Temporary blockade of contractility during reperfusion elicits a cardioprotective effect of the p38 MAP kinase inhibitor SB-203580

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SUMMARY

SB-203580, a p38 MAP kinase inhibitor, increased creatine kinase release and infarct size. Cotreatment with SB-203580 and the contractile blocker 2,3-butanedione monoxime (BDM, 20 mM) or the ultra-short-acting kinase inhibitor SP-600125 (10 μM) for 30 min during reperfusion, but not the c-Jun NH2-terminal kinase inhibitor SP-600125 (10 μM), improved contractility but increased creatine kinase release and infarct size. Cotreatment with SB-203580 and the contractile blocker 2,3-butanedione monoxime (BDM, 20 mM) or the ultra-short-acting β-blocker esmolol (0.15 mM) for the first 30 min during reperfusion significantly reduced creatine kinase release and infarct size. In vitro mitochondrial ATP generation and myocardial ATP content were significantly increased in the heart cotreated with SB-203580 and BDM during reperfusion. Dystrophin was translocated from the sarcolemma during ischemia and reperfusion. SB-203580 increased accumulation of Evans blue dye in myocytes depleted of sarcolemmal dystrophin during reperfusion, whereas cotreatment with BDM facilitated restoration of sarcolemmal dystrophin and mitigated sarcolemmal damage after withdrawal of BDM. These results suggest that treatment with SB-203580 during reperfusion aggravates myocyte necrosis but concomitant blockade of contractile force unmasks cardioprotective effects of SB-203580.

THE ROLE OF p38 MAP kinase in myocardial protection or injury during ischemia and reperfusion remains a controversial issue (1, 30). Studies using p38 MAP kinase inhibitors and manipulating p38 MAP kinase gene expression have provided evidence that ischemia-induced activation of p38 MAP kinase is involved in myocardial injury (3, 24, 28, 35, 39). Although the mechanism by which p38 MAP kinase activation induces cell death is not fully understood, it has been shown that p38 MAP kinase mediates mitochondrial dysfunction and triggers activation of the cell death pathway (7, 12, 43). Thus p38 MAP kinase inhibition has emerged as a therapeutic means to prevent ischemia-reperfusion injury. In particular, developing a strategy to mitigate myocardial damage by employing p38 MAP kinase inhibitors at the moment of reperfusion is of prime importance in the clinical setting of cardioprotection.

Besides the involvement of p38 MAP kinase in the cell death pathway, it has been demonstrated that its activation mediates a negative inotropic effect through the modulation of Ca2+ sensitivity of the myofilaments (6, 8, 16, 20), suggesting that p38 MAP kinase activation is involved in postsischemic contractile dysfunction. Delaying the recovery of contractile function after reperfusion may negatively affect the clinical outcome in patients undergoing percutaneous coronary interventions and heart surgery. Conversely, it is possible that contractile dysfunction contributes to the prevention of contractile force-induced reperfusion injury. Fragility of the sarcolemmal membrane is a characteristic feature of myocardial reperfusion injury (14). Indeed, protection of the myocytes from mechanical stress has become a therapeutic strategy against reperfusion injury. Studies using the contractile blocker 2,3-butanedione monoxime (BDM) demonstrated that temporary blockade of contractile activity during reperfusion prevents myocardial necrosis in certain experimental models (36, 40).

The pathogenesis of sarcolemmal membrane injury during reperfusion has been a target of extensive research for many years. Contractile force generated by actin-myosin interaction is transmitted to the sarcolemma at the lateral costamere junction. The mechanical stress imposed on the fragile membrane causes its breakage at the site of Z-band attachment. Thus it has been thought that alterations of structural proteins that link the Z band and the sarcolemma are involved in membrane fragility and are responsible for the membrane damage on reintroduction of the contractile force (2, 10). Of these, we have focused on dystrophin as a potential target of research for myocardial reperfusion injury induced by physical stress. Dystrophin forms part of an integral membrane protein termed the dystrophin-glycoprotein complex in the skeletal and cardiac muscles. Dystrophin provides a mechanically strong physical linkage between the sarcolemmal membrane and the costameric cytoskeleton in the cardiac muscle (32), thereby stabilizing the sarcolemmal membrane from shear stresses imposed during eccentric muscle contraction (29, 37). The absence of the dystrophin gene is associated with vulnerability of the sarcolemma to mechanical force (25, 38). We recently
demonstrated that sarcolemmal dystrophin is translocated to the myofibril fraction during ischemia and is subsequently lost during reperfusion (17, 19). Reintroduction of contractile activity during reperfusion produced necrosis in myocytes depleted of sarcolemmal dystrophin, but not in myocytes replenished with sarcolemmal dystrophin. Accordingly, we have hypothesized that loss of sarcolemmal dystrophin is causally related to reperfusion injury.

