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Over-expression of a modified bifunctional apoptosis regulator protects against cardiac injury and doxorubicin-induced cardiotoxicity in transgenic mice

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Aims Bifunctional apoptosis regulator (BAR) is an endoplasmic reticulum protein that interacts with both the extrinsic and intrinsic apoptosis pathways. We hypothesize that over-expression of BARΔRING prevents apoptosis and injury following ischaemia/reperfusion (I/R) and attenuates doxorubicin (DOX)-induced cardiotoxicity.

Methods and results We generated a line of transgenic mice that carried a human BARΔRING transgene under the control of the mouse α-myosin heavy chain promoter. The RING domain, which binds ubiquitin conjugating enzymes, was deleted to prevent auto-ubiquitination of BAR and allow accumulation of the BAR protein, which binds apoptosis-regulating proteins. High levels of human BARΔRING transcripts and 42 KDa BARΔRING protein were expressed in the hearts of transgenic mice. When excised hearts were reperfused *ex vivo* for 45 min as Langendorff preparations after 45 min of global ischaemia, the functional recovery of the hearts, expressed as left ventricular developed pressure $\times$ heart rate, was $23 \pm 1.7\%$ in the non-transgenic hearts compared with $51.5 \pm 4.3\%$ in the transgenic hearts ($P < 0.05$). For *in vivo* studies, mice were subjected to 50 min of ligation of the left descending anterior coronary artery followed by 4 h of reperfusion. The infarct sizes following I/R injury, expressed as the percentage of the area at risk, were significantly smaller in the transgenic mice than in the non-transgenic mice (29 ± 4 vs. $55 \pm 4\%$, $P < 0.05$). In hearts of mice subjected to cardiac I/R injury, BAR transgenic hearts had significantly fewer *in situ* oligo-ligation-positive cardiac cells (5.0 ± 0.4 vs. $13.4 \pm 0.5\%$, $P < 0.05$). Over-expression of BARΔRING also significantly attenuated DOX-induced cardiac dysfunction and apoptosis.

Conclusion Our results demonstrate that over-expression of BARΔRING renders the heart more resistant to I/R injury and DOX-induced cardiotoxicity, and this protection correlates with reduced cardiomyocyte apoptosis.

1. Introduction

Apoptosis, or programmed cell death, has been implicated in a number of cardiac diseases such as heart failure[1–3] and anthracycline-induced cardiotoxicity.[4–6] Myocardial ischaemia/reperfusion (I/R) also leads to cell death via both apoptosis and necrosis.[7–10] Kajstura *et al.*[11] showed that apoptosis was the predominant mode of cardiac cell death induced by coronary artery occlusion.

Two major pathways leading to apoptosis have been investigated in detail.[12–14] The extrinsic apoptotic pathway is mediated by the death receptor Fas/FasL and involves the activation of caspase-8. The intrinsic pathway involves mitochondrial dysfunction, cytochrome c release, and activation of caspase-9. Various proteins have been identified, which block one of the two apoptotic pathways, such as Bcl-2 and c-FLIP,[15,16] known for their suppression of the intrinsic and extrinsic pathways, respectively.

Bifunctional apoptosis regulator (BAR) is a 450 amino acid protein that blocks both the extrinsic and intrinsic pathways of apoptosis. BAR contains four recognizable domains: (i) an N-terminal zinc-binding RING domain (24–86); (ii) an SAM domain (180–254); (iii) a coiled-coil domain (273–345); and (iv) a C-terminal transmembrane (TM) domain (400–428). The TM domain inserts in membranes of

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the endoplasmic reticulum (ER). The RING domain binds ubiquitin conjugating enzymes (E2s). The coiled-coil domain of BAR, which has limited sequence homology to death effector domains (DEDs), reportedly associates directly or indirectly with procaspases-8 or -10, blocking Fas-induced activation of the extrinsic apoptosis pathway. The SAM domain facilitates interaction with Bcl-2 and Bcl-X_L, which in turn suppresses Bax-induced cell death, thus blocking the intrinsic apoptosis pathway.[17] BAR therefore may represent a protein capable of bridging two major apoptosis pathways.

Doxorubicin (DOX) is an anthracycline antibiotic that has been widely used for the treatment of acute leukaemia, malignant lymphoma, and solid tumours.[18] Unfortunately, its effectiveness is limited by its severe cardiotoxicity.[19,20] DOX treatment of the heart leads to increased iNOS expression, which in turn elevates NO levels.[21] NO and superoxide anion react to generate peroxynitrite, which is a potent oxidant responsible for DOX-induced cardiotoxicity. Peroxynitrite has been found to cause apoptosis in cardiomyocytes.[22] Recent studies suggest that apoptosis plays an important role in DOX-induced cardiotoxicity.[23–25] We and several other investigators demonstrated that both intrinsic and extrinsic apoptotic pathways are involved in DOX-induced cardiac apoptosis.[24,26] To further support this hypothesis, we decided to study how over-expressing modified BAR affects DOX-induced cardiotoxicity and apoptosis.

