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# An Essential Role of the Antioxidant Gene Bcl-2 in Myocardial Adaptation to Ischemia: An Insight with Antisense Bcl-2 Therapy

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## ABSTRACT

**Reperfusion of ischemic myocardium results in apoptotic cell death, which can be blocked by adapting the heart to ischemic stress induced by cyclic episodes of brief periods of ischemia and reperfusion. In concert, the anti-apoptotic gene bcl-2 is decreased by ischemia/reperfusion, but increased in the ischemically adapted myocardium. To examine if bcl-2 plays a crucial role in cardioprotection, adaptive cardioprotection was further examined in the hearts treated with antisense bcl-2 oligodeoxynucleotides (ODN). Isolated Langendorff-perfused rat hearts were divided into three groups: control (perfused with Krebs–Henseleit bicarbonate buffer for 210 min); 30-min ischemia followed by 2-h reperfusion; ischemic adaptation followed by 30-min ischemia and 2-h reperfusion. The last (adapted heart) group was subdivided into another two groups: one was transfected 48 h earlier with antisense bcl-2 ODN, whereas the other group was transfected with sense bcl-2 ODN. Cardioprotection was examined by determining cardiomyocyte death due to necrosis and apoptosis. Antisense gene therapy almost completely abolished bcl-2 protein expression in the hearts. Bcl-2 mRNA was down-regulated in the ischemic/reperfused heart, but up-regulated in the adapted myocardium. Adapted myocardium showed decreased infarct size and reduced number of apoptotic cardiomyocytes. Ischemia/reperfusion resulted in increased oxidative stress as evidenced by increased malonaldehyde formation. Adapted myocardium had a reduced amount of malonaldehyde. Antisense bcl-2 ODN completely abolished the cardioprotective effects of adaptation by eliminating the antideath signal of bcl-2. In concert, reduced oxidative stress in the adapted myocardium no longer persisted. The results suggest an antioxidant role of bcl-2 that appeared to be essential for the cardioprotection achieved by ischemic adaptation.** Antioxid. Redox Signal. 3, 403–413.

## INTRODUCTION

RECENT STUDIES have demonstrated that myocardial ischemia and reperfusion result in apoptotic cell death in addition to tissue necrosis (5, 15, 18, 19). Translocation of phosphatidylserine and phosphatidylethanolamine, a hallmark for apoptosis, has been found to occur during ischemia, but apoptosis does not become apparent until hearts are reperfused following an ischemic insult (20). Myocardial adaptation to ischemia induced by repeated cyclic episodes of reversible short durations of ischemia each followed by another short duration of reperfusion was found to be effective in reducing apoptotic cell death (21).

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Bcl-2 is an oncogene that inversely regulates apoptosis (29). Bcl-2 is located downstream to p53 in the apoptotic pathway, and it contains negative or positive p53 response elements (35). Bcl-2 can also protect cells by acting as an antioxidant (16). Several reports exist in the literature to show a diminished bcl-2 activity in the hearts subjected to ischemia and reperfusion (22, 25). A recent study from our laboratory showed up-regulation of bcl-2 by ischemic adaptation (26). Induction of bcl-2 was found to be associated with reduction of apoptotic cell death and DNA fragmentation.

To gain further insight into the possible cardioprotective role of bcl-2, rat hearts were transfected with antisense bcl-2 oligodeoxynucleotides (ODN) to block bcl-2 gene expression. The ischemically adapted hearts transfected with antisense bcl-2 ODN remained vulnerable to cardiomyocyte death to the same degree as nonadapted hearts, whereas the adapted hearts that were transfected with the sense bcl-2 remained resistant to the cell death. These results suggest the crucial role of bcl-2 in cardioprotection achieved through adaptation.

## MATERIALS AND METHODS

### *Transfection of antisense and sense bcl-2 ODN into rat myocardium*

To verify the role of bcl-2 in ischemia/reperfusion-induced apoptotic cell death, rat hearts were transfected with both antisense [5′-CAGCGTGCGCCATCCTTCCC-3′ (20 mer)] and sense [5′-GGGAAGGATGGCGCACGCTG-3′ (20 mer)] bcl-2 ODN (modified) into rat myocardium. Successful transfer of antisense and sense bcl-2 was confirmed by western blotting of the bcl-2 protein in the hearts.

### *Preparation of HVJ liposomes*

HVJ (hemagglutinating virus of Japan) liposomes were prepared as described previously (34). Liposomes containing oligonucleotides and high-mobility group 1 were constituted. In brief, dried lipid (phosphatidylserine, phosphatidylcholine, and cholesterol combined at a weight ratio of 1:4.8:2) was mixed with oligonucleotides (30 ng/ml; previously incubated at 20°C for 1 h with high-mobility group 1), shaken vigorously, and sonicated to form the liposome. Purified HVJ (Z strain) was inactivated by ultraviolet irradiation (110 erg/mm$^2$/s) for 3 min just before use. The liposome suspension mixed with HVJ was incubated at 4°C for 10 min and at 37°C for 30 min. The HVJ-liposome complex was collected for use after removal of free HVJ.

### *Animal preparation*

Sprague–Dawley male rats weighing 275–300 g were used in this study. The experiments were conducted in accordance with the *Guidelines for the Care and Use of Laboratory Animals* (NIH publication no. 86-23, revised in 1985). Endotracheal intubation was performed under general anesthesia with sodium pentobarbital (80 mg/kg of body weight, intraperitoneal injection; Abbott Laboratories, North Chicago, IL, U.S.A.). Artificial respiration was maintained with the respiration volume at 10 ml/kg, the respiration rate at 70 times/min, and positive end-expiratory pressure at 1 cm $H_2O$.

The rats were placed in the supine position, and the right common carotid artery was exposed. PE 50 cannula was introduced into the right common carotid artery and passed as far as the aortic valve through the ascending aorta. Then the rats were held in the left lateral position, and right lateral third intercostal thoracotomy was performed. The right lung was compressed, and the ascending aorta was exposed. 3-0 silk thread was placed around the ascending aorta, positioned above the top of the cannula. The thread was tightened for 20 s, and 0.7 ml of HVJ liposome-DNA complex (including 5 $\mu$g of bcl-2 DNA) was infused into the coronary artery (coronary direct perfusion) at this time. The chest incision was closed with a three-layer suture using 3-0 nylon thread, and the endotracheal tube was removed after wakening.

