MATERIALS AND METHODS

Ex Vivo Ischemia Reperfusion (I/R) Treatment

NTG (n = 3) were treated with 500 U/kg heparin and anesthetized with pentobarbital 150 mg/kg pentobarbital sodium. Hearts were excised and placed in ice cold Krebs-Henseleit buffer (KHB, Sigma) with aortas cannulated and perfused using Langendorff perfusion procedure by gravity flow. Hearts were then perfused at 80 mmHg in an isolated perfused heart apparatus where a water-filled balloon connected to a pressure transducer (Gould Stathem P23 ID) was inserted into the left ventricle where left ventricular developed pressure was collected using Powerlab and AD Instruments Chart 4 v4.12. During perfusion treatment, hearts were submersed in 37°C KHB. Hearts were paced solely during perfusion at 400 Hz using Powerlab Stimulus Isolator (AD Instruments) at 0.5 mA. During I/R treatment, hearts were equilibrated for 30 minutes before being subjected to 30 minutes of ischemia followed by increasing increments of reperfusion up to 120 minutes.

Neonatal Rat Cardiomyocyte (NRCM)s Culture/Infections

NRCMs were harvested from 2-3 day old Sprague-Dawley rats. Rats were anesthetized with 400 mg/kg chloral hydrate followed by excision of the heart and left ventricular myocytes enzymatically dissociated. Retroperfusion was performed on the myocardium through the aorta at 37°C with a Ca^{++} free solution gassed with 85% O_2 and 15% N_2 for 5 minutes. After, 0.1 mmol/L $CaCl_2$, 274 U/ml collagenase (type 2, Worthingon Biochemical Corp) and 0.57 U/ml protease (type XIV, Sigma) were added to with solution containing 126 mmol/L NaCl, 4.4 mmol/L KCl, 5 mmol/L Pyruvate, and 5 mmol/L NaH_2PO_4 (pH 7.4).

Isolated NRCMs were cultured in M199 media (Mediatech Inc.) containing 10% fetal bovine serum (FBS) overnight. The following day, NRCMs were infected with adenoviral constructs expressing green fluorescent protein (GFP) or Pim-1 (Pim WT) (23) for 2 hours (MOI = 150) in M199 media containing 2% FBS, washed in PBS, and refed with M199 media containing 2% FBS and 50 µg/ml Penicillin-Streptomycin and 100 µmol/L glutamine overnight. GFP adenoviral constructs were received from Pittsburgh Viral Corp. Pim WT adenoviral constructs were designed and purified as previously described (19). Nhe/SmaI fragments were subcloned from pEGFP-C1 Pim-1 plasmids into the pDC315io (Microbix) adenoviral shuttle vector. Shuttle vectors were sequenced for verification and cotransfrected with genomic pBHGloxΔE1,3Cre into 293iq™ cells (Microbix). GFP and Pim WT purified plaques were then isolated and expanded for use.

Quantification of fragmented DNA in cell death by enzyme-linked immunosorbent assay (ELISA)

Custom-designed synthetic Bcl-X_L and Bcl-2 small interfering RNA (siRNA) and negative control scrambled siRNA negative control were purchased from Applied Biosystems. NRCMs were transfected with Bcl-X_L, Bcl-2, Bcl-X_L together with Bcl-2 or control siRNA (NT-SCR) oligonucleotides (5nM) using HiPerfect transfection reagent according to the manufacturer's instructions (QIAGEN). NRCMs were then transfected with either GFP or Pim WT adenovirus. The efficiency of the knockdown of the BCL-2 family members was determined using western blot analysis. Following 48 hours after transfection, apoptosis was induced in the NRCM samples using 0.5 µmol/L staurosporine for 16 hours. NRCM samples were analyzed for the presence of DNA fragmentation in cell death using a cell death detection ELISA kit (Roche) according to the manufacturer’s instructions.

