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Bax ablation protects against myocardial ischemia-reperfusion injury in transgenic mice

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Bax-deficient hearts; heart; apoptosis

APOPTOSIS OF CARDIAC MYOCYTES has been demonstrated in several myocardial states, including ischemic heart disease and congestive heart failure (29). Inhibition of the apoptotic process was shown to prevent or slow down the loss of contractile cells and thus was suggested as an additional therapeutic approach to cardiac disease (9). A proapoptotic stimulus initiates a cell-autonomous cascade of events that activates caspases, apoptosis-specific endonucleases, and the apoptosis-inducing factor APAF (32). The apoptotic mechanism is regulated by a number of proteins, such as the Bcl-2 family, which can either inhibit or promote apoptosis (ced-9, Bcl-w, Bcl-2, Bcl-xl vs. Bax, Bcl-x_S, and Bak, respectively) (3). The suppressor Bcl-2 and the inducer Bax proteins can function autonomously in regulating cell death (14).

The involvement of Bcl-2 family members in ischemia and oxidative stress has been shown in several studies (21, 28, 30). Direct involvement of Bcl-2 in myocardial ischemia is also supported by alterations in control of the apoptotic cascade under anaerobic conditions (2, 18). Bcl-2 overexpression in transgenic mice or elevation of Bcl-2 protein expression through ischemic preconditioning result in superior recovery from cardiac ischemia-reperfusion (I/R) injury (4, 8).

In line with these studies, we aimed to determine the effect of decreased expression of the proapoptotic Bax gene. We used Bax knockout mice to assess the role and importance of the Bax gene in cardiac function during I/R and to determine the effect of Bax deficiency on Bcl-2 activity and apoptotic markers. With the use of the Langendorff preparation, hearts of Bax-deficient mice ($Bax^{-/-}$) were compared with the heterozygotic group ($Bax^{+/-}$) and with their controls ($Bax^{+/+}$) to determine the mechanical, biochemical, and morphological parameters of injury in conjunction with assays of Bcl-2, caspases, and apoptotic markers. The results indicated that Bax plays a role in cardiac function, and the absence of this gene renders the heart more resistant to necrosis and apoptosis after I/R injury.

MATERIALS AND METHODS

Animals. Bax-deficient mice were kindly provided by Dr. S. J. Korsmayer (15). They were originally inbred from C57Bl. Experiments were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at Tel Aviv University (Tel Aviv, Israel).

As male mice proved infertile, we mated the hemizygote for the Bax gene ($Bax^{+/-}$). To verify genetic identity for genotyping, DNA was isolated from the tail using the high pure template preparation kit (Roche) and then subjected to standard PCR (MJ Research). The primers used were as

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follows: 5′-GTTGACCAGATGGCGTAGG-3′ (Bax in), 5′-GAGCTGATCAGAACCATFATG-3′ (Bax ex), and 5′-CCGCT-TCCATTGCTCAGCGG-3′ (Neo R) for the exogenous neo gene (15).

Isolated mouse heart preparation and measurement of the contractile functions and creatine kinase release. Seventy-two mice (12–15 wk old), weighing 23–28 g, were divided into the following three groups (n = 24 in each group): transgenic knockout homozygotic ($Bax^{-/-}$), transgenic heterozygotic ($Bax^{+/-}$), and wild-type homozygotic control ($Bax^{+/+}$). Heparin sulfate (500 U/kg) was injected intraperitoneally to prevent intravascular coagulation of blood, and, 30 min later, mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). Hearts were excised by a bilateral thoracotomy and immersed in cold heparinized saline. The aorta was cannulated, and the heart was perfused in a retrograde Langendorff preparation at a pressure of 96 cmH_2O with oxygenated Krebs-Henseleit bicarbonate (KHB) buffer solution containing (in mM) 118 NaCl, 4.7 KCl, 1.2 $MgSO_4 \cdot 7H_2O$, 2.5 $CaCl_2$, 0.5 disodium EDTA, 25 $NaHCO_3$, 11 glucose, and 2 sodium pyruvate. A small incision was made in the main trunk of the pulmonary artery to drain the coronary effluent from the right ventricle. A force transducer (Grass FT03 mechanical transducer) was secured to the left ventricle using a 4-0 silk suture, and a hook was attached through the apex of the ventricle. The difference between the peak developed and end-diastolic contractile force, presented as left ventricular developed force (LVF) of contraction of the heart, was recorded and analyzed in real time using the CODAS data-acquisition system (San Diego, CA). The temperature of the heart (sensed in the right ventricle) was maintained at 37 ± 0.2°C throughout the experiment by a microthermocouple connected to a digital thermometer (Webster Laboratories; Altadena, CA). The isolated heart was stabilized for 20 min and then was either continuously perfused with KHB solution for 150 min (n = 4 in each group) or subjected to 30-min normothermic ischemia by clamping the aortic cannula and 120-min reperfusion (10, 22). LVF, heart rate (HR), and coronary flow rates (CF) were continuously recorded. The preischemic baseline parameters were measured after a 20-min stabilization period. Coronary effluent was collected at 1-min intervals before and after ischemia at various time points (1, 30, 60, and 120 min of reperfusion) and analyzed for creatine kinase (CK) activity (Boehringer Mannheim).

Western blotting. Heart tissue samples (20 mg) were homogenized in lysis buffer and quantified for protein levels using a commercial assay (Bio-Rad). Proteins (60 μg/sample) were separated using SDS-polyacrylamide gels (12.5%) under denaturing conditions and electrotransferred onto nitrocellulose (Bio-Rad) for 1 h at 100 V. Membranes were blocked with 5% nonfat milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST) overnight at 4°C. Primary antibodies [anti-Bcl-2 polyclonal from Oncogene (Cambridge, MA); anti-Bax from Cell Signaling (Nottingham, UK); and anti-α-actin from Sigma (St. Louis, MO)] were used at 1:1,000 dilution in TBST with 5% nonfat milk. After incubation with the primary antibodies (overnight at 4°C), horseradish peroxidase-conjugated secondary antibodies were added (1:4,000 dilution) for 1 h at room temperature. Films were developed using enhanced chemiluminescence methods and exposed to X-ray films (Amersham; Arlington Heights, IL) (17, 23).

