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ORIGINAL ARTICLE

Bax Deficiency Reduces Infarct Size and Improves Long-Term Function After Myocardial Infarction

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Abstract

We have previously found that, following myocardial ischemia/reperfusion injury, isolated hearts from bax gene knockout mice [Bax(−/−)] exhibited higher cardioprotection than the wild-type. We here explore the effect of Bax(−/−), following myocardial infarction (MI) in vivo. Homozygotic Bax(−/−) and matched wild-type were studied. Mice underwent surgical ligation of the left anterior descending coronary artery (LAD). The progressive increase in left-ventricular end diastolic diameter, end systolic diameter, in Bax(−/−) was significantly smaller than in Bax(+/+) at 28 d following MI ($p < 0.03$) as seen by echocardiography. Concomitantly, fractional shortening was higher (35 ± 4.1% and 27 ± 2.5%, $p < 0.001$) and infarct size was smaller in Bax(−/−) compared to the wild-type at 28 days following MI (24 ± 3.7 % and 37 ± 3.3%, $p < 0.001$). Creatine kinase and lactate dehydrogenase release in serum were lower in Bax(−/−) than in Bax(+/+) 24 h following MI. Caspase 3 activity was elevated at 2 h after MI only in the wild-type, but reduced to baseline values at 1 and 28 d post-MI. Bax knockout mice hearts demonstrated reduced infarct size and improved myocardial function following permanent coronary artery occlusion. The Bax gene appears to play a significant role in the post-MI response that should be further investigated.

Index Entries: Bax-deficient hearts; myocardial infarction; caspase; apoptosis.

INTRODUCTION

The process of remodeling following an acute ischemic insult to the heart has been extensively investigated in mouse models *(1,2)*. Shortly after the injury, the infarcted muscle expands to create a dilated left-ventricular chamber, increasing mechanical stress on the surviving myocytes. Removal of the dead myocytes and assumption of function of the surviving cells along with fibrosis lead to mural thinning and left-ventricular dilatation *(3)*. Weeks to months after infarction, the process of remodeling is ongoing in remote areas of the left ventricle, which are required to compensate for the loss in mechanical work. This excessive demand results in further myocardial hypertrophy, left-ventricular dilation, and decompensation of the systolic pump function *(4)*.

It is difficult to accurately distinguish between the necrosis/apoptosis process that leads to death of myocytes after myocardial infarction (MI). It has been suggested that apoptosis is an independent mechanism of cardiomyocyte death during ischemia and reperfusion *(5,6)*. For example, treatment with a caspase inhibitor led to the inhibition of cardiomyocyte apoptosis, which was associated with a reduction in infarct size and improved hemodynamic function *(7)*. In addition, upregulation of Bcl-2 (anti-apoptotic) in viable myocytes and upregulation of Bax in nonviable myocytes shortly after MI has been interpreted as a

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significant contribution of apoptosis to ischemic cell loss *(5,6)*. In contrast, it has been suggested that apoptosis in the infarcted myocardium was attributed to necrotic myocytes that displayed DNA fragmentation *(8)*. It is likely that both forms of cell death occur in the infarcted myocardium *(6,8)*.

Both necrosis and apoptosis are responsible for acute cell death in the infarcted area *(9)*. Cell loss is also observed in the border zone of the infarcted myocardium, which contributes to progressive left-ventricular dilatation and dysfunction *(9)*. Involvement of the apoptotic machinery is sensed through the upregulation of proapoptotic genes after myocardial infarction and in failing hearts *(10)*. Various interventions aiming to inhibit apoptosis have been shown to prevent and slow down the decline in contractile function *(11)*. Suppression of apoptosis by Bcl-2 and its enhancement by Bax proteins modulate the extent of cell death *(12–15)*. Bcl-2 overexpression in transgenic mice, or elevation in Bcl-2 expression induced by ischemic preconditioning, resulted in superior recovery from ischemia/reperfusion (I/R) injury *(16–18)*. Inhibition of caspase 3, the common effector of apoptosis, improved myocardial structure and function after infarction *(19)*. Using the isolated heart preparation, we showed that Bax deficiency renders the heart more resistant to necrosis and apoptosis immediately after acute I/R injury *(20)*. In this study we aimed to monitor the effects of Bax-deficiency in mice on the remodeling process in the first month postinfarction.

