Inhibition of p38 Mitogen-Activated Protein Kinase Decreases Cardiomyocyte Apoptosis and Improves Cardiac Function After Myocardial Ischemia and Reperfusion

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Background—Activation of p38 mitogen-activated protein kinase (MAPK) plays an important role in apoptotic cell death. The role of p38 MAPK in myocardial injury caused by ischemia/reperfusion, an extreme stress to the heart, is unknown.

Methods and Results—Studies were performed with isolated, Langendorff-perfused rabbit hearts. Ischemia alone caused a moderate but transient increase in p38 MAPK activity (3.5-fold increase, \(P<0.05\) versus basal). Ischemia followed by reperfusion further activated p38 MAPK, and the maximal level of activation (6.3-fold, \(P<0.01\)) was reached 10 minutes after reperfusion. Administration of SB 203580, a p38 MAPK inhibitor, decreased myocardial apoptosis (14.7±3.2% versus 30.6±3.5% in vehicle, \(P<0.01\)) and improved postischemic cardiac function. The cardioprotective effects of SB 203580 were closely related to its inhibition of p38 MAPK. Administering SB 203580 before ischemia and during reperfusion completely inhibited p38 MAPK activation and exerted the most cardioprotective effects. In contrast, administering SB 203580 10 minutes after reperfusion (a time point when maximal MAPK activation had already been achieved) failed to convey significant cardioprotection. Moreover, inhibition of p38 MAPK attenuated myocardial necrosis after a prolonged reperfusion.

Conclusions—These results demonstrate that p38 MAPK plays a pivotal role in the signal transduction pathway mediating postischemic myocardial apoptosis and that inhibiting p38 MAPK may attenuate reperfusion injury. (Circulation. 1999;99:1685-1691.)

Key Words: reperfusion • signal transduction • apoptosis • contractility • myocardial infarction

Growing evidence from both animal experiments and clinical observations indicates that apoptosis, a form of cell death that is distinct from necrosis, plays a key role in myocardial reperfusion injury.1–5 The signal transduction pathway that leads to this posts ischemic myocardial apoptosis, however, remains largely unexplored. Recent cell biology studies have demonstrated that c-Jun N-terminal kinases (JNKs)/stress-activated protein kinases (SAPKs) and p38 mitogen-activated protein kinase (MAPK), 2 members of the MAPK family, are activated by a variety of cellular stresses6–7 and that their activation results in apoptosis and cell death.8–14 It has also been demonstrated recently that myocardial ischemia/reperfusion, an extreme pathological stress to the heart, activates p38 MAPK.15–17 However, whether activation of p38 MAPK plays an important role in posts ischemic myocardial apoptosis has not been determined.

Given that apoptosis is an active, gene-directed process, inhibition of apoptosis could be achieved more successfully than prevention of necrosis, a passive form of cell death that is inflicted by the acute stimulus.18 However, it is well recognized that apoptosis proceeds through a genetically programmed series of biochemical and morphological steps designed to avoid the indiscriminate release of cytosolic contents and the ensuing inflammatory response; removal of damaged myocytes through apoptosis may be a mechanism by which the heart limits the extent of the potentially more destructive process of necrosis.19 Thus, there exists a need for further clarification as to whether or not inhibition of myocyte apoptosis might result in improved recovery of left ventricular function after myocardial ischemia and reperfusion.

The objectives of the present studies were to (1) determine the time course of p38 MAPK activation in isolated, perfused rabbit hearts subjected to ischemia and reperfusion in a quantitative manner; (2) establish a direct link between p38 MAPK activation and myocyte apoptosis after myocardial ischemia and reperfusion; (3) determine the time course of myocardial apoptosis; and (4) determine whether inhibition of p38 MAPK might attenuate reperfusion injury.
ischemia and reperfusion; and (3) define the effects of inhibition of p38 MAPK activation and ischemia-induced apoptosis by specific pharmacological agents on functional damage in the rabbit heart.