### *Isolated rat heart preparation*

Forty-eight hours after the first operation, the rats were anesthetized again with sodium pentobarbital (80 mg/kg i.p.) and heparin (500 IU/kg) infused via the femoral vein. Then

the chests were opened and the hearts were rapidly excised and mounted on a Langendorff perfusion apparatus (9). Retrograde perfusion was established at a pressure of 100 cm $H_2O$ with an oxygenated normothermic Krebs–Henseleit bicarbonate (KHB) buffer (in m*M*: 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 $KH_2PO_4$, 25 $NaHCO_3$, 10 glucose, and 1.7 $CaCl_2$), gassed with 95% $O_2$/5% $CO_2$, pH 7.4 at 37°C. After 10 min of KHB perfusion for stabilization, the hearts (both antisense and sense bcl-2 ODNs) were subjected to ischemic adaptation, followed by 30 min of global ischemia and 120 min of reperfusion (Fig. 1).

In a separate set of experiments, isolated rat hearts were subjected to (i) 210 min of perfusion with the KHB buffer (control), (ii) 30 min of ischemia followed by 2 h of reperfusion (ischemia/reperfusion), or (iii) adaptation by subjecting them to four cyclic episodes of 5 min of ischemia each followed by 10 min of reperfusion, which were then followed by 30 min of ischemia and 2 h of reperfusion. The experimental protocol is depicted in Fig. 1.

*Measurement of myocardial infarct size*

Hearts to be used for infarct size calculations were taken upon termination of the experiment and immersed in 1% triphenyltetrazolium solution in phosphate buffer (88 m*M* $Na_2HPO_4$, 1.8 m*M* $NaH_2PO_4$) for 10 min at 37°C and then stored at −70°C for later processing (33). Frozen hearts (including only ventricular tissue) were sliced transversely in a plane perpendicular to the apico-basal axis into ~0.5-mm-thick sections, blotted dry, placed in between microscope slides, and scanned on a Hewlett–Packard Scanjet 5p single-pass flatbed scanner (Hewlett–Packard, Palo Alto, CA, U.S.A.). Using the NIH 1.61 image processing software, each digitized image was subjected to equivalent degrees of background subtraction, brightness, and contrast enhancement for improved clarity and distinctness. Risk (equivalent to total left ventricle muscle mass) as well as infarct zones of each slice were traced and the respective areas were calculated in terms of pixels. The weight of each slice was then recorded to facilitate the expression of total and infarct masses of each slice in grams. The risk and infarct volumes (in cubic centimeters) of each slice were then calculated on the basis of slice weight to remove the introduction of any errors due to nonuniformity of heart-slice thickness. The risk volumes and infarct volumes of each slice were summed to obtain the risk and infarct volumes for the whole heart. Infarct size was taken to be the percent infarct volume/risk volume for any one heart.

*Evaluation of apoptosis*

Immunohistochemical detection of apoptotic cells was carried out using TUNEL in which residues of digoxigenin-labeled dUTP are catalytically incorporated into the DNA by terminal deoxynucleotidyltransferase II, an enzyme that catalyzes a template-independent addition of nucleotide triphosphate to the 3′-OH ends of double- or single-stranded DNA (32). The incorporated nucleotide was incubated with a

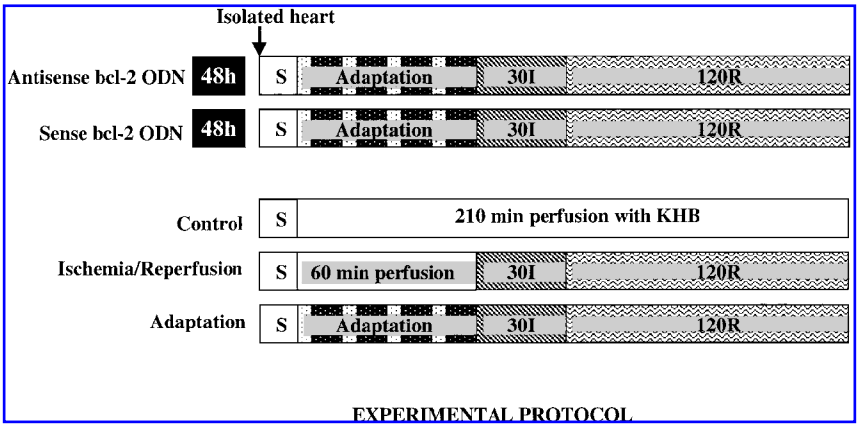


**FIG. 1. Experimental protocol.** 30I, 30 min of ischemia; 120R, 120 min of reperfusion.

sheep polyclonal anti-digoxigenin antibody followed by a fluorescein isothiocyanate-conjugated rabbit anti-sheep IgG as a secondary antibody as described by the manufacturer (Apop Tag Plus, Oncor Inc., Gaithersburg, MD, U.S.A.). The sections were washed in phosphate-buffered saline three times, blocked with normal rabbit serum, and incubated with mouse monoclonal antibody recognizing cardiac myosin heavy chain (Biogenesis Ltd., Poole, U.K.) followed by staining with tetramethylrhodamine isothiocyanate-conjugated rabbit anti-mouse IgG (200:1 dilution; Dako Japan, Tokyo, Japan). The fluorescence staining was viewed with a confocal laser microscope (Olympus Co., Tokyo, Japan). The number of apoptotic cells was counted and expressed as a percentage of total myocyte population.

*Determination of bcl-2 mRNA by northern blot analysis*

Total RNA was extracted from the heart tissues by the acid–guanidinium thiocyanate–phenol–chloroform method as described previously (20). Total RNA was electrophoresed in 1% agarose formaldehyde-formamide gel and transferred to Gene Screen Plus hybridization transfer membrane (Biotech Systems, NEN Research Products, Boston, MA, U.S.A.). The membrane was then baked under vacuum at 80°C for 1 h. Each hybridization was repeated at least three times using different membranes. After each hybridization, the residual cDNA was removed and rehybridized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe, the results of which served as a loading control. The autoradiograms were quantitatively evaluated by computerized $\beta$-scanner. The results of densitometric scanning were normalized relative to the signal obtained by using GAPDH cDNA.