Ischemic-reperfusion (I/R) injury induces cardiac cell death through a combination of apoptotic and necrotic mechanisms. Several studies have implicated mitochondria and the intrinsic apoptosis pathway in I/R-induced cell death.[13] However, I/R also reportedly causes loss of c-FLIP from cardiomyocytes, thus removing a block to the extrinsic pathway.[27] Thus, I/R injury may induce or sensitize cells to both intrinsic and extrinsic apoptosis pathways. In addition, injury is a well-known inducer of ER stress, due to redox-based disturbances in disulfide bonding of luminal ER proteins, which results in proteins unfolding and which triggers an evolutionarily conserved signal transduction response termed the 'unfolded protein responses' (UPR). Prolonged ER stress triggers cell death, principally via the intrinsic apoptosis pathway.[28]

We hypothesized that over-expression of BARΔRING in transgenic mice could attenuate I/R injury and DOX-induced cardiotoxicity. To investigate this possibility, we generated a line of transgenic mice that over-expresses BAR in the heart. Because BAR possesses an E2-binding RING domain resulting in its auto-ubiquitination and proteasome-dependent destruction,[17] we expressed BAR devoid of its RING, thus promoting the protein's accumulation. We examined the effect of BARΔRING over-expression on apoptosis, cardiac injury, and DOX-induced cardiotoxicity.

2. Methods

2.1 Generation of bifunctional apoptosis regulator transgenic mice

BAR expression vector was constructed by initially inserting the *Sac*I to *Sal*I fragment of clone 22 (provided by Dr J. Robbins, University of Cincinnati, Cincinnati, OH, USA), which contains the sequence from the last intron of the mouse α-myosin heavy chain (α-MHC) gene to exon 3 of the α-MHC gene, into *Sac*I to *Sal*I sites in plasmid pMSG (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). *Bam*HI digestion of the resultant plasmid allowed isolation of the DNA fragment containing SV40 early splicing and polyadenylation sites downstream from the mouse α-MHC sequence. This DNA fragment was then inserted into the *Bam*HI site of plasmid pKS-S, a modified pKS vector (Stratagene, La Jolla, CA, USA) in which the *Sal*I site was destructed by insertion of an SfiI linker, to generate plasmid pMHC. Human BAR cDNA (ΔRING), which had previously been flanked by *Sal*I sites using linker ligation, was subsequently inserted into the *Sal*I site in plasmid pMHC. The entire expression sequence was isolated by *Cla*I plus *Not*I digestion of the resultant plasmid, and it was utilized in the generation of transgenic mice using fertilized mouse eggs isolated from mating of B6C3 F1 hybrid mice according to standard procedures.

All the animal protocols were approved by the East Tennessee State University's Animal Care and Use Committee. All experiments conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

2.2 RNase protection assay

To detect the expression of human BAR in heart, total RNA was extracted from the heart of non-transgenic and transgenic mice using the acid guanidinium thiocyanate-phenol-$CHCl_3$ extraction method. A 369 bp fragment corresponding to nucleotides 2162–2531 of human BAR cDNA was used as a template. BAR cDNA template was prepared by inserting the *Eco*RI-*xba*I digestion fragment of human BAR cDNA (2 Kb) into pCI-neo vector which was linearized with *Bam*H1. RNA (10 μg) was hybridized overnight with the [^{32}P]-labelled BAR riboprobe. The protected mRNAs were resolved on a 5% denaturing polyacrylamide gel. GAPDH was used as an internal control (Ambion, Austin, TX, USA). The protected fragments for BAR and GAPDH were 369 and 316 bp, respectively.

2.3 Immunoblot analysis of bifunctional apoptosis regulator

Hearts from non-transgenic and transgenic animals were homogenized in a lysis buffer as described previously.[29] Immunoblot analysis was carried out by incubating the membrane with antibodies against BAR (Chemicon, Temecula, CA, USA), Bcl-2, Bcl-X_L (Santa Cruz Biotechnology, Santa Cruz, CA, USA), caspase-8 (Cell Signaling Technology, Beverly, MA, USA), and α-actin (Sigma, St Louis, MO, USA). Immunoblots were developed using ECL detection system (GE Healthcare, Piscataway, NJ, USA) and exposed to X-ray films.

2.4 Immunoprecipitation

Heart lysates of non-transgenic or transgenic animals (300 μg) were incubated with anti-BAR antibodies (Chemicon) in 200 μL of lysis buffer at 4°C for 3 h. Protein A/G (Calbiochem, La Jolla, CA) was added and incubated for 2 h. The immunoprecipitate was washed five times, resuspended in 2× SDS gel sample buffer, and subjected to immunoblot analysis with antibodies specific for caspase-8 (Cell Signaling Technology), Bcl-2, and Bcl-X_L (Santa Cruz Biotechnology).

2.5 DNA immunization with *in vivo* electroporation

Female Lewis (LEW/SSN) rats (8 weeks old) were anaesthetized. Plasmid-encoding BAR cDNA (50 μg) was injected into the anterior tibialis muscles of both legs. The two-needle array electrode (10 mm apart) was inserted into the muscle encompassing the DNA injection site to a depth of 4 mm. Six pulses of length 20 ms with 200 ms intervals at 160 V were delivered using the BTX ECM830 square wave electroporator (BTX, San Diego, CA, USA).[30] Boost injections were done on days 14 and 28. Sera were collected on day 35.

2.6 Immunohistochemistry

Hearts were removed, and a 2 mm section near the mid-ventricle was sliced and embedded. Paraffin-embedded myocardial sections (5 μm) were mounted on superfrost slides and dried at 37°C overnight. Immunostaining was carried out with a rat anti-BAR antibody or polyclonal Bcl-X_L antibody (Cell Signaling Technology) at 4°C overnight. Pre-immune serum was included as background level. Antigen–antibody complexes were detected by the Super sensitive alkaline phosphatase kit (BioGenex, San Ramon, CA, USA), using Fast Red as a chromogen. Haematoxylin was used as a counterstain.