Measurement of irreversible ischemic injury. After 120 min of reperfusion, the heart was infused into the coronary vasculature through the sidearm of the aortic cannula with a 10% solution of 2,3,5-triphenyl tetrazolium chloride (TTC) in phosphate buffer at 37°C and cut into sections (width 0.8 mm). TTC stained the viable tissue red, whereas the necrotic tissue remained discolored. Sections were fixed overnight in 2% paraformaldehyde. The sections were then placed between two coverslips, digitally photographed using a Fugi Finepixs1pro camera (resolution of 1,400 × 960 pixels), and quantified with IMAGE J 5.1 software. The area of irreversible injury (TTC negative) is presented as a percentage of the entire area of the section (22).

Evaluation of apoptosis. Immunohistochemical detection of apoptotic cells was carried out using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method. Residues of digoxigenin-labeled dUTP are catalytically incorporated into DNA by terminal deoxynucleotidyl transferase II, an enzyme that catalyzes the template-independent addition of nucleotide triphosphate to the 3′-OH ends of double- or single-stranded DNA. The incorporated nucleotide was incubated with a sheep polyclonal anti-digoxygenin antibody, followed by FITC-conjugated rabbit anti-sheep IgG as a secondary antibody, as described by the manufacturer (Apop Tag Plus, Oncor; Gaithersburg, MD). Sections were washed three times in phosphate-buffered saline, blocked with normal rabbit serum, and incubated with mouse monoclonal antibody recognizing α-sarcomeric actin (Sigma), followed by staining with tetramethylrhodamine isothiocyanate-conjugated rabbit anti-mouse IgG (1:200 dilution, Dako Japan; Tokyo, Japan). The fluorescence staining was viewed with a confocal laser microscope (Olympus; Tokyo, Japan). The number of apoptotic cells was counted in 100 high-power (×60) fields observed from the endocardium through the epicardium midventricular section and expressed as a percentage of the total myocyte population (8). The TUNEL assay was significantly more reliable, reproducible, and sensitive for detection of apoptosis than assays of DNA fragmentation by gel electrophoresis.

Caspase 3 activity. Hearts were tested for caspase 3 activity at the following different time points: *1*) when excised and washed with KHB solution (n = 3); *2*) 20 min after perfusion with KHB solution (n = 3); *3*) after being subjected to 30-min ischemia (n = 3); and *4*) after being subjected to 30-min ischemia followed by 2-h reperfusion (n = 6). Hearts were frozen in liquid nitrogen and stored until used. The myocardium was homogenized with a polytron homoginizer and centrifuged at 16,000 *g* for 20 min at 4°C. Protein extracts (60 μg/sample) were used to measure caspase 3 activity using a fluorescence substrate (CaspACE, Progmega). Briefly, the fluorogenic substrates for caspase 3 were labeled with the fluorochrome 7-amino-4-methyl coumarin (AMC). They generated blue fluorescence that could be detected by exposure to ultraviolet light at 360 nm, revealing the release of AMC from the substrates upon cleavage by caspase 3. Free AMC emits a yellow-green fluorescence, which was measured with a fluorometer at 460 nm (Fluostar) (26).

Electron microscopy. Tissue samples were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate for 1 h, postfixed in 1% osmium tetroxide in the same buffer for 1 h, and stained in 0.5% uranyl acetate. The samples were then dehydrated in ascending concentrations of alcohol, infiltrated in Epon-Araldite epoxy resin, and heat polymerized. The sections were cut, poststained with uranyl acetate and lead citrate, and examined by electron microscopy (31). The volume density of cellular components was determined by the point-count method (34). Briefly, a transparent grid of test points was laid over each micrograph. The number of test points falling on an individual structure was recorded, as was the total number of test points available on the test grid. The volume occupied by each component was equivalent to the number of points falling on that structure divided by the total

number of test points available on the test grid. This value was expressed as a percentage. Three types of mitochondria were analyzed: *1*) mature mitochondria (M_m), containing well-developed cristae; *2*) vacuolated mitochondria (M_v), containing a membrane-bound vesicle; and *3*) irreversibly damaged mitochondria (M_{Ca}), with electron-dense deposits containing calcium. The ratio between the volume density of heterochromatin and the volume density of nuclei (N_{hcr}) was also determined.

Statistical analysis. Results are expressed as means ± SE. Values during the stabilization period were defined as 100%. Statistical differences between the groups were assessed by ANOVA with repeated measurements using the multiple-comparison option of Duncan. If differences were established, values were compared using Student's *t*-test. $P < 0.05$ was considered significant.

RESULTS

Genetic identity by PCR. Figure 1 shows the genetic identity of the various mice tested. One band of Bax gene (300 bp) was seen in the $Bax^{+/+}$ mice, and two bands (300 and 500 bp) were seen in the hemizygotic $Bax^{+/-}$ mice, whereas in the $Bax^{-/-}$ mice only one band (500 bp) was seen in the neo gene.

Weight. There were no differences in either body weight or the ratio of heart weight to body weight (0.0044–0.005) in $Bax^{-/-}$ compared with $Bax^{+/-}$ and $Bax^{+/+}$ mice.

Bcl-2 and Bax content. Similar levels of Bcl-2 protein expression were detected in the three groups of hearts when analyzed with Western blots (Fig. 2). As expected, Bax protein was not expressed in Bax-deficient hearts and was detected in $Bax^{+/+}$ normal hearts (Fig. 2).