*Determination of bcl-2 protein by western blot analysis*

For western blot analysis, heart tissues exposed to various experimental conditions were homogenized and suspended (5 mg/ml) in sample buffer (10 m*M* HEPES, pH 7.3, 11.5% sucrose, 1 m*M* EDTA, 1 m*M* EGTA, diisopropyl fluorophosphate, 0.7 mg/ml pepstatin A, 10 mg/ml leupeptin, 2 mg/ml aprotinin] (26). The homogenates were centrifuged at 3,500 rpm, and cytosolic fractions were used for protein analysis. The total protein concentration was determined by using BCA (bicinchoninic acid) protein assay kit (Pierce, Rockville, IL, U.S.A.). The cytosolic proteins (10 $\mu$g) were then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed under reducing conditions on 14% Tris glycine gels (Novex, San Diego, CA, U.S.A.). Proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, U.S.A.). Bcl-2 antigen was detected by incubation of the blots in 5 ml of solution containing 1–5 $\mu$g/ml of the relevant primary antibody and using 1 $\mu$g/ml concentration of rabbit anti-mouse secondary horseradish peroxidase-conjugated antibody. Enhanced chemiluminescence reagents (Amersham) served as substrate solution according to the recommendation of the manufacturer.

*Estimation of malondialdehyde (MDA) in heart*

For MDA determination, heart was homogenized followed by derivatization using 2,4-dinitrophenylhydrazine as described previously (9). The aqueous phase was extracted with pentane, blown down with nitrogen, and reconstituted in 200 $\mu$l of acetonitrile. Aliquots of 25 $\mu$l were injected onto a Beckman Ultrasphere $C_{18}$ (3 mm) column in a Waters HPLC. The products were eluted isocratically with a mobile phase containing acetonitrile $H_2O$ $CH_3COOH$ (34:66:0.1, by volume). The amount of MDA was determined by performing peak area analysis using the Maxima software program (Waters).

*Statistical analysis*

For statistical analysis, a two-way analysis of variance followed by Scheffé's test was first carried out using Primer Computer Program (McGraw–Hill, 1988) to test for any differences between groups. If differences were established, the values were compared using Student's $t$ test. The values were expressed as means $\pm$ SEM. The results were considered significant if $p$ was $<0.05$.

## RESULTS

### *Bcl-2 mRNAs and protein activities*

Bcl-2 mRNAs were present in all hearts. Thirty minutes of ischemia did not change the level of bcl-2 mRNAs (Fig. 2). The amount of bcl-2 gene was decreased significantly after 2 h of reperfusion following 30 min of ischemia. A significant 2.5-fold increase in the induction of bcl-2 expression was found in the preconditioned hearts. The results thus demonstrated a reduction of bcl-2 gene expression in the ischemic/reperfused myocardium and an increase in the induction of the gene expression in the preconditioned hearts.

To verify bcl-2 transfection, bcl-2 protein activity was determined in the hearts 48 h after the transfection with antisense or sense bcl-2 ODN. As shown in Fig. 3, antisense bcl-2 prevented the induction of bcl-2 protein levels observed in sense-transfected preconditioned hearts or control nontransfected preconditioned hearts.

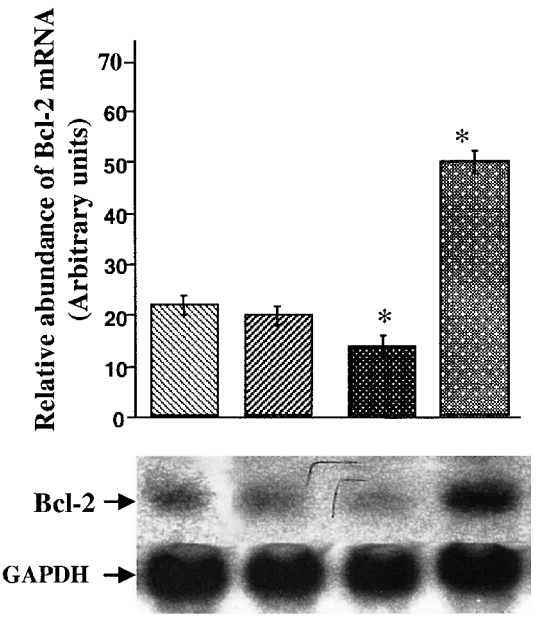


**FIG. 2. Northern blot analysis of bcl-2 mRNA.** Northern blot analysis reveals induction of bcl-2 in preconditioned rat myocardium. Total RNA was isolated, and northern hybridization was performed as described in Materials and Methods. (**Bottom**) Representative northern blots show decrease in bcl-2 mRNA in the ischemic/reperfused myocardium and increase in mRNA in the preconditioned heart. GAPDH served as housekeeping gene. (**Top**) Results of densitometric scanning (means ± SEM) for six different experiments at each time point are shown for each blot. *$p < 0.05$ compared with nonischemic or ischemic control. (▧), baseline; (▩), 30-min ischemia; (■), 30-min ischemia follwed by 2 h of reperfusion; (▩), adapted.

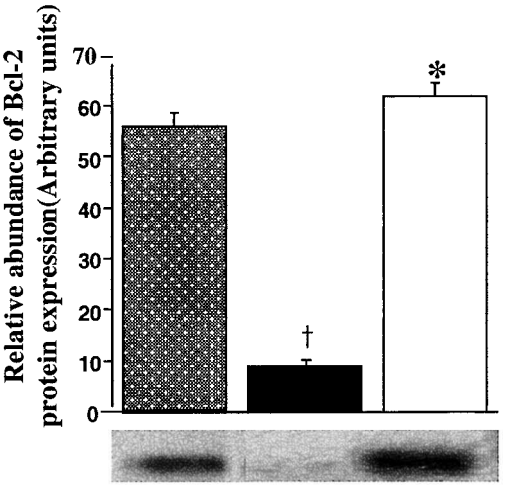


**FIG. 3. Western blot analysis of bcl-2 in rat myocardium transfected (2 days after transfection) with both antisense and sense bcl-2 ODN.** (**Bottom**) Representative western blots show a decrease in bcl-2 protein in the adapted heart transfected with bcl-2 antisense ODN. (**Top**) Results of densitometric scanning (means ± SEM) for six different experiments at each time point are shown for each blot. Results are expressed as means ± SEM of six different rats per group. *$p < 0.05$ compared with the transfected antisense ODN myocardium. (▩), adapted (nontransfected control); (■), transfected with antisense ODN; (□), transfected with sense ODN.

### *Myocardial infarction*

Cardiomyocytes die of both necrosis and apoptosis during the reperfusion following an ischemic insult. No infarct was visible in the hearts perfused for 210 min (Fig. 4). Thirty minutes of ischemia also did not produce any infarction. A significant amount of infarction was noticed in the hearts subjected to 30 min of ischemia followed by 2 h of reperfusion. About 50% reduction in the infarct size was found in the adapted hearts.