MATERIALS AND METHODS

Bax-deficient male mice proved infertile. We therefore mated heterozygote (+/–) mice to obtain a Bax-deficient mouse (–/–). To determine genetic identity, we used PCR *(21)*. Wild-type Bax(+/+) and knockout mice matched for body weight, aged 12–18 wk, were used. There were 20 animals in each group. Heterozygote (+/–) mice were used for breeding.

Microsurgery

Experimental procedures were approved by the local animal experimentation Ethics Committee. Mice were anesthetized (mixture of 8 mg/100 g ketamine, 5 mg/100 g xylazine) then intubated and ventilated with Harvard Rodent Ventilator Model 383 (respiratory rate: 100/min, respiratory volume: 0.5 mL). A left thoracotomy in the third intercostal space was performed to expose the heart using a surgical microscope. The location of the left descending coronary artery was identified and then occluded with a 7-0 silk suture *(22)*. Less than 5 min is required to expose the heart to ligation. The thorax was closed immediately after surgery and mice were returned to their cages. Occlusion was confirmed by monitoring the pallor of region at risk and electrocardiogram was used to observe changes such as widening of QRS and ST-T segment elevation.

Two-Dimensional Guided M-Mode Echocardiography

Animals were lightly anesthetized by inhaling isoflurane. Two-dimensional (2D) guided M-mode echocardiography was performed using an echocardiogram (Siemens 512, Sequoia) equipped with a 15-MHz linear transducer. The heart was imaged in the 2D mode in the parasternal long-axis view. From this view, an M-mode cursor was positioned perpendicular to the interventricular septum and posterior wall of the left ventricle (LV) at the level of the papillary muscles. An M-mode image was obtained at a sweep speed of 100 mm/s. Diastolic and systolic left-ventricular wall thickness, left-ventricular end-diastolic dimensions (LVDD), and left-ventricular end-systolic chamber dimensions (LVSD) were measured. The percentage of left-ventricular fractional shortening (FS) was calculated as $[(\text{LVDD} - \text{LVSD})/\text{LVDD}] \times 100$ *(22)*.

Experimental Protocol

After the initial assessment using echocardiography, mice were either sacrificed ($n = 4$ for each group) or subjected to left anterior descending coronary artery (LAD) ligation, tested with echo at 1 d, and then sacrificed ($n = 8$ for each group) or echo was repeated at 4 wk after surgery [$n = 8$ for Bax(+/+) and $n = 7$ for Bax(–/–)].

Measurement of Infarct Size

Midventricular heart sections 0.8 mm thick were put in a 1% solution of 2,3, 5-triphenyl tetrazolium chloride in phosphate buffer (TTC) for 30 min at 37°C. TTC stained the viable tissue with red while the necrotic tissue remained discolored. Sections were fixed overnight in 2% paraformaldehyde. The sections were then placed between two cover slips and digitally photographed using a Fugi Finepixs1pro camera, with a resolution of 1400×960 pixels and quantified by IMAGE J 5.1 software. The area of irreversible injury (TTC-negative) is presented as a percentage of the entire area of the section *(20)*.

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 sodium dodecyl sulfate (SDS) polyacrylamide gel (7.5–12%) 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 with 0.1% Tween 20 (TBST) overnight at 4°C. Primary antibodies were used in a 1:1000 concentration in TBST with 5% nonfat milk for 2 h at room temperature. The Bcl-$_2$ and actin antibodies were obtained from PharMingen (San Diego, CA). The Bax antibody is polyclonal and was obtained from Oncogene (Cambridge, MA). Immunodetection of actin with monoclonal anti-actin antibody was performed as an internal control. Horseradish peroxidase-conjugated secondary antibodies were added at a concentration of 1:2000 for 1 h at room temperature. Films were developed using the enhanced chemiluminescence method (Amersham, Arlington Heights, IL) *(23–24)*.