It is anticipated, therefore, that the reversal of contractile dysfunction at the time of reperfusion by p38 MAP kinase inhibition may instantaneously produce necrosis in myocytes depleted of sarcolemmal dystrophin before implementation of any cardioprotective maneuvers. However, concomitant blockade of contractile force could enhance the protective effect of p38 MAP kinase inhibition, if dystrophin is restored in the sarcolemma during contractile arrest, leading to the prevention of myocyte necrosis after reintroduction of contractile activity.

MATERIALS AND METHODS

Perfusion technique. Male Sprague-Dawley rats (250–300 g body wt) were used in accordance with National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). The study protocol was approved by the Animal Committee of Kansai Medical University. Perfusion of the isolated rat heart was performed as described elsewhere (22). Perfusion pressure of the heart was kept constant at 70–75 mmHg. When the reperfusion buffer contained 20 mM BDM, an equimolar concentration of NaCl in the buffer was reduced.

Experimental protocols. Figure 1 shows the experimental protocol. The control heart was subjected to 20 min of global ischemia followed by 120 min of reperfusion (control). SB-203580 (SB), SP-600125 (SP), 2,3-butanedione monoxime (BDM), or SB-203580 for 30 min. SB-203580 and SP-600125 were obtained from Calbiochem, BDM from Sigma, and esmolor from Maruishi Pharmaceutical.

Measurements of myocardial ATP content. The heart was quickly frozen and stored in liquid nitrogen until use. ATP content in the LV was measured as described elsewhere (18).

Immunofluorescence microscopy of dystrophin. Immunofluorescence microscopy of dystrophin in the frozen myocardial section was performed as described elsewhere (17, 19). To quantitate sarcolemmal dystrophin, 500 myocytes were randomly selected from the endocardium through the epicardium of the mid-LV free wall by a blind observer. The sarcolemma was identified by immunofluorescence analysis of tetramethylrhodamine isothiocyanate-conjugated wheat germ agglutinin. The area of the sarcolemma and the fluorescence intensity of dystrophin were quantified using image-analyzing software (Win Roof, Mitani).

Evan's blue dye perfusion and identification of cardiomycocyte necrosis. To detect sarcolemmal damage of myocytes, Evans blue dye (EB; Sigma Chemical) was given for 15 min at a concentration of 0.1% during normal perfusion or on reperfusion or after the withdrawal of BDM. Double-fluorescence laser microscopic images of dystrophin and EB were obtained as described elsewhere (17, 19).

Infarct size measurements. Infarct size was measured by a triphenyltetrazolium chloride staining method as described elsewhere (17).

Measurements of p38 MAP kinase and JNK activities. The heart was removed from the perfusion apparatus at the indicated time, and the LV was rapidly frozen in liquid nitrogen. p38 MAP kinase activity in the muscle was measured as described elsewhere (35) using a p38 MAP kinase assay kit (Cell Signaling). The same samples were used to measure phosphorylation of heat shock protein 27 (HSP-27) as described elsewhere (33) using antibodies specific for phosphorylated HSP-27 (phospho-HSP-27; Santa Cruz Biotechnology).

JNK activity was measured using a JNK assay kit (Cell Signaling). Briefly, the sample proteins were incubated with c-Jun fusion protein beads. The precipitates were suspended in a kinase buffer supplemented with ATP. The reaction was terminated by the addition of SDS sample buffer. The samples were analyzed for phosphorylated c-Jun by Western blotting. The same samples were immunoblotted for the quantification of JNK to normalize JNK activity.