The amount of infarct remained high in the adapted hearts that had been transfected with antisense bcl-2 gene (Fig. 4). In contrast, a reduced amount of infarction was found in the adapted myocardium transfected with sense bcl-2, indicating that inhibition of bcl-2 abolished the infarct size, lowering abilities due to adaptation.

*Cardiomyocyte apoptosis*

Apoptotic cardiomyocytes were detected using TUNEL staining in conjunction with staining with antibody against $\alpha$-myosin heavy chain. No apoptotic cells were visible in the hearts perfused for 210 min or in the hearts subjected to 30 min of ischemia (Fig. 5). A large number of cardiomyocytes were found to undergo apoptosis after 2 h of reperfusion following 30 min of ischemic insult, indicating that reperfusion, but not ischemia, induces apoptosis. Myocardial adaptation to ischemia significantly lowered the cardiomyocyte death due to apoptosis.

The apoptotic cardiomyocytes reappeared in the adapted myocardium when they were transfected with bcl-2 antisense (Fig. 5). Only a small amount of cells underwent apoptosis in the adapted hearts transfected with sense bcl-2, indicating that inhibition of bcl-2 abolished adaptive cardioprotection.

*MDA formation*

MDA formation is the presumptive marker for the development of oxidative stress and production of reactive oxygen species. As expected, the amount of MDA in the heart increased progressively during the reperfusion (Fig. 6). A significantly reduced amount of MDA was found in the adapted myocardium, indicating decreased oxidative stress. Bcl-2 antisense ODN increased the amount of MDA in the adapted heart, suggesting that bcl-2 functioned as an antioxidant during myocardial adaptation to ischemia.

## DISCUSSION

In this report, we demonstrate that ischemia/reperfusion down-regulates, whereas myocardial adaptation to ischemia up-regulates, the bcl-2 gene. Prolonged reperfusion following an ischemic insult results in cardiac cell death due to both necrosis and apoptosis. Ischemic adaptation by cyclic episodes of short durations of reversible ischemia and reperfusion reduced myocardial infarct size and almost abolished apoptotic cell death. Inhibition of the bcl-2 gene by infecting the hearts with antisense bcl-2 blocked the cardioprotective abilities of adaptation as the infected cardiomyocytes underwent both necrotic and apoptotic cell death. In concert, bcl-2 antisense ODN increased the amount of oxidative stress as indicated by the increased amount of MDA formation in the adapted hearts. The results indicated that bcl-2 plays a crucial role in cardioprotection by functioning as an intracellular antioxidant during myocardial adaptation to ischemia.

Evidence is rapidly accumulating to support the notion that both necrosis and apoptosis contribute to the pathophysiology of ischemic and reperfusion injury. A recent study showed that apoptotic and necrotic myocyte cell deaths independently contribute to infarct size in rats, but apoptosis did not become evident for up to 2 h of ischemia (11). A growing body of evidence indicates that cardiomyocytes undergo apoptotic cell death in a variety of coronary diseases, including heart failure, myocardial infarction, and ischemia/reperfusion (5, 15, 18, 19, 26, 30). Characteristic signs of apoptosis appear in the ischemic myocardium only after several hours of ischemia. In the case of the rat heart, apoptosis first occurs after 2 h of ischemia, and a significant number of cells undergo apoptotic cell death after prolonged ischemia. In contrast, reperfusion even after a brief period of ischemia results in apoptosis. Recent

**FIG. 5. Effect of ischemia, ischemia/reperfusion (I/R), ischemic adaptation, and antisense and sense bcl-2 ODN transfection on myocardial apoptosis by double antibody staining (TUNEL assay).** Hearts were perfused for 150 min and served as control, or hearts were subjected to 30 min of ischemia or 30 min of ischemia followed by 120 min of reperfusion. The ischemic preconditioned group was subjected to four episodes of, 5 min of ischemia followed by 10 min of reperfusion. (**Bottom**) Panel shows significant number of apoptotic cardiomyocytes in the ischemic/reperfused rat myocardium, whereas only a few apoptotic cardiomyocytes were detectable in the adapted heart compared with I/R hearts. In baseline control and 30-min ischemic hearts, no apoptotic cells were detected. A reduced number of apoptotic cells in the adapted hearts (nontransfected or sense ODN-transfected) was increased in the antisense-transfected hearts. (**Top**) The percent apoptotic cardiomyocyte cell death of control (▧), ischemia (▩), ischemia and reperfusion (■), adapted (▩), and antisense bcl-2 (■) and sense bcl-2 (□) transfected rat myocardium. Results are expressed as means ± SEM of six different rats per group. *$p < 0.05$ compared with the control group of sample.

