Transgenic Expression of Bcl-2 Modulates Energy Metabolism, Prevents Cytosolic Acidification During Ischemia, and Reduces Ischemia/Reperfusion Injury

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Abstract—The antiapoptotic protein Bcl-2 is targeted to the mitochondria, but it is uncertain whether Bcl-2 affects only myocyte survival after ischemia, or whether it also affects metabolic functions of mitochondria during ischemia. Hearts from mice overexpressing human Bcl-2 and from their wild-type littermates (WT) were subjected to 24 minutes of global ischemia followed by reperfusion. During ischemia, the decrease in pH, and the initial rate of decline in ATP were significantly reduced in Bcl-2 hearts compared with WT hearts ($P<0.05$). The reduced acidification during ischemia was dependent on the activity of mitochondrial $F_0F_1$-ATPase. In the presence of oligomycin (Oligo), an $F_0F_1$-ATPase inhibitor, the decrease in pH, was attenuated in WT hearts, but in Bcl-2 hearts, Oligo had no additional effect on pH, during ischemia. Likewise, addition of Oligo to WT hearts slowed the rate of decline in ATP during ischemia to a level similar to that observed in Bcl-2 hearts, but addition of Oligo had no significant effect on the rate of decline in ATP in Bcl-2 hearts during ischemia. These data are consistent with Bcl-2–mediated inhibition of consumption of glycolytic ATP. Furthermore, mitochondria from Bcl-2 hearts have a reduced rate of consumption of ATP on uncoupler addition. This could be accomplished by limiting ATP entry into the mitochondria through the voltage-dependent anion channel, and/or the adenine nucleotide transporter, or by direct inhibition of the $F_0F_1$-ATPase. Immunoprecipitation showed greater interaction between Bcl-2 and voltage-dependent anion channel during ischemia. These data indicate that Bcl-2 modulation of metabolism contributes to cardioprotection. (Circ Res. 2004;95:734-741.)

Key Words: transgenic models ■ magnetic resonance spectroscopy ■ mitochondria ■ reperfusion injury

Bcl-2 is an antiapoptotic protein that was originally found to be overexpressed in B-cell lymphoma because of a chromosomal translocation. Recent studies suggest that Bcl-2 may have a more general role in regulating mitochondrial metabolism and function, and that its protective effect may not be limited to an antiapoptotic role. Bcl-2 is the founding member of a family of proteins involved in apoptosis, which includes antiapoptotic members such as Bcl-2 and Bcl-x,$^L,^S$ and proapoptotic members such as BAX, BAK, and Bid.$^L,^R,^S$ Emerging data suggest that proapoptotic family members such as BAX enter the outer mitochondrial membrane and form a large conductance channel that allows release of cytochrome c.$^L,^R$ The precise mechanism by which BAX mediates release of cytochrome c is not well understood, but has been suggested to involve channels formed by BAX or by BAX in association with other mitochondrial proteins such as the voltage-dependent anion channel (VDAC), or the mitochondrial permeability transition pore (MPTP), which is suggested to be composed of the adenine nucleotide transporter (ANT), VDAC, and cyclophilin.$^L,^R,^S$ However, recent data have questioned the role of ANT in the MPTP.$^L$ It has been suggested that antiapoptotic family members such as Bcl-2 oppose release of cytochrome c either by binding and sequestering proapoptotic members,$^R$ or by binding to proteins such as VDAC and blocking the formation or opening of a cytochrome c release pathway.$^L,^R,^S$ Bcl-2 is localized to the outer mitochondrial membrane and Bcl-2 family proteins have been reported to interact with VDAC.$^L,^R,^S$ There is controversy as to whether Bcl-2 binding to VDAC promotes the opening or closing of VDAC.$^R,^S$ Thompson and coworkers$^L,^R$ report that Bcl-2 maintains VDAC in an open state, whereas Shimizu et al$^L,^R,^S$ report that Bcl-2 promotes VDAC closure.