Measurements of mitochondrial ATP generation and cytochrome oxidase activity. The heart was removed from the perfusion apparatus at the indicated time. The LV was trimmed free from the atria and the right ventricle and immersed in an ice-cold mitochondria isolation buffer consisting of 250 mM sucrose, 20 mM MOPS (pH 7.2), 2 mM EGTA, and 0.1% bovine serum albumin. The LV was finely minced and gently homogenized with a hand-held Teflon homogenizer in the isolation buffer. The homogenate was centrifuged at 800 g for 5 min at 4°C. The supernatant was retrieved and centrifuged at 7,000 g for 10 min. The resulting mitochondrial pellet was resuspended in the buffer. The mitochondrial suspension was supplemented with 5 mM KH2PO4, 5 mM MgCl2, and 10 mM succinate in the presence of 0.5 μM rotenone. In some experiments, cytochrome c (50 μM) was added to supplement this respiratory chain intermediate of mitochondria. ATP generation was started by addition of 5 mM ADP, and the reaction was kept at 30°C for 15 min. The reaction was terminated with an equal volume of 12% trichloroacetic acid, and the reaction mixture was centrifuged at 1,200 g for 15 min at 4°C. The supernatant was neutralized, and the ATP content was measured by an enzymatic assay method. Cytochrome oxidase activity of mitochondria was measured as described previously (42).

Measurements of left ventricular function. Isovolumic left ventricular (LV) function was measured as described elsewhere (22). A latex balloon was inserted into the LV through the left atrium and filled with saline to produce an LV end-diastolic pressure of 5–10 mmHg at baseline, and the balloon volume was kept constant throughout the experiment. The heart was paced at 330 rpm throughout the experiments.

Creatine kinase assay. Coronary effluent was collected at the indicated time, and creatine kinase (CK) activity was measured as described elsewhere (22).

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Evan’s blue dye perfusion and identification of cardiomycocyte necrosis. To detect sarcolemmal damage of myocytes, Evans blue dye (EB; Sigma Chemical) was given for 15 min at a concentration of 0.1% during normal perfusion or on reperfusion or after the withdrawal of BDM. Double-fluorescence laser microscopic images of dystrophin and EB were obtained as described elsewhere (17, 19). The number of EB-positive and EB-negative myocytes was counted on 60 high-power fields (magnification ×600) from the endocardium through the epicardium of the mid-LV free wall, and the percentage of EB-positive myocytes was calculated.
Statistical analysis. Values are means ± SE. Statistical analysis was performed by one-way ANOVA or two-way repeated-measures ANOVA followed by Bonferroni’s post hoc test.

RESULTS

Time course of p38 MAP kinase activation during ischemia and reperfusion. p38 MAP kinase activity, as evaluated by in vitro phosphorylation of ATF and phospho-HSP-27 content in the heart, was significantly increased 20 min after ischemia and 10 min after reperfusion but returned to the basal level by 30 min after reperfusion (Fig. 2).

Effect of SB-203580 on p38 MAP kinase and JNK activity. Next, we examined the effect of p38 MAP kinase inhibition during ischemia and reperfusion on the recovery of contractility and the extent of myocardial necrosis during reperfusion. We employed SB-203580 as an inhibitor of p38 MAP kinase (15). SB-203580 does not block the phosphorylation of p38 MAP kinase but binds reversibly to its catalytic site and can be washed away during the p38 MAP kinase activity assay (44). Therefore, we determined phospho-HSP-27 to evaluate the effect of SB-203580 on p38 MAP kinase activity. Treatment with SB-203580 inhibited the phosphorylation of HSP-27 10 min after reperfusion (Fig. 3A).

Because SB-203580 is also a potent inhibitor of JNK (9), we tested the effect of the specific JNK inhibitor SP-600125 on the postischemic recovery of contractility and myocyte necrosis. JNK was significantly activated 10 min after reperfusion, but, in contrast to p38 MAP kinase, JNK remained activated 60 min after reperfusion (not shown). JNK activation was significantly inhibited by SB-203580 and, to a greater extent, by SP-600125 (Fig. 3B).