2.7 Global ischaemia *in vitro*

Male B6C3 non-transgenic and transgenic littermates ($n = 6$) weighing between 25–30 g were injected with sodium heparin (500 U/kg body weight, i.p.) 30 min prior to anaesthetization with tribromoethanol (275 mg/kg, i.p.). Hearts were rapidly excised and perfused retrogradely at 60 mmHg by the Langendorff technique with Krebs–Henseleit bicarbonate buffer, as described previously.[31]

2.8 Regional ischaemia *in vivo*

Male B6C3 non-transgenic and transgenic littermates (six in each group) weighing between 25–30 g were anaesthetized with tribromoethanol (275 mg/kg i.p.). Left anterior descending coronary artery (LAD) ligation was performed. After 50 min of LAD ligation, the heart was reperfused for 4 h. Mice were anaesthetized with tribromoethanol (275 mg/kg, i.p.), and hearts were perfused as Langendorff preparations for 5 min. The left coronary artery was re-occluded and 1% Evans blue was infused into the aorta and coronary arteries to determine the risk area. Macroscopic staining with triphenyltetrazolium chloride (TTC) was used to quantify the infarct sizes. The area at risk was expressed as the percentage of the left ventricle (LV) and the area of infarct was expressed as the percentage of the area at risk as described previously.[31]

2.9 Cardiac troponin I release

An ELISA kit (Life Diagnostics, Inc., West Chester, PA, USA) was used to assay the plasma levels of cardiac troponin I (cTnI), a sensitive and specific biomarker of cardiac injury. Results were expressed as ng/mL.

2.10 *In situ* oligo-ligation analysis

Our results showed that mouse hearts subjected to 60 min of ischaemia developed more than 50% infarction. The heterogeneity of mixed populations of live and dead cells hampers the interpretation of apoptosis results in the risk area. Therefore, a period of 30 min of ischaemia and 3 h of reperfusion in the absence of cell death based on TTC staining was chosen for apoptosis analysis.

Hearts were harvested from non-transgenic ($n = 6$) and transgenic ($n = 6$) mice after LAD ligations or DOX treatment. *In situ* staining of DNA strand breaks in 5 μm section of each specimen was detected by the ApopTag *in situ* oligo-ligation (ISOL) kit (Chemicon) using oligo-A according to manufacturer's instructions with some modifications.[31] ISOL-positive myocytes were determined by counting 10 fields of approximately 1000 nuclei. Myocyte nuclei are characterized by their sizes, shapes, and locations. The apoptosis index was calculated (number of apoptotic myocytes/total number of myocytes counted $\times 100$).

2.11 Analysis of *in vivo* cardiac function in acute doxorubicin-induced cardiotoxicity

A single dose of DOX (20 mg/kg, i.p.) or an equivalent volume of saline was injected. Five days after DOX or saline administration, non-transgenic ($n = 6$) and transgenic ($n = 6$) mice were injected with heparin (500 U/kg, i.p.) and anaesthetized with 2% isoflurane. At this dose, none of the mice from this strain died. Each mouse was intubated with a 22-gauge soft catheter and ventilated with a rodent ventilator (Columbus Instruments International Corp., Columbus, OH, USA) at a tidal volume of 0.3–0.5 mL and a respiratory rate of 120 breaths/min. After left thoracotomy, the pericardium was dissected to expose the heart. A microtip pressure–volume catheter (SPR-839; Millar Instruments, Houston, TX, USA) was inserted through a 25-gauge apical stab into the LV to measure the steady-state cardiac function. To change the cardiac pre-load, the inferior vena cava was occluded for 1 s. At the completion of the study, 10 μL of hypertonic saline (15%) was injected into the right atrium to calibrate *V*p, the parallel volume. The signals were continuously recorded at a sampling rate of 1000 s^{-1} using an ARIA pressure–volume conductance system (Millar Instruments) coupled to a Powerlab/4SP A/D converter (AD Instruments, Mountain View, CA, USA). All pressure–volume loop data were analyzed with a cardiac pressure–volume analysis program (PVAN3.4; Millar Instruments). At the end of functional analysis, animals were anaesthetized with tribromoethanol (275 mg/kg, i.p.). Hearts were removed and perfused for 2 min as Langendorff preparations to remove the remaining blood. Portions of the mid-ventricle were fixed for immunological and apoptosis studies.

In this study, an acute DOX toxicity model was chosen because apoptosis developed rapidly on the third day and severe cardiac dysfunction was observed on the fifth day. The rationale for using an acute, high dose of DOX stemmed from the fact that DOX-induced apoptosis is time-dependent; as such, activity of these cascades may be easily missed in a chronic model.

2.12 Statistical analysis

All values were expressed as mean ± SE. One-way ANOVA was used followed by Student–Newman–Keuls multiple-range test if there were significant differences between groups. Significance of differences between two groups was established by Student's *t*-test. Significance was indicated if $P < 0.05$.

3. Results

We generated a line of transgenic mice that carried a human BARΔRING transgene under the control of an α-MHC promoter. No significant differences were observed in body weight or heart protein content between the non-transgenic and transgenic groups at all ages examined. In addition, all transgenic mice were healthy and showed no apparent phenotypic differences. Histological analysis by H & E staining indicated that the hearts of transgenic animals were normal (results not shown).

A detailed expression study was performed with the offspring of the founder mouse. Total RNA was isolated from the hearts of non-transgenic or transgenic animals and probed for human BAR mRNA using a ribonuclease protection assay. A higher level of BAR transcripts was detected in the heart of the transgenic animals (*Figure 1A*). We could not detect any BAR expression in the lung, kidney, or liver (results not shown). Immunoblot analysis showed that the 42 KDa BARΔRING protein was expressed in the transgenic heart (*Figure 1B*). Native BAR migrates at a molecular weight of 50 KDa; the smaller size observed is due to the deletion of the RING domain. In contrast, transgenic and non-transgenic mouse hearts did not differ in their levels of Bcl-2, Bcl-X_L, and caspase-8.