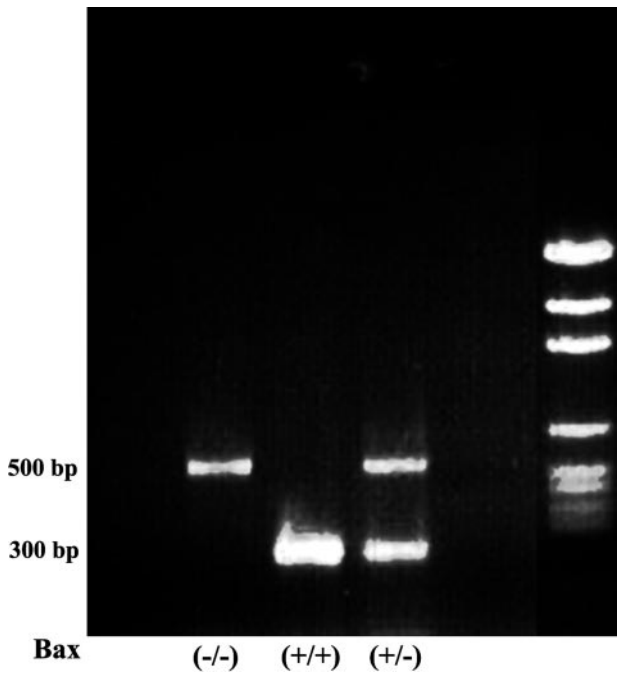

Fig. 1. Genetic identity by PCR. One band of Bax gene (300 bp) was seen in the homozygous control ($Bax^{+/+}$), and two bands (300 and 500 bp) were seen in the hemizygotic ($Bax^{+/-}$) mice, whereas in Bax-deficient ($Bax^{-/-}$) mice only one band (500 bp) was seen in the neo gene. To verify genetic identity for genotyping, DNA was isolated from the tail using the high pure template preparation kit and then subjected to standard PCR.

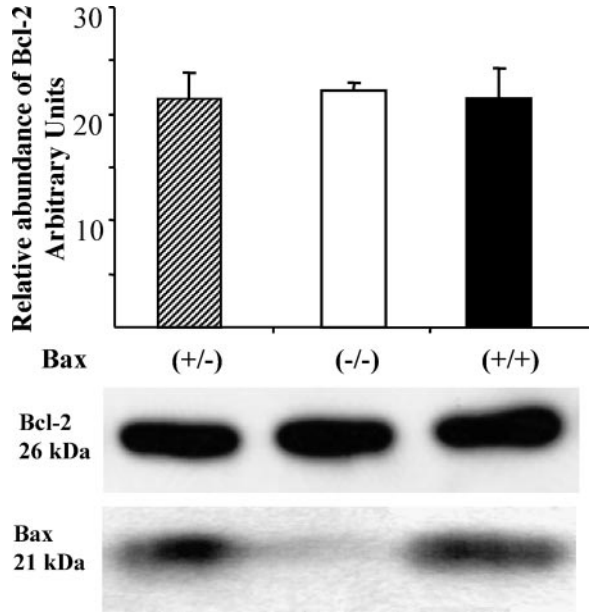

Fig. 2. Western blot analysis of Bcl-2 and Bax protein levels in $Bax^{+/+}$, $Bax^{+/-}$, and $Bax^{-/-}$ myocardium. *Top*: densitometric scan of Bcl-2 protein in the heart muscle of the three groups of mice. Values are means ± SE. *Bottom*: Western blots of Bcl-2 and Bax protein measured with anti-Bcl-2 and anti-Bax polyclonal antibodies. Proteins (60 μg/sample) were layered on a SDS-polyacrylamide gel (12.5%) under denaturing conditions and electrotransferred onto nitrocellulose for 1 h at 100 V.

Mechanical recovery. LVF of hearts from the $Bax^{-/-}$, $Bax^{+/+}$, and $Bax^{+/-}$ groups was continuously monitored using the isolated heart Langendorff preparation. During the initial stabilization period, there were no significant differences in the mechanical activity of hearts from normal and knockout mice (Table 1). There was, however, a trend of increased developed force in $Bax^{-/-}$ hearts. Continuous perfusion for 170 min of $Bax^{-/-}$, $Bax^{+/-}$, and $Bax^{+/+}$ hearts resulted in a decrease in LVF to 78 ± 3%, 75 ± 4%, and 77 ± 3%, respectively. LVF of Bax-deficient hearts were consistently slightly higher than the wild-type $Bax^{+/+}$ hearts. During 120 min of reperfusion after 30 min of global no-flow ischemia, LVF decreased to 37 ± 3% and 37 ± 5% vs. 27 ± 5% of baseline in $Bax^{-/-}$, $Bax^{+/-}$, and $Bax^{+/+}$ hearts, respectively ($P < 0.05$; Fig. 3, *A* and *B*).

Table 1. *Basal cardiac parameters*

	Left Ventricular Developed Force, mg	Coronary Flow, ml/min	Heart Rate, beats/min
$Bax^{+/+}$	1,161 ± 137	3.2 ± 0.41	405 ± 22
$Bax^{+/-}$	1,178 ± 144	2.8 ± 0.19	400 ± 14
$Bax^{-/-}$	1,411 ± 177	3.2 ± 0.77	413 ± 14
P values	NS	NS	NS

Values are expressed as means ± SE; $n = 18$ in each group. NS, not significant.