VDAC, the most abundant protein in the outer mitochondrial membrane, allows transport of adenine nucleotides, metabolites, and ions across the outer mitochondrial membrane. In vitro, VDAC has been shown to decrease conductance when either a positive or negative potential is applied.$^R$ VDAC in concert with ANT controls transport of adenine nucleotides in and out of the mitochondria, a function that is extremely important for a cardiomyocyte, which is dependent on mitochondrial oxidative phosphorylation and where mito-
imahashi et al Bcl-2 and F1F0-ATPase in Cardioprotection 735

Mitochondrial generation of ATP occurs via the F1F0-ATPase, which uses the proton gradient generated by electron transport to provide the driving force for converting ADP to ATP. Cell energetics depends on the appropriate transport of ADP into the mitochondria and the transport of ATP to the cytosol. Thus, Bcl-2-mediated alteration in adenine nucleotide transport could have important implications for cardiac energetics. During ischemia, when lack of oxygen inhibits mitochondrial electron transport and mitochondrial generation of ATP, the F1F0-ATPase can run in reverse and consume glycolytically generated ATP. In fact, many cells contain a natural inhibitor of the F1F0-ATPase, which binds the F1F0-ATPase during ischemia to inhibit breakdown of glycolytic ATP. There are also data showing that ischemia and anoxia lead to a preferential decrease in cytosolic versus mitochondrial ATP, suggesting that cells may regulate mitochondrial ATP exchange during ischemia.

The aim of this study was to obtain insights into the mechanism by which cardiac-specific overexpression of Bcl-2 suppresses cell death as well as potential mechanisms by which Bcl-2 might modulate cellular metabolism. We found that Bcl-2 overexpression, in addition to reducing ischemia/reperfusion injury, also reduced the rate of decline in ATP during ischemia and reduced ischemic acidification; these data are consistent with Bcl-2-mediated inhibition of consumption of glycolytically generated ATP. Consistent with this hypothesis, we further showed that the reduction in acidification and the rate of decline in ATP during ischemia was dependent on the activity of the mitochondrial F1F0-ATPase. Bcl-2-mediated inhibition of glycolytically generated ATP could be accomplished by limiting ATP entry into the mitochondria through VDAC or ANT, or by direct inhibition of the F1F0-ATPase. Consistent with this hypothesis, we find that Bcl-2 overexpression reduces the rate of mitochondrial ATP consumption under conditions in which ATP hydrolysis is stimulated.

Materials and Methods

Animals
All animals (Baylor College of Medicine, Houston, Tex) were treated in accordance with National Institutes of Health guidelines. Fifty-six mice of either sex were used. We originally analyzed males and females separately but because there were no significant differences between males and females, the data from both sexes were combined. Two experimental groups were studied: cardiac-specific expression of human Bcl-2-αMHC (Bcl-2) and age-matched wild-type littermates (WT).

Heart Perfusion and Protocols
Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (80 mg/kg body weight), and hearts were excised and perfused as described previously. Briefly, hearts were cannulated with pentobarbital (80 mg/kg body weight), and hearts were excised and perfused with Krebs-Henseleit buffer containing (in mmol/L) NaCl 120, KCl 5.9, MgSO4 1.2, CaCl2 1.75, NaHCO3 25, and glucose 11. Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (80 mg/kg body weight), and hearts were excised and perfused with Krebs-Henseleit buffer containing (in mmol/L) NaCl 120, KCl 5.9, MgSO4 1.2, CaCl2 1.75, NaHCO3 25, and glucose 11. The buffer was aerated with 95% O2 and 5% CO2, to give a pH of 7.4 at 37°C.

All hearts were perfused for a stabilization period of 20 to 30 minutes. Hearts then were subjected to a 24-minute period of ischemia at 37°C and 2 hours of reperfusion. Left ventricular developed pressure (LVPD), ±dP/dt, and heart rate were continuously monitored via a water-filled latex balloon inserted into the left ventricle. Recovery of contractile function was assessed by measurement of LVDP and rate-pressure product (RPP) during reperfusion and was expressed as a percentage of preischemic LVDP or RPP. Two baseline (preischemic) 31P-NMR spectra were obtained. During 24 minutes of ischemia, we acquired five NMR spectra. Spectra were obtained every 5 minutes during the 2 hours of reperfusion. In some hearts, oligomycin (~3 µg/mL, 0.3% DMSO) was administered 4 minutes before ischemia to estimate the role of F1F0-ATPase during ischemia. After the experiment, hearts were weighed, and used for TTC staining, TUNEL staining, or Western blot analysis.