Effect of BDM and esmolol on myocardial contractility and CK release. To test the hypothesis that the increased necrosis resulting from treatment with SB-203580 is attributed to increased contractility, the hearts were cotreated with SB-203580 and the contractile blocker BDM or the ultra-short-acting β-blocker esmolol for the first 30 min during reperfusion. BDM and esmolol had no effect on basal p38 MAP kinase activity, nor did they significantly affect the increase in p38 MAP kinase activity during reperfusion (not shown). Reperfusion with BDM and esmolol decreased LV developed pressure (Fig. 5A) and LV dP/dt (Fig. 5B) to <10% of the basal level.
within 3 min, although contractility was inhibited more promptly with BDM treatment (not shown). CK release was abolished during reperfusion with BDM and esmorol irrespective of the presence or absence of SB-203580 (Fig. 5C). Withdrawal of BDM from the buffer immediately restored contractility to the basal level, but contractility declined thereafter. Withdrawal of esmorol also restored contractility to ~80% of the basal level within 5 min. CK release was markedly increased 5 min after the withdrawal of BDM and esmorol. As a consequence, infarct size of these hearts (Fig. 5D) was not significantly different from that observed in the nontreated heart. Cotreatment with SB-203580 and BDM or esmorol significantly improved contractility after withdrawal of these drugs compared with treatment with BDM or esmorol alone, and CK release and infarct size were significantly reduced.

Effect of SB-203580 on mitochondrial ATP generation and myocardial ATP content. In vitro mitochondrial ATP generation was measured 15 min after reperfusion (Fig. 6A). Because the loss of cytochrome c is involved in mitochondrial dysfunc-

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Fig. 4. A: left ventricular (LV) developed pressure (LVDP). B: LV maximum dP/dt. C: creatine kinase (CK) release. Control; ○, SB-203580; ▲, SP-600125. SB-203580 or SP-600125 was administered for 30 min during reperfusion. DW, dry weight. D: infarct size. Control, control ischemia-reperfusion; SB-203580, reperfusion with SB-203580; SP-600125, reperfusion with SP-600125. Values are means ± SE of 6 experiments. *P < 0.05 vs. control.

Fig. 5. A: LV developed pressure. B: LV maximum dP/dt. C: CK release. BDM (●) or esmorol (▲), alone or in combination with SB-203580 (○), or esmorol (△) was administered for 30 min during reperfusion. Values are means ± SE of 6 experiments. *P < 0.05 vs. BDM alone. †P < 0.05 vs. esmorol alone. D: infarct size. Control, control ischemia-reperfusion; BDM, reperfusion with BDM; esmorol, reperfusion with esmorol; SB-203580 + BDM, reperfusion with SB-203580 + BDM; SB-203580 + esmorol, reperfusion with SB-203580 + esmorol. Values are means ± SE of 6 experiments. *P < 0.05 vs. control. †P < 0.05 vs. BDM alone. *P < 0.05 vs. esmorol alone.
Cytochrome oxidase activity was also significantly more decreased in the SB-203580-treated heart after reperfusion than in the control ischemic-reperfused heart in the absence of cytochrome c (Fig. 6B). Exogenous cytochrome c completely restored cytochrome oxidase activity in both groups. Treatment with BDM alone during reperfusion did not significantly improve cytochrome oxidase activity in the absence of cytochrome c. Exogenous cytochrome c completely restored cytochrome oxidase activity also in this group of hearts. Cotreatment with SB-203580 and BDM during reperfusion significantly increased cytochrome oxidase activity to the level comparable to that in the control heart in the absence of cytochrome c. No further increase in cytochrome oxidase activity was noted by addition of cytochrome c in this group.

Myocardial ATP content was significantly decreased 30 min after reperfusion (Fig. 6C). Treatment with SB-203580 or BDM alone during reperfusion had no significant effect on myocardial ATP content. However, cotreatment with SB-203580 and BDM during reperfusion significantly increased ATP content.

Effect of SB-203580 on sarcolemmal dystrophin and EB uptake. Dystrophin was translocated from the sarcolemma to the cytoplasmic region in myocytes 20 min after ischemia and was not restored in the sarcolemma during reperfusion (Fig. 7, A and B). Treatment with SB-203580 or BDM alone during reperfusion had no significant effect on the loss of sarcolemmal dystrophin. However, sarcolemmal dystrophin was significantly increased by cotreatment with SB-203580 and BDM during reperfusion.