Caspase 3

Caspase 3 activity was performed pre- and post-MI. The enzymatic activity of caspase 3 in hearts was determined in myocardial extracts from the peri-infarcted area after 120 min of LAD ligation, after 2 h 1 and 30 d post-MI. Samples were tested for caspase 3 activity at different time points: (1) before MI (time 0) (n = 3), (2) after being subjected to LAD ligation (n = 3) (2 h); (3) after 1 d (n = 3), and after 1 mo (n = 4). Hearts were frozen in liquid nitrogen and stored until used.

The myocardium (25 mg) was homogenized with a Teflon homogenizer in a lysis buffer (0.5 mL) which contained 50 m*M* HEPES buffer (pH 7.4), 0.1 m*M* EDTA, 1 m*M* dithiothreitol (DTT), 0.1% CHAPS, and protease inhibitors from Bohringer Manheim 1836145 (1:25). The homogenate was centrifuged at 30,000*g* for 30 min at 4°C. Protein content was determined by the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL), which enables the quantification of proteins in the presence of detergents. Protein extracts (100 µg/sample) were used to measure caspase 3 activity using fluorescence substrate DEVD-AMC [*N*-Acetyl-Asp-Glut-Val-Asp-AMC (7-amino-4-methylcoumarin); Biomol, UK]. The reaction buffer contained 2 m*M* DTT and 10% glycerol in 20 m*M* HEPES buffer (pH 7.4). The fluorogenic substrate, ac-DEVD-AMC, was added just before the reaction to a final concentration of 50 µ*M*. Assays were carried out in duplicates. The reaction was started by addition of the extract to the reaction mixture (100 µL) and monitored using a fluorescence plate reader (FLUOstar, BMG LabTechnologies, Offenburg, Germany). The fluorogenic substrate for caspase 3 generated a blue fluorescence that could be detected by exposure to ultraviolet light at 360 nm, revealing the release of AMC from the substrate upon cleavage by caspase-3. Free AMC emits a blue fluorescence that was measured with a fluorometer at 460 nm. Specific caspase 3 activity was expressed as a ratio between the reaction rate (increase in fluorescence over time) and protein content as determined by the BCA. Pan-caspase inhibitor Z-Asp-2,6,dichlorobenzoyloxymethyl ketone was added to 1 of the duplicate wells (50 µ*M*) together with AC-DEVD-AMC to determine the specificity of the caspase reaction *(20,25)*.

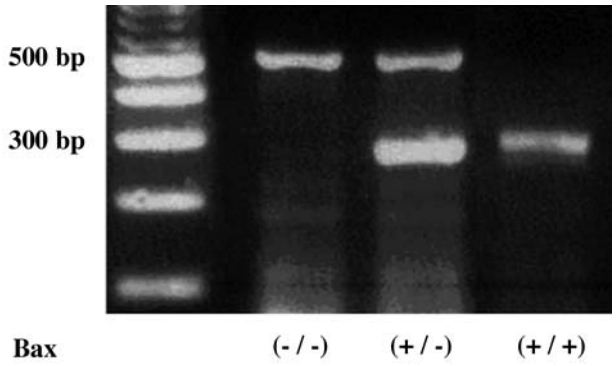

Fig. 1. Genetic identity by PCR: one band of bax gene (300 bp) was seen in Bax(+/+) and two bands (300 bp) were seen in the hemizygotic Bax(+/–), whereas in Bax (–/–), only one band (500 bp) was seen in the neo gene. To verify genetic identity for genotyping, DNA was isolated from the tail using high pure template preparation kit and then subjected to standard PCR reaction.

Statistical Analysis

Results are expressed as means ± standard error of the mean (SEM). Values during stabilization period were defined as 100%. A statistical difference between the groups was assessed by analysis of variance (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 represents the genetic identity of the various mice tested. One band of bax gene of 300 bp was seen in Bax(+/+), whereas in Bax(–/–), only one band of 500 bp was seen in the neo gene.

