suggestion that p38 MAPK protects the heart against ischemia. The addition of SB 203580, a selective inhibitor of p38 MAPK (22, 23), during a preconditioning treatment abolishes the protective effect of preconditioning, supporting the initial observation (21). Similar results, showing that SB 203580 inhibits the protection afforded by ischemic preconditioning against myocardial infarction, were obtained using isolated rat hearts (24).

Most previous ischemia studies have investigated MAPK activation in whole heart, which contains a large proportion of non-myocyte cells, mainly fibroblasts and endothelial cells. In the present study, we used primary cultures of neonatal rat cardiac myocytes and confirmed that p38 MAPK is activated in...
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a model of ischemia which uses a glucose-free hypoxic incubation. We report that activation of p38 MAPK occurred in two distinct phases and that inhibition of p38 MAPK during the second phase protected cardiac myocytes from ischemic injury. These results are consistent with the hypothesis that sustained p38 MAPK mediates ischemia-induced cell injury and death in neonatal rat cardiac myocytes.

EXPERIMENTAL PROCEDURES

Reagents—All antibodies were used according to manufacturers’ protocols. Amylase (Sigma) was dissolved in dimethyl sulfoxide (Me2SO). 5 mg were added to give a final Me2SO concentration less than 0.1%. SB 203580 (Calbiochem) was dissolved in Me2SO at 10 mg and used to give final a Me2SO concentration less than 0.1%

Culture of Ventricular Myocytes—Primary cultures of ventricular myocytes from I-day-old Sprague-Dawley rats were performed by gentle, serial trypsinization, as described previously (25) with modifications (26). A preplating step was included to reduce the number of contaminating non-myocytes. Myocytes were plated at 800 cells/mm² in 35- or 60-mm dishes (Falcon). Myocytes represented 90–95% of total adhering cells. Division of non-myocytes was prevented by the addition of 0.1 mg bromodeoxyuridine for the first 4 days of culture. Cells were maintained at 37 °C in a 1% CO2 incubator in M-199 medium (Life Technologies, Inc.) containing 10% fetal bovine serum (Hyclone), maintained with 0.2–0.5% O2, 1% CO2, and the balance N2. Medium was aspirated and discarded. Cells were washed once with cold phosphate-buffered saline and cell lysate (retained LDH) using a spectrophotometric assay stored at 4 °C. LDH activity was measured from both medium (released LDH) and cell lysate (retained LDH) and used to give final a Me2SO concentration less than 0.1%

Induction of Ischemia—Ischemia was induced in a humidified 37 °C incubator within an air-tight Plexiglas glove box (Anaerobic Systems) maintained with 0.2–0.5% O2, 1% CO2, and the balance N2. Medium (defined minimal essential medium and Hank’s balanced salt solution without glucose) was equilibrated to low O2 within the glove box for at least 90 min before commencing experiments. Inside the glove box, cells were washed twice with warm, preequilibrated medium before the addition of incubation medium (1.5 ml/35-mm dish). O2 was measured using an electronic gas analyzer OXOR®II or Fyrite® (both from Bacharach). Lactate Dehydrogenase (LDH) Assay—After ischemic or normoxic treatments, incubation medium was stored at 4 °C, and the same volume of cold buffer (10 ml Tris-HCl, pH 7.4, 1 ml EDTA) was added to the cells. The cells were scraped and lysed by trituration. Lysates were centrifuged at 4 °C at 16,000 × g for 15 min, and the supernatant was stored at 4 °C. LDH activity was measured from both medium (released LDH) and cell lysate (retained LDH) using a spectrophotometric assay (Sigma). Briefly, filters were probed with anti-p38 MAPK. The ratio of phospho-ATF2 to total ATF2 was determined for each sample, and then results were expressed as fold activation over control.