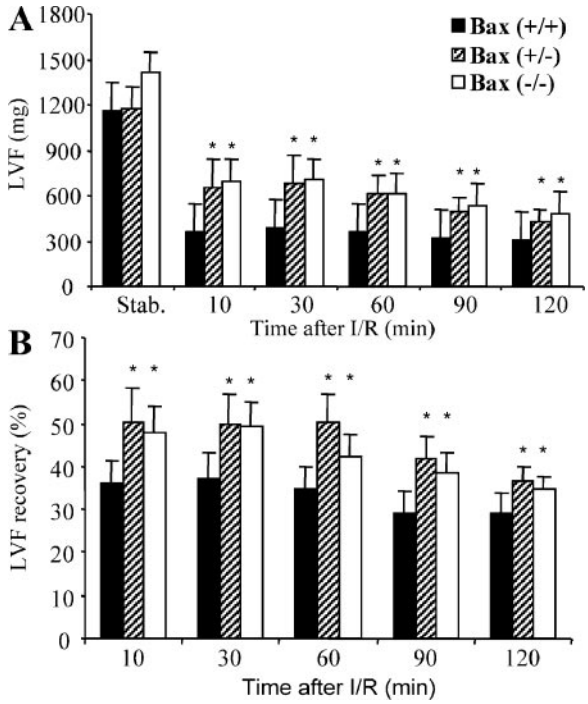

Fig. 3. Left ventricular developed force (LVF) expressed in absolute values (*A*) or as a percentage of the preischemic value (*B*) in $Bax^{+/+}$, $Bax^{+/-}$, and $Bax^{-/-}$ hearts. LVF is shown during the stabilization (Stab) period and at various time points after 30 min of ischemia (37°C). I/R, ischemia-reperfusion. Values are means ± SE; $n = 14$ hearts in each group. *$P < 0.05$ vs. $Bax^{+/+}$.

CF and HR were essentially the same in the all groups during the stabilization period (Table 1). CF rate progressively deteriorated during the reperfusion period in all groups. CF in the nonischemic hearts declined to 90 ± 8% after 170 min of continuous perfusion. In the ischemic hearts, CF rate deterioration during the reperfusion period was higher for $Bax^{+/+}$ than Bax-deficient mice after 120 min of reperfusion (63 ± 5% vs. 83 ± 8% of baseline, $P < 0.01$, and 68 ± 7% for $Bax^{+/-}$ mice; Fig. 4). All hearts resumed beating spontaneously after 30 min of global ischemia, with no significant differences in rates between the experimental groups.

Biochemical markers of ischemia and reperfusion damage. CK activity in the effluent increased in all groups, but in the $Bax^{+/+}$ group it was higher than in both the $Bax^{-/-}$ and $Bax^{+/-}$ groups. CK release to the coronary effluent at 1-min reperfusion was 2.2 times higher in the $Bax^{+/+}$ hearts than in the $Bax^{-/-}$ or $Bax^{+/-}$ hearts (270 ± 45 vs. 120 ± 10 and 110 ± 10 units/l, $P < 0.01$; Fig. 5).

In other studies, we performed similar measurements of contractile recovery from ischemia, coronary flow, and CK release in hearts of BALB/c mice. The values were not significantly different from those of the normal C57Bl mice, in this study termed as $Bax^{+/+}$ controls.

Irreversible ischemic injury. TTC staining revealed that Bax deficiency of the isolated heart subjected to 30 min of global ischemia and 120 min of reperfusion was accompanied with reduced regions of irreversible ischemic injury compared with the $Bax^{+/+}$ group (22.3 ± 6% vs. 43.6 ± 12%, respectively, $P = 0.001$; Fig. 6). The extent of ischemic injury in the hemizygotic $Bax^{+/-}$ mice was moderate (33.6 ± 6%).

Cardiomyocyte apoptosis. Apoptotic cardiomyocytes were detected using TUNEL staining in conjunction with staining with antibody against sarcomeric actin to identify myocytes. In the wild-type group, a large number of cardiomyocytes was found to undergo apoptosis after 2 h of reperfusion after 30 min of ischemic insult. Bax deficiency lowered cardiomyocyte death 10-fold due to apoptosis compared with the $Bax^{+/+}$ group and by 3-fold compared with the $Bax^{+/-}$ group (7.52 ± 2.36%, 0.74 ± 0.4%, and 2.27 ± 1.1%, respectively; Fig. 7).

Caspase 3 activity. Caspase 3 activity was performed in pre- and postischemic hearts to verify whether the protective effect of Bax deficiency was produced via antiapoptotic pathways. Enzymatic activity of caspase 3 in hearts was determined in myocardial extracts in vivo (0 min), after 20 min of perfusion (20 min stab), and after 30 min of total normothermic ischemia and 1

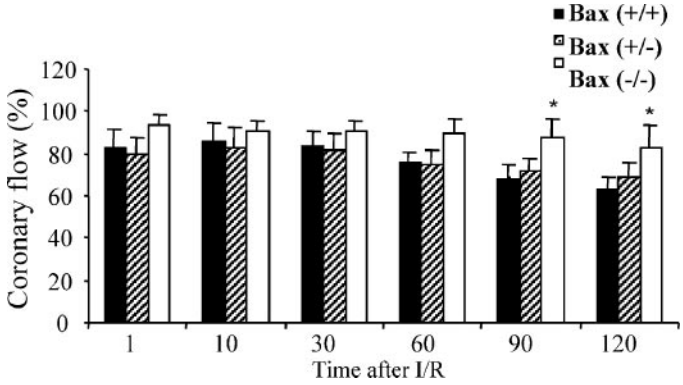

Fig. 4. Coronary flow rate of $Bax^{+/+}$, $Bax^{+/-}$, and $Bax^{-/-}$ hearts. Coronary flow rate is expressed as a percentage of the baseline value after 30 min of ischemia (37°C) and at various time points after reperfusion. Values are means ± SE; $n = 14$ hearts in each group. *$P < 0.05$ vs. $Bax^{+/+}$.

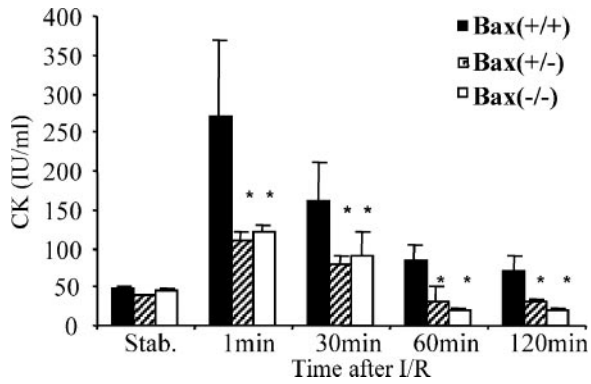

Fig. 5. Release of creatine kinase (CK) in $Bax^{+/+}$, $Bax^{+/-}$, and $Bax^{-/-}$ hearts. Release of CK to the coronary effluent is shown during the stabilization (stab) period and at various time points after reperfusion. Values are means ± SE; $n = 14$ hearts in each group. *$P < 0.05$ vs. $Bax^{+/+}$.