Transmission Electron Microscopy (TEM)

Sixty µg of isolated mouse heart mitochondria from NTG, Pim WT, and Pim DN (n = 2) in swelling buffer were incubated with 150 µmol/L $CaCl_2$ in a final volume of 200 µl for 10 minutes. Mitochondria were spun down to form a pellet at 3000x g for 5 minutes. The mitochondrial pellet was fixed in 4% glutaraldehyde and 2% low melting point agarose in 0.1 mol/L sodium cacodylate buffer (pH 7.4) for 2 hours. Samples were washed in sodium cacodylate buffer and postfixed for 1 hour on ice with 1% Osmium tetroxide and 3% Potassium ferricyanide in sodium cacodylate buffer. Samples were then washed several times in ice-cold double distilled water and en bloc-stained with ice cold 1% aqueous uranyl acetate overnight. Samples were dehydrated in ascending concentrations of ethanol (50%, 75%, 95% at 10 minutes incubation each) and dehydrated twice with 100% acetone for 10 minutes. After dehydration, the samples were infiltrated with Eponate- 12 kit (Pelco) starting with a concentration of 2 acetone: 1 epon mix for several hours followed by an infiltration in a concentration of 1 acetone: 2 epon mix overnight. Samples were then fully infiltrated in 100% epon for 24 hours and placed in an oven to polymerize at 60° C at -10 psi for at least 48 hours. Thin sections were collected from the embedded samples and stained with 1% uranyl acetate and lead citrate. Micrographs were captured using FEI Tecnai 12 transmission electron microscope operated at 120 kV with images recorded on a Tietz 214 CCD camera, magnification at 2700X.

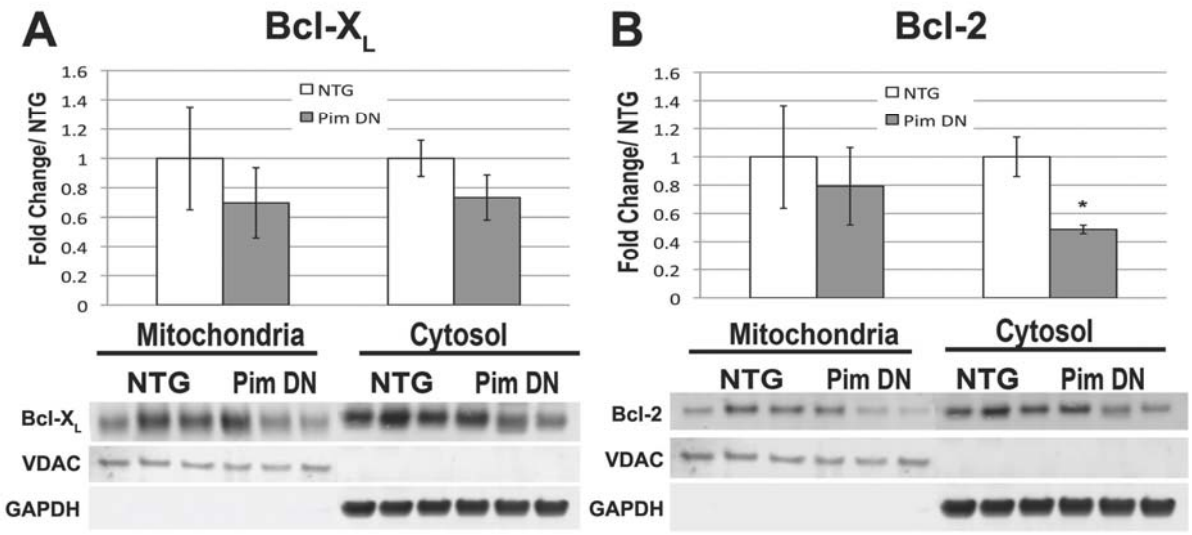
Immunoblot analyses.

Samples were separated on a 4-12% Bis-tris mini-gel (Invitrogen) followed by transfer to PVDF membrane. Membranes were blocked in 10% milk in TBST (150 mmol/L NaCl, 50 mmol/L Tris-pH 7.5, 0.1% Tween) for one hour and probed with primary antibody overnight (Table 1). The following day, blots were washed in TBST three times for 10 minutes each, and incubated in secondary antibody (see table below) in blocking solution. Blots were scanned using a Typhoon 9410 (GE Healthcare) and quantitated for pixel intensity using ImageQuant 5.2 software (GE Healthcare) and normalized to corresponding loading control