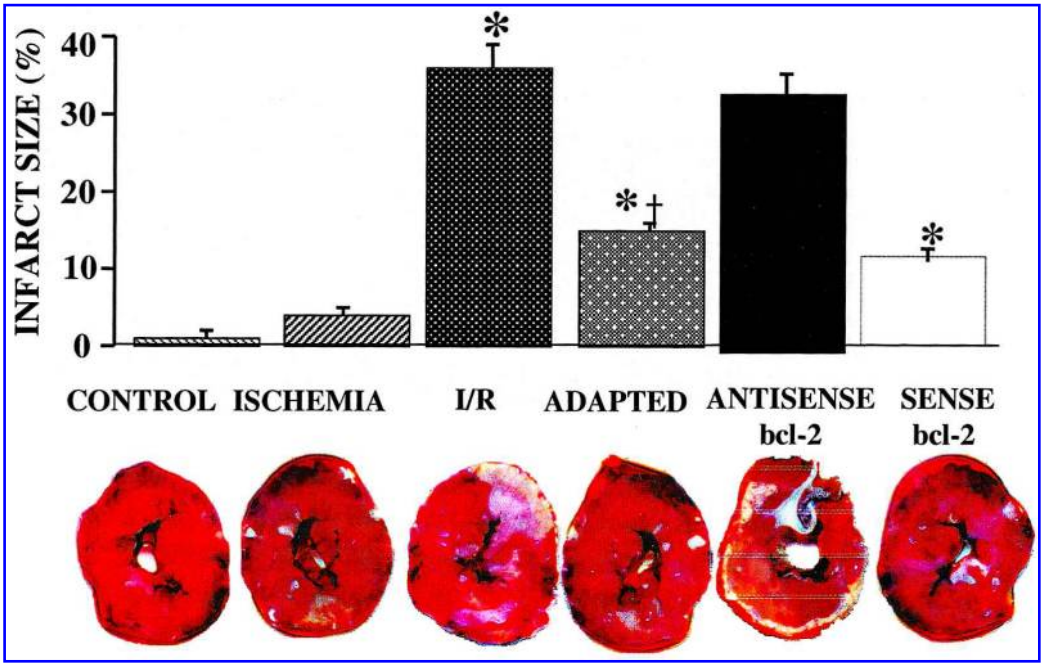


**FIG. 4. Effect of ischemia, ischemia/reperfusion (I/R), ischemic adaptation, and antisense and sense bcl-2 ODN transfection on myocardial infarction.** Hearts were perfused for 150 min and served as control, or hearts were subjected to 30 min of ischemia or 30 min of ischemia followed by 120 min of reperfusion. The ischemic preconditioned group was subjected to four episodes of, 5 min of ischemia followed by 10 min of reperfusion. (**Bottom**) Representative hearts revealing various degrees of myocardial infarction (white to yellowish zones after TTC staining). (**Top**) The percent infarct size of control (■), ischemia (■), ischemia and reperfusion (■), adapted (■), antisense bcl-2 (■), and sense bcl-2 (□) transfected rat myocardium. Results are expressed as means ± SEM of six different rats per group. $*p < 0.05$ compared with the control group of sample.

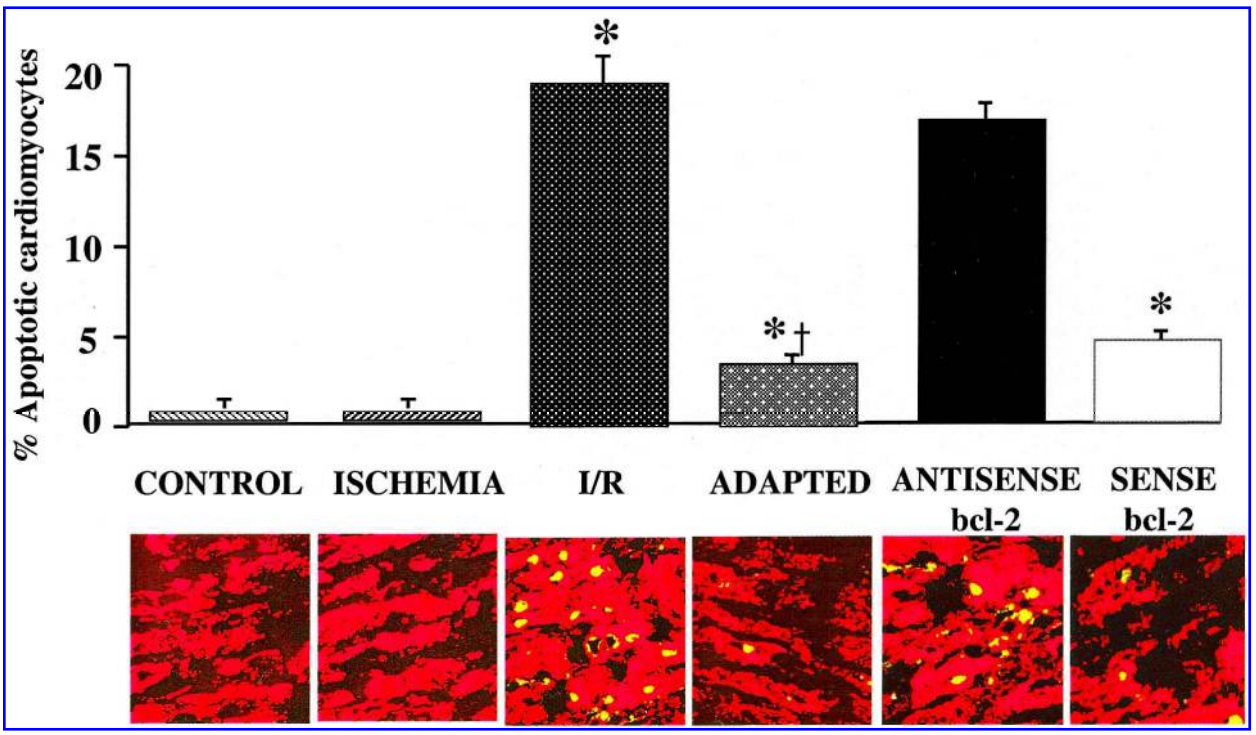


FIG. 5.

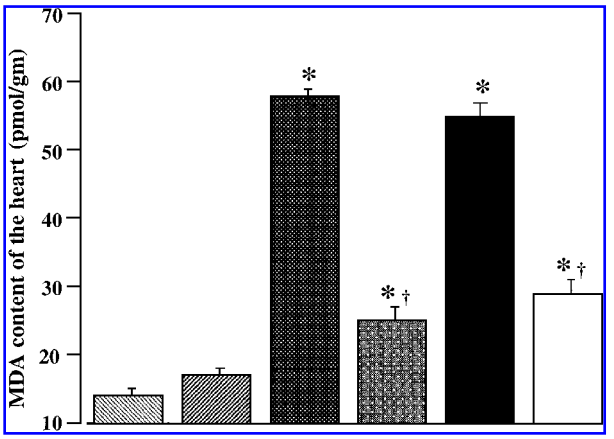


**FIG. 6. Effect of antisense and sense bcl-2 ODN transfection on the MDA content of the heart during ischemia and reperfusion.** (▧), control; (■), ischemia; (■), ischemia and reperfusion; and (■), adapted heart. (■), antisense-bcl-2, and (□) sense bcl-2 transfected rat myocardium. Results are expressed as means ± SEM of six different rats per group. *$p < 0.05$ compared with the control group of sample.

studies from our laboratory also demonstrated that in the rat heart apoptosis does not occur with up to 2 h of ischemia, but a significant number of myocytes are subjected to apoptotic cell death and DNA fragmentation after even 90 min of reperfusion following 15 min of ischemia (20). These results suggest that although apoptosis occurs during prolonged ischemia or in purely infarcted myocardium, reperfusion triggers a distinct signal for apoptosis.