Methods

Experimental Preparation

Rabbits were anesthetized with sodium pentobarbital (35 mg/kg IV) and heparinized with sodium heparin (1000 U/kg IV). Five minutes after heparin injection, a midsternal thoracotomy was performed, and the heart was rapidly excised and placed into ice-cold Krebs-Henseleit (KH) buffer solution consisting of (in mmol/L) NaCl 118, KCl 4.75, KH2PO4 1.19, MgSO4 1.19, CaCl2 2H2O 2.54, NaHCO3 25, EDTA 0.5, and glucose 11. Within 30 seconds, the heart was mounted onto a Langendorff heart perfusion apparatus (Radnoti Glass Technology, Inc). The hearts were perfused in a retrograde fashion via the aorta at a constant pressure of 60 mm Hg (Radnoti Glass Technology, Inc). The hearts were perfused in a retrograde fashion via the aorta at a constant pressure of 60 mm Hg (Radnoti Glass Technology, Inc). The hearts were perfused in a retrograde fashion via the aorta at a constant pressure of 60 mm Hg (Radnoti Glass Technology, Inc).

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Experimental Protocol

After a 15-minute equilibration time, hearts were subjected to complete global ischemia (MI) for 30 minutes (unless otherwise indicated) by turning off the perfusion system. After 30 minutes of ischemia the perfusion system was restarted, and the hearts were reperfused (R) for 0 to 60 minutes (for studying the time course of p38 MAPK activation, formation of apoptosis, and cardiac function) for 120 minutes (for myocardial necrosis). Hearts were randomly divided into 4 major treatment groups. In the first group, hearts were subjected to MI/R but received vehicle only (0.9% NaCl, MI+vehicle). The second group of hearts was treated with SB 203580 (a pyridinyl imidazole inhibitor of p38 MAPK; final concentration, 1 or 10 μmol/L) before ischemia (for 10 minutes) and during the entire period of reperfusion (MI+SB/IR). In the third group of hearts, treatment with SB 203580 (10 μmol/L) was initiated at the onset of reperfusion and continued for the rest of the reperfusion period (MI+SB/IR). In the fourth group of hearts, SB 203580 (10 μmol/L) treatment was delayed, beginning 10 minutes after reperfusion was initiated and continuing for the rest of the reperfusion period (MI+SB/DT). Drugs were infused into the heart via a side arm in the perfusion line located just proximal to the heart cannula. The rate of infusion (1/1000 of CF) was adjusted on the basis of the CF rate so that the desired final concentration was obtained. Sham I/R hearts were continuously perfused with KH solution for 2.5 hours.

SAPK, p38 MAPK, and MAPK-Activated Protein Kinase 2 (MAPKAP K2) Kinase Assay

At the completion of the perfusion protocol, the hearts were frozen in LN2 and used within 1 week. The hearts were “freeze-clamped” by using precooled aluminum tongs and pulverized under LN2.20 The powder was resuspended in ice-cold lysis buffer, and the protein content in the detergent-soluble supernatant fraction was measured as described previously.7 SAPK, p38 MAPK, and MAPKAP K2 kinases were immunoprecipitated with antibodies specific for JNK1 and JNK2 (Santa Cruz), p38 MAPK (SmithKline Beecham), or MAPKAP K2 (Dr J. Landry, University of Quebec, Montreal) and assayed by using glutathione-S-transferase–c-Jun1–81, glutathione-S-transferase–activating transcription factor–2, or heat shock protein 27 as the substrate, respectively, as described in our previous studies.20–22

DNA Fragmentation (DNA Ladder)

The frozen hearts (stored at −70°C) were minced while being thawed in lysis buffer (50 mmol/L Tris-HCl, pH 8.0; 20 mmol/L EDTA; and 1% SDS) on ice, and proteinase K (100 μg/mL) was then added. After incubation at 55°C with shaking for 18 hours, DNA was extracted with phenol/chloroform 3 times, precipitated in ethanol, treated with DNA-free RNase, reextracted, and precipitated again. Five micrograms of DNA was then loaded onto a 1.8% agarose gel, and DNA ladder formation was detected as described in our previous study.20

Terminal Deoxynucleotidyl Transferase–Mediated dUTP-Biotin In Situ Nick-End Labeling (TUNEL)

The TUNEL assay was performed using ApopTag (Oncor) according to the manufacturer’s instructions.20 Cardiomyocytes from at least 4 slides per block that were randomly selected were evaluated immunohistochemically to determine the number and percentage of cells exhibiting positive staining for apoptosis. For each slide 10 fields were randomly chosen, and by using a defined rectangular field area (×20 objective), a total of 100 cells per field were counted. The index of apoptosis was determined (ie, number of apoptotic myocytes divided by the total number of myocytes counted ×100) from a total of 40 fields per heart, and the assays were performed in a blinded manner.