BAR was strongly expressed in the myocytes of transgenic hearts, as demonstrated by diffuse cytoplasmic staining in measuring immunoreactivity (*Figure 2B*), while little BAR staining was detected in non-transgenic hearts (*Figure 2A*). There was no difference of Bcl-X_L immunostaining between non-transgenic (*Figure 2C*) and transgenic hearts

(*Figure 2D*), thus showing the specificity of these findings. Also, no staining was observed using the pre-immune serum (*Figure* 2E). Though BAR is known to associate with ER membranes, the resolution of these light-microscopy studies using colourimetric immunostaining is insufficient to comment on the subcellular location of the transgenic BARΔRING protein.

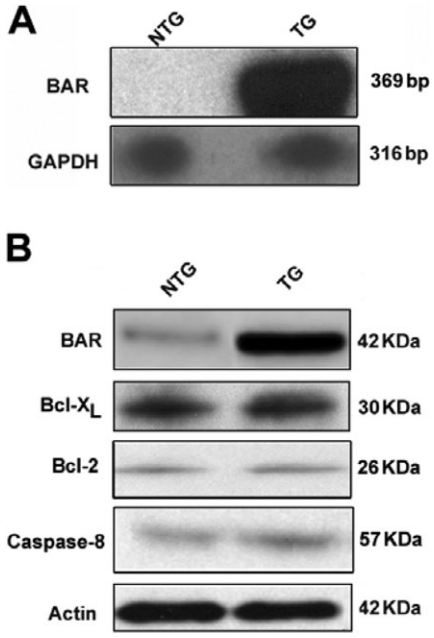

Figure 1 Bifunctional apoptosis regulator (BAR) is expressed in transgenic heart tissue. (*A*) Ribonuclease protection assay of human BAR mRNA in mouse tissues. RNA was isolated from the hearts of non-transgenic (NTG) and transgenic (TG) animals and probed with a human BAR riboprobe. (*B*) Immunoblot analysis of BAR hearts from non-transgenic and transgenic mice. Aliquots of 50 μg of heart homogenates from non-transgenic and transgenic mice were separated on 12% SDS–PAGE and were transferred to nitrocellulose membranes. Immunoblot was carried out with antibodies against BAR, Bcl-2, Bcl-X_L, and caspase-8. α-Actin was used as loading control.

The interaction of BAR with caspase-8 and Bcl-2/Bcl-X_L was assessed by immunoprecipitation experiments. Non-transgenic and transgenic heart homogenates were immunoprecipitated with a rat anti-BAR antibody and immunoblotted with caspase-8, Bcl-2, or Bcl-X_L antibodies. More caspase-8, Bcl-2, and Bcl-X_L were pulled down with BAR immune-complexes in transgenic compared with non-transgenic hearts, consistent with the elevated levels of BAR protein in the transgenic hearts (*Figure 3*).

We compared the cardiac parameters of BAR transgenic mouse hearts with non-transgenic hearts. After 30 min of equilibration perfusion, cardiac basal parameters were measured. Maximum rates of pressure development during both contraction and relaxation ($\pm dP/dt$) were essentially the same in the two groups. Left ventricular developed pressures (LVDPs) were 113 ± 17 mmHg in non-transgenic mice and 106 ± 5 mmHg in BAR transgenic mice. Heart rates and coronary flow rates were also similar in both groups (*Table 1*).

Compared with non-transgenic hearts, there was a significantly improved functional recovery of transgenic hearts subjected to 40 min of ischaemia and 45 min of reperfusion (*Table 1*). Functional recovery, expressed as LVDP × HR, was higher in BARΔRING transgenic mice than in non-transgenic mice after 45 min of reperfusion ($P < 0.05$). In addition, LVDP and $\pm dP/dt$ were also higher in the transgenic group. Heart rates and coronary flow rates were not significantly different between the two groups. Post-ischaemic end-diastolic pressure of the transgenic hearts was significantly lower than non-transgenic hearts after 45 min of post-ischaemic reperfusion. Pre-ischaemic end-diastolic pressures of the two groups were not statistically different (*Figure 4*).

To study the effect of BARΔRING over-expression on regional I/R injury *in vivo*, mice were subjected to 50 min of LAD ligation followed by 4 h of reperfusion. The risk area, expressed as the percentage of the LV between the non-transgenic and transgenic hearts, was comparable. The infarct size of the LV

