Pim-1 Kinase Protects Mitochondrial Integrity in Cardiomyocytes

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Rationale: Cardioprotective signaling mediates antiapoptotic actions through multiple mechanisms including maintenance of mitochondrial integrity. Pim-1 kinase is an essential downstream effector of AKT-mediated cardioprotection but the mechanistic basis for maintenance of mitochondrial integrity by Pim-1 remains unexplored. This study details antiapoptotic actions responsible for enhanced cell survival in cardiomyocytes with elevated Pim-1 activity.

Objective: The purpose of this study is to demonstrate that the cardioprotective kinase Pim-1 acts to inhibit cell death by preserving mitochondrial integrity in cardiomyocytes.

Methods and Results: A combination of biochemical, molecular, and microscopic analyses demonstrate beneficial effects of Pim-1 on mitochondrial integrity. Pim-1 protein level increases in the mitochondrial fraction with a corresponding decrease in the cytosolic fraction of myocardial lysates from hearts subjected to 30 minutes of ischemia followed by 30 minutes of reperfusion. Cardiac-specific overexpression of Pim-1 results in higher levels of antiapoptotic Bcl-X_L and Bcl-2 compared to samples from normal hearts. In response to oxidative stress challenge, Pim-1 preserves the inner mitochondrial membrane potential. Ultrastructure of the mitochondria is maintained by Pim-1 activity, which prevents swelling induced by calcium overload. Finally, mitochondria isolated from hearts created with cardiac-specific overexpression of Pim-1 show inhibition of cytochrome *c* release triggered by a truncated form of proapoptotic Bid.

Conclusion: Cardioprotective action of Pim-1 kinase includes preservation of mitochondrial integrity during cardiomyopathic challenge conditions, thereby raising the potential for Pim-1 kinase activation as a therapeutic interventional approach to inhibit cell death by antagonizing proapoptotic Bcl-2 family members that regulate the intrinsic apoptotic pathway. (*Circ Res*. 2010;106:1265-1274.)

Key Words: Pim-1 ■ mitochondria ■ cardiomyocyte ■ apoptosis

Cardiovascular disease is the leading cause of death among men and women and affects approximately 33% of the US population.[1] A direct correlation between the decline in heart function and loss of cardiomyocytes via apoptosis involving the mitochondria occurs in cardiomyopathy, myocardial ischemia/reperfusion (I/R), and congestive heart failure.[2–9] Specifically, myocardial I/R injury generates calcium overload and oxidative stress, which initiate the intrinsic apoptotic pathway through activation of the mitochondrial permeability transition pore (mPTP). The ensuing chain of events result in dramatic changes to mitochondrial morphology associated with uncoupling of the electron transport chain, depolarization of the inner membrane, matrix swelling, unfolding of the cristae, and ultimately outer membrane rupture, with release of proapoptotic cytochrome *c*.[10–15] Release of cytochrome *c* into the cytosol consequently activates apoptotic protease-activating factor, which mediates caspase cascade programmed cell death.[16] Thus, preservation of mitochondrial integrity is essential in designing molecular strategies to enhance cardiomyocyte cell survival by blunting injury attributed to cardiomyopathic insult.

Cardioprotection mediated by survival kinase signal transduction acts through multiple mechanisms including preservation of mitochondrial integrity.[17] Numerous studies have documented antiapoptotic actions of the serine/threonine kinase AKT, which acts in part through protecting mitochon-

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Non-standard Abbreviations and Acronyms	
ΔΨm	mitochondrial inner membrane potential
CCCP	carbonyl cyanide 3-chlorophenylhydrazone
CsA	cyclosporin A
D	maximum shift of mitochondrial diameter distribution
GFP	green fluorescent protein
I/R	ischemia/reperfusion
MOMP	mitochondrial outer membrane permeabilization
mPTP	mitochondrial permeability transition pore
NRCM	neonatal rat cardiomyocyte
NRCM-NI	noninfected neonatal rat cardiomyocyte
NTG	nontransgenic wild type
Pim DN	expressing K79M dominant negative mutant of Pim-1
Pim WT	expressing 34-kDa Pim-1 transgene
SERCA2a	sarco-/endoplasmic reticulum Ca^{+2} ATP-ase 2a
siRNA	small interfering RNA
tBid	activated truncated Bid
TMRE	tetramethylrhodamine ethyl ester
VDAC	voltage-activated anion channel
WT	wild type

drial structure and function.[17] However, the cardioprotective action of AKT signaling depends, at least in part, on downstream induction of Pim-1 kinase.[18] Pim-1 overexpression in cardiomyocytes results in enhanced cell survival, whereas loss of Pim-1 results in increased apoptotic cell death.[18–20] Pim-1 antagonizes mitochondrial outer membrane permeabilization (MOMP) associated with release of several proapoptotic factors, including cytochrome *c*. Pim-1 suppresses MOMP by impacting on BCL-2 family members through a combination of inhibiting proapoptotic proteins as well as activating antiapoptotic proteins. Specifically, cardiomyocytes overexpressing Pim-1 exhibit increased levels of antiapoptotic members Bcl-2 and Bcl-X_L in conjunction with elevated phosphorylation and inactivation of proapoptotic Bad.[18] These data suggest that cardioprotective effects of Pim-1 are linked to mitochondrial preservation, but fail to directly assess the impact of Pim-1 activity on mitochondrial structure and function. Therefore, the role of Pim-1 in protection of mitochondrial integrity in cardiomyocytes was examined by multiple approaches. Taken together, our findings indicate that Pim-1 translocates to the mitochondria in response to I/R injury, enhances mitochondrial resistance to inner membrane depolarization, attenuates mitochondrial swelling, and inhibits cytochrome *c* release. These findings have important implications for AKT-mediated cardioprotective signaling as well as molecular therapeutic interventional strategies to blunt cell death via preservation of mitochondrial integrity.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Generation of Transgenic Animals

Transgenic mice were generated as previously described.[21] Transgenics generated in the FVB/NJ strain (nontransgenic [NTG]) express wild-type 34-kDa human Pim-1 transgene (Pim WT) fused downstream of enhanced green fluorescent protein (GFP). Similarly, the K79M kinase dead mutant (Pim DN) was fused downstream of enhanced GFP. Transgene expression is regulated by the α myosin heavy chain promoter (a kind gift from Dr Jeffrey Robbins, Cincinnati Children's Hospital Medical Center, Ohio) for cardiomyocyte specificity. All experimental procedures were approved by San Diego State University and the Institutional Animal Care and Use Committee.

Ex Vivo I/R Treatment

I/R ex vivo I/R treatment of mouse hearts was performed as previously described,[22] with additional details provided in the Online Data Supplement.