Mortality

Two-eighths of the mice (25%) died in each group within 24 h of surgery. Two-eighths of the Bax(–/–) group of the Bax(+/+) group died during the following 2 wk (25%) ($p > 0.05$). Death in the first postoperative week was mainly due to LV rupture (within 4 d), massive infarct area, congested lungs, and dilated left ventricle.

Table 1
Background Data on the Mice in the Present Study

	Group	BW (g)	HW (g)	HW/BW
Preoperation	Bax(+/+)	24.9 ± 1.25	0.140 ± 0.0008	0.00571 ± 0.0006
	Bax (−/−)	22.15 ± 1.07	0.114 ± 0.0045	0.00514 ± 0.0006
MI—1 d	Bax(+/+)	22.5 ± 1.05	0.149 ± 0.006[b]	0.00661 ± 0.00021[b]
	Bax (−/−)	22.35 ± 1.5	0.119 ± 0.007	0.00534 ± 0.0023
MI—4 wk	Bax(+/+)	26.43 ± 1.15	0.197 ± 0.005[a][b]	0.00735 ± 0.0015[a][b]
	Bax (−/−)	24.9 ± 0.25	0.123 ± 0.005	0.00494 ± 0.0012

MI, myocardial infarction; BW, body weight; HW, heart weight. Data represent means ± SEM,
[a]$p < 0.005$ vs its own control at baseline.
[b]$p < 0.005$ vs Bax (−/−) at the same time.

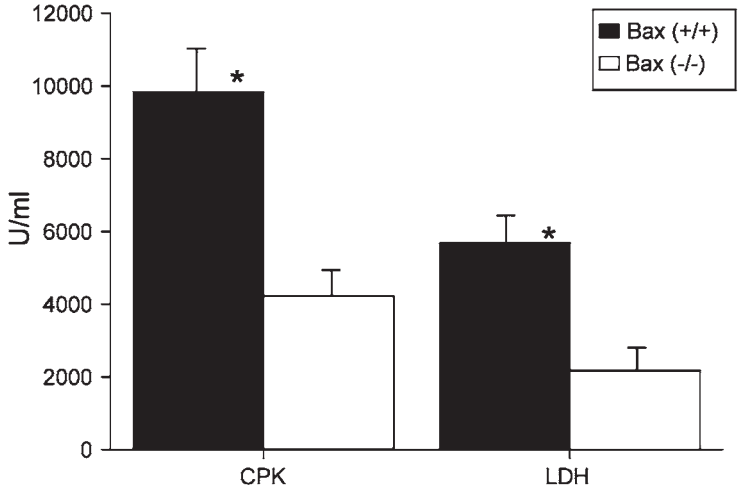

Fig. 2. Release of creatine kinase (CK) and lactate dehydrogenase (LDH)from Bax(+/+) and Bax (−/−) hearts: release of CK and LDH to the serum 24 h post-left anterior descending coronary artery ligation. Values represent means ± SE, $n = 8$ hearts in each group. * $p < 0.03$ vs Bax (−/−).

Weight

At baseline, body weight or heart-to-body weight ratio in Bax(−/−) compared to Bax (+/+) mice was similar. Four weeks after LAD ligation, this ratio was decreased in Bax(−/−) and increased in Bax(+/+) mice, $p < 0.05$ (Table 1). Heart weight increase was significantly higher when compared to body weight in the Bax(+/+) group, compared to Bax(−/−) group at 4 wk after surgery (*see* Table 1).

Biochemical Markers of Ischemia and Reperfusion Damage

Creatine kinase (CK) and lactate dehydrogenase (LDH) activity in the serum, assessed at 1 d after infarction, increased in both groups, but in Bax(+/+), it was higher than in Bax(−/−), $p < 0.003$ (Fig. 2).

Irreversible Ischemic Damage

Using TTC staining, the mean infarct size assessed 1 d and 4 wk after infarction and was significantly smaller in the Bax(−/−) group compared to Bax(+/+). Four weeks post-MI, it was 37 ± 3.6% vs 25 ± 1.9%, $p < 0.03$ (Fig. 3).