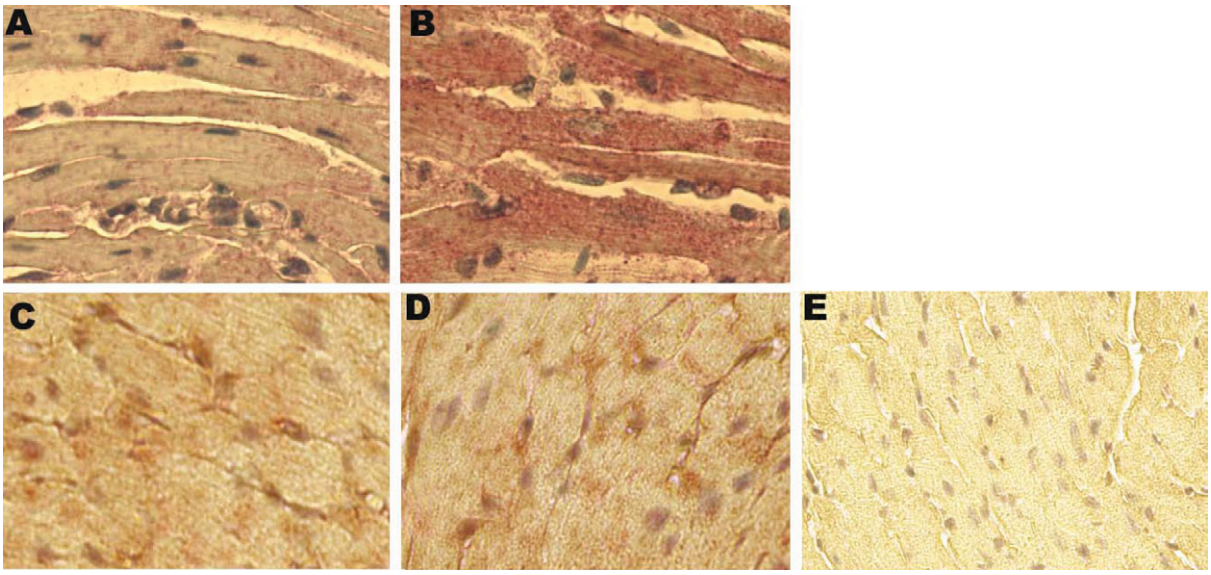

Figure 2 Immunohistochemical staining of BAR and Bcl-X_L in hearts of non-transgenic and transgenic mice. Paraffin-embedded sections of non-transgenic (*A*) and transgenic heart (*B*) were immunostained with a rat anti-BAR antibody. (*B*) BAR immunoreactivity (in red) was demonstrated by diffused cytoplasmic staining in the myocytes. (*C* and *D*) Bcl-X_L immunostaining of non-transgenic and transgenic hearts, respectively. (*E*) Staining pattern of transgenic heart with pre-immune serum. Magnification, ×200.

was significantly larger in the non-transgenic hearts than the transgenic hearts. Infarct sizes, expressed as the percentage of the risk area, were smaller in the transgenic hearts than in the non-transgenic hearts (*Table 2*).

We compared troponin I levels in the sera of non-transgenic and transgenic animals after 4 h of reperfusion following LAD ligation. The total release of cTnI of the non-transgenic and transgenic group was 168 ± 14 and 93.5 ± 11.9 ng/mL ($P < 0.05$), respectively, indicating a 1.8-fold reduction in mice over-expressing BARΔRING.

Next, we evaluated markers of apoptosis in hearts of mice subjected to LAD ligation. As TUNEL analysis is known to detect non-specific DNA fragmentation due to necrosis, a more specific *in situ* ligation assay for identification of apoptotic nuclei using hairpin oligo-nucleotide probes was performed. As shown in *Figure 5*, little labelling of myocyte nuclei was observed in sham-operated animals. In contrast, the LV of non-transgenic animals and transgenic animals contained 14.43 ± 0.60 and 6.21 ± 0.53% ISOL-positive myocyte nuclei, respectively ($P < 0.05$). These results demonstrate that myocyte apoptosis was attenuated in BARΔRING transgenic mice.

Finally, experiments were designed to determine whether DOX-induced myocardial dysfunction is attenuated by over-expression of a modified BAR in the heart. Our studies revealed no significant differences in baseline cardiac function between non-transgenic and transgenic mice (*Table 3*). Cardiac function was significantly depressed in DOX-treated non-transgenic mice, as evidenced by a significant decrease in heart rate (24%), cardiac index (74%), stroke index (54%), and ejection fraction (48%). In contrast, cardiac performance was better in transgenic mice treated with DOX. DOX treatment in non-transgenics led to lower end-systolic pressure, dP/dt_{max}, $dP/dt_{max} - V_{ed}$, and PRSW. In terms of diastolic function, end-diastolic pressure and τ increased, whereas end-diastolic volume and dP/dt_{min} decreased. In contrast, cardiac performance was markedly improved in transgenic mice treated with DOX. Thus, over-expression of BARΔRING in the heart significantly protected against DOX-induced cardiac dysfunction. No significant difference was observed between transgenic and non-transgenic mice with respect to changes in DOX-induced body weight loss or heart rate. Altogether, these results indicate that over-expression of BARΔRING greatly improves LV contractility in DOX-treated mice.

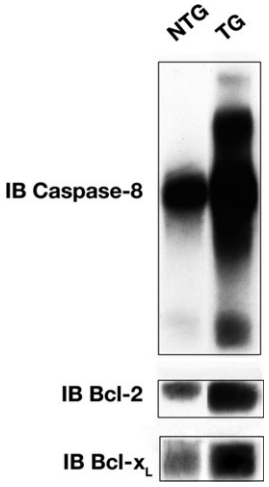

Figure 3 Interaction of BAR with caspase-8, Bcl-2 and Bcl-X_L. Aliquots of 300 μg of heart homogenates from non-transgenic and transgenic mouse hearts were immunoprecipitated with BAR antibodies, followed by immunoblotting with caspase-8, Bcl-2, or Bcl-X_L antibodies.

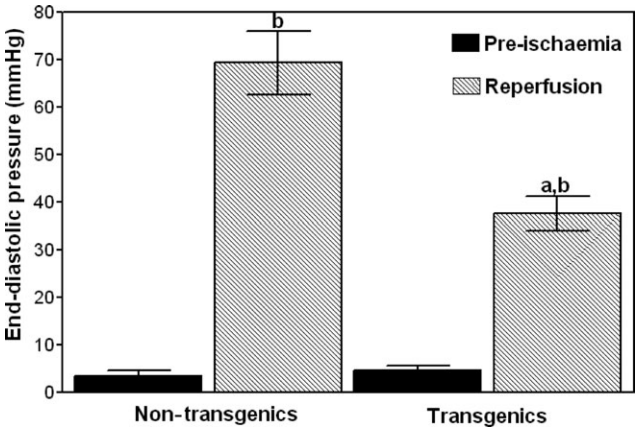

Figure 4 *Ex vivo* studies of BAR-mediated protection from cardiac ischaemia/reperfusion injury. Improvement of left ventricular end-diastolic pressure after 45 min of global ischaemia and 45 min of reperfusion in isolated non-transgenic and transgenic hearts. Values are mean ± SEM of six hearts. a, $P < 0.05$ vs. reperfusion group in non-transgenics, b, $P < 0.05$ vs. pre-ischaemic groups.