Immunoprecipitation of Dual Phosphorylated p38 MAPK—Cardiac myocytes (one 100-mm dish/treatment) were treated, and then the incubation medium was aspirated and discarded. Cells were washed once with cold phosphate-buffered saline and then scraped into 800 µl of lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2 mg sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 25 µg/ml aprotinin, 25 µg/ml leupeptin, and 20 µg/ml soybean trypsin inhibitor) and lysed by trituration. Samples were extracted on ice for 15 min, and then cell debris was removed by centrifugation at 15,000 × g for 10 min. A sample of supernatant was retained for electrophoresis. 20 µl of dual phospho-p38 MAPK (Thr180 Tyr182) antibody (New England Biolabs) was added to the remainder of the supernatant, and samples were rotated overnight at 4 °C. 60 µl of a 50% slurry of protein A-Sepharose CL-4B (Amersham Pharmacia Biotech) was added, and the samples were rotated for 2 h at 4 °C. The immunoprecipitates were washed four times with phosphate-buffered saline, electrophoresed on 10% low ratio bisacrylamide SDS-PAGE, and Western blot analysis was performed. ATP2 was detected using electrophoresis state-independent antibody from Santa Cruz Biotechnology.

Densitometric Analysis—Autoradiographs were scanned using an ArcusII flatbed scanner (AGFA) with FotoLookPS 2.07.2, and the band density was analyzed by NIH Image™.

ATP2 Phosphorylation—Preparation of nuclear proteins from cardiac myocytes was performed exactly as described by Clerk and Sugden (28). Approximately 50 µg of nuclear protein was electrophoresed on 8% SDS-PAGE, and Western blot analysis was performed. ATP2 was detected using phosphorylation state-independent antibody from Santa Cruz Biotechnology.

Cell Viability Assay—Cell death from ischemic or normoxic incubations was assessed using two dyes that distinguish between live and dead cells. Calcein acetoxyethyl ester (calcein AM; Molecular Probes) and propidium iodide (PI) were added to the incubation medium at final concentrations of 2 µM and 1 µg/ml, respectively, and dishes incubated at 37 °C for 15 min (ischemic samples were maintained under ischemic conditions during this incubation). Cells were viewed using a Zeiss microscope and a 40 × objective and were scored as live (green cytosolic fluorescence from calcein AM) or dead (red nuclear fluorescence from PI).

Isolation of DNA and Agarose Gel Electrophoresis—After ischemic or normoxic incubation, cells were scraped into incubation medium to allow retention of any floating cells and were harvested by centrifugation. DNA was prepared by standard techniques (29). Identical amounts of DNA (2 µg) were electrophoresed through 1.8% agarose and DNA visualized on a UV transilluminator.

RESULTS

Activation of MAPKs and Stress-activated Kinases by Ischemia—Activation of MAPKs in primary neonatal rat cardiac myocytes in response to simulated ischemia was investigated.
The model used combines two properties of ischemia: decreased energy source, because incubations are performed in the absence of glucose, and hypoxia, with oxygen levels between 0.2 and 0.5%. p38 MAPK activation was estimated by Western blot analysis using an antibody that specifically recognizes the dual phosphorylated (on residues Thr180 and Tyr182), active form of the enzyme. Antibody recognizing p38 MAPK regardless of its phosphorylation state was used to normalize for differences in protein loading. This antibody is specific for the α isoform of p38 MAPK, and the level detected remained constant throughout ischemia. Dual phosphorylation of p38 MAPK was observed within 10 min of ischemia, remained maximal until 30 min, then decreased but remained above basal until 180 min of ischemia (Fig. 1, A and B). At later time points, dual phospho-
 phosphorylation increased again with a peak at 240 min and remained high for 420 min (Fig. 1, A and B).

We and others (30) have observed that when cardiac myocytes are fed with fresh medium, they cease contracting for a period of time. In our study, cells stopped contracting for approximately 20 min, then spontaneous contraction recovered gradually to a normal rate within 60–90 min. Because this corresponds with the timing of the first phase of p38 MAPK activation, and a change of medium is required to induce ischemic conditions, we examined p38 phosphorylation levels after changing incubation medium and maintaining cells under normoxic conditions. Transient phosphorylation of p38 MAPK was observed after simply feeding fresh medium (Fig. 1, C and D). Cardiac myocytes can be maintained healthily in culture for up to 8 days with multiple changes of medium. Although this does not show that the initial ischemic p38 MAPK activation and that induced by changing medium are equivalent, these results do demonstrate that transient activation of p38 MAPK can occur without long term harmful effects to cardiac myocytes.