Assessment of Myocardial Contractile Function

Cardiac contractile function was assessed as described previously.23 In brief, a latex balloon was inserted into the left ventricular cavity and connected to a pressure transducer. The balloon was initially inflated with water to produce an end-diastolic pressure of 8 to 10 mm Hg. Left ventricular pressure was continually recorded on a Power Macintosh computer via a data acquisition system (MacLab, AD Instruments, Inc). The left ventricular systolic pressure, left ventricular diastolic pressure, left ventricular diastolic pressure, left ventricular generated pressure (LVGP; systolic minus diastolic), the maximal value of the first derivative of left ventricular pressure (dP/dtmax), heart rate, the pressure-rate product (PRP; heart rate times generated pressure), and CF were automatically analyzed.

Determination of Myocardial Necrotic Injury

Myocardial necrotic injury was assessed by measuring myocardial creatine kinase (CK) loss and nitro blue tetrazolium (NBT) staining as previously reported.24 In brief, ~100 mg myocardial tissue was taken at the end of each experiment and homogenized in cold 0.25 mol/L sucrose (1:10, wt/vol) containing 1 mmol/L EDTA and 0.1 mmol/L mercaptoethanol. Homogenates were centrifuged and the supernatant was decanted. CK activity was analyzed using a Beckman DU 640 spectrophotometer as reported previously.24 Protein concentration was determined by the bicinchoninic acid method (Pierce). CK loss was calculated by subtracting CK activity of MI/R hearts from the CK activity of sham ischemic hearts and expressed in IU per 100 mg protein. The remainder of the heart tissue was cut into slices ~2 mm thick. Slices were incubated in 0.1% NBT in phosphate buffer at pH 7.4 and 37°C for 15 minutes. The unstained portion (which is the irreversibly injured, necrotic region) was then separated from the stained (nonnecrotic) portion. Both sections were weighed, and the results were expressed as a percentage of necrotic tissue over total ventricular mass.

Statistical Analysis

All values in the text, tables, and figures are presented as mean±SEM of n independent experiments. All data were subjected to ANOVA followed by the Bonferroni correction for post hoc t tests. Probabilities of P≤0.05 were considered statistically significant.
Results

Time Course of p38 MAPK Activation by Ischemia and Ischemia/Reperfusion and Its Inhibition by SB 203580

To determine the time course of p38 MAPK activation by ischemia and reperfusion, the hearts were subjected to 0 (control), 15, 30, 40, 50, 60, or 90 minutes of ischemia alone or 30 minutes of ischemia followed by 10, 20, 30, or 60 minutes of reperfusion (at least 5 hearts per time point). Ischemia alone caused a modest (3- to 4-fold), transient (peaked at 15 minutes, declined at 30 minutes of ischemia, and maintained at low levels thereafter) increase in p38 MAPK activity (Figure 1). However, when myocardial tissue was reperfused after 30 minutes of ischemia, p38 MAPK was markedly reactivated. A 6.3-fold increase in p38 MAPK activity was observed at 10 minutes after reperfusion and remained significantly elevated at 20 minutes throughout reperfusion. At 30 and 60 minutes of reperfusion, p38 MAPK activity returned to a level that was not significantly different from control (Figure 1). Administration of 1 μmol/L SB 203580, a specific p38 MAPK inhibitor, only slightly reduced p38 MAPK activity (maximal activity at 10 minutes of reperfusion: 5.8±0.95-fold increase in 1 μmol/L SB 203580—treated hearts versus 6.3±0.83-fold increase in vehicle-treated hearts, P>0.05). In contrast, when 10 μmol/L SB 203580 was administered, p38 MAPK activity was markedly inhibited (1.26±0.62-fold increase, P<0.001 versus vehicle-treated hearts, P>0.05 versus sham MI/R hearts; Figure 2).