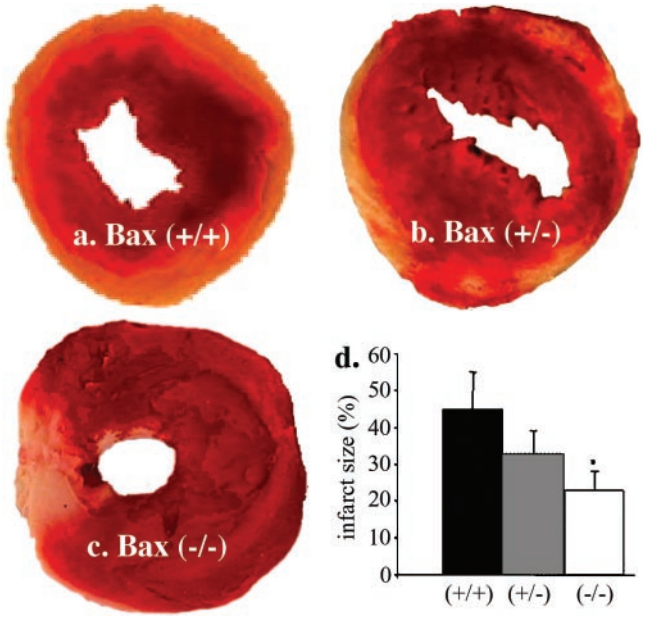

Fig. 6. Effect of I/R on myocardial infarction. Irreversible ischemia was determined by scanning the images of mice heart ventricular sections with triphenyltetrazolium chloride (TTC). *a–c*: representative images of the three different groups, revealing various degrees of myocardial ischemia (white to yellowish zones after TTC staining). Hearts were subjected to 30 min of ischemia, followed by 120 min of reperfusion. A significant size of damaged tissue is noticed in the I/R myocardium of the $Bax^{+/+}$ heart (*a*) compared with the $Bax^{-/-}$ heart (*c*) ($P < 0.05$). The extent of ischemic damage in the $Bax^{+/-}$ group is moderate (*b*). *d*: Percentage of irreversible injury from the total area of the section. Values are means ± SE; $n = 14$ hearts in each group. *$P < 0.05$ vs. $Bax^{+/+}$.

min (1 min rep) or 120 min of reperfusion (120 min rep). Caspase 3 activity was low at all measured time points except for 120 min postischemia, where both the $Bax^{-/-}$ and $Bax^{+/-}$ groups exhibited decreased caspase 3 activity compared with the $Bax^{+/+}$ group ($P < 0.0005$), representing a 1.5- or 2-fold increase compared with 4.5-fold, respectively (Fig. 8).

Morphological markers of ischemia and reperfusion damage. The ultrastructure of $Bax^{-/-}$ and $Bax^{+/+}$ myocytes before ischemia is similar (Fig. 9, *A* and *D*). These myocytes have well-ordered myofibrils with a distinct sarcomeric registry and dark mitochondria with tightly arranged cristae. Ischemia and 120-min reperfusion caused markedly higher damage in the $Bax^{+/+}$ group compared with the $Bax^{-/-}$ group. Whereas in the $Bax^{+/+}$ hearts many nuclei were irregularly shaped and showed clumped chromatin and increased amounts of heterochromatin and pyknosis (Table 2), in $Bax^{-/-}$ hearts the nuclei had finely dispersed chromatin and pleomorphism and heterochromatin were significantly less expressed (Fig. 9, *B* and *E*, and Table 2). The mitochondria of many of the $Bax^{+/+}$ hearts were distorted with the swelling of the matrix, and many of them contained electron-dense deposits (Ca-phosphate electron-dense deposits), which were significantly lower in Bax-deficient hearts (Fig. 9, *C* and *F*, and Table 2).

DISCUSSION

The use of transgenic and knockout mice to study the role of various genes in the cardiovascular system has been popular in recent years (12). For example, transgenic mice overexpressing Bcl-2 were used to illustrate the protective role of Bcl-2 against I/R injury in the heart (4), brain (20), and intestine (6). This is the first report that evaluates the role of the antiapoptotic Bax gene in myocardial ischemia and reperfusion. Our results indicate that the suppression of the Bax gene improves myocardial tolerance to I/R injury. Cardioprotection was achieved by the amelioration of necrotic as well as apoptotic cell death. The improved contractile function, CF rates, and reduced irreversible ischemic injury were accompanied by consistent differences in muscle ultrastructure, apoptosis of cardiomyocytes, and proteolytic activity of caspase 3. In general, these markers of injury showed moderate differences in hearts of heterozygous mice, suggesting that Bax activity may be quantitatively related to cardiac tolerance to I/R.

Both necrotic and apoptotic cell death contribute to the pathophysiology of I/R injury (4). The extended period of reperfusion (120 min) employed in this study has been used in previous studies (19, 22). The time period of 2 h is recommended because activation of the apoptotic mechanism requires this time period to maximize the detection of apoptotic cell death. Minimal evidence of apoptotic markers was found using shorter periods of reperfusion (30 and 60 min).