When EB was given for the first 15 min after reperfusion in the control ischemic-reperfused heart, EB accumulated in myocytes depleted of sarcolemmal dystrophin (Fig. 7, C and D). Treatment with SB-203580 during reperfusion markedly increased the accumulation of EB in sarcolemmal dystrophin-depleted myocytes. EB accumulation was not observed during reperfusion with BDM, despite the depletion of sarcolemmal dystrophin. However, withdrawal of BDM resulted in robust accumulation of EB in myocytes depleted of sarcolemmal dystrophin. In contrast, cotreatment with SB-203580 and BDM during reperfusion restored dystrophin in the sarcolemma and prevented EB accumulation after withdrawal of BDM.

DISCUSSION

The present study highlighted for the first time that p38 MAP kinase activation plays a dual role in modulating contractility and myocardial injury during reperfusion. The major findings of this study were as follows. 1) p38 MAP kinase was activated 20 min after ischemia and early during reperfusion. 2) Treatment with SB-203580, but not SP-600125, temporally improved contractility during reperfusion but increased CK release and infarct size. 3) Cotreatment with SB-203580 and the contractile blocker BDM or the ultra-short-acting β-blocker esmolol during reperfusion reduced CK release and infarct size and improved LV function after withdrawal of these drugs. 4) Cotreatment with SB-203580 and BDM, but not each alone, during reperfusion improved mitochondrial function and increased tissue content of ATP. 5) Cotreatment with SB-203580 and BDM, but not each alone, during reperfusion restored sarcolemmal dystrophin and mitigated sarcolemmal damage after withdrawal of BDM. These results are consistent with the
hypothesis that inhibition of p38 MAP kinase reverses contractile dysfunction early after reperfusion and aggravates myocyte necrosis, but concomitant blockade of contractile force un-masks the beneficial effects of p38 MAP kinase inhibition on cardioprotection.

The results of our present study differ at some points from other recent studies that have clearly shown a protective effect of SB compounds administered during ischemia and reperfusion or only during reperfusion on myocardial function and necrosis (21, 23, 35). The reason for this discrepant observation is unknown. The involvement of different p38 MAP kinase isoforms is a possible explanation. Accordingly, the use of SB-203580 as an inhibitor of p38 MAP kinase may be problematic, because SB-203580 can inhibit the α- and the β-isoforms, and these isoforms are known to exert a distinct effect on myocyte function and survival (41). It has been reported that neonatal rat cardiomyocytes expressing the dominant negative p38α are resistant to lethal simulated ischemia (34). Thus it is likely that p38α is involved in cardiomyocyte injury, whereas p38β may be involved in the negative inotropic effect. More importantly, the temporal role of p38 MAP kinase in contractility and the development of infarction during reperfusion differ between experimental models. Although our study utilizing a relatively brief period of ischemia resulted in a marked increase in contractility at the time of reperfusion with SB-203580 associated with enhanced myocyte necrosis, this injurious component of reperfusion with SB-203580 may be attenuated, and its cardioprotective properties become apparent when the duration of ischemia is prolonged. In this context, the previous studies lacked evaluation of the temporal relation between contractility and myocyte necrosis. It is, therefore, possible in the previous studies that myocyte necrosis was increased temporarily early during reperfusion by p38 MAP kinase inhibition, even when gross necrosis measured by a triphenyltetrazolium chloride staining method 120 min after reperfusion was significantly less than that in the nontreated heart.

Such a paradoxical increase in myocyte necrosis in the face of improved functional recovery at the onset of reperfusion is likely when the sarcolemmal membrane is weakened during reperfusion unmasking the beneficial effects of p38 MAP kinase inhibition on cardioprotection.