Table 1 *Ex vivo* heart ischaemia/reperfusion injury studies show protection by over-expression of BARΔRING

Parameter	Non-transgenics		Transgenics	
	Pre-ischaemia	Post-ischaemia	Pre-ischaemia	Post-ischaemia
Heart rate (b.p.m.)	227 ± 18	210 ± 17	199 ± 17	220 ± 19
$+dP/dt$ (mmHg/s)	3634 ± 605	903 ± 150^{b}	3533 ± 227	$1671 \pm 107^{a,b}$
$-dP/dt$ (mmHg/s)	2903 ± 510	757 ± 133^{b}	2439 ± 65	$1143 \pm 30^{a,b}$
LVDP (mmHg)	113 ± 17	31.5 ± 4.7^{b}	106 ± 5	$51.3 \pm 2.4^{a,b}$
LVDP × heart rate	$24\,657 \pm 3015$	5695 ± 696^{b}	$20\,799 \pm 1388$	$10\,711 \pm 714^{a,b}$
Coronary flow (mL/min)	1.54 ± 0.12	1.39 ± 0.10	1.23 ± 0.12	1.18 ± 0.12

Improvement of the post-ischaemic recovery of cardiac function after 45 min of global ischaemia and 45 min of reperfusion in isolated non-transgenic and transgenic hearts. Values are mean ± SEM of six hearts.There was no difference in cardiac function between pre-ischaemic non-transgenics and transgenics. $\pm dP/dt$, maximum rates of pressure development; LVDP, left ventricular developed pressure.

[a] $P < 0.05$, post-ischaemic transgenics vs. post-ischaemic non-transgenics.

[b] $P < 0.05$, post-ischaemia vs. pre-ischaemia.

Table 2 Over-expression of BARΔRING protects against myocardial infarction in mice

Parameter (% recovery)	Non-transgenics	Transgenics
Infarct/LV	16.76 ± 1.86	7.98 ± 3.6^a
Infarct/risk area	55.35 ± 4.15	29.61 ± 3.64^a
Risk area/LV	30.34 ± 2.29	27.50 ± 3.53

Animals were subjected to 50 min of LAD coronary artery ligation followed by 4 h of reperfusion. Infarct sizes were evaluated by TTC staining. Values are mean ± SEM of six hearts.
[a] $P < 0.05$ vs. non-transgenics.

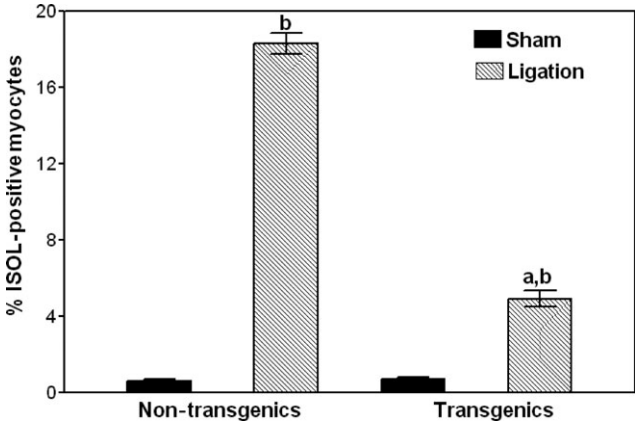

Figure 5 BAR reduces apoptosis in heart following left anterior descending coronary artery (LAD) ligation. Paraffin-embedded sections of non-transgenic and transgenic hearts subjected to 30 min of LAD ligation followed by 3 h of reperfusion were stained by the *in situ* oligo-ligation (ISOL) procedure. Immunolabelled nuclei of myocytes were determined by random counting of 10 fields per section. Each bar represents mean ± SEM of six hearts. a, $P < 0.05$ vs. LAD ligation group in non-transgenic mice, b, $P < 0.05$ vs. sham groups.

ISOL was used to identify apoptotic myocytes in DOX-treated mouse hearts. We detected $11.3 \pm 1.3\%$ ISOL-positive cardiomyocytes in DOX-treated non-transgenic mice, compared with $3.7 \pm 0.63\%$ ISOL-positive in DOX-treated transgenic mice ($P < 0.05$) (*Figure 6*). Thus, BARΔRING over-expression reduces DOX-induced apoptosis of cardiomyocytes *in vivo*.

4. Discussion

We created transgenic mice over-expressing BARΔRING in the heart under the control of the mouse α-MHC promoter. Previous studies have shown that BAR or BARΔRING over-expression can protect several types of cells, including neuronal, epithelial, and haematopoietic, against a variety of apoptotic stimuli.[32] However, this is the first study that shows that BARΔRING over-expression can inhibit apoptosis of cardiomyocytes and the first to demonstrate BAR-mediated protection against I/R injury and DOX-induced cardiotoxicity.

Previously, we reported that over-expression of the anti-apoptotic Bcl-2 and c-IAP2 genes renders mouse hearts more resistant to apoptosis and I/R injury.[29,31] In the present study, we demonstrate that over-expression of BARΔRING confers cardioprotection against I/R injury *in vivo*, as well as against I/R injury of isolated hearts *ex vivo*. We showed that transgenic hearts had fewer ISOL-positive cardiomyocytes than non-transgenic hearts following LAD ligation *in vivo*, suggesting that the cardioprotective effect of BARΔRING over-expression is related to inhibition of cardiac apoptosis.