Detection of DNA Fragmentation (DNA Ladder) in Ischemic/Reperfused Hearts and Its Inhibition by the p38 MAPK Inhibitor

In myocardial tissue from sham ischemic hearts, no DNA ladder was detected (Figure 3, lanes 1 and 7). In contrast, the formation of DNA nucleosome ladders was clearly detected in myocardial tissues obtained from all 5 I/R hearts receiving vehicle only (Figure 3, lanes 2 through 6). In the 5 rabbit hearts treated with 10 μmol/L SB 203580, DNA ladder formation was absent in 3 heart samples and significantly reduced in the other 2 (Figure 3, lanes 8 through 12).

In Situ Determination of Apoptotic Myocytes in Ischemic/Reperfused Rabbit Hearts and Its Inhibition by SB 203580

Consistent with the absence of DNA ladders, few myocytes stained positive in tissue from sham ischemic hearts (Figures 4A and 5). In contrast, TUNEL-positive nuclei were prevalent in tissues from I/R hearts receiving vehicle only (Figures 4B and 5). Most notably, SB 203580 treatment markedly reduced the numbers of myocytes that were stained positive by TUNEL (Figures 4C and 5). Therefore, after 30 minutes of ischemia and 60 minutes of reperfusion, TUNEL-positive myocytes were reduced to 14.7±3.2% in SB 203580–treated hearts compared with 30.6±3.5% in vehicle-treated hearts (P<0.01, Figure 5).

Effect of SB 203580 on Myocardial Necrotic Injury After Ischemia/Reperfusion

As summarized in the Table, 30 minutes of global ischemia and 120 minutes of reperfusion resulted in significant necrotic injury, as evidenced by a large area of negative NBT staining and significant CK loss. Administration of 1 μmol/L SB 203580 had no significant effects on either myocardial CK loss or the size of negative NBT staining. However, when 10 μmol/L SB 203580 was administered 10 minutes before ischemia and during reperfusion, a significant reduction in both myocardial CK loss and size of negative NBT staining was observed (Table 1).

Effect of Inhibition of p38 MAPK on Cardiac Function Recovery: Dose-Protection Relationship

Global ischemia decreased CF to zero, and functional myocardial contraction was completely absent 30 minutes after ischemia and reperfusion. Administration of SB 203580 to isolated, perfused rabbit hearts during reperfusion of ischemic myocardium was associated with significant recovery of CF (Figure 6). Inhibition of p38 MAPK at 1 μmol/L reduced the recovery of CF to 50% of control, whereas inhibition at 10 μmol/L SB 203580 (Figure 6B) reduced recovery of CF to 20% of control. Inhibition of p38 MAPK at 10 μmol/L SB 203580 was associated with a significant reduction in both myocardial CK loss and size of negative NBT staining.
ischemia. When perfusion was restored, functional contraction usually resumed within 5 minutes. CF, LVGP, dP/dt max, and PRP all gradually recovered and reached a maximal level 20 to 40 minutes after reperfusion. In vehicle-treated hearts, LVGP and PRP declined again 60 minutes after reperfusion while CF and dP/dt max remained at a relatively stable level. Treatment with 10 μmol/L SB 203580 slightly increased LVGP 40 minutes after reperfusion and enhanced dP/dt max 40 and 60 minutes after reperfusion. However, none of these differences were statistically significant. In contrast, when 10 μmol/L SB 203580 was administered, a significant improvement in cardiac contractile function was observed. At 60 minutes of reperfusion, LVGP, dP/dt max, and PRP were significantly higher compared with vehicle-treated hearts (P < 0.001 for LVGP and dP/dt max and P < 0.05 for PRP). Moreover, although SB 203580 exerted no direct vasorelaxation effects in control hearts or MI/R hearts when infused up to 30 μmol/L for 1 minute (data not shown), treatment with 10 μmol/L SB 203580 before ischemia and during reperfusion significantly improved CF 60 minutes after reperfusion (P < 0.05, Figure 6).