Mitochondrial Isolation

NTG, Pim WT, and Pim DN mice were anesthetized with 120 mg/kg ketamine and 5 mg/kg xylazine followed by excision of the heart. Isolated hearts were washed in sterile PBS (Mg- and Ca^{2+}-free), minced in 2 mL of homogenization buffer (250 mmol/L sucrose, 10 mmol/L MOPS-pH 7.4, 1 mmol/L EGTA, 2mmol/L $MgCl_2$, 0.1% BSA-fatty acid free) and briefly homogenized using a glass-Teflon dounce. The homogenized mixture was centrifuged at 600*g* for 5 minutes, and the supernatant was decanted and centrifuged at 3000*g* for 10 minutes. The mitochondrial pellet was resuspended in 100 μL homogenization buffer. All procedures were performed on ice. Total mitochondria protein was quantified using a Bradford assay.

Neonatal Rat Cardiomyocyte Culture/Infections

Isolation of neonatal rat cardiomyocytes (NRCMs) were performed as previously described,[23–25] with details provided in the Online Data Supplement.

Mitochondrial Swelling Assay

Sixty μg of isolated mouse heart mitochondria from NTG, Pim WT, and Pim DN (n=at least 5) in swelling buffer (250 mmol/L sucrose, 10 mmol/L MOPS, 5 μmol/L EGTA, 2 mmol/L $MgCl_2$, 5 mmol/L KH_2PO_4, 5 mmol/L pyruvate, 5 mmol/L malate) were incubated with 150 μmol/L of calcium chloride ($CaCl_2$) or 1 μmol/L cyclosporin A (CsA) in a final volume of 200 μL in a 96-well plate for 20 minutes. Absorbance was read every 30 seconds at 520 nm.

Transmission Electron Microscopy

The protocol was adapted from a previous fixative protocol for isolated mitochondria,[26] with additional details in the Online Data Supplement.

Cytochrome *c* Assay

Isolated mouse heart mitochondria from NTG, Pim WT, and Pim DN (60 μg, (n=5) in swelling buffer were incubated with 50 μL activated truncated Bid (tBid) protein at a final volume of 200 μL on a 96-well plate. The plate was immediately spun down at 3000*g* for 20 minutes to separate treated mitochondria mixture into supernatant and mitochondria pellet fractions. Sample buffer (1mol/L Tris-HCl, pH 6.8, 50% glycerol, 10% SDS, 0.288 mol/L β-mercaptoethanol, 1% bromophenol blue) was added to the fractions and the samples were boiled for 10 minutes to denature.

Western Blot Analysis

Immunoblotting was performed as described previously,[27] with additional details in the Online Data Supplement.

Statistical Analysis

Statistical analysis was performed using Student's *t* test and ANOVA (1-way and 2-way) for comparison where indicated. Komogorov–Smirnov test was performed to compare the distribution of mitochondrial diameter size obtained from transmission electron microscopy, reported as the maximum difference between

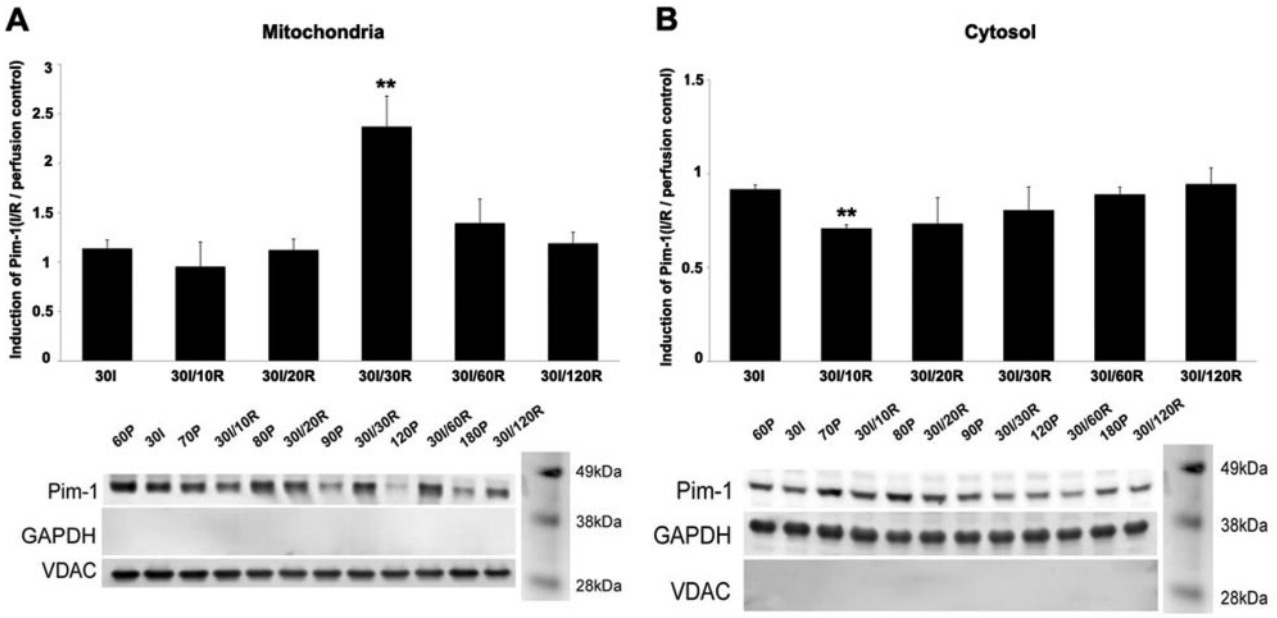

Figure 1. Induction of Pim-1 in response to ex vivo I/R injury. Mitochondrial **(A)** and cytosolic **(B)** fractions from NTG whole hearts challenged by ischemia (I), I/R, or perfusion (P) as control probed for Pim-1 levels by immunoblot analyses. Corresponding quantitative graphs represent Pim-1 induction (fold change) determined by comparing Pim-1 levels (normalized to loading control, mitochondrial fraction [voltage-activated anion channel, VDAC] and cytosolic fraction [GAPDH]) to corresponding perfusion control. Results represented as means±SEM; N=3. Student's *t* test: $^{**}P<0.02$ for 30I vs 30I/30R in the mitochondrial fraction and 30I vs 30I/10R in the cytosolic fraction.

the cumulative distribution (D). Probability values of <0.05 were considered statistically significant.

Results

Pim-1 Translocates to Mitochondria in Response to I/R Injury

Endogenous Pim-1 (44 kDa) level in mitochondrial and cytosolic fractions of NTG heart lysates was assessed under conditions of ischemia or I/R with perfusion samples served as the control (Figure 1). The mitochondrial fraction exhibited a significant 2.3-fold increase of Pim-1 expression at 30I/30R (Figure 1A). A corresponding decrease of Pim-1 levels was also observed in the cytosolic fraction at 30I/10R up to 30I/120R compared with 30I. Specifically at 30I/10R in the cytosol, Pim-1 levels significantly decreased by 25% (Figure 1B).