Nuclear Magnetic Resonance Spectroscopy
Relative changes in the concentration of high-energy phosphate metabolites were measured during the ischemia/reperfusion protocol by acquiring consecutive 5-minute 31P-NMR spectra using a Varian 500 MHz spectrometer with an 11.7-Tesla superconducting magnet at the 31P resonance frequency of 202.47 MHz. Intracellular pH was estimated from the chemical shift between inorganic phosphate (P1) and phosphocreatine (PCr) as described previously. In some hearts, extracellular pH was determined by the shift difference between phenylphosphonic acid (PPA) and PCr. An expanded Materials and Methods section can be found in the online data supplement available at http://circres.ahajournals.org.

Measurement of Infarct Size and Apoptosis
At the end of 2 hours of reperfusion, the hearts were cut into thin cross-sectional slices and then were incubated in a 0.08% solution of 2,3,5-triphenyltetrazolium chloride (TTC) dissolved in Krebs-Henseleit buffer at 37°C for 30 minutes. The slices were then fixed in formalin. The area of necrosis was quantitated by measuring the stained area (live tissue) versus the unstained area (necrotic) using a digital camera mounted on an Olympus stereomicroscope. To measure apoptotic cell death, immunoperoxidase TUNEL staining was performed. The sections were counterstained with hematoxylin to identify cardiac myocytes and TUNEL-positive cardiac myocyte nuclei were counted in 10 randomly selected fields by a blinded observer.

Heart and Mitochondria Preparation for Western Blot Analysis
Whole cell samples, the mitochondria-enriched fraction, or cytosolic fraction were prepared by differential centrifugation from the frozen heart. Details can be found in the online data supplement. For determination of Bcl-2, VDAC, Bak, Bcl-xL, and apoptosis-inducing factor (AIF), 50 µg of homogenate protein was separated by electrophoresis. After transfer to a nitrocellulose membrane, the membrane was incubated with anti-Bcl-2 antibody (Transduction Laboratories), anti-VDAC antibody (Calbiochem), anti-Bak antibody (Upstate Biotechnology), anti-Bcl-xL antibody (Cell Signaling), or anti-AIF antibody (BD Pharmingen) in TBS-T with 5% BSA/milk at 4°C overnight. Membranes were incubated with the secondary antibody, appropriate horseradish peroxidase–conjugated IgG in TBS-T with 5% dry fat milk for 1 hour at room temperature. Immunoreactive protein was visualized using an enhanced chemiluminescence analysis kit (Amersham Pharmacia Biotech Inc).

Immunoprecipitation Study
The mitochondrial fraction (500 µg) was incubated with 1 µg of VDAC antibody or Bcl-2 (Santa Cruz) in a buffer containing (in mmol/L) NaCl 150, HEPES 20 (pH 7.4), EDTA 10, NaVO4 1, 0.1% Nonidet-P40, and protease inhibitors for 2 hours at 4°C. Protein G agarose (30 µL) was added and was incubated at 4°C overnight. The beads were washed four times and suspended with sample buffer, boiled, centrifuged, and the supernatants were subjected to immunoblotting [Bcl-2 mouse monoclonal (Transduction Laboratory) or VDAC mouse monoclonal (Calbiochem)].
Isolated Mitochondria Study

Freshly isolated mitochondria (25 mg/mL) were prepared from the ventricles of two hearts by differential centrifugation. Measurement of mitochondrial respiration was performed at 25°C in a chamber (600 μL) connected with a Clark-type O2 electrode (Instech) and O2 monitor (Model 5300, YSI, Inc). The mitochondria were incubated in a chamber containing respiration buffer (in mmol/L) KCl 120, MOPS 5, EGTA 1, KH2PO4 5, and 0.2% BSA. After addition of glutamate/malate (10 mmol/L), state 3 respiration was measured by addition of ATP (0.125 to 0.5 mmol/L). On depletion of ATP, state 4 respiration was determined by adding an uncoupler, 2,4-dinitrophenol (DNP, 50 μmol/L). The maximum ADP-stimulated respiration (Vmax) was estimated from a linear regression of double-reciprocal plots.