Assessment of LV Remodeling Post-MI

Echocardiography

At baseline, there were no significant differences in the echocardiographic measurements of cardiac structure or function between all groups. Induction of MI resulted in more pronounced increased left-ventricular end-systolic and left-ventricular end-diastolic diameters in the wild-type compared to the bax-deficient mice ($p < 0.03$). The

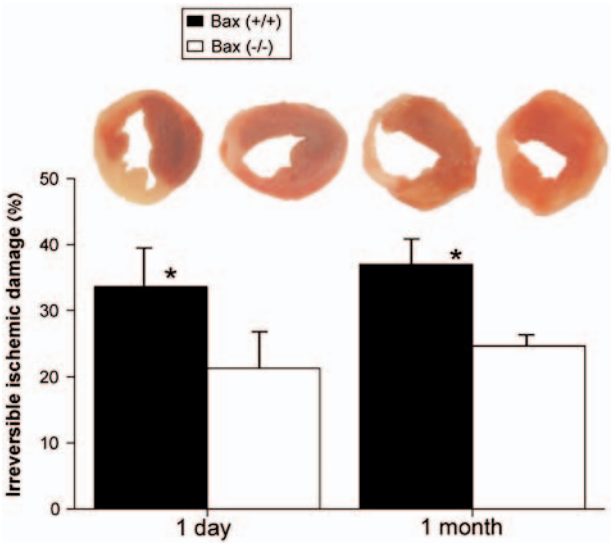

Fig. 3. The effect of left anterior descending coronary artery (LAD) ligation on the percent of irreversible injury was determined by scanning the images of mice hearts ventricular sections with triphenyltetrazolium (TTC). Representative images of the two different groups, revealing various degrees of myocardial ischemia (white to yellowish zones after TTC staining). Hearts were subjected to 1 or 30 d of LAD ligation. A significant size of damaged tissue is noticed in the myocardium of Bax (+/+) compared to Bax (–/–) heart, $p < 0.03$. This figure represents the percent of irreversible injury from the total area of the section at 1 and four weeks post-LAD ligation. Values represent means± SE, n=6 hearts in each group. *P<0.03 vs. Bax (–/–) hearts.

infarcted wild-type mice also demonstrated significantly reduced cardiac function with decreased fractional shortening, compared to the bax(–/–) ($p < 0.01$). Posterior wall thinning was significantly higher in the Bax(+/+) hearts compared to Bax(–/–) hearts at 4 wk post-MI, $p < 0.05$, (Fig. 4A–D). No differences were observed in the heart rate of both infarcted groups, (data not shown).

Bcl-2 and Bax Protein Expression

Figure 5 is a representation of the levels of Bax and Bcl-2 in hearts of wild-type and Bax-deficient mice, before and after ischemia. When data were assayed as changes in reference to actin levels, similar levels of Bcl-2 expression in hearts of both groups before ischemia were shown using planimetric analysis of the gels. A significant reduction in the expression of Bcl-2 compared to the bax-deficient hearts 2 h postischemia (60 ± 13% vs 28 ± 2%) was observed in wild-type mice. As expected, the bax protein was not expressed in bax-deficient hearts but was detected in wild-type hearts. Bax protein expression was increased by 19 ± 10% above baseline levels 2 h postischemia.

Caspase 3 activity was low at all time points measured, except for 2 h postischemia when Bax-deficient hearts exhibited decreased caspase-3 activity, compared with Bax(–/–),($p < 0.001$, Fig. 6).