Pim-1 Enhances Expression of Antiapoptotic Bcl-X_L and Bcl-2

BCL-2 family members Bcl-X_L and Bcl-2 were assayed for protein expression level in mitochondrial and cytosolic fractions of lysates prepared from either NTG or Pim WT hearts (Figure 2). Bcl-X_L show a significant 2-fold increase in the mitochondrial fraction prepared from Pim WT compared to NTG samples, although levels of Bcl-X_L were comparable in the cytosol of both NTG and Pim WT samples (Figure 2A). In comparison, Bcl-2 levels show a significant 2-fold increase in the cytosolic fraction of Pim WT relative to NTG samples, whereas Bcl-2 levels remain similar in the mitochondria (Figure 2B). To address the adverse effects of Pim-DN, mitochondrial and cytosolic fractionations were compared between NTG and Pim-DN for Bcl-2 and Bcl-X_L expression. As shown in Online Figure I, Bcl-2 levels show a significant 0.5-fold decrease in the cytosolic fraction of Pim DN relative to NTG samples. In addition, Bcl-X_L and Bcl-2 show a 1.4-fold increase in both mitochondrial and cytosolic fraction of Pim WT compared to NTG samples in response to I/R injury (Online Figure II). To further investigate the mechanistic role of the BCL-2 family members in mediating the protective effects of Pim-1 in cardiomyocytes, we used small interfering RNA to knock down the expression of Bcl-X_L, Bcl-2 or both proteins. Knockdown of Bcl-X_L and Bcl-2 led to a significant increase of apoptotic cells in GFP overexpressing cells after initiation of apoptosis with 0.5 mmol/L staurosporine. Interestingly, Pim-1 overexpressing cardiomyocytes were still protected from apoptosis after knockdown of Bcl-2, Bcl-X_L, or both proteins (Online Figure III). Collectively, these findings indicate that Pim-1 elevates expression of antiapoptotic BCL-2 family members in both mitochondrial and cytoplasmic cellular compartments, although a significant inhibition of BCL-2 family members is still possible without diminution of the antiapoptotic action of Pim-1.

Mitochondria Show Inhibition of Inner Membrane Depolarization by Pim-1

Noninfected cardiomyocytes (NRCM-NI) and cardiomyocytes infected with adenovirus expressing either GFP or GFP-tagged wild-type Pim-1 (Pim WT) were labeled with tetramethylrhodamine ethyl ester (TMRE) and challenged by oxidative stress (100 μmol/L H_2O_2) to assess preservation of mitochondrial inner membrane potential ($\Delta\Psi_m$). Maintenance of $\Delta\Psi_m$ in cardiomyocytes from Pim WT, GFP, and the NRCM-NI groups under normal conditions is evident by TMRE fluorescence intensity above 80% throughout the

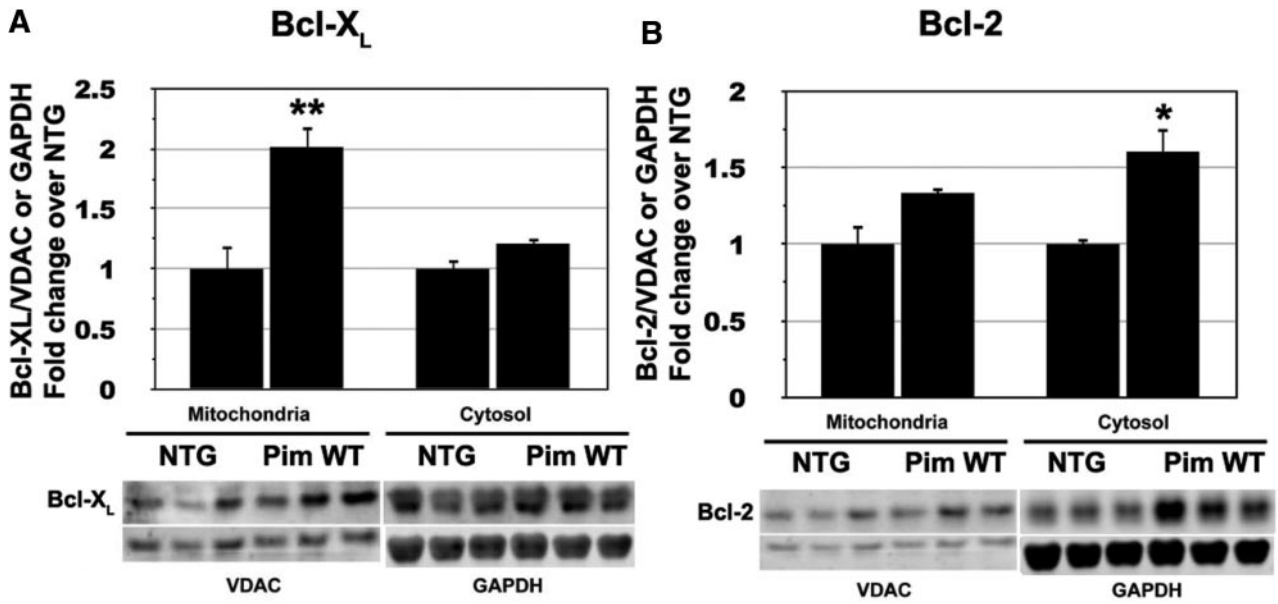

Figure 2. Antiapoptotic Bcl-2 family members are upregulated in Pim WT hearts. Bcl-X_L **(A)** and Bcl-2 **(B)** from mitochondrial and cytosolic fractions of NTG mouse hearts probed by immunoblot analyses to determine protein expression levels. Quantitative graphs represent relative Bcl-X_L and Bcl-2 levels normalized to VDAC (mitochondrial fraction) or GAPDH (cytosolic fraction). Results are represented as means±SEM; n=at least 3 hearts. Student's *t* test: $^{**}P<0.02$ for NTG vs Pim WT Bcl-X_L levels in the mitochondria fraction; $^{*}P<0.05$ for NTG vs Pim WT Bcl-2 levels in the cytosolic fraction.