To confirm that dual phosphorylation of p38 MAPK during ischemia truly reflected activation, p38 MAPK was immunoprecipitated and used in an in vitro kinase assay with recombinant ATF2 as a substrate. We observed more than a 6-fold increase in the phosphorylation of ATF2 over basal after 20 min of ischemia (Fig. 1E) and more than a 4-fold increase after 25 or 30 min of ischemia (n = 1, data not shown). A similar activation ratio was obtained by incubating cells with anisomycin, an activator of p38 MAPK (Fig. 1E). Thus, p38 MAPK is indeed activated during ischemia.

To determine if ischemia-induced phosphorylation is unique to p38 MAPK, we examined phosphorylation of other MAPKs. Phosphorylation of ERK2 (p42 MAPK) was estimated using a gel electrophoresis mobility shift assay with an antibody that detects both inactive (non-phosphorylated) and active (dual phosphorylated) ERK2. The reduced mobility form of ERK2, indicating phosphorylation as seen with 4β-phorbol 12-myristate 13-acetate treatment, was not observed at any time during prolonged ischemia (Fig. 2A). Similarly, probing with anti-active JNK, which detects the dual phosphorylated active forms of both JNK1 and JNK2, showed little or no activation of JNK in 10–240 min of ischemia (Fig. 2B) and no activation in 300, 360, or 420 min of ischemia (n = 1, data not shown).

Taken together, our results indicate that changing medium transiently activates p38 MAPK, whereas ischemia results in a transient, then sustained activation of p38 MAPK, but not ERK2 or JNK, in primary cultures of neonatal rat cardiac myocytes; these results are consistent with previous studies performed in whole heart (6–8).

**Significance of p38 MAPK Activation in Ischemia**—Release of the cytosolic enzyme LDH, caused by cell membrane leakage, was used to assess cell damage resulting from ischemia. Under the conditions used in this study, 7–9 h of ischemic incubation resulted in the release of between 45 and 60% of cellular LDH,
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Results were expressed as LDH released into the medium as a percent of the total LDH activity and then normalized to 100% for vehicle-treated samples. Panel B, as in panel A, except that for the left three bars, 10 μM SB 203580 (SB) was added 45 min after the start of ischemic incubation or at the start of ischemia for comparison. For the right panel, 10 μM SB 203580 or 0.1% Me2SO vehicle was added at the start of ischemia. After 46 min, the incubation medium was removed from all dishes, and the cells were washed twice with fresh medium, pre-equilibrated to hypoxic conditions. Cells were then incubated with vehicle or 10 μM SB 203580 for the remainder of ischemia, as indicated. For both panels, data represent the mean (±S.E.) from three experiments performed in duplicate. *p < 0.001 versus vehicle-treated sample. ns indicates that the difference is not significant. Panel C, cardiac myocytes were incubated in ischemia or normoxia in the presence of 10 μM SB 203580 or 0.1% Me2SO vehicle for 7–8 h, as indicated. Cells were stained with calcein AM and PI and then scored as live (green cytosolic fluorescence) or dead (red nuclear fluorescence). Data represent the percent of viable cells and are plotted as the mean (±S.E.) from three experiments. Each experiment, more than 800 cells were counted for each treatment. *p < 0.05.
from cell death, we used a cell viability assay. Live cells are distinguished by the conversion of calcein AM to fluorescent calcein by intracellular esterases, but the intact membrane excludes PI. Dead cells are distinguished by entry of PI through damaged membrane and fluorescence of PI on binding to nucleic acid. We demonstrated that coinubcation of myocytes with 10 μM SB 203580 during ischemia significantly decreased cell death at two time points (Fig. 4C). It is important to note that cell death is delayed but not prevented; after 8 h of ischemia, cell death is reduced significantly by the presence of the inhibitor, but it is increased from basal cell death under normoxic conditions. Thus, inhibition of p38 MAPK delays cell injury and death resulting from ischemia.