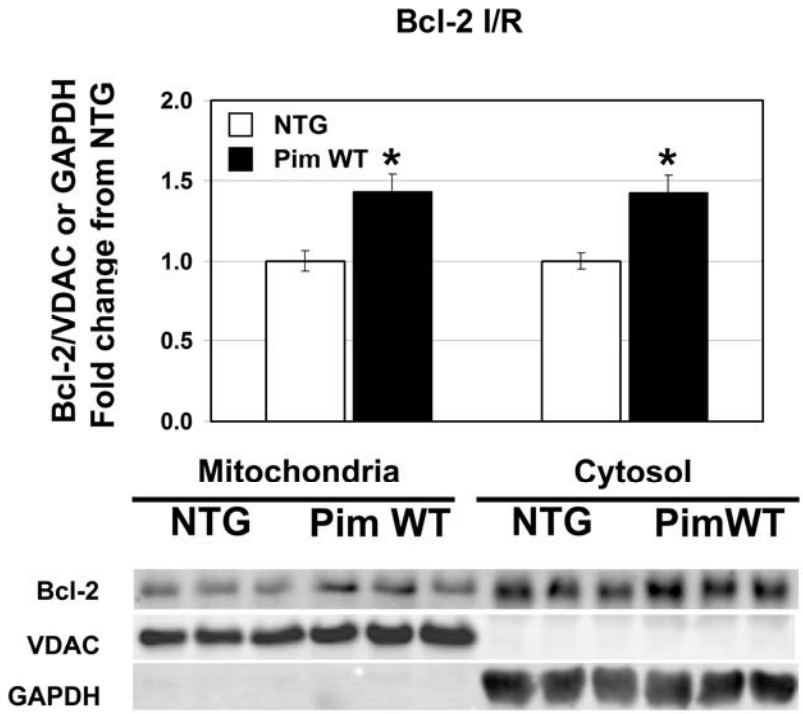
Table I. List of Antibodies

Primary Antibody	Species	Dilution	Manufacturer	Corresponding Secondary Antibody & Dilution
Bcl-X_L	Mouse	1:300	Santa Cruz Technology	AP*, 1:2000
Bcl-2	Mouse	1:500	Biosource	AP*, 1:2000
Pim-1	Rabbit	1:10000	Gift from Dr. Nancy Magnuson (Washington State University)	AP*, 1:2000
Cytochrome c	Mouse	1:1000	Pharmingen	HRP**, 1:1000
Loading Controls				
GAPDH[#]	Rabbit	1:3000	Chemicon	Bcl-X_L & Bcl-2: AP*, 1:2000 Pim-1: CY5***, 1:5000
VDAC[##]	Rabbit	1:1000	Cell Signaling Technology	Bcl-X_L & Bcl-2: AP*, 1:2000 Pim-1: CY5***, 1:5000 Cytochrome c: HRP**, 1:1000
Nucleoporin	Rabbit	1:1000	Cell Signaling Technology	Pim-1: CY5***, 1:5000

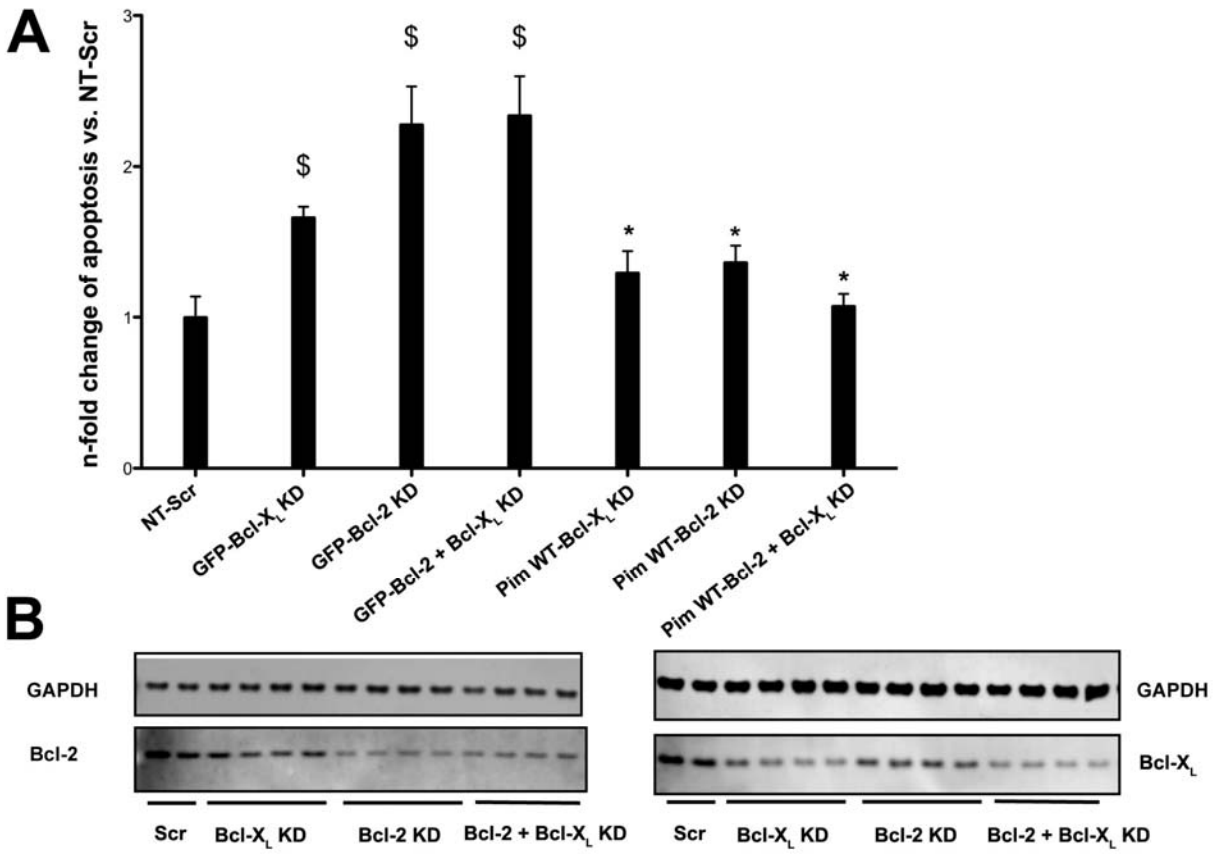
*AP = Alkaline phosphatase
**HRP = horseradish peroxidase
***CY5 = cyanine5
#GAPDH = glyceraldehyde-3-phosphate dehydrogenase
##VDAC = voltage activated anion channel