10-minute time course (NRCM-NI=90.3%, GFP=80.8%, Pim WT=89.0%; Figure 3A through 3C). NRCM and GFP groups challenged with H_2O_2 show dramatic decreases in fluorescence within 2 minutes leading to significantly lower fluorescence intensities at all time points examined indicative of mitochondrial inner membrane depolarization (Figure 3A and 3B; Figure 3D and 3E). Strikingly, TMRE fluorescence intensity in Pim WT exposed to H_2O_2 is also maintained above 80% for up to 8 minutes, after which 75.2% of fluorescence intensity remained at 10 minutes of exposure (Figure 3C). A control experiment using NRCM treated with CCCP (carbonyl cyanide 3-chlorophenylhydrazone), an un-

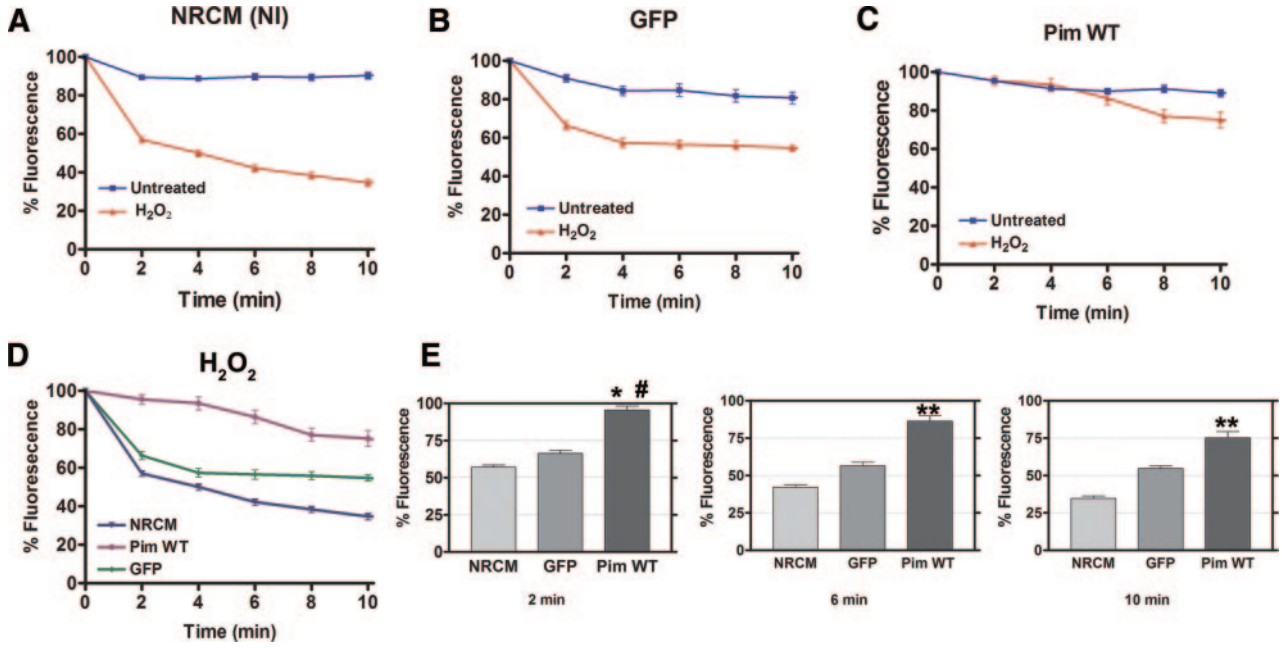

Figure 3. Mitochondrial inner membrane depolarization in cardiomyocytes induced by H_2O_2 is delayed by Pim-1 expression. TMRE fluorescence of NRCMs under control conditions (untreated) vs oxidative stress challenge (H_2O_2; 100 μmol/L). NRCM (NI) indicates noninfected NRCM **(A)**; GFP, NRCM infected with GFP adenovirus **(B)**; Pim WT, NRCM infected with Pim-1 adenovirus **(C)**; and combined plot comparing results of **A**, **B**, and **C** is shown in **D**. Quantitative graphs comparing H_2O_2-treated NRCM, GFP, and Pim WT at 2, 6, and 10 minutes **(E)**. Results are represented as means±SEM; N=at least 3 independent experiments. One-way ANOVA: $^{*}P<0.001$ for NRCM (NI) vs Pim WT at 2 minutes; #$P<0.001$ for Pim WT vs GFP at 2 minutes; $^{**}P<0.001$ for NRCM (NI) vs GFP vs Pim WT at 6 and 10 minutes.