Because it appeared that the consequence of the first and second phases of p38 MAPK activation differed, we attempted to determine whether the same or different isoforms of p38 MAPK are activated during these two different phases. By immunoprecipitation of dual phospho-p38 MAPK (Thr180 Tyr182; this antibody detects both activated α and β isoforms of p38 MAPK) at different times during ischemia, we determined that p38α is activated in both phases during ischemia (Fig. 5). p38β was only weakly detected in cell lysates, and therefore we could not determine if p38β was also present in any of the immunoprecipitates.

**Ischemia Results in Apoptosis in Neonatal Rat Cardiac Myocytes**—Staining with PI as described above in the cell viability assay allows visualization of nuclei and could potentially be used to distinguish morphologically between apoptotic and necrotic cells. Apoptotic cells generally show condensed, fragmented nuclei, whereas necrotic cells have normal nuclei. However, after ischemia in this cell type, many nuclei appeared slightly condensed but not fragmented and therefore were difficult to classify clearly as apoptotic or necrotic.

Previous studies have documented both apoptosis and necrosis induced by myocardial infarction (12, 13). To examine if our model of ischemia induces apoptosis in cardiac myocytes, cells were subjected to a DNA laddering assay to assess the extent of DNA fragmentation. Identical amounts of DNA, from cells treated under either normoxic or ischemic conditions for 8 h, were visualized after agarose gel electrophoresis. In control samples incubated under normoxic conditions, a low level of basal DNA fragmentation was observed (Fig. 6A). However, in the ischemic sample, there was a decrease in high molecular weight DNA and a corresponding increase in low molecular weight DNA when compared with the normoxic sample (Fig. 6A). The low molecular weight DNA both in normoxia and ischemia showed the hallmark intranucleosomal laddering of apoptosis. Therefore, although it was not possible to quantitate apoptosis versus necrosis, we clearly demonstrated that ischemia induces apoptosis in neonatal cardiac myocytes. These data do not rule out the possibility that some cells undergo necrosis during ischemia.

**SB 203580 Inhibits Activation of Caspase-3**—To confirm that inhibition of p38 MAPK by SB 203580 delayed apoptosis, we examined the activation state of caspase-3 (also known as CPP32, Yama, or apopain). This enzyme has been identified as a key protease during the early stages of apoptosis and is activated by degradation of the 32-kDa proenzyme to approximately 17- and 12-kDa subunits that heterodimerize to give active enzyme (32). Using Western blot analysis, we determined that caspase-3 degradation is delayed in cardiac myocytes treated with SB 203580 compared with vehicle-treated cells (Fig. 6B). Therefore, apoptosis is delayed by inhibition of...
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In response to ischemia, cells activate biochemical pathways that allow adaptation to this stressful environment. However, on prolonged ischemia, these protective mechanisms may not be sufficient to maintain normal cellular function, and cell injury and death follow. Therefore, signal transduction pathways activated during ischemia may be either protective or part of the signal that leads to cell death. In this study, we demonstrated that ischemia induces both a transient and sustained activation of p38 MAPK in neonatal rat cardiac myocytes. In contrast, ERK2 and JNK are not activated during ischemia. By using the specific p38 MAPK inhibitor SB 203580, we demonstrated that sustained activation of p38 MAPK is deleterious to the cells and at least partially mediates apoptosis.

At least four members of the p38 MAPK family have been identified, and it is probable that different isoforms have specific physiological functions (1, 22, 34–37). The isoforms α (also termed p38, CSBP, or RK) and β are both expressed in heart tissue. Although these isoforms share approximately 74% sequence identity, they have been suggested to have opposing functions in cardiac myocytes (17). Expression of an activated mutant of MAPK kinase 3b, an upstream activator of both α and β isoforms of p38 MAPK, results both in a hypertrophic response and to apoptosis in neonatal rat cardiac myocytes (17). By using coexpression of individual activated p38 MAPK isoforms or of dominant negative inhibitory fragments, the same study showed that apoptosis appears to be mediated by the α isoform, whereas the hypertrophic response is mediated by the β isoform. In addition, suppression of p38β using the dominant negative fragment results in an increase in cell death, suggesting that this isoform can function to promote cell survival. In another study, overexpression of MAPK kinase 6, a different selective activator of p38 MAPK, protects neonatal cardiac myocytes against apoptosis (18). Thus, in cardiac myocytes it appears that p38β protects against, whereas p38α promotes, apoptotic cell death.