Apoptosis, as a distinct type of cell death, is governed by number of regulating genes and mediated by apoptotic signals. Bax plays a crucial role in the induction phase of apoptosis, whereas Bcl-2 and Bcl-xL are known to counteract the proapoptotic action of Bax (16). A positive correlation between the expression of Bcl-2 family proteins and protection of the heart against I/R injury has been suggested. Acute myocardial infarction after coronary artery ligation upregulates Bcl-2 expression in viable myocytes at the border areas of the infarcted myocardium, whereas nonviable myocytes upregulate their Bax expression (5, 13). It has been suggested that apoptosis is an independent contributor to cardiomyocyte death during ischemia and reperfusion (13, 33). Studies have shown that apoptosis was triggered in 2.8 million myocytes within 2 h of coronary artery occlusion, whereas during the same period only 90,000 cells were necrotic, and the number of the latter increased with prolongation of ischemia (13). On the basis of these data, the authors concluded that apoptosis was the major form of myocardial injury after occlusion of a major coronary artery, whereas postapoptotic necrotic myocyte death contributed to progressive loss of cells (13). Consistent with this hypothesis is the fact that treatment with a caspase inhibitor led to the inhibition of apoptosis and was associated with a reduction in infarct size and improved hemodynamic function (35). In contrast, it was suggested that apoptotic cardiomyocytes observed in the infarcted myocardium after ischemia and reper-

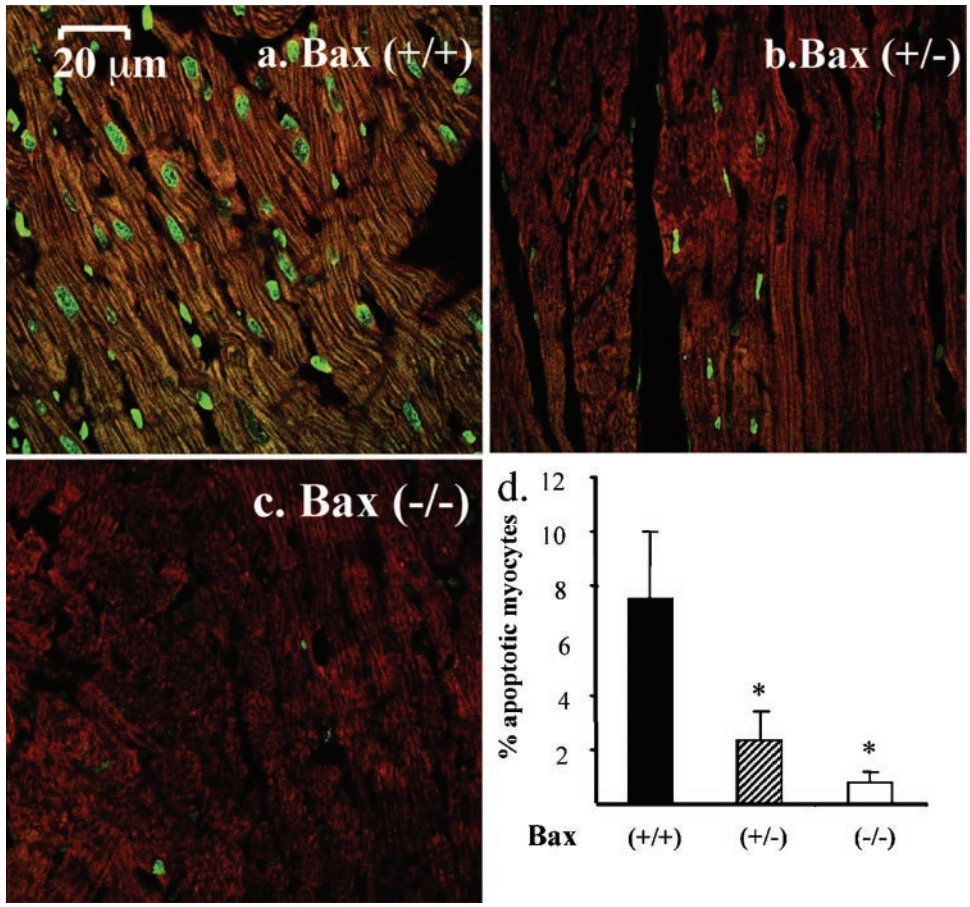

Fig. 7. Effect of ischemia and I/R on myocardial apoptosis by double antibody staining [terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay]. Hearts were subjected to 30 min of ischemia, followed by 120 min of reperfusion. The number of apoptotic cells was counted in 100 high-power (×60) fields of the midventricular section and expressed as a percentage of the total myocyte population. This figure is for demonstration purposes only and does not represent the quantitative differences between experimental groups. There were significant numbers of apoptotic cardiomyocytes in the I/R $Bax^{+/+}$ myocardium (*a*), whereas only a few apoptotic cardiomyocytes were detectable in the $Bax^{-/-}$ heart (*b*) ($P < 0.01$). The amount of apoptotic cardiomyocyte cell death in the $Bax^{+/-}$ group was moderate (*c*). *d*: Percentage of apoptotic cardiomyocytes. Values are means ± SE. *$P < 0.01$ vs. $Bax^{+/+}$.

fusion were in fact necrotic myocytes that displayed DNA fragmentation (25). Despite this controversy, it is clear that myocardial ischemia and reperfusion provoke both forms of cell death (25, 33). By eliminating the Bax gene or by reducing it in the heterozygotic group, we succeeded in reducing the damage caused by global ischemia and reperfusion via both the necrotic and the apoptotic pathways.

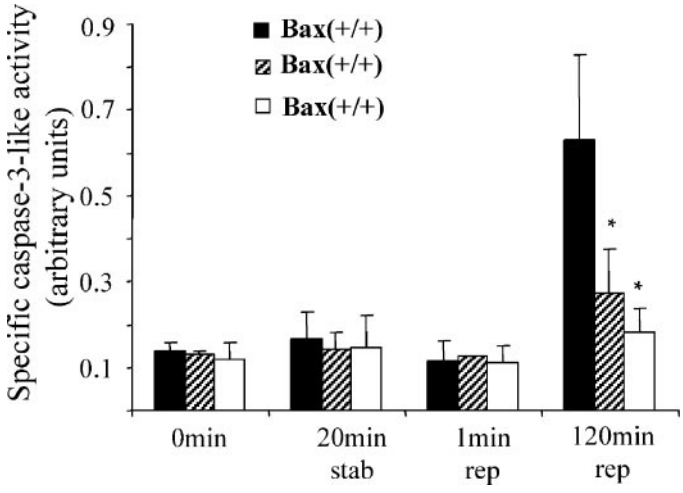

Fig. 8. Enzymatic activity of caspase 3 in hearts subjected to 30 min of ischemia, followed by 120 min of reperfusion. Caspase 3 activity was determined in myocardial extracts in vivo (0 min), after 20 min of perfusion (20 min Stab), after 30 min of total normothermic ischemic and 1 min (1 min rep) or 120 min reperfusion (120 min rep). Values are means ± SE. *$P < 0.05$ vs. $Bax^{+/+}$.