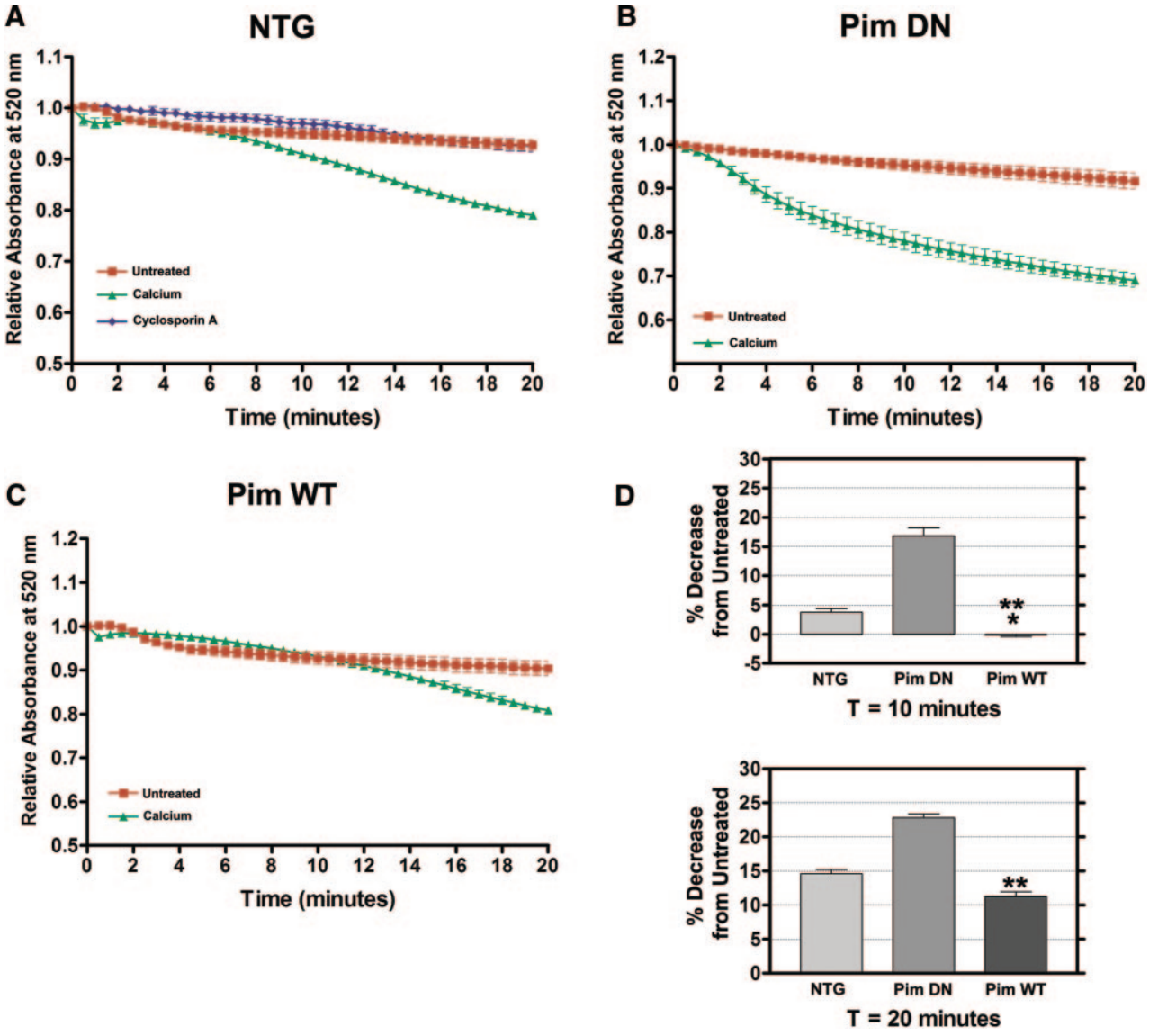

Figure 4. Pim-1 overexpression attenuates calcium-induced mitochondrial swelling. Rate of mitochondrial swelling of untreated, calcium-treated (150 μmol/L $CaCl_2$) and CsA-treated (1 μmol/L CsA) isolated NTG mitochondria **(A)**. Rate of mitochondrial swelling of untreated and calcium-treated mitochondria from Pim DN **(B)** or Pim WT **(C)**. Quantitative graphs comparing calcium-treated NTG, Pim DN, and the percentage decrease of 520-nm absorbance reading of Pim WT at 10 minutes and 20 minutes **(D)**. Results represented as means±SEM; N=at least 5. One-way ANOVA: *$P<0.01$ for NTG vs Pim WT at 10 minutes; **$P<0.001$ for NTG vs Pim DN and Pim DN vs Pim WT at 10 minutes and NTG vs Pim DN vs Pim WT at 20 minutes.

coupler (100 nmol/L), evokes a response similar to H_2O_2-induced oxidative stress (65.4% TMRE florescence intensity within 4 minutes), confirming the decrease of TMRE fluorescence can be attributed to mitochondrial inner membrane depolarization (Online Figure IV).

Mitochondrial Swelling Is Attenuated by Pim-1

Isolated mitochondria from Pim WT, Pim DN, and NTG hearts were challenged with calcium overload stimulus (150 μmol/L $CaCl_2$) and assessed for rate of mitochondrial swelling determined by light scattering. Samples of untreated Pim WT, Pim DN, and NTG mitochondria show stable absorbance at 520 nm throughout the 20-minute time course (Figure 4A through 4C). However, Pim DN and NTG mitochondria challenged with calcium overload show decreased absorbance at 8 minutes and 2 minutes, respectively, indicative of swelling (Figure 4A and 4B). At 8 minutes, calcium-treated Pim DN mitochondria showed a dramatic 14.3% decrease in absorbance compared to untreated Pim DN mitochondria, whereas NTG had a 1.5% decrease compared to untreated NTG mitochondria. In contrast, Pim WT mitochondria maintain relatively stable absorbance indicative of resistance to calcium-induced swelling reading for up to 12 minutes after which absorbance starts to decrease (Figure 4C). The greatest difference in swelling is observed between the calcium-treated isolated mitochondrial samples (NTG, Pim WT, and Pim DN) at 14 minutes. Calcium-treated Pim WT mitochondria exhibited a 4.1% decrease of absorbance compared to untreated Pim WT mitochondria, whereas calcium-treated NTG mitochondria had an 8.7% decrease

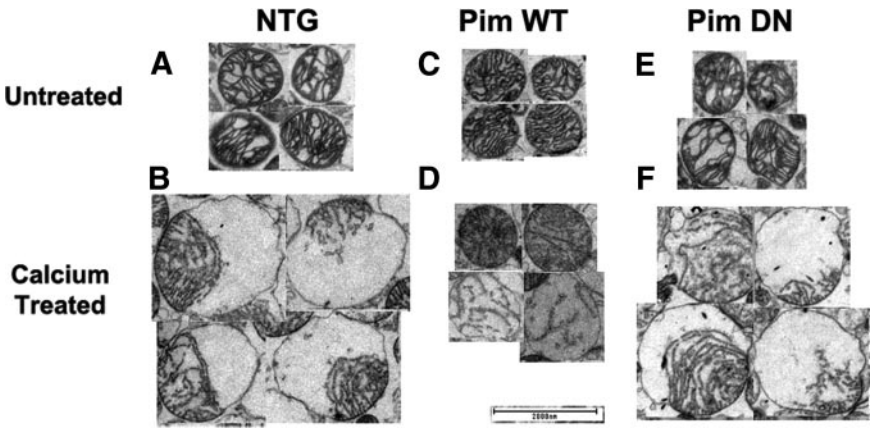

Figure 5. Representative electron micrographs of mitochondria under normal or calcium overload conditions. Untreated mitochondrial preparations from NTG **(A)**, Pim WT **(C)**, or Pim DN **(E)** compared to calcium challenged NTG **(B)**, Pim WT **(D)**, or Pim DN **(F)**. Magnification at ×2700; **scale bar:** 2000 nm.

compared to untreated NTG mitochondria and calcium-treated Pim DN mitochondria with 19.9% compared to untreated Pim DN mitochondria. By the end of the 20-minute time course, the Pim WT mitochondria exposed to calcium exhibit only an 11.3% decrease of absorbance relative to untreated Pim WT mitochondria (Figure 4D), whereas calcium-treated NTG mitochondria had a 14.7% decrease compared to untreated NTG mitochondria and calcium-treated Pim DN mitochondria had the highest rate of calcium-induced mitochondrial swelling with a 22.1% decrease (Figure 4D). As a control, NTG mitochondria treated with the mPTP inhibitor CsA (1 μmol/L) prevented mitochondrial swelling and maintained a stable absorbance reading (Figure 4A).

Mitochondrial ultrastructure was directly visualized to confirm the effects of calcium-induced swelling in the NTG, Pim WT, and Pim DN samples. Untreated mitochondrial samples isolated from Pim WT, Pim DN, or NTG hearts show intact cristae uniformly distributed across the organelle (Figure 5A, 5C, and 5E). However, calcium challenged NTG and Pim DN mitochondria show evidence of matrix swelling represented by unfolded cristae localized at one pole of the organelle (Figure 5B and 5F). Interestingly, examples of distressed mitochondria could be observed in Pim DN preparations that include disfigured mitochondria in untreated samples, as well as massive swelling with inner membrane ruptured through the outer membrane in calcium-treated Pim DN mitochondria (Online Figure V). In comparison, protective effects were evident in samples from Pim WT mitochondria that resist calcium-induced matrix swelling and contain either intact or only partially folded cristae following challenge (Figure 5D).