ATP uptake into mitochondria and hydrolysis was measured by adding ATP (875 μmol/L) to mitochondria followed by addition of DNP (50 μmol/L). After the indicated time, oligomycin (1 μmol/L) was added to block ATP hydrolysis and the supernatant was obtained by brief centrifugation. ATP remaining in the supernatant was measured. To confirm that ATP hydrolysis was dependent on ATP transport into the mitochondria, an ANT blocker, atractylloside (3676 μmol/L) connected with a Clark-type O2 electrode (Instech) and O2 monitor (Model 5300, YSI, Inc). The mitochondria were incubated in a chamber containing respiration buffer (in mmol/L) KCl 120, MOPS 5, EGTA 1, KH2PO4 5, and 0.2% BSA. After addition of glutamate/malate (10 mmol/L), state 3 respiration was measured by addition of ATP (0.125 to 0.5 mmol/L). On depletion of ATP, state 4 respiration was determined by adding an uncoupler, 2,4-dinitrophenol (DNP, 50 μmol/L). The maximum ADP-stimulated respiration (Vmax) was estimated from a linear regression of double-reciprocal plots.

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Statistical Analysis

Results are expressed as mean±SEM. For comparison between 2 groups, significance was determined by Student t test. For comparison among 4 groups, ANOVA and a Fisher post hoc test analysis was used. A value of P<0.05 was considered significant.

Results

Basal Contractile Function and Bcl-2 Expression

The Table shows that there were no differences in the baseline hemodynamic parameters between Bcl-2 and WT hearts. Table 1 also shows that the heart/body weight ratio was not significantly different between Bcl-2 and WT mice (14.0±0.1×10^-3 in Bcl-2 versus 14.0±0.1×10^-3 in WT; P>0.05). Western blot analysis confirmed that the expression of Bcl-2 protein was higher in Bcl-2 hearts than in WT hearts (Figure 1A).

Bcl-2 Overexpression Reduces Infarct Size and Improves Postischemic Function

As shown in Figure 1B, infarct size, measured with TTC staining, was significantly smaller in Bcl-2 hearts (18±3%) than in WT hearts (38±5%; P<0.05). This 50% reduction in infarct size in the Langendorff model was similar to that observed in these Bcl-2 overexpressing hearts using an in vivo model of coronary occlusion and reperfusion (Schneider et al, unpublished data, 2004). We also confirmed that the TUNEL-positive apoptotic cardiac myocyte death was reduced by 73% in our model (4.3±1.7% myocytes showed apoptosis in WT versus 1.2±0.9% in Bcl-2). Figure 1C shows that after 2 hours of reperfusion, Bcl-2 transgenic hearts had significantly improved postischemic contractile function as measured by recovery of rate pressure product (RPP; left ventricular developed pressure (LVDP)×heart rate, expressed as % initial RPP, 55±4% in Bcl-2 hearts versus 27±3% in WT hearts; P<0.05), or recovery of LVDP (78.9±7.8 cmH2O in Bcl-2 hearts versus 38.0±3.1 cmH2O in WT hearts; P<0.05). On reperfusion, the rate of contraction (+dP/dt) and rate of relaxation (−dP/dt) were also significantly better in Bcl-2 hearts compared with WT hearts (2392±239 in Bcl-2 versus 2312±295, −1193±203 in WT; P<0.05). Figure 1D shows that release of the proapoptotic factor, AIF, from the mitochondria to the cytosol was attenuated in Bcl-2 hearts compared with WT hearts. AIF release from the mitochondria...
has been used as a marker for apoptosis. AIF is released from the mitochondria to the cytosol and nucleus; it has been reported to cause chromatin condensation in the nucleus.28 These data indicate that Bcl-2 overexpression inhibits both necrotic and apoptotic death pathways during ischemia/reperfusion.

High-Energy Phosphate Metabolites and pH
Figure 2 shows the changes in [ATP], [PCr], and pH at baseline, during ischemia and during reperfusion in WT and Bcl-2 hearts. At baseline, there were no significant differences in pH between WT and Bcl-2 hearts (WT, 7.28±0.04 versus Bcl-2, 7.27±0.01; P>0.05). During ischemia, the decrease in pH was significantly reduced in Bcl-2 hearts (6.49±0.06) compared with WT hearts (6.26±0.07; P<0.05). During reperfusion, pH was similar in Bcl-2 and WT hearts. At baseline, there were no significant differences in creatine phosphate (PCr) or ATP content between Bcl-2 and WT hearts. At the end of ischemia there were no significant differences in ATP between the groups, although the rate of ATP decline during the first 10 minutes of ischemia was slower in the Bcl-2 hearts than in WT hearts. During reperfusion, ATP recovered similarly in both Bcl-2 hearts and WT hearts. PCr declined to similar levels in both WT and Bcl-2 hearts during ischemia. After 2 hours of reperfusion, the postischemic recovery of PCr was significantly better in Bcl-2 hearts (85±5% of preischemic level) than in WT hearts (60±4%; P<0.05), consistent with smaller infarcts in the Bcl-2 hearts and therefore better retention of creatine. These results indicate that Bcl-2 overexpression reduced cytosolic acidification during ischemia, slowed the rate of decline in ATP, and allowed improved PCr recovery during reperfusion.