It is difficult to compare the extent of necrosis and apoptosis after I/R using two different techniques to assay cell death. If we compare TTC and TUNEL staining in normal hearts, the necrotic and apoptotic markers accounted for 44% and 7.5% of the injury, respectively. Necrotic cell death was reduced twofold in Bax-deficient hearts compared with controls, and CK release was consistently reduced by the same amount. Compared with normal hearts, the Bax-deficient hearts displayed a remarkable 10-fold decrease in myocyte apoptosis, as determined by the TUNEL assay. Corroborating these differences, the Bax-deficient hearts recovered better than controls, the contractile function of these hearts after ischemia improved, and significant differences were observed in myocardial morphology. In particular, mitochondrial structure, as evident from the amount of intramitochondrial electron-dense deposits and vacuoles, and nuclear structure were better preserved in Bax-deficient hearts than in controls. These data suggest that the lack of the Bax gene renders the heart less susceptible to necrotic injury, which in itself improved recovery in the long run.

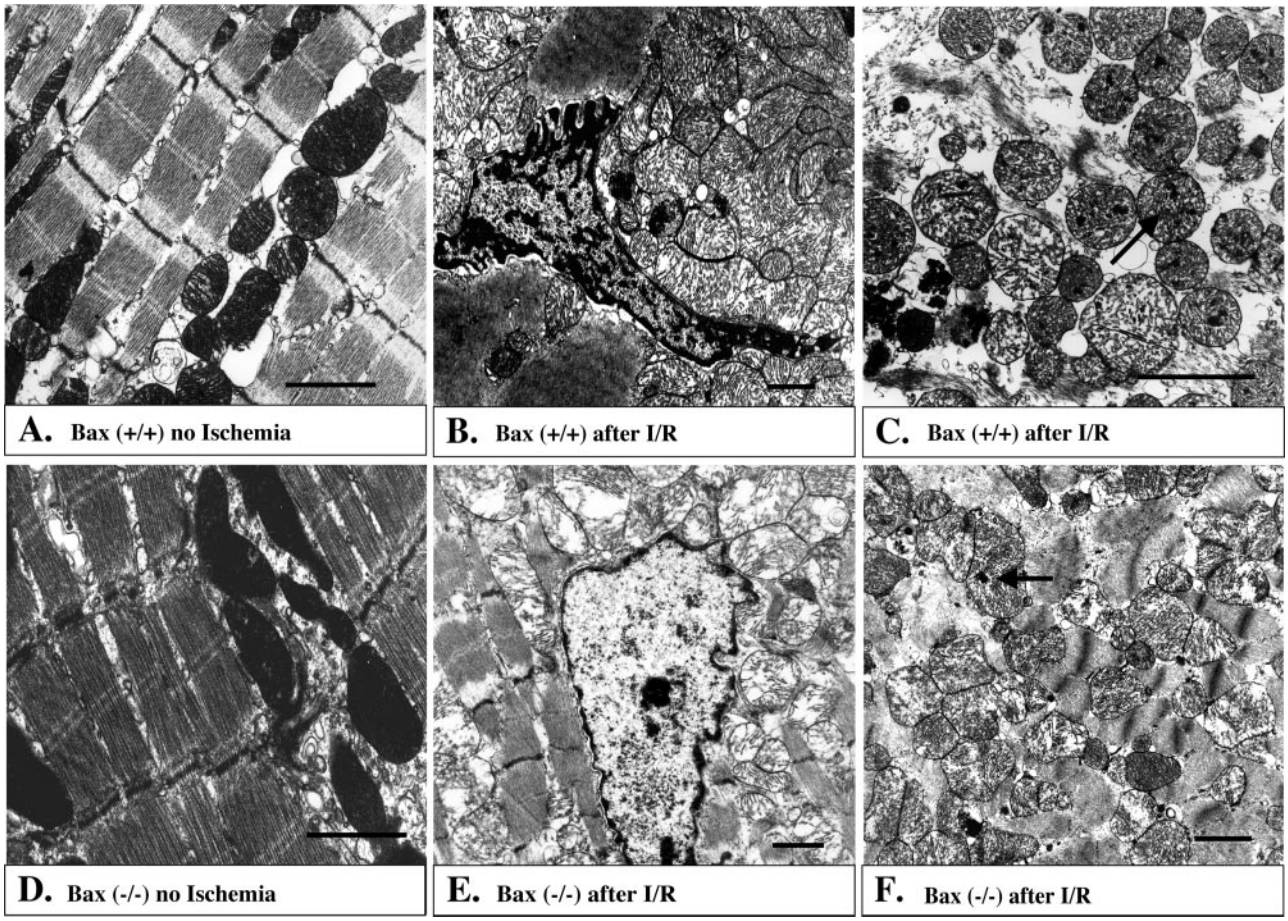

Fig. 9. Electron micrographs subjected to 30 min of ischemia, followed by 120 min of reperfusion. Electron micrographs are of cardiac muscle of $Bax^{+/+}$ and $Bax^{-/-}$ mice before ischemia (*A* and *D*, respectively), after 30 min of ischemia (*B* and *E*, respectively), and 120 min of reperfusion (*C* and *F*, respectively). *A* and *D* show normal sarcomers and dark mitochondria with tightly arranged cristae in both groups. *B–E* show damaged cells in the two groups. Whereas many nuclei of the $Bax^{+/+}$ cardiac muscle had an irregular shape with blebbing and clumped chromatin (*B*), $Bax^{-/-}$ cardiac muscle had finely dispersed nuclear chromatin (*E*). Mitochondria of $Bax^{+/+}$ mice contained a larger number of electron-dense deposits (arrows, *C*) than those of $Bax^{-/-}$ mice (arrow, *F*). The images show sections from 5 mice in each group. Bar = 1 μm.