Morphometric determinations were assessed by measuring the diameter (nm) of each mitochondria from transmission electron microscopy micrographs and comparing the maximum shift of diameter distribution (D) between untreated and calcium-treated samples using Komogorov–Smirnov test (Figure 6). Mitochondria from untreated NTG samples possess a median diameter of 825.0 nm, but exposure to calcium resulted in a 34.5% increase in diameter (D=0.2983) indicative of matrix swelling (Figure 6A and 6D). The median diameter of Pim DN mitochondria is 737.6 nm with a 33.2% increase in diameter size (D=0.2904) (Figure 6C and 6D). Although Pim WT mitochondria normally show a median diameter of 824.0 nm, comparable to NTG samples, the calcium-treated Pim WT mitochondria show inhibition of swelling relative to NTG samples with a 23.9% average increase in diameter (D=0.1999) (Figure 6B and 6D). Although the diameter size between calcium-treated Pim WT and calcium-treated Pim DN appear to be relatively similar (calcium-treated Pim WT=1083.99±17.29, calcium-treated Pim DN=1045.38±13.86 nm), untreated Pim DN have a significantly smaller initial size in diameter compared to untreated Pim WT (untreated Pim WT=875.27±12.60, untreated Pim DN=785.10±9.05) (Figure 6D). This resulted in calcium-treated Pim DN with a larger percentage increase of 33.2% in diameter size compared to Pim WT with 23.9% (Figure 6D), consistent with our data that showed calcium-treated Pim DN with a significantly greater rate of swelling compared to calcium-treated NTG and calcium-treated PIM WT (Figure 4D).

Cytochrome *c* Release From Mitochondria Is Prevented by Pim-1

Mitochondrial preparations from NTG or Pim WT hearts were challenged with tBid to induce cytochrome *c* release from mitoplasm into the cytosolic fraction indicating loss of membrane integrity and proapoptotic signaling (Figure 7). Mitochondrial preparations from Pim WT possess 99.4% of the total cytochrome *c* and show modest release after tBid challenge (91.9% versus 8.1% in the mitochondrial pellet versus supernatant, respectively). In comparison, whereas untreated NTG mitochondria retain 89.0% of total cytochrome *c* in the assay, the tBid challenge prompts release into the supernatant and decreases the percentage retained in the mitochondrial pellet to 58.3%, with a corresponding increase to 41.6% in the supernatant fraction (Figure 7C). Collectively, these results demonstrate a higher degree of resistance to tBid induced release in mitochondria isolated from Pim WT hearts.

Discussion

Mitochondria are key regulators in the intrinsic apoptotic pathway and recent studies also view mitochondria as a target for cardioprotection.[2–9] In response to a variety of stress signals to the heart, mitochondria undergo dramatic changes in morphology that ultimately result in the release of several proapoptotic factors including cytochrome *c* to trigger acti-

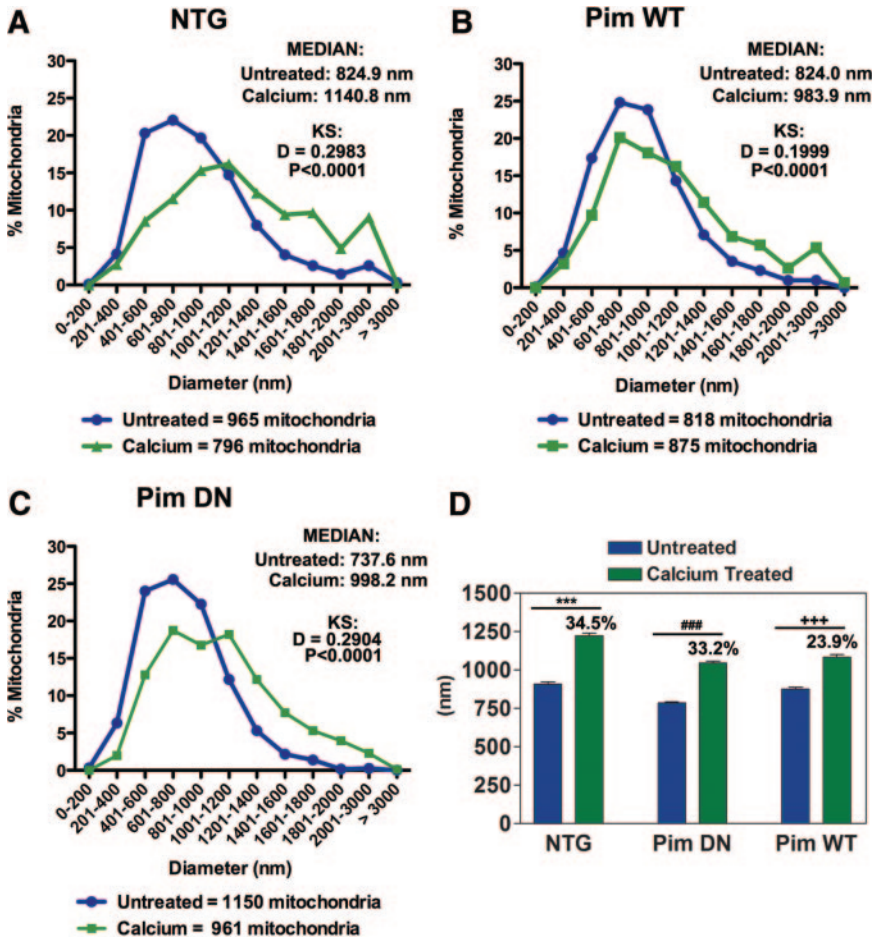

Figure 6. Morphometric comparison of mitochondrial diameter resulting from exposure to calcium overload. Quantitative graphs displaying the percentage of total untreated and calcium-treated mitochondria with corresponding diameter in nm **(A through C)**. The median of each sample is listed in the **upper right corner** of each **graph** with reported diameter shift (D) and significance (P) value using Komogorov–Smirnov test for NTG **(A)**, Pim WT **(B)**, and Pim DN **(C)** mitochondrial preparations from 2 separate hearts. Average diameter length represented as means±SEM with corresponding percentage increase when treated with calcium **(D)**. One-way ANOVA: ***$P<0.001$ for untreated NTG vs calcium-treated NTG; ###$P<0.001$ for untreated Pim DN vs calcium-treated Pim DN; +++$P<0.001$ for untreated Pim WT vs calcium-treated Pim WT.