Time-Dependent Inhibition of p38 MAPK on Cardiac Function Recovery

Previous experiments by other investigators as well as our own data have demonstrated that transient p38 MAPK activation can be detected after 10 to 20 minutes of ischemia without reperfusion. However, the influence of this ischemia activated p38 MAPK on myocardial injury after reperfusion remains unknown. Moreover, our time course study has shown that maximal p38 MAPK activity was observed 10 minutes after reperfusion and declined thereafter. It was not clear whether this peak increase of p38 MAPK in early reperfusion played a key role in subsequent myocardial injury. To address this issue, 2 additional experiments were performed. In the first experiment, SB 203580 (10 μmol/L, a concentration that exerted significant protective effects when given both before ischemia and after reperfusion), was given during reperfusion only (MI+SB/R). As illustrated in Figures 7 and 8, administration of SB 203580 during reperfusion only markedly improved myocardial function recovery and signifi-

Effects of SB 203580 on Necrotic Size and Myocardial CK Loss in Isolated, Perfused Rabbit Hearts Subjected to 30 Minutes of Global Ischemia and 120 Minutes of Reperfusion

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>SB 203580, 1 μmol/L</th>
<th>SB 203580, 10 μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Necrotic, %</td>
<td>44.3±2.1</td>
<td>41.2±2.4</td>
<td>29.2±2.3*</td>
</tr>
<tr>
<td>CK loss, IU/mg protein</td>
<td>465±37</td>
<td>421±24</td>
<td>305±21*</td>
</tr>
</tbody>
</table>

*P < 0.01 vs vehicle.
icantly reduced myocardial necrotic injury, as evidenced by a reduction in necrotic area and a decrease in myocardial CK loss. In the second experiment, administration of SB 203580 was delayed until 10 minutes after reperfusion, a time point at which maximal p38 MAPK activation had already occurred. No significant protective effects were observed (Figures 7 and 8A). Taken together, these 2 experiments suggest that the peak increase in p38 MAPK activation soon after reperfusion is a critical early event that plays an important role in the determination of the final extent of myocardial reperfusion injury.

To provide further evidence that the cardioprotective effects of SB 203580 described above were directly related to its inhibition of p38 MAPK, activation of MAPKAP kinase 2 (MAPKAP K2), a specific downstream target of p38 MAPK, was measured in the myocardial tissues from the 5 experimental groups. In sham ischemic tissue, no MAPKAP K2 activity was detected (Figure 8B, lane 1). However, MAPKAP K2 activity was markedly enhanced in myocardial tissues from the hearts subjected to 30 minutes of ischemia and 10 minutes of reperfusion (Figure 8B, lane 2). Treatment with SB 203580 both before ischemia and reperfusion completely blocked the activation of MAPKAP K2 (Figure 8B, lane 3). Administration of SB 203580 during reperfusion only also markedly inhibited MAPKAP K2 activity (Figure 8B, lane 4). In contrast, when treatment with SB 203580 was delayed until 10 minutes after reperfusion (∼100 mg myocardial tissue from the left ventricular free wall was taken 10 minutes after reperfusion but before initiating SB 203580 infusion), MAPKAP K2 activity was comparable to that observed in vehicle-treated hearts (Figure 8B, lane 5).

To investigate whether or not SB 203580 treatment also inhibited SAPK activity, thereby exerting cardiovascular...

**Figure 6.** Dose-dependent effects of SB 203580 on myocardial function recovery after reperfusion. Hearts were subjected to sham MI/R or MI/R treated with vehicle or SB 203580. SB 203580 was initiated 15 minutes before ischemia and continued during reperfusion. Each point represents mean±SE of 9 through 11 animals. *P<0.05, **P<0.01 vs vehicle.

**Figure 7.** Time-dependent effects of SB 203580 on myocardial function recovery at 60 minutes of reperfusion. SB 203580 (10 μmol/L) was given (1) 15 minutes before ischemia and during reperfusion (MI/R+SB 203580/IR); (2) only during reperfusion (MI/R+SB 203580/R); or (3) 10 minutes after reperfusion (MI/R+SB 203580/DT). Each point represents mean±SE of 9 through 11 animals. *P<0.05, **P<0.01 vs vehicle.
protection, we determined the time course of SAPK activation in the presence and absence of 10 μmol/L SB 203580. Consistent with our previous report, 20 SAPK activity was markedly elevated after reperfusion; peak activation occurred 20 minutes after reperfusion (Figure 9, open circles and representative autoradiogram A). Treatment with SB 203580 had no effect on SAPK activity at any time point observed (Figure 8C, lanes 2 and 3 and Figure 9, filled circles and representative autoradiogram B). This result suggests that the cardiovascular protection observed with SB 203580 treatment was not related to SAPK activation.