I/R injury is known to induce cardiac cell death through a combination of apoptosis, necrosis, and possibly necroptosis.[33,34] Moreover, multiple pathways for each of these types of cell death may be induced in parallel. It is likely that BAR only blocks some of these cell death pathways. In addition to cell death, I/R may compromise organ function by other non-cell death mechanisms, which BAR does not alleviate. Thus, it may not be surprising that cardiac function is still depressed (*Figure 4*).

DOX-induced heart failure was also attenuated in BARΔRING transgenic mice, as evidenced by measurements of cardiac index, stroke index, ejection fraction, and pre-load recruitable stroke work. Both systolic and diastolic dysfunctions induced by DOX were significantly ameliorated in BARΔRING mice. Although we do not have direct evidence for the relation between cardiac function and calcium handling, we speculate that the DOX-induced decrease in $dP/dt_{max} - V_{ed}$ may reflect alterations in calcium cycling proteins during isovolumic contraction, while the DOX-induced increase in τ may reflect effects on SERCA2a and/or phospholamban during isovolumic relaxation. These aspects of cardiac performance therefore could be relevant to the location of BAR in the ER, where Bcl-2, Bcl-X_L, and other BAR-interacting proteins have been shown to regulate Ca^{2+} homeostasis and dynamics.[32] BARΔRING over-expression did not prevent DOX-induced body weight loss, suggesting that DOX-induced weight loss is not directly related to cardiac damage.

We did not observe a change in Bcl-2 or Bcl-X_L protein levels, which is consistent with previously published results from cultured cells where either full-length or RING-deleted BAR was co-expressed with these anti-apoptotic proteins in cells.[32] Thus, Bcl-2 and Bcl-X_L are probably not substrates of the E3 ligase activity of BAR. Further studies will be required to understand at a mechanistic level the interactions of BAR with Bcl-2 and Bcl-X_L as pertains to regulation of cell death, ER Ca^{2+} handling, and other molecular events.

DOX, similar to I/R injury, induces cardiac cell death through a combination of apoptotic and necrotic mechanisms. Over-expression of BARΔRING is able to restore cardiac function, and this correlates with reduced ISOL-positive cells, implicating apoptosis. However, because BAR binds Bcl-2 family proteins and because those proteins are known to control both apoptotic and non-apoptotic cell death, we cannot surmise whether reducing apoptosis is the only contribution that BAR makes to preserving cardiac function following DOX exposure.

Another multidomain anti-apoptotic protein, named ARC (apoptosis repressor with CARD),[35] is similar to BAR in that it can block both Bax-induced and Fas-induced cell death. Like BAR, over-expression of ARC blocks both the extrinsic and intrinsic pathways. Conversely, inactivation of ARC increases myocyte apoptosis during myocardial I/R and hemodynamic overload.[36] Thus, ARC is an important endogenous regulator of cardiomyocyte cell survival. ARC, however, is a soluble cytosolic protein, while BAR is tethered to ER membranes, implying differences in their cytoprotective mechanisms.

Table 3 *In vivo* cardiac function of non-transgenic and transgenic mice treated with and without doxorubicin

Parameter (units)	NTG	TG	NTG+DOX	TG+DOX
Weight (g)	28.6 ± 0.61^{a}	28.3 ± 0.91^{a}	23.9 ± 0.36	24.0 ± 0.79^{b}
HR (b.p.m.)	489 ± 18^{a}	462 ± 14^{a}	370 ± 36	415 ± 20
CI (μL/g)	178 ± 9^{a}	178 ± 7^{a}	46 ± 11	$136 \pm 11^{a,b}$
SI (μL/g)	0.37 ± 0.01^{a}	0.39 ± 0.02^{a}	0.17 ± 0.02	$0.33 \pm 0.02^{a,b}$
EF (%)	62.8 ± 0.51^{a}	63.6 ± 0.86^{a}	32.6 ± 3.06	$52.5 \pm 1.35^{a,b}$
Systolic function and contractility				
P_{es}(mmHg)	93.0 ± 1.63^{a}	100.2 ± 3.59^{a}	69.4 ± 5.51	90.4 ± 3.26^{a}
V_{es}(μL)	6.17 ± 0.07^{a}	6.22 ± 0.12^{a}	8.06 ± 0.13	$7.00 \pm 0.10^{a,b}$
dP/dt_{max}(mmHg/s)	9446 ± 661^{a}	9783 ± 1127^{a}	4334 ± 607	9096 ± 1019^{a}
$dP/dt_{max} - V_{ed}$(mmHg/μL)	650 ± 88^{a}	605 ± 73	381 ± 90	740 ± 118^{a}
PRSW (mmHg)	96.2 ± 1.65^{a}	96.1 ± 1.75^{a}	26.0 ± 8.14	84.2 ± 3.27^{a}
Diastolic function				
P_{ed}(mmHg)	3.09 ± 0.45^{a}	3.94 ± 0.28^{a}	5.47 ± 0.64	3.60 ± 0.45^{a}
V_{ed}(μL)	16.58 ± 0.15^{a}	17.10 ± 0.25^{a}	12.08 ± 0.54	$14.88 \pm 0.47^{a,b}$
dP/dt_{min}(mmHg/s)	8832 ± 444^{a}	8397 ± 633^{a}	3862 ± 630	7123 ± 763^{a}
τ (Weiss) (ms)	6.43 ± 0.18^{a}	7.07 ± 0.45^{a}	11.51 ± 1.13	8.79 ± 0.98^{a}
β (beta) (mmHg/μL)	0.32 ± 0.03	0.45 ± 0.06	0.45 ± 0.11	0.40 ± 0.04