In our study, we observed that transient activation of p38α does not have a deleterious effect on the cell, whereas sustained activation of p38α induces apoptosis. However, we could not rule out activation of p38β or other p38 MAPK isoforms during one or both phases of p38 MAPK activation seen during ischemia. Therefore, it is possible that the two phases differ not only in duration, but also in the balance of isoforms activated. A short period of ischemia is not detrimental to cardiac myocytes and paradoxically protects against subsequent prolonged ischemia, as discussed in the Introduction. In models similar to that used in our study, preconditioning neonatal rat cardiac myocytes with 25 or 30 min of ischemic incubation followed by 30 min of normoxic recovery was shown to protect cultured neonatal cardiac myocytes from ischemia (30, 38). Thus, from the data presented here, preconditioning would be expected to result in only the first phase of p38α activation, and previous studies have shown that p38 MAPK is necessary for the protective effect of preconditioning (21, 24). It is intriguing that p38 MAPK appears to play a role both in protection of myocytes against injury and in mediating cellular apoptosis, and we propose that transient versus sustained activation of p38 MAPK, possibly in combination with activation of different isoforms, determines these different cellular effects. The most likely explanation is that the transient p38 MAPK activation that occurs on initiation of ischemia represents an adaptive response of the cell. Cardiac myocytes clearly can adapt to ischemic stress as shown by the fact that they can be protected by preconditioning. This hypothesis provides an explanation for the apparent discrepancy between this study, where SB 203580 protects against ischemia, and the previous studies in which SB 203580 inhibits preconditioning protection (21, 24).

Differential cellular effects of transient versus prolonged activation have been demonstrated previously for MAPK family members, for ERK2 in primary rat hepatocytes (39), and for JNK in rat mesangial cells (40). In addition, transient p38 MAPK activation is not sufficient to induce neuronal differentiation in rat pheochromocytoma PC12 cells, whereas sustained activation is sufficient (41). Interestingly, a transient activation of the ERK/MAPK pathway is additionally required to allow differentiation (41). In a similar way, it is likely that other proteins activated or inactivated at different times during ischemia regulate or act in combination with p38 MAPK.
For example, the prolonged phase of p38 MAPK activation may be caused by inactivation of specific regulatory phosphatases or, alternatively, degradation of an antiapoptotic protein may be required before p38 MAPK-mediated apoptosis can proceed. These possibilities can now be explored.

Although adult and neonatal cardiac myocytes can differ in their responses, it is unlikely that the protection seen here with SB 203580 would not translate to adult cells. A study performed in isolated adult rat hearts demonstrates that the presence of SB 203580 during ischemia preserves cardiac function during ischemia and improves posts ischemic recovery of cardiac function (42). Therefore, it is predicted that SB 203580, or a novel selective inhibitor of p38 MAPK, will prove useful to protect against damage from ischemic episodes in adult animals, both by decreasing cell death and by improving cardiac function.

We cannot rule out the possibility that SB 203580 targets another kinase, in addition to p38 MAPK, which mediates cell death. Selectivity has been demonstrated against other kinases (22, 23), but it has recently been reported that SB 203580 inhibits cardiac JNK2-related isoforms, albeit with a higher IC50 than for p38 MAPK (43). It is unlikely that inhibition of JNK explains the protection reported here because we and others (6, 7) have shown that JNK2 is not activated by ischemia.

In conclusion, our results strongly support a role for sustained p38 MAPK activation in mediating apoptosis induced by ischemia. This study used an experimental design that does not require overexpression of the proteins involved but rather inhibition of endogenous kinase. In addition, we suggest that transient activation can have very different cellular consequences from sustained activation of p38 MAPK in cardiac myocytes.

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