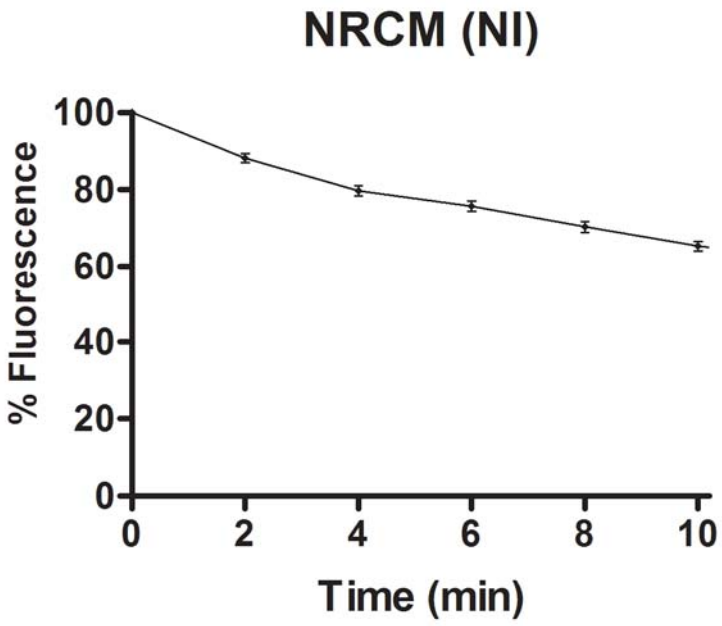
Supplemental Figure I. Pim DN hearts resulted in lower Bcl-2 levels compared to NTG in the cytosol. Bcl-X_L (**A**) and Bcl-2 (**B**) from mitochondrial and cytosolic fractions of Pim DN and NTG mouse hearts probed by immunoblot analyses to determine protein expression levels. Quantitative graphs represent Bcl-X_L and Bcl-2 fold change from NTG (normalized to loading control, mitochondrial fraction = VDAC and cytosolic fraction = GAPDH). Results are represented as mean ± SEM, N = 5. Student's T test: *P<0.05 for Pim DN vs. NTG Bcl-2 levels in the cytosolic fraction.