**Fig. 7.** Sarcolemmal dystrophin and Evans blue (EB) uptake. A: confocal images of dystrophin (left) and wheat germ agglutinin (right). Scale bars, 20 μm. Control, nonischemic control; CI, 20 min after control ischemia; CR, 15 min after control reperfusion; SBR, 15 min after reperfusion with SB-203580; BDM, 15 min after reperfusion with BDM; SBBDM, 15 min after reperfusion with SB-203580 + BDM. B: quantitative analysis of sarcolemmal dystrophin. Values are means ± SE of 5 hearts. *P < 0.05 vs. control. **P < 0.05 vs. CR. C: confocal images of dystrophin (green fluorescence) and EB (red fluorescence). Scale bars, 20 μm. CR, 15 min after control reperfusion; SBR, 15 min after reperfusion with SB-203580; BDM, 15 min after reperfusion with BDM; SBBDM, 15 min after reperfusion with SB-203580 + BDM; BDM/WD, 15 min after withdrawal of BDM; SBBDM/WD, 15 min after withdrawal of SB-203580 + BDM. D: quantitative analysis of EB-positive myocytes. Values are means ± SE of 5 hearts. *P < 0.05 vs. control. **P < 0.05 vs. CR.
the preceding ischemia. Although this point must be clarified in the future, our observations suggest that contractility at the time of reperfusion is a crucial determinant of myocyte survival or death when p38 MAP kinase is inhibited. Consistent with this notion is the fact that, in contrast to SB-203580 treatment alone, CK release was abolished during cotreatment with SB-203580 and BDM or esmolol, and CK release and infarct size after withdrawal of these drugs were significantly reduced. The benefit from temporary blockade of contractility during reperfusion was not obtained by BDM treatment alone, which provoked robust CK release immediately after its withdrawal and had no significant effect on ultimate infarct size. The inability of BDM or esmolol treatment alone to limit infarct size in our model is presumably the result of a similar protective effect against contractile force-induced myocyte necrosis exerted by the p38 MAP kinase-mediated decrease in contractility.

The finding that cotreatment with SB-203580 and BDM or esmolol limited myocyte necrosis after withdrawal of these drugs indicates that sarcolemmal fragility was repaired during contractile arrest concomitant with p38 MAP kinase inhibition. Because cotreatment with SB-203580 and BDM during reperfusion improved mitochondrial function and increased tissue content of ATP, it is speculated that when sarcolemmal damage is prevented early after reperfusion by increased contractility, p38 MAP kinase inhibition improves mitochondrial function and ATP availability and promotes a reparative mechanism for sarcolemmal fragility, thereby preventing necrosis on the introduction of contractile activity after withdrawal of BDM or esmolol.

The mechanism by which p38 MAP kinase inhibition promotes a reparative process against sarcolemmal fragility under contractile arrest is unclear. We recently hypothesized that the loss of sarcolemmal dystrophin is responsible for sarcolemmal fragility and reperfusion injury (17, 19). In those studies, we demonstrated that sarcolemmal dystrophin was translocated to the myofibril fraction during ischemia and subsequently lost during reperfusion. Reintroduction of contractile activity during reperfusion produced necrosis in myocytes depleted of sarcolemmal dystrophin but not in myocytes replenished with dystrophin in the sarcolemma. Ischemic preconditioning, which is known to improve mitochondrial function (34), facilitated restoration of sarcolemmal dystrophin, and this was correlated with cardioprotection against myocyte necrosis. The present study is consistent with these previous reports suggesting that loss of sarcolemmal dystrophin is involved in contractile force-induced sarcolemmal damage early during reperfusion and that improvement of mitochondrial function correlates with the restoration of sarcolemmal dystrophin during reperfusion, although the cause-and-effect relation between mitochondrial dysfunction, loss of dystrophin, and occurrence of reperfusion injury remains to be elucidated. The idea that improvement of mitochondrial function and increased ATP availability during reperfusion facilitate redistribution of dystrophin to the sarcolemma comes from the evidence that dystrophin is a phosphoprotein that is phosphorylated at the COOH-terminal region near the cysteine-rich domain, where it is associated in the sarcolemma with other membrane proteins such as β-dystroglycan (26, 27). Because it is conceivable that the phosphorylation status of dystrophin determines its intracellular localization and phosphorylation of many intracellular proteins depends on the availability of ATP, improvement of mitochondrial function by p38 MAP kinase inhibition may be necessary for phosphorylation and redistribution of dystrophin to the sarcolemma during reperfusion.