**Discussion**

The present studies provide crucial information in clarifying the role of stress-response MAPK in response to ischemic injury. Our results demonstrated that ischemia alone resulted in a transient, moderate (3.5-fold) increase in p38 MAPK activation. On reperfusion, however, marked activation of p38 MAPK was observed, with a 6.3-fold activation achieved at 10 minutes after reperfusion. These results demonstrate for the first time in a quantitative manner that, although ischemia alone can activate p38 MAPK, ischemia followed by reperfusion results in a more profound p38 MAPK activation. These results also lead us to hypothesize that the strong activation of p38 MAPK by reperfusion may play a more significant role in subsequent myocardial injury than previously realized.

A novel finding in our study is that administration of a p38 MAPK inhibitor, SB 203580, markedly reduced postischemic myocardial apoptosis. Although previous studies have demonstrated that p38 MAPK plays a key role in apoptosis in a variety of cell culture systems 8,9,14,25 and that ischemia/reperfusion activates p38 MAPK in animal models, 15,16 whether or not p38 MAPK activation in ischemic myocardial tissue is involved in postischemic myocardial apoptosis has not been directly determined. To our knowledge, our results provide the first direct evidence that p38 MAPK is a key factor in signal transduction leading to apoptotic myocyte death, thus attenuating postischemic myocardial injury and improving cardiac functional recovery. This conclusion is supported by our time-dependent study. Our results demonstrated that the improvement in cardiac function was closely correlated with the inhibition of p38 MAPK.

**Figure 8.** Effects of SB 203580 given at different times on necrotic area size and myocardial CK loss (A, n=8 through 10 in each group), myocardial MAPKAP K2 activity (B), and SAPK activity (C) after myocardial ischemia and reperfusion. SB 203580 (10 μmol/L) was given (1) 15 minutes before ischemia and during reperfusion (MI/R+SB 203580/IR); (2) only during reperfusion (MI/R+SB 203580/R); or (3) 10 minutes after reperfusion (MI/R+SB 203580/DT). Myocardial necrosis and myocardial CK loss were measured 120 minutes after reperfusion, and myocardial MAPKAP K2 activity was measured 10 minutes after reperfusion. *P<0.05, **P<0.01 vs vehicle.

**Figure 9.** Effects of SB 203580 on activation of SAPK in rabbit hearts subjected to 30 minutes of ischemia alone or 30 minutes of ischemia followed by various periods of reperfusion as indicated in figure. Top panel summarizes experimental results of 3 through 6 animals in each group; bottom panels are representative autoradiograms: A, MI/R + vehicle; B, MI/R + 10 μmol/L SB 203580.
MAPK. Administration of SB 203580 both before ischemia and during reperfusion completely blocked p38 MAPK activation. This treatment also exerted the best cardioprotective effect. In contrast, when SB 203580 was administered 10 minutes after reperfusion, a time point at which maximal p38 MAPK has already been reached, no protective effect against postischemic cardiac dysfunction was observed. Taken together, our results provide the first direct evidence that inhibition of p38 MAPK and reduction of subsequent myocardial apoptosis are capable of improving heart function after myocardial ischemia and reperfusion.

In addition, we have also demonstrated that administration of SB 203580 significantly attenuated postischemic myocardial necrotic injury. We cannot precisely determine the mechanisms by which SB 203580 reduces myocardial necrosis in this severe pathological model. Although we do not have any direct evidence to support a hypothesis that apoptosis may be followed by necrosis in the same population of cells, it cannot be ruled out that the protective effect of SB 203580 against necrotic injury is related to its ability to reduce early apoptosis in ischemic/reperfused hearts.

In summary, we have demonstrated that myocardial ischemia and reperfusion, a real pathological stress to the heart, results in significant activation of p38 MAPK. We have provided the first direct evidence that activation of p38 MAPK plays a key role in the signal transduction pathway mediating myocardial apoptosis after ischemia and reperfusion. Most interestingly, we have found that inhibiting p38 MAPK, which reduces myocardial apoptosis associated with p38 MAPK activation, significantly improves postischemic cardiac functional recovery. These results provide a likely answer to the critical question of whether or not a reduction of apoptosis can translate into meaningful cardiac functional improvement in ischemic/reperfused hearts. Moreover, these results are potentially of great clinical significance because they may suggest a new treatment for ischemic heart disease.

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References

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