We added a caspase inhibitor (zD-DCB**)** to the reaction to show that the measured increase in fluorescence is caspase 3-dependent. Treatment with the pan-caspase inhibitor in the reaction mixture of Bax(+/+) heart resulted in an immediate decrease in caspase-3 activity in the ischemic group, demonstrating caspase-reaction specificity**.**

DISCUSSION

Transgenic animal models are useful in identifying the role of various genes in the pathophysiology of cardiac diseases *(26)*. In this study, we assessed the role of the bax gene, one of the participants in transduction and regulation of apoptotic signals. In prior studies we showed that suppression of the bax gene improves myocardial tolerance to acute ischemia and reperfusion injury *(20)*. Here, we extend this observation and show that postischemic remodeling is also improved in the absence of Bax, suggesting that its silencing may be a useful approach to achieve cardioprotection after MI. Bax-deficient hearts had smaller infarct sizes, increased muscle posterior wall thickness, and superior diastolic and systolic functions, as compared to the wild-type controls.

The development of heart failure after MI is linked to impaired LV contractility, ventricular remodeling including LV dilatation and hypertrophy, and scarring of the remote and noninfarcted myocardium *(1)*. The presence of the bax gene in the Bax(+/+) group encouraged the higher rate of remodeling process. Signs of left-ventricular hypertrophy were observed in Bax(+/+) hearts subjected to myocardial infarction as seen by heart-to-body-weight ratio. In contrast, in the bax-deficient hearts this process was not seen. At the same time, the myocardial functional parameters in Bax-deficient hearts were superior to the control group. Both groups showed similar body growth rate. This suggests that heart-to-bodyweight index is not a good predicting parameter for functional recovery from acute ischemia of the heart.

Apoptosis in the ischemia border-zone myocardium is thought to be critical to the remodeling process, and limitation of apoptosis may mitigate postischemic ventricular remodeling and dysfunction *(27–29)*. Treatment with caspase 3 inhibitors resulted in improved survival and decreased ventricular dilatation *(19)*. On the other hand, heart-targeted overexpression of caspase 3 increased infarct size and depressed cardiac function *(30)*. Considering a net contribution of apoptosis of 10%