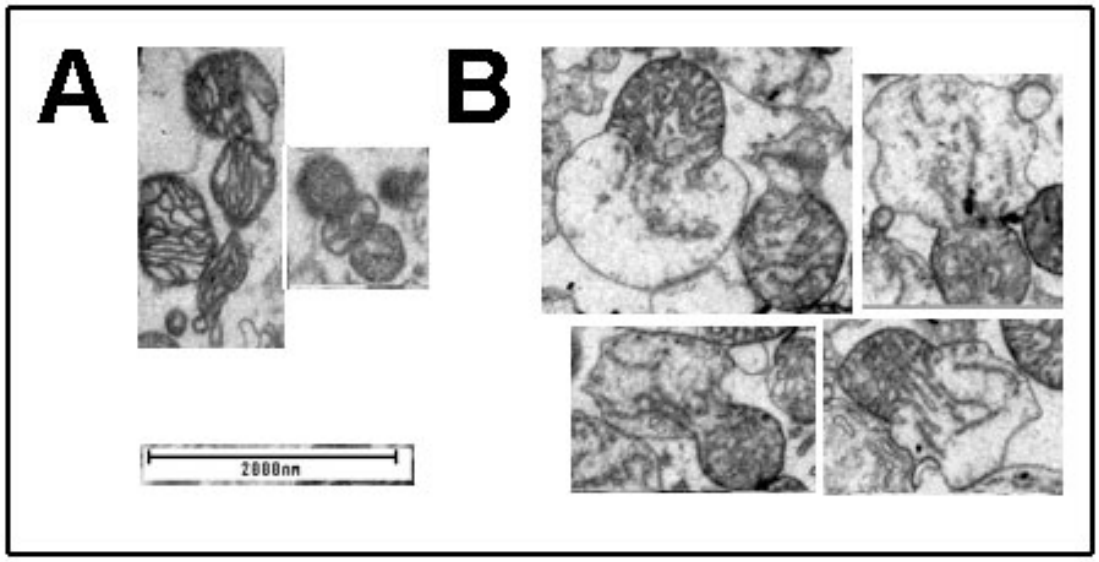
Supplemental Figure II. Pim WT hearts exhibit higher levels of Bcl-2 in both mitochondrial and cytosolic fractions in response to ex vivo I/R injury. Mitochondrial and cytosolic fractions from Pim WT and NTG whole hearts challenged by ischemia/reperfusion (I/R) and probed for Bcl-2 levels by immunoblot analyses. Quantitative graphs represent Bcl-2 fold change from NTG (normalized to loading control, mitochondrial fraction = VDAC and cytosolic fraction = GAPDH). Results are represented as mean ± SEM, N = 3. Student's T test: *P<0.05 for Pim WT vs. NTG in the mitochondrial and cytosolic fraction.



Supplemental Figure III. Mechanistic Role of Bcl proteins in staurosporine induced apoptosis in cardiomyocytes. (A) NRCM (Non-infected = NT, NRCM over expressing Pim-1 = Pim WT, and NRCM over expressing GFP = GFP) transfected with siRNA (control siRNA = Scr, Bcl-X_L siRNA = Bcl-X_L KD, Bcl-2 siRNA = Bcl-2 KD, or siRNA for both BCL family proteins = Bcl-2 + Bcl-X_L KD) and treated with 0.5 µmol/L staurosporine. Quantitative graph represent n-fold change of cell apoptosis compared to NT-Scr control. Cell apoptosis was quantitated using Cell Death Detection ELISA (Roche). Results are represented as mean ± SEM, N = 2 independent experiments. Student's T test: \$p<0.05 for GFP-Bcl-X_L KD, GFP-Bcl-2 KD, and GFP-Bcl-2 + Bcl-XL KD vs. NT-Scr, *p<0.05 for Pim WT-Bcl-X_L KD vs. GFP-Bcl-X_L KD, Pim WT-Bcl-2 KD vs. GFP-Bcl-2 KD, and Pim WT Bcl-2 + Bcl-X_L KD vs. GFP Bcl-2 + Bcl-X_L KD. (B) Representative western blot confirmed transfection of NRCM with siRNA (Scr, Bcl-X_L KD, Bcl-2 KD, or Bcl-2 + Bcl-X_L KD) resulted in significant reduction of targeted protein expression.



Supplemental Figure IV. Mitochondria membrane depolarization occurs when cardiomyocytes are treated with an uncoupler. TMRE fluorescence of non-infected NRCM (NI) treated with 100 nmol/L CCCP. Results are represented as mean ± SEM. N = 5 independent experiments.



Supplemental Figure V. Electron micrographs of distressed mitochondria from Pim DN hearts. (A) Untreated Pim DN (B) Pim DN treated with calcium. Magnification at 2700X with scale bar set at 2000 nm.