NTG and TG mice were injected with DOX (20 mg/kg, i.p.) or saline. Five days later, *in vivo* cardiac function was measured by the Millar conductance catheter system. Values are mean $\pm$ SEM of six to seven mice. HR, heart rate; V_{ed}, end-diastolic volume; V_{es}, end-systolic volume; SI, stroke index (SI°=°stroke volume/body wt); EF, ejection fraction; CI, cardiac index (HR $\times$ stroke volume/body wt); PRSW, preload recruitable stroke work; P_{es}, end-systolic pressure; P_{ed}, end-diastolic pressure; dP/dt_{max} (or $_{min}$), the maximal rate of pressure increasing (or decreasing); $dP/dt_{max} - V_{ed}$, slope describing isovolumic contraction; τ_{Weiss}, the mono-exponential time constant of relaxation; β, slope of end-diastolic pressure–volume relationship.

[a] $P < 0.05$ vs. NTG+DOX.

[b] $P < 0.05$, TG+DOX vs. TG.

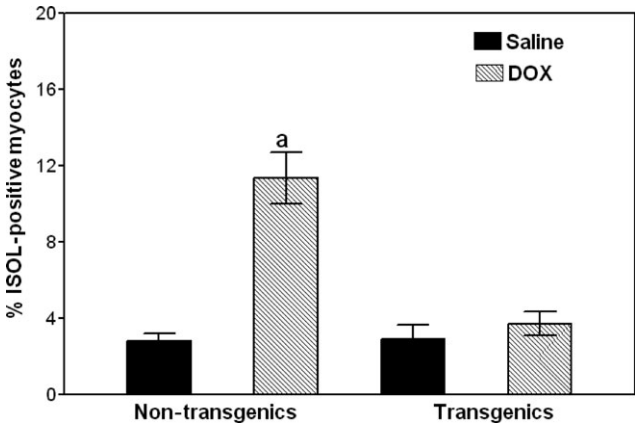

Figure 6 Effect of DOX on apoptosis in hearts from non-transgenic and transgenic mice. ISOL procedure was performed in paraffin-embedded myocardial sections. Immunolabelled nuclei of myocytes were determined by random counting of 10 fields per section. Each bar represents mean $\pm$ SEM of six hearts. a, $P < 0.05$, non-transgenics+DOX vs. other groups.

How precisely BARΔRING inhibits apoptosis of cardiomyocytes during I/R or DOX treatment remains to be determined. BARΔRING has been reported to bind anti-apoptotic proteins Bcl-2 and Bcl-X_L, as well as associating directly or indirectly with caspase-8. Indeed, we confirmed by co-immunoprecipitation assay that the BAR protein in transgenic heart tissue associates with these apoptosis-regulating proteins. With respect to Bcl-2 and Bcl-X_L, these anti-apoptotic proteins have been shown to insert into both ER and mitochondrial membranes. At ER membranes, Bcl-2 and Bcl-X_L regulate ER Ca^{2+} handling, reducing resting ER Ca^{2+} concentrations and decreasing cytosolic Ca^{2+} concentrations following release of Ca^{2+} from ER.[37] Given the importance of ER Ca^{2+} in cardiac performance and cardiomyocyte cell death, it is possible that BAR's interactions with Bcl-2 and Bcl-X_L in ER membranes enhance the ability of these anti-apoptotic proteins to regulate ER Ca^{2+} homeostasis. With respect to caspase-8, this apoptotic protease has been reported to not only associate with BAR-containing protein complexes, but also with Bap31, another ER membrane protein implicated in cell survival and ER Ca^{2+} regulation.[38] Both BAR and Bap31 contain variant DED-like domains, recently shown to form dimeric coiled-coil structures[39] rather than the DED fold. BAR and Bap31 bind each other, presumably via these variant DED-like domains, and Bap31 also associates with Bcl-2 and Bcl-X_L[40] suggesting the possibility of a multiprotein complex that contains minimally BAR, Bap31, Bcl-2, and Bcl-X_L. In turn, Bcl-2 and Bcl-X_L have been reported to interact in ER membranes with BI-1, a multitransmembrane protein implicated in ER Ca^{2+} regulation and with inositol-triphosphate receptors, Ca^{2+} channels of the ER.[41]

Altogether, these intriguing protein interactions raise the possibility that BAR's cytoprotective activity in the heart relates at least in part to effects on ER Ca^{2+} regulation. However, it should be noted that we employed a ΔRING mutant of BAR for these studies, due to the autoubiquitination of BAR mediated by the binding of E2s to its RING domain. Expression of BARΔRING therefore may operate as a dominant-negative antagonist of endogenous BAR with respect to its E3 ligase activity. Other than BAR itself, the substrates of BAR's E3 ligase activity are unknown. In this regard, we did not observe a difference in heart levels of BAR-interacting proteins Bcl-2, Bcl-X_L, or caspase-8. Future studies of the proteosomes of transgenic and non-transgenic BARΔRING mice may reveal candidate substrates of BAR and also yield additional insights into the cytoprotective mechanisms of this interesting protein.

In summary, over-expression of BARΔRING protects the heart against I/R injury and DOX-induced cardiotoxicity. These findings suggest that BARΔRING over-expression strategies may be helpful for promoting cardiomyocyte survival. The precise mechanism of BAR's ability to inhibit myocardial I/R injury and DOX-induced cardiotoxicity requires further investigation.

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