Table 2. *Morphometric analysis of myocardial cells in Bax knockout and wild-type mice*

	N_{hcr}	M_m	M_v	M_{Ca}
Control				
$Bax^{+/+}$	9.7 ± 1.4	26.8 ± 2.4	4.6 ± 2.1	None
$Bax^{-/-}$	9.5 ± 1.9	29.7 ± 2.1	2.1 ± 1.8	None
I/R				
$Bax^{+/+}$	23.7 ± 2.6*	2.3 ± 1.8*	29.3 ± 4.6*	14.6 ± 3.1*
$Bax^{-/-}$	15.8 ± 1.7*†	6.8 ± 2.8*†	23.1 ± 3.1*†	8.4 ± 3.3*†

Values are expressed as means ± SE. The morphometric analysis of cardiac cells in wild-type $Bax^{+/+}$ and knockout $Bax^{-/-}$ mice before (control) and after 30 min of ischemia and 120 min of reperfusion (I/R) is shown. The analysis included the percentage of volume density of heterochromatin per volume density of nuclei (N_{hcr}) and percent changes in mitochondrial morphology [mature mitochondria, containing well-developed cristae (M_m); vacuolated mitochondria, containing a membrane-bound vesicle (M_v); and irreversible damaged mitochondria, containing Ca phosphate electron-dense deposits (M_{Ca})]. Control values (none) were considered as 0.3 ± 0.1. *$P < 0.05$ vs. control $Bax^{+/+}$; †$P < 0.05$ vs. I/R $Bax^{+/+}$.

Reduced cardiomyocyte apoptosis and DNA fragmentation were observed after repeated cyclic episodes of intermittent brief ischemia and reperfusion (ischemic preconditioning), a process associated with the increased expression of Bcl-2 mRNA and activation of nuclear factor-κB (21). Ischemic preconditioning was also reported to induce a decrease in Bax expression and limit the extent of apoptotic cell death (24). Changes in the expression levels of regulatory factors of apoptosis are known to occur in failing hearts (27) and as an adaptive mechanism of aging (17). In this respect, upregulation of the activity of the antiapoptotic Bcl-2 gene may be considered as a natural defense mechanism. The ability of Bax to block apoptosis is critically dependent on the ratio of Bcl-2 to Bax. When excess Bax/Bcl-2 heterodimers are formed, cells are protected, whereas in hearts with predominantly Bax, the Bax homodimers render the myocytes susceptible to apoptosis (17). Our data indicate that the absence of the Bax gene in knockout mice per se did not induce

alterations in cardiac Bcl-2 expression. Thus, at similar levels of Bcl-2, Bax deficiency protects against I/R injury.

In this study, the presence of the Bax gene in normal hearts was associated with impaired structure of mitochondria compared with Bax-deficient hearts. In the absence of Bax protein, the defensive role of Bcl-2 is intensified, protecting mitochondria from ischemic damage. Bcl-2 has been shown to act as a free radical scavenger in both myocardial and neural tissues, offering protection against I/R injury (2, 11). The generation of oxygen-derived free radicals is considered to be one of the mechanisms responsible for I/R injury, and superoxide is known to induce apoptosis/necrosis (36). There is evidence that Bcl-2 regulates mitochondrial transition pores, promoting contacts between the inner and outer membranes of mitochondria and initiating the apoptotic process (1, 16). Bcl-2 homodimers have been shown to inhibit pore formation, whereas Bax homodimers open these pores, allowing a cascade of events leading to I/R injury.

Another factor involved in these events is the relationship between Bcl-2 and Ca^{2+} pumps in the mitochondria, endoplasmic reticulum, and nuclear membranes (7, 37), which prevent calcium overload during I/R. The important role of Bcl-2 in regulation of the activity of mitochondrial transition pores and prevention of Ca^{2+} overload in the mitochondria reduces cellular injury both via the apoptotic and necrotic pathways (16). These mechanisms are suggested to be at work in Bax-deficient hearts, preserving mitochondrial integrity and function.

Studies of the Bax gene using knockout technology requires caution in interpretation of the apparent pinpoint mutation and careful extrapolation of the experimental data to normal tissue. The Bax-deficient mice were viable and their body weight and myocardial mass-to-body weight ratio were similar to those of the wild-type mice, without signs of developmental abnormalities or hypertrophy. It is likely, however, that deletion of an important gene activates adaptive processes that allowed normal development and function. Although we found no significant alterations in the activity of apoptosis-associated proteins (including Bcl-2 and caspase) in the hearts of homozygous and heterozygous Bax-deficient mice, it remains unclear why these hearts displayed a slightly stronger contraction before ischemia compared with hearts of normal mice. We cannot exclude the possibility that, in addition to long-term adaptation to the absence of the Bax gene, acute compensatory mechanisms were also activated in our experimental conditions. Despite these limitations, deletion and overexpression of genes using knockout and transgenic technologies allow us to investigate their function. An additional concern is the limited physiological relevance of the isolated heart preparation, in particular perfusion of mouse hearts for extended periods of time. Future experiments referring to the role of genes in the pathogenesis of myocardial ischemia should be performed in vivo.

In summary, myocytes lacking the Bax gene reduced I/R injury through prevention of the necrotic and apoptotic pathways. Although the precise mechanism for the inhibition of I/R injury in Bax knockout mice remains unclear, it appears from the present study that downregulation of the Bax gene plays a crucial role in ameliorating ischemic damage. Any treatment inducing downregulation of the Bax gene or elevating the level of Bcl-2 proteins offers a promising approach to alleviating I/R injury.

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