Myocardial cells possess a remarkable ability to adapt themselves to any stressful events by increasing resistance to the adverse conditions. When stress is induced by ischemia/reperfusion or by subjecting the heart to heat or other kinds of oxidative challenge, the myocardium is able to meet the future stress challenge by up-regulating its cellular defense through direct accumulation of intracellular mediators that constitute the material basis of increased adaptation to stress (8). Several recent studies demonstrated that myocardial adaptation to ischemia, also known as ischemic preconditioning, by repeated brief episodes of ischemia and reperfusion renders the heart resistant to subsequent lethal ischemic reperfusion injury (2, 10). Such adaptation has been found to be associated with the reduction in tissue necrosis, as well as apoptosis (21). Very few apoptotic cells exist in the ischemic/reperfused hearts that had been adapted previously to ischemic stress.

In various systems, bcl-2 functions as an antideath gene by inhibiting both necrotic and apoptotic cell death (6). Recent analysis of the bcl-2 gene family reveals a complex network of this gene regulating apoptosis. Within this bcl-2 gene family, some of the candidates can suppress apoptosis, whereas the others can induce apoptosis (1). Apoptosis initiated by various different stimuli can be blocked by overexpressing bcl-2. Apoptosis induced by c-Myc overexpression has been found to be p53-dependent, and these cells can be rescued by bcl-2 gene expression (3). Bax, a bcl-2-associated x protein, on the other hand, possesses powerful death-promoting abilities and enhances apoptotic cell death (13). Bax has been reported to be positively regulated by p53 on a transcriptional level (27). The ability of bcl-2 to block apoptosis is critically dependent on the ratio of bcl-2 to bax. In the presence of excess bcl-2, the formation of bax/bcl-2 heterodimers protects the cells from undergoing apoptosis.

Previous studies documented a down-regulation of bcl-2 gene expression in the ischemic/reperfused myocardium. In contrast, myocardial adaptation to ischemia resulted in a severalfold induction of the expression of the bcl-2 gene. In the present study, the bcl-2 gene remained unaffected after 30 min of ischemia, but significantly down-regulated after 2 h of reperfusion. The activation of bcl-2 in the adapted hearts was associated with the inhibition of apoptosis and necrosis. Hearts infected with bcl-2 antisense could not be adapted because these hearts were no longer resistant to the cell death due to apoptosis and necrosis. The results thus indicate an essential role of bcl-2 in ischemic adaptation.

A large number of studies exist in the literature indicating a role of reactive oxygen species in myocardial ischemic reperfusion injury (17, 31). The presence of oxygen free radicals has been confirmed directly using electron spin resonance and HPLC techniques and indirectly by MDA formation (36). The amount of MDA increases progressively as a function of reperfusion time, suggesting a steady increase in the development of the oxidative stress during the reperfusion. Consistent with these previous re-

ports, our results determined an increased amount of MDA formation during the reperfusion of ischemic myocardium. The amount of MDA decreased significantly in the adapted hearts indicating a reduction of the oxidative stress. The extent of ischemic reperfusion injury can be reduced by pretreating the hearts with free radical scavengers or antioxidants (7).

Interestingly, free radicals and/or oxidative stress are also common mediators of apoptosis, perhaps via the formation of lipid peroxidation and lipid hydroperoxides. A direct role of oxygen free radicals has also been implicated in the pathogenesis of apoptosis. For example, superoxide dismutase (SOD) or an expression vector containing SOD cDNA was found to delay apoptotic cell death, and free radical-mediated apoptosis was found to be modulated by protooncogene expression (12). The results of a recent study demonstrated that a selenoperoxide mimic, ebselen, could reduce apoptotic cell death and DNA fragmentation in concert with reduction of myocardial ischemic reperfusion injury (21).

A large number of studies exist in the literature to indicate that bcl-2 lies in an antioxidant pathway where it protects mitochondrial integrity by reducing the cellular reactive oxygen species (37). Overexpression of bcl-2 resulted in a lower amount of hydroxyl radical production (39) and blocks the formation of reactive oxygen species during mitochondrial dysfunction (4). Bcl-2 has also been suggested to be involved in modulating redox signaling by manipulating cellular thiol status (28). In another related study, bcl-2 was found to prevent lipid peroxidation and reduced damage to lipids and proteins (14, 38). The results of our study confirmed these previous results and further demonstrated that the decreased cardiomyocyte apoptosis in the adapted heart was associated with an increase in bcl-2 activity and a reduced amount of oxidative stress. Inhibition of bcl-2 by antisense bcl-2 ODN eliminated the anti-death signal of adaptation by reducing bcl-2 and increasing oxidative stress in the heart.

In summary, our results revealed a crucial role of bcl-2 in myocardial adaptation to ischemic stress. Recurrent episodes of myocardial ischemia are commonly observed in patients with coronary artery disease who suffer from frequent angina pectoris or angioplasty of the left anterior descending coronary artery. Reversibly injured myocardium (by a short episode of ischemia followed by another short period of reperfusion) renders the heart more resistant to a longer ischemic reperfusion period. Such adaptation is mediated through the up-regulation of the heart's own cellular defense via the accumulation of intracellular mediators and reprogramming of gene expression. It appears from the present study that bcl-2 is an essential member of the antioxidant defense of the heart and plays a vital role in dictating cardiomyocytes whether to live or die.

## ACKNOWLEDGMENTS

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## ABBREVIATIONS

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HVJ, hemagglutinating virus of Japan; KHB, Krebs–Henseleit bicarbonate; MDA, malondialdehyde; ODN, oligodeoxynucleotides; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SOD, superoxide dismutase; TUNEL, terminal deoxynucleotidyltransferase-mediated nick end-labeling.

## REFERENCES

1. Adams JM and Cory S. The Bcl-2 protein family. Arbiters of cell survival. *Science* 281, 1312–1316, 1998.
2. Banerjee A, Locke-Winter C, Rogers KB, Mitchell MB, Bensard DD, Brew EC, Cairns CB, and Harken AH. Transient ischemia preconditions against subsequent cardiac ischemia-reperfusion injury by an alpha-1 adrenergic mechanism. *Circ Res* 73: 656–670, 1993.
3. Beham A, Marin MC, Fernandez A, Herrmann J, Brian S, Tari AM, Lopez-Berestein G, Lozano G, Sarkiss M, and McDonnell M. Bcl-2 inhibits p53 nuclear import following DNA damage. *Oncogene* 15: 2767–2772, 1997.
4. Bogdanov MB, Ferrante RJ, Mueller G, Ramos LE, Martinou JC, and Beal MF. Oxidative stress is attenuated in mice overexpressing BCL-2. *Neurosci Lett* 262: 33–36, 1999.
5. Bromme HJ and Holz J. Apoptosis in the heart; when and why. *Mol Cell Biochem* 163–164: 261–275, 1996.