We evaluated mitochondrial function in vitro in the absence or presence of exogenous cytochrome c. Cytochrome c is known to be released from the intermembrane space of mitochondria during ischemia and reperfusion associated with the opening of a permeability transition pore leading myocytes to apoptosis and necrosis (5). The present study demonstrated that treatment with SB-203580 during reperfusion significantly aggravated mitochondrial ATP generation and that exogenous cytochrome c was more effective in improving mitochondrial ATP generation in this group of hearts. Although we did not measure mitochondrial content of cytochrome c, this observation seems to indicate that the loss of cytochrome c from mitochondria was greater in the heart treated with SB-203580. The loss of cytochrome c is not attributed to the isolation procedures, because exogenous cytochrome c had no effect on ATP generation in the normally perfused control heart. Instead, it is likely that massive loss of cytochrome c from mitochondria in the SB-203580-treated heart occurred as a result of increased sarcolemmal damage during reperfusion before isolation of mitochondria, because sarcolemmal damage allows unlimited entry of Ca2+ and other solutes into the cytosol, which causes matrix swelling and outer membrane rupture of mitochondria, leading to massive loss of cytochrome c. However, necrotic mitochondrial damage is not a sole mechanism of loss of cytochrome c and impairment of ATP generation, because impaired ATP generation was also observed in the absence of necrosis by treatment with BDM, and exogenous cytochrome c significantly improved ATP generation in this group of hearts. Furthermore, the evidence that cotreatment with SB-203580 and BDM significantly improved mitochondrial ATP generation over BDM treatment alone and exogenous cytochrome c had no significant effect on ATP generation in this group of hearts suggests that p38 MAP kinase activation is involved at least in part in the loss of cytochrome c and the impairment of ATP generation during reperfusion. The failure of exogenous cytochrome c to completely restore ATP generation is not attributed to general defects of the respiratory chain or the insufficient amount of exogenous cytochrome c, because the same amount of exogenous cytochrome c completely restored cytochrome oxidase activity of mitochondria in all groups of hearts. Therefore, it is conceivable that, in addition to the loss of cytochrome c, other mechanism(s) must also be involved in impaired ATP generation of mitochondria isolated from the ischemic-reperfused heart. Although caution must be taken to extrapolate these in vitro observations to in vivo conditions, significantly increased ATP content in the heart cotreated with SB-203580 and BDM during reperfusion reinforces the hypothesis that p38 MAP kinase inhibition during reperfusion could improve mitochondrial function when contractile force-induced sarcolemmal damage is prevented.

The exact mechanism of p38 MAP kinase activation-mediated contractile dysfunction has not been fully elucidated. Although mitochondrial dysfunction and contractile dysfunction are tightly coupled, impaired generation of ATP may not be the primary cause of p38 MAP kinase-mediated contractile dysfunction. A growing body of evidence indicates that reduced sensitivity of myofilaments to Ca2+ is responsible for
the negative inotropic effect mediated by p38 MAP kinase activation (6, 8, 16, 20). Moreover, the present study suggests that p38 MAP kinase activation contributes to contractile dysfunction only early during reperfusion. Persistent depression of contractility thereafter may be attributed to the loss of functional myocardium because of the progression of necrosis or by other mechanisms of myocardial stunning, such as intracellular Ca\(^{2+}\) overload and degradation of contractile proteins (4, 11). Because the mechanism of contractile dysfunction during reperfusion is multifactorial, limited contribution of p38 MAP kinase to functional deterioration during ischemia and reperfusion may explain why p38 MAP kinase inhibition was not sufficient to rescue contractile dysfunction in mice with low-flow ischemia (13).

In summary, the present study demonstrated that treatment with the p38 MAP kinase inhibitor SB-203580 during reperfusion improved contractility but aggravated necrosis and that temporary blockade of contractility by co-treatment with BDM or esmolar elicits a cardioprotective effect of p38 MAP kinase inhibition. Thus, in our experimental model, SB-203580 exerts cardioprotection only when contractile force-induced necrosis is prevented. However, in other experimental models where p38 MAP kinase inhibitors alone are sufficient to confer cardioprotection, enhanced cardioprotection against reperfusion injury could be achieved by combined addition of p38 MAP kinase inhibitors and negative inotropic drugs.

GRANTS

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