**Effect of F1F0-ATPase Inhibition During Ischemia**
To explore the mechanism responsible for the reduced acidification observed during ischemia in Bcl-2 hearts, we performed a series of studies to investigate the role of the F1F0-ATPase. During global ischemia, ATP is generated by anaerobic glycolysis and if this glycolytic ATP is consumed by the F1F0-ATPase, this would accelerate the decline in ATP content and increase acidification during ischemia. To test whether the F1F0-ATPase is involved in the reduced acidification observed in Bcl-2 hearts, we treated hearts with oligomycin, a specific inhibitor of the F1F0-ATPase. Oligomycin has been reported to decrease acidification during ischemia because it blocks the breakdown of glycolytic ATP.15 We reasoned that if the effects of Bcl-2 were mediated by a pathway that included the F1F0-ATPase, then the reduced acidification observed with Bcl-2 and oligomycin should not be additive. We selected an oligomycin concentration that was in the lower range of those previously used16 (~3 μmol/L), and it was administered for 4 minutes before ischemia. As shown in Figure 3, consistent with the data in Figure 2, we found reduced acidification during ischemia in untreated Bcl-2 hearts. We also found that F1F0-ATPase inhibition by oligomycin significantly reduced cytosolic acidification in WT hearts to a level similar to that observed in Bcl-2 transgenic hearts. Figure 3 further shows that in Bcl-2 transgenic hearts oligomycin had no additional effect on pH during ischemia. Thus, there was no significant difference in pH between WT and Bcl-2 hearts treated with oligomycin (Figure 3, P>0.05). To exclude the possibility that the reduced acidification in Bcl-2 hearts was attributable to the enhanced proton efflux across the plasma membrane, we also measured pH during ischemia by adding phenylphosphonic acid to the perfusate. If the reduced pH in Bcl-2 hearts was attributable to enhanced efflux, we would expect a lower pH in Bcl-2 hearts; instead we found that pH at the end of ischemia was slightly higher in Bcl-2 hearts (6.4±0.1) than in WT hearts (6.2±0.1). These data are entirely consistent with our previous findings that preconditioning results in a parallel attenuation of the decrease in both intracellular and extracellular pH during ischemia.27 These data confirm that the reduced acidification in Bcl-2 hearts is not attributable to enhanced proton efflux from the cell, and are consistent with...
a role for the F$_1$F$_0$-ATPase in the reduced cytosolic acidification during ischemia in Bcl-2 hearts.

As discussed, inhibition of the F$_1$F$_0$-ATPase would be expected to reduce the rate of decline in ATP during ischemia. We therefore measured the initial rate of decline in ATP during the first 10 minutes of ischemia. As shown in Figure 4, the rate of decline in ATP was slower in Bcl-2 hearts than in WT hearts ($P<0.05$). Oligomycin reduced the rate of decrease in ATP in WT hearts but addition of oligomycin had no additional effect in Bcl-2 hearts, suggesting that Bcl-2 and oligomycin are affecting a common pathway. Because oligomycin cannot be readily removed from the perfused heart, it is not possible to measure the recovery of ATP or LVDP during reperfusion.