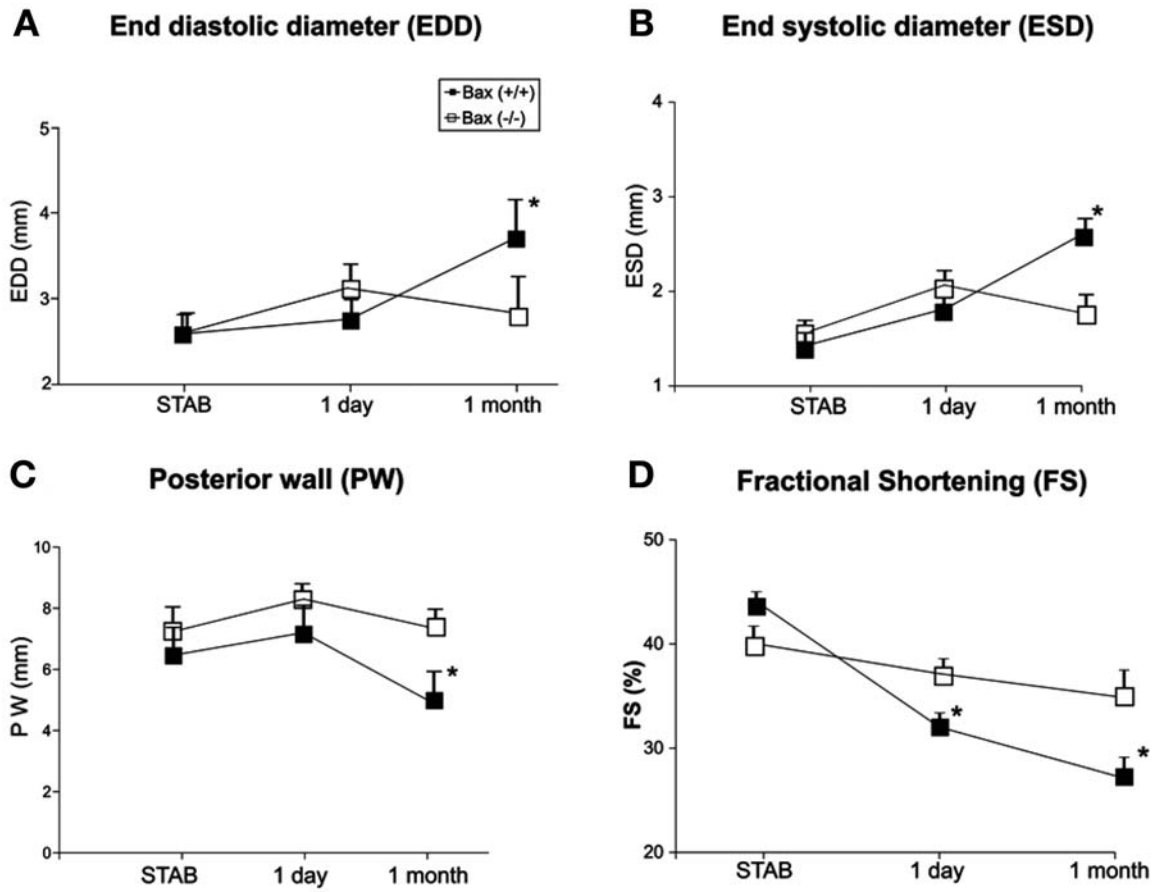

Fig. 4. Echocardiographic studies at baseline, 1 d, and 4 wk after myocardial infarction demonstrating increased left-ventricular end-diastolic diameter **(A)**, left-ventricular end-systolic diameter **(B)**, thinning of the left-ventricular posterior wall **(C)**, and a decreased fractional shortening (FS) in all infarcted animals **(D)**. Four weeks after infarction, the Bax (−/−) group showed lesser degree of left ventricle dilation [both EDD ($p < 0.03$) and ESD ($p < 0.03$)], posterior wall thinning ($p < 0.05$), and a better FS ($p < 0.01$) when compared to the Bax(+/+) hearts. Values represent means± SE, $n = 6$ hearts in each group.

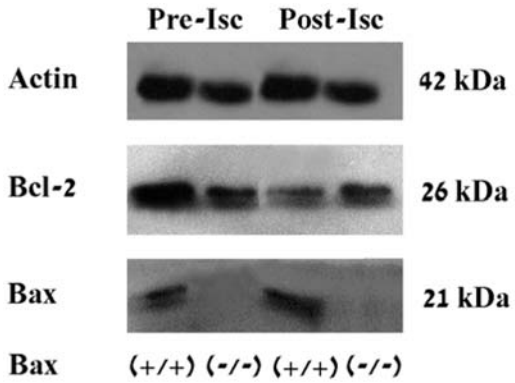

Fig. 5. Representative Western blot analysis of Bcl-2, Bax protein levels in Bax(+/+) and Bax (−/−) myocardium subjected to left anterior descending coronary artery ligation. Western blot of Bcl-2 and Bax protein measured with anti-Bcl-2 and anti Bax polyclonal antibodies. Proteins (60 µg/sample) were layered on a sodium dodecyl sulfate polyacrylamide gel (12.5%) under denaturing conditions and electrotransferred onto nitrocellulose for 1 h at 100 V. A representative sample of Western blotting showing that similar levels of Bcl-2 protein expression were detected in the two groups of hearts. Bax protein was not expressed in Bax-deficient hearts but was detected in Bax(+/+) hearts before (Pre-Isc) and after 2 h of ischemia (Post-Isc). Immunodetection of actin with monoclonal anti-actin antibody was performed as internal control.