vation of caspase cascade programmed cell death.[9] Dynamic regulation of this intrinsic apoptotic pathway depends on Bcl-2 family proteins.[28] When activated, proapoptotic members Bax and Bak translocate from the cytosol to the outer mitochondrial membrane to mediate cytochrome *c* release from the mitochondria through MOMP.[9] Bad and Bid are proapoptotic BH3-only proteins that also localize to the outer mitochondrial membrane on activation, assisting MOMP activation by antagonizing antiapoptotic effects of Bcl-2 and Bcl-X_L.[9,29] Bcl-2 and Bcl-X_L sequester proapoptotic tBid and Bad at the mitochondria, resulting in the prevention of Bax and Bak translocation and activation.[30] Bak can also be directly sequestered by Bcl-X_L at the mitochondria.[31]

Pim-1 is part of a family of survival kinases that function downstream of JAK/STAT and AKT signaling.[17–18,32] Pim-1 enhances cell survival by targeting proapoptotic Bcl-2 family members and acting as an upstream regulator of Bcl-2 and Bcl-X_L expression.[33–35] Bcl-X_L and Bcl-2 expression levels elevated in mitochondrial versus cytosolic fractions (respectively) of Pim WT hearts as shown in Figure 2 are consistent with initial findings using cultured cardiomyocytes with elevated Pim-1 activity.[18] Although the protection of the mitochondria by Pim-1 is mediated by the upregulation of BCL-2 family proteins that are primarily located in the cytosol, an upregulation of Bcl-X_L was observed in the mitochondrial fraction (Figure 2A). It has been previously reported that the 33-kDa version of Pim-1 upregulated Bcl-2 levels, whereas the 44-kDa version was shown to antagonize the proapoptotic effects in cells overexpressing Bax.[36] The additional presence of Pim-1 expression in the mitochondria could therefore supplement the inhibition of the apoptotic intrinsic pathway by targeting Bcl-X_L in the mitochondria and further preventing Bax/Bak activation of MOMP. Previous studies have also reported that Bcl-2 can directly interact with Bax in the cytosol and prevent Bax translocation to the mitochondria.[37] The upregulation of cytosolic Bcl-2 observed in Pim WT hearts (Figure 2B; Online Figure II) could play a role of sequestering Bax in response to stress and I/R injury. Pim-1 cardioprotection also may involve inhibition of proapoptotic Bad via increased phosphorylation[18] as previously reported to occur at both residues Ser112[34] and Ser136.[35] Phosphorylation at the regulatory site Ser112 prevents Bad from binding and inhibiting Bcl-X_L and Bcl-2,[34,35] whereas phosphorylation at Ser136 signals Bad to be sequestered by 14-3-3 and prevent activation of apoptosis.[38,39] Because Pim-1 expression inhibits tBid induced cytochrome *c* release (Figure 7), blunting of MOMP activation is a plausible mechanism for the observed preservation of mitochondrial integrity by Pim-1 through combined inhibition of proapoptotic molecules and enhancing expression levels of antiapoptotic Bcl-2 family members.

Pim-1 mediates an antiapoptotic effect through Bcl-2 family members including increasing expression as well as phosphorylation of proapoptotic proteins, but knockdown

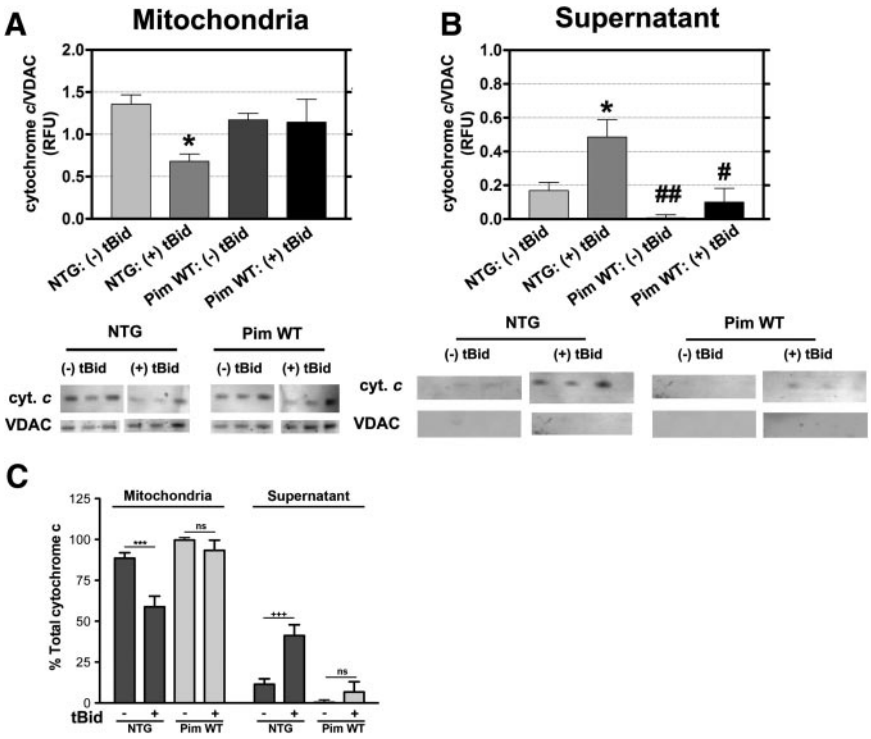

Figure 7. Pim-1 overexpression decreases cytochrome *c* release from mitochondria. Preparations of NTG vs Pim WT under normal conditions (-tBid) or challenged with tBid (+tBid) and subsequently separated into mitochondrial pellet **(A)** or supernatant **(B)** fractions for immunoblot analysis to quantitate cytochrome *c* levels. Corresponding quantitative graph is normalized to VDAC as a loading control. Results are represented as means±SEM; N=5 hearts. Two-way ANOVA: **P*<0.05 for untreated NTG vs tBid-treated NTG in mitochondrial and supernatant fraction; #*P*<0.01 for tBid-treated NTG vs tBid-treated Pim WT in supernatant fraction; ##*P*<0.001 for tBid-treated NTG vs untreated Pim WT in supernatant fraction. **C,** Corresponding percentage of total cytochrome *c* in mitochondrial and supernatant fractions of untreated and tBid-treated isolated NTG and Pim WT mitochondria. Two-way ANOVA: ****P*<0.001 for untreated NTG vs tBid-treated NTG in mitochondrial fraction; +++*P*<0.001 for untreated NTG vs tBid-treated in supernatant fraction; ns indicates not significant for untreated Pim WT vs tBid-treated Pim WT in mitochondrial and supernatant fraction.

experiments of Bcl-2 and Bcl-X_L suggest that Pim-1 may work through both Bcl-2 dependent and independent mechanisms that could not be deciphered in our experiments. It is possible that Bcl-2 and Bcl-X_L can still provide a substantial protective effect if levels are not completely eliminated by small interfering RNA treatment in our studies. Pim-1 phosphorylation of Bad in combination with residual Bcl-2 and Bcl-X_L in our system could supply enough protective effect to inhibit staurosporine-mediated apoptosis. Alternatively, Bcl-2 independent mechanisms may also contribute to Pim-1–mediated protection, such as phosphorylation of mitochondrial hexokinase II.[17] Alternate mechanisms of protection and cell integrity mediated by Pim-1 are a subject of ongoing studies.