6. Chao DT, and Korsmeyer SJ. Bcl-2 family: regulators of cell death. *Annu Rev Immunol* 16: 395–419, 1998.
7. Das DK and Maulik N. Evaluation of antioxidant effectiveness in ischemia-reperfusion tissue injury methods. *Methods Enzymol* 233: 601–610, 1994.
8. Das DK and Maulik N. Cellular and molecular basis of stress heart. In: *Environmental Stressors and Gene Responses,* edited by Storey KB and Storey J. CT: Jay Press, 2000, pp. 193–218.
9. Engelman DT, Watanabe M, Engelman RM, Rousou JA, Kisin E, Kagan VE, Maulik N, and Das DK. Hypoxic preconditioning preserves antioxidant reserve in the working rat heart. *Cardiovasc Res* 29: 133–140, 1995.
10. Flack J, Kimura Y, Engelman RM, and Das DK. Preconditioning the heart by repeated stunning improves myocardial salvage. *Circulation* 84 (Suppl III): 369–374, 1991.
11. Fliss H and Gattinger D. Apoptosis in ischemic and reperfused rat myocardium. *Circ Res* 79: 949–956, 1996.
12. Greenlund LJ, Deckwerth TL, and Johnson EM, Jr. Superoxide dismutase delays neuronal apoptosis: a role for reactive oxygen species in programmed neuronal death. *Neuron* 14: 303–315, 1995.
13. Gross A, Jockel J, Wei MC, and Korsmeyer SJ. Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. *EMBO J* 17: 3878–3885, 1998.
14. Hockenbery DM, Oltavi ZN, Yin XM, Milliman CL, and Korsmeyer SJ. Bcl-2 functions in an antioxidant pathway to prevent programmed cell death. *Cell* 75: 241–251, 1993.
15. Kajstura J, Cheng W, Reiss K, Clark WA, Sonnenblick EH, Krajewski S, Reed JC, Olivetti G, and Anversa P. Apoptotic and necrotic myocyte cell deaths are independent contributing variables of infarct size in rats. *Lab Invest* 74: 86–107, 1996.
16. Korsmeyer SJ, Yin XM, Oltvai ZN, Veis-Novack DJ, and Linette GP. Reactive oxygen species and the regulation of cell death by the Bcl-2 gene family. *Biochim Biophys Acta* 1271: 63–66, 1995.
17. Kukreja RC, Kontos MC, and Hess ML. Free radicals and heat shock protein in the heart. *Ann NY Acad Sci* 793: 108–122, 1996.
18. Maclellan WR and Schneider MD. Death by design. Programmed cell death in cardiovascular biology and disease. *Circ Res* 81: 137–144, 1997.
19. Maulik N and Das DK. Apoptosis, heart failure, ischemic disease. *Heart Failure Rev* 4: 165–173, 1999.
20. Maulik N, Kagan VE, and Das DK. Redistribution of phosphatidylserine and phosphatidylethanolamine precedes reperfusion-induced apoptosis. *Am J Physiol* 274: H242–H248, 1998.
21. Maulik N, Yoshida T, Engelman RM, Deaton D, Flack JE, Rousou JA, and Das DK. Ischemic preconditioning attenuates apoptotic cell death associated with ischemia/reperfusion. *Mol Cell Biochem* 186: 139–145, 1998.
22. Maulik N, Engelman RM, Rousou JA, Flack JE, Deaton DW, and Das DK. Ischemic preconditioning suppresses apoptosis by upregulating the anti-death gene bcl-2. *Surg Forum* 48: 209–211, 1998.
23. Maulik N, Yoshida T, Zu YL, Banerjee A, and Das DK. Ischemic stress adaptation of heart triggers a tyrosine kinase regulated signaling pathway: a potential role for MAPKAP kinase 2. *Am J Physiol* 275: H1857–H1864, 1998.
24. Maulik N, Yoshida T, and Das DK. Oxidative stress developed during the reperfusion of ischemic myocardium induces apoptosis. *Free Radic Biol Med* 24: 869–875, 1998.
25. Maulik N, Goswami S, Galang N, and Das DK. Differential regulation of Bcl-2, AP-1 and NF$\kappa$B on cardiomyocyte apoptosis during myocardial ischemic stress adaptation. *FEBS Lett* 443: 331–336, 1999.
26. Maulik N, Engelman RM, Rousou JA, Flack JE, Deaton D, and Das DK. Ischemic preconditioning reduces apoptosis by upregulating anti-death gene Bcl-2. *Circulation* 100: II369–II375, 1999.
27. McCurrach ME, Conner TM, Knudson CM, Korsmeyer SJ, and Lowe SW. Bax-deficiency promotes drug resistance and oncogenic transformation by attenuating p53-dependent apoptosis. *Proc Natl Acad Sci U S A* 94: 2345–2349, 1997.
28. Mirkovic N, Voehringer DW, Story MD, McConkey DJ, McDonnell TJ, and Meyn RE. Resistance to radiation-induced programmed cell death in Bcl-2-expressing cells is reversed by depleting cellular thiols. *Oncogene* 15: 1461–1470, 1997.
29. Misao J, Hayakawa Y, Ohno M, Kato S, Fujiwara T, and Fujiwara H. Expression of bcl-2 protein, an inhibitor of apoptosis, and Bax, an accelerator of apoptosis, in ventricular myocytes of human hearts with myocardial infarction. *Circulation* 94: 1506–1512, 1996.
30. Olivetti G, Abbi R, Quaini F, Kajstura J, Cheng W, Nitahara JA, Quaini E, Di Loreto C, Beltrami CA, Krajewski S, Reed JC, and Anversa P. Apoptosis in the failing human heart. *N Engl J Med* 336: 1131–1141, 1997.
31. Otani H, Engelman RM, Rousou JA, Breyer RH, Lemeshow S, and Das DK. Cardiac performance during reperfusion improved by pretreatment with oxygen free radical scavengers. *J Thorac Cardiovasc Surg* 91: 290–295, 1986.
32. Ray PS, Martin JL, Swanson EA, Otani H, Dillmann WH, and Das DK. Transgene overexpression of alphaB crystallin confers simultaneous protection against cardiomyocyte apoptosis and necrosis during myocardial ischemia and reperfusion. *FASEB J* 15: 393–402, 2001.
33. Sato M, Engelman RM, Otani H, Maulik N, Rousou JA, Flack JE, Deaton DW, and Das DK. Myocardial protection by preconditioning of heart with losartan, AT1 receptor blocker. *Circulation* 102: III346–III351, 2000.
34. Sawa Y, Suzuki K, Bai HZ, Shirakura R, Morishita R, Kaneda Y, and Matsuda H. Efficiency of in vivo gene