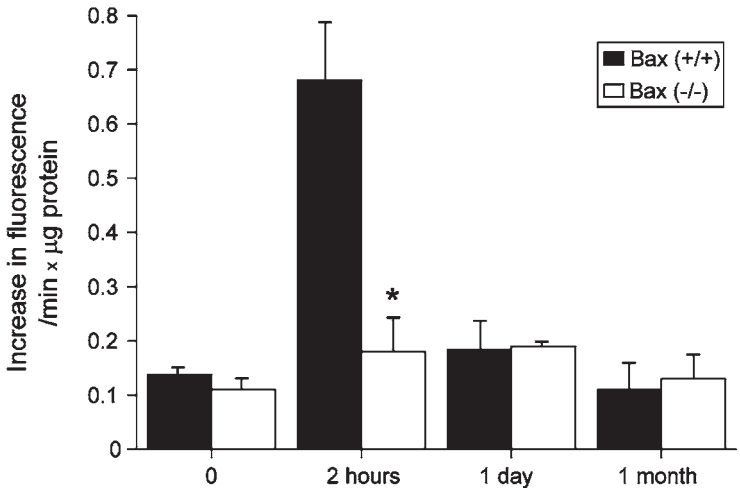

Fig. 6. represents enzymatic activity of caspase 3 in hearts subjected to left anterior descending coronary artery (LAD) ligation. Specific caspase activity was expressed as a ratio between the reaction rate (increase in fluorescence) / min*µg protein. Caspase 3 activity was determined in myocardial extracts from the perinfarcted zone before, at 2 h, 1 d, and 30 d post-LAD ligation. Values represent means ± SE. $p < 0.05$ vs Bax(+/+).

to cell death after ischemia, our data indicate that bax deficiency reduced both necrotic and apoptotic cell death. This protective effect persisted throughout the remodeling period, as is evident from the improved end-systolic and end-diastolic functions in the bax-deficient hearts as compared to the wild-type hearts. In addition, the posterior wall thinning induced by MI was less prominent in the bax-deficient hearts. On the basis of these criteria, we assume that the absence of bax ameliorated the long-term detrimental processes inflicted to the heart by acute ischemia.

The ability of Bax to block apoptosis is considered to depend on the Bax-to-Bcl-2 ratio *(23)*. Bax deficiency in the knockout mice was not accompanied by apparent adaptive changes in Bcl-2 levels *(20)* (Fig. 5). However, Bax deficiency blunted the decrease in Bcl-2 characteristic of wild-type hearts shortly after ischemia *(23,28)*. This superior outcome observed immediately after ischemia persisted and affected the cardiac remodeling during the postischemic period. Despite a very short increase in caspase activity, LAD ligation resulted in dilated cardiomyopathy, as determined by increased diastolic and systolic dimensions, marked thinning of the posterior left ventricular wall, and the decreased LV function in wild-type mice. In the Bax-deficient mice, the postischemic course was ameliorated in all measured parameters. These data raise the question of whether the superior remodeling in the Bax-deficient hearts was triggered during and shortly after the time of infarct, or the persistent absence of Bax altered the process of remodeling itself. These questions can be answered by a model that silences the expression of the Bax gene for limited periods of time, using small interfering (si)RNA. Such a model is not yet available in an in vivo system.

The important role of Bcl-2 in regulating 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 *(31)*. In addition, hearts overexpressing Bcl-2 have a slower rate of decline in ATP during ischemia and reduced ischemic acidification *(32)*. In the absence of the Bax protein, the role of Bcl-2 may be more pronounced in preserving the mitochondrial integrity and function during ischemic damage.

Despite the reduction in infarct size and the better overall LV function in the post-MI period in bax-deficient mice, there were no differences in mortality rates of both groups. When knockout technology is used, caution is required in the interpretation of the apparent mutation and careful extrapolation of the experimental data to normal tissue. The bax- deficient mice at baseline were viable, their body weight and myocardial mass-to-body-weight ratio were similar to those of the wild-type. It is likely, however, that deletion of an important gene activates adaptive processes that enabled normal development and function. 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 by our experimental conditions. Despite these limitations, deletion and overexpression of genes using the knockout and transgenic technologies allow us to examine their function.

Although the apoptotic process postinfarct is very small as compared to the predominant necrotic damage in the infarct territory, it appears that downregulation of the BAX gene plays a crucial role in reducing myocardial ischemic damage, both in vitro and in vivo. In Bax-deficient mice, the post-MI course was ameliorated in all measured parameters. The superior short-term outcome from ischemic injury observed immediately after LAD ligation was sustained throughout the first month after the insult. The precise mechanism remains unclear. Treatment aimed at down regulating the BAX or elevating Bcl-2 proteins, may offer a potential approach to decrease ischemic injury.

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