In addition to MOMP, activation of mPTP during calcium overload-dependent necrotic cell death can also triggers release of cytochrome *c* from mitochondria.[40,41] I/R injury generates an increase in cytosolic calcium and oxidative stress in cardiomyocytes that subsequently triggers opening of the mPTP pore, inner membrane depolarization and mitochondrial swelling, and eventual rupture of the outer membrane and release of cytochrome *c*.[10–15,40] Pim-1 may play a direct role in preventing calcium overload during reperfusion by decreasing cell calcium loading. Overexpression of Pim-1 enhances calcium handling and reuptake in part by increased sarco-/endoplasmic reticulum Ca^{2+} ATP-ase 2a (SERCA2a) and sodium/calcium exchanger expression.[18] Although it is uncertain whether Pim-1 interferes with reactive oxygen species formation, previous studies have shown that the presence of oxidative stress induced Pim-1 protein and mRNA expression,[42] as well as increased phosphorylated Pim-1 protein levels.[43] In addition, recent findings have shown Pim-1's protective effects from oxidative stress-induced apoptosis by inhibiting apoptosis signaling kinase 1 and the caspase-3 cascade.[44]

Although mPTP activation and MOMP independently regulate the intrinsic apoptotic pathway, recent studies suggest a connection between the two during key events of inner membrane depolarization and mitochondrial swelling. Bcl-X_L and Bcl-2 not only promote cell survival by binding and inhibiting proapoptotic Bcl-2 family members,[31,45] but also prevent cytochrome *c* release by regulating the inner mitochondrial membrane potential.[46] Indeed, Pim-1 overexpression in cardiomyocytes protects $\Delta\Psi_m$ in the face of oxidative stress challenge (Figure 3). Thus, the enhancement of Bcl-X_L and Bcl-2 expression levels by Pim-1 may also participate in prevention of inner membrane depolarization in both MOMP and mPTP activation.

Preservation of morphology and size in Pim WT isolated mitochondria (Figures 4 through 6) reinforces the postulate that overexpression of Pim-1 protects mitochondrial structural integrity. Recent studies show that Bax also plays a role in regulating calcium concentrations at the endoplasmic reticulum and sarcoplasmic reticulum. Consequently, activation of Bax results in overabundance of calcium taken up by mitochondria and ultimately triggering activation of mPTP.[46] Bcl-X_L expression raised by Pim-1 also functions to inhibit the generation of calcium overload by Bax, thereby suppressing mitochondrial swelling and disruption of the outer mitochondrial membrane.

Loss of Pim-1 activity has also been found to produce deleterious consequences in the context of cardiac-specific Pim DN expression in transgenic mice.[47] The participation of mitochondria in the cardiomyopathic phenotype of Pim DN hearts is supported by observations in this report of increased rate of calcium induced swelling and disruption of structural

integrity in mitochondria when Pim DN was present (Figures 4 and 5; Online Figure V). Future studies may uncover additional links between the loss of inducible cardioprotection in Pim-1 knockout mice[18] and the destabilization of mitochondrial integrity in this report.

Another subject to be explored is Pim-1's relation to mitochondrial fusion and fission events. Recent studies point to regulation of mitochondrial morphology as a mechanism to mediate apoptosis where fission can occur during apoptosis resulting in formation of "small and round mitochondrial fragments."[48] Proapoptotic Bcl-2 family members are implicated in this process, because Bax colocalizes and activates dynamin-related protein-1, a fission protein located at scission sites at the foci of the outer mitochondrial membrane.[9,48] Although our findings provide initial insights regarding Pim-1's inhibition of Bax activity by enhancing Bcl-X_L expression levels and preventing tBid-induced cytochrome *c* release (Figures 1 and 7), ongoing studies are still needed to correlate Pim-1's cardioprotective role with mitochondrial fusion and fission.

Pim-1 offers many intriguing cardioprotective actions that may prove useful as a therapeutic agent for myocardial repair and protection from cardiac failure. For example, overexpression of Pim-1 blunts infarction injury in the myocardium, whereas inactivation of Pim-1 increases infarction injury and fibrosis.[17,18,21] In addition, hypertrophic remodeling is inhibited by Pim-1 leading to improved hemodynamic function[17,21] that may be attributable, in part, to enhanced calcium dynamics and cardiac contractility through increased expression of SERCA2a.[18,21] We now add preservation of mitochondrial structure and function to the mechanistic basis for Pim-1–mediated cardioprotection that promotes cardiomyocytes survival by inhibition of MOMP and mPTP activation. By targeting the mitochondria, Pim-1 can serve as a therapeutic intervention in the treatment of cardiomyopathy damage by blunting cell death through the intrinsic apoptotic pathway.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Pim-1 is a cardioprotective kinase that inhibits cell death and cardiomyocyte hypertrophy induced by pathologic injury without apparent maladaptive side effects.
- Myocardial regeneration is enhanced using cardiac stem cells genetically engineered to overexpress Pim-1.
- Protection of mitochondrial integrity is critical for cellular function and survival.

What New Information Does This Article Contribute?

- Pim-1 kinase preserves mitochondrial integrity, thereby enhancing cellular survival., Multiple types of pathologic challenge including oxidative stress, calcium overload, and pro-apoptotic cascades are similarly inhibited by Pim-1 activity.
- The protective effects of Pim-1 kinase are mediated by both mitochondrial-dependent and mitochondrial-independent mechanisms.

Identification of the Pim-1 cardioprotective kinase challenges us to examine long-standing observations regarding the anti-apoptotic effects of the Akt signaling cascade. Now that we appreciate the role of Pim-1 for enhancing survival downstream of Akt, understanding the mechanism of Pim-1-mediated cellular protection in the cardiac context is critical to assess therapeutic utility and potential clinical relevance to treat heart disease. In this report we demonstrate that Pim-1 activity enhances resistance to pathologic insults that compromise mitochondrial integrity, such as calcium overload, oxidative stress, and pro-apoptotic signaling. Enhanced mitochondrial integrity was observed in both intact cardiomyocytes and purified mitochondrial preparations. Furthermore, loss of Pim-1 activity correlated with enhanced susceptibility to pathologic challenge. The protective effect of Pim-1 appears mediated through a combination of mitochondrial dependent and independent actions. Based on these findings, preservation of mitochondrial integrity is an important mechanism for the cardioprotective actions of Pim-1. This salutary influence provides a basis for using Pim-1 as a molecular interventional strategy to protect myocardial structure and function and may explain enhanced regenerative and reparative capacity of hearts and stem cells engineered to express Pim-1 kinase.