transfection into transplnted rat heart by coronary infusion of HVJ liposome. *Circulation* 92: II479–II482, 1995.
35. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. *Science* 267: 1456–1462, 1995.
36. Tosaki A, Bagchi D, Hellegouarch D, Pali T, Cordis GA, and Das DK. Comparisons of ESR and HPLC methods for the detection of hydroxyl radicals in ischemic/reperfused hearts. A relationship between the genesis of oxygen free radicals and reperfusion-induced arrhythmias. *Biochem Pharmacol* 45: 961–969, 1993.
37. Tyurina YY, Tyurin, VA, Carta G, Quinn PJ, Schor NF, and Kagan VE. Direct evidence for antioxidant effect of Bcl-2 in PC12 rat pheochromocytoma cells. *Arch Biochem Biophys* 344: 413–423, 1997.
38. Virgili F, Santini MP, Canali R, Polakowska RR, Heads A, and Perozzi G. Bcl-2 overexpression in the HaCat cell line is associated with a different fatty acid composition and sensitivity to oxidative stress. *Free Radic Biol Med* 24: 93–101, 1998.
39. Wiedau-Pazos M, Trudell JR, Altenbach C, Kane A, Hubbell WT, and Bredesen DE. Expression of bcl-2 inhibits cellular radical generation. *Free Radic Res* 24: 205–212, 1998.

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2. Hiroyuki Kobayashi, Shinji Yasuda, Narentuoya Bao, Masamitsu Iwasa, Itta Kawamura, Yoshihisa Yamada, Takahiko Yamaki, Syohei Sumi, Hiroaki Ushikoshi, Kazuhiko Nishigaki, Genzou Takemura, Takako Fujiwara, Hisayoshi Fujiwara, Shinya Minatoguchi. 2009. Postinfarct Treatment With Oxytocin Improves Cardiac Function and Remodeling via Activating Cell-survival Signals and Angiogenesis. *Journal of Cardiovascular Pharmacology* **54**:6, 510-519. [CrossRef]
3. Nuno G. Machado, Marco G. Alves, Rui A. Carvalho, Paulo J. Oliveira. 2009. Mitochondrial Involvement in Cardiac Apoptosis During Ischemia and Reperfusion: Can We Close the Box?. *Cardiovascular Toxicology* **9**:4, 211-227. [CrossRef]
4. 2009. The Effect of Swimming and Minocycline on Motor Function and Expression of Bcl-2 after Spinal Cord Injury in the Rats. *Journal of Life Science* **19**:10, 1489-1494. [CrossRef]
5. H. Kobayashi, S. Minatoguchi, S. Yasuda, N. Bao, I. Kawamura, M. Iwasa, T. Yamaki, S. Sumi, Y. Misao, H. Ushikoshi, K. Nishigaki, G. Takemura, T. Fujiwara, Y. Tabata, H. Fujiwara. 2008. Post-infarct treatment with an erythropoietin-gelatin hydrogel drug delivery system for cardiac repair. *Cardiovascular Research* **79**:4, 611-620. [CrossRef]
6. Hong-zhu Li, Li-ping Han, Chun-ming Jiang, Hong Li, Ya-jun Zhao, Jun Gao, Yan Lin, Shu-xia Ma, Ye Tian, Bao-feng Yang, Chang-qing Xu. 2008. Effect of Dopamine Receptor 1 on Apoptosis of Cultured Neonatal Rat Cardiomyocytes in Simulated Ischaemia/Reperfusion. *Basic & Clinical Pharmacology & Toxicology* **102**:3, 329-336. [CrossRef]
7. WEI Sun, HONG Li, SHI-JIE Yang. 2008. A triterpene saponin from Tribulus terrestris attenuates apoptosis in cardiocyte via activating PKC signalling transduction pathway. *Journal of Asian Natural Products Research* **10**:1, 39-48. [CrossRef]
8. Sandra B. Haudek, George E. Taffet, Michael D. Schneider, Douglas L. Mann. 2007. TNF provokes cardiomyocyte apoptosis and cardiac remodeling through activation of multiple cell death pathways. *Journal of Clinical Investigation* **117**:9, 2692-2701. [CrossRef]
9. Fabio Di Lisa, Marcella Canton, Roberta Menabò, Nina Kaludercic, Paolo Bernardi. 2007. Mitochondria and cardioprotection. *Heart Failure Reviews* **12**:3-4, 249-260. [CrossRef]
10. Antigone Lazou, E. K. Iliodromitis, D. Cieslak, K. Voskarides, S. Mousikos, E. Bofilis, D. T. Kremastinos. 2006. Ischemic but not mechanical preconditioning attenuates ischemia/reperfusion induced myocardial apoptosis in anaesthetized rabbits: The role of Bcl-2 family proteins and ERK1/2. *Apoptosis* **11**:12, 2195-2204. [CrossRef]
11. M TANAKA, G MOKHTARI, R TERRY, F GUNAWAN, L BALSAM, G HOYT, K LEE, P TSAO, R ROBBINS. 2005. Prolonged Cold Ischemia in Rat Cardiac Allografts Promotes Ischemia–Reperfusion Injury and the Development of Graft Coronary Artery Disease in a Linear Fashion. *The Journal of Heart and Lung Transplantation* **24**:11, 1906-1914. [CrossRef]
12. Dipak K Das, Nilanjana Maulik. 2005. Mitochondrial function in cardiomyocytes: target for cardioprotection. *Current Opinion in Anaesthesiology* **18**:1, 77-82. [CrossRef]
13. S GHOSH. 2004. Role of dietary fatty acids and acute hyperglycemia in modulating cardiac cell death*1. *Nutrition* **20**:10, 916-923. [CrossRef]
14. Christopher L Murriel, Daria Mochly-Rosen. 2003. Opposing roles of # and #PKC in cardiac ischemia and reperfusion: targeting the apoptotic machinery. *Archives of Biochemistry and Biophysics* **420**:2, 246-254. [CrossRef]

15. Albert Amberger, Stefan Schneeberger, Gerald Hernegger, Gerald Brandacher, Peter Obrist, Peter Lackner, Raimund Margreiter, Walter Mark. 2002. Gene expression profiling of prolonged cold ischemia and reperfusion in murine heart transplants. *Transplantation* **74**:10, 1441-1449. [CrossRef]