Interaction of Bcl-2 With VDAC During Ischemia

The oligomycin data suggest that Bcl-2 can reduce consumption of glycolytic ATP via the reverse mode of the F$_1$F$_0$-ATPase. Bcl-2 could accomplish this by limiting entry of cytosolic ATP into the mitochondria (through VDAC or ANT) or by direct inhibition of the F$_1$F$_0$-ATPase. Because Bcl-2 and VDAC are both located on the outer mitochondrial membrane and it has previously been demonstrated that Bcl-xL interacts with VDAC, we examined whether an interaction between Bcl-2 and VDAC occurs in heart. Western blot data show that a similar amount of VDAC was present in Bcl-2 and WT mitochondria (Figure 5A). There was also no apparent difference in Bak or Bcl-xL levels between WT and Bcl-2 mitochondria. As shown in Figure 5B, immunoprecipitation with VDAC showed a significant association with Bcl-2 in the Bcl-2 hearts. Furthermore, immunoprecipitation with Bcl-2 confirms an association with VDAC. As shown in Figure 5C, we found increased binding of Bcl-2 to VDAC before and during ischemia in Bcl-2 hearts ($P<0.05$) in both WT and Bcl-2 hearts, but the increase was only significant in the Bcl-2 hearts ($P<0.05$).

ATP/ADP Flux in Bcl-2 Overexpressed Mitochondria

Figure 6A shows reciprocal plots of ADP-stimulated (state 3) oxygen consumption rates under normal conditions, indicating no inhibition of ADP flux under normal conditions between WT and Bcl-2 mitochondria ($V_{max}$, $145\pm13$ [nmol O$_2$/mg/min] in WT, $124\pm6$ in Bcl-2; $P>0.05$). Figure 6B showed that under normoxic condition, Bcl-2 does not limit ADP flux into mitochondria. Interestingly, with addition of the mitochondrial uncoupler DNP, the rate of ATP consumption was significantly less in mitochondria isolated from hearts that overexpress Bcl-2 than in WT mitochondria (Figures 6C and 7A). This is consistent with the reduced cytosolic ATP depletion during ischemia observed in Bcl-2 hearts. Mitochondrial ATP breakdown was completely blocked by inhibition of ATP transport using an inhibitor of ANT, ATR or an inhibitor of VDAC, DIDS (Figure 7A). We also examined whether addition of recombinant Bcl-2 (rBcl-2) to isolated mitochondria would alter the rate of ATP uptake and consumption. As shown in Figure 7B, rBcl-2 bound to isolated mitochondria and remained following washing. Furthermore, mitochondria containing rBcl-2 protein showed the reduced ATP uptake and hydrolysis (Figure 7B), providing additional support for the hypothesis.

Discussion

In this study, we found that hearts overexpressing Bcl-2 showed improved postischemic recovery of left ventricular developed pressure, reduced infarct size, and improved postischemic recovery of phosphocreatine. These data are consistent with previous studies showing that cardiac-specific
overexpression of Bcl-2 reduced ischemia/reperfusion injury. In addition, this study provides new insights into the mechanism by which Bcl-2 mediates cardioprotection. We show that hearts overexpressing Bcl-2 have a slower rate of decline in ATP during ischemia and reduced ischemic acidification, and these were dependent on the activity of the mitochondrial F$_1$F$_0$-ATPase, suggesting that oligomycin and Bcl-2 affect a common pathway. These data add to the emerging concept that Bcl-2 modulates mitochondrial physiology and function.

With a slower rate of decline in ATP, during early ischemia there would be more ATP and therefore less ionic alterations and less activation of degradative enzymes. The reduced acidification has been shown to be beneficial because there is less stimulation of Na$^+$/H$^+$ exchange, and therefore less of a rise in Na$^+$, resulting in less Na$^+/Ca^{2+}$ exchange and less of a rise in Ca$^{2+}$. The reduction in Ca$^{2+}$ during ischemia has been shown in many studies to reduce ischemic injury. Furthermore, if lethal cell injury is related to the time interval after ATP reaches a critically low value, then slowing the rate of ATP depletion would be protective.

There are considerable data in the literature suggesting that mitochondrial membrane potential during ischemia or metabolic inhibition of sufficient duration to cause significant depletion of ATP. During simulated ischemia is faster and more complete with addition of oligomycin. These data are interpreted as showing that glycolytic ATP is used to maintain the mitochondrial membrane potential during ischemia via reverse mode F$_1$F$_0$-ATPase activity. Furthermore, during ischemia, the F$_1$F$_0$-ATPase consumes glycolytic ATP, thus contributing to depletion of ATP and cytosolic acidification caused by increased generation of lactate. We find that Bcl-2 slows the rate of decline in ATP and reduces ischemic acidification during ischemia, consistent with Bcl-2 induced inhibition of consumption of glycolytically generated ATP. This could be accomplished by Bcl-2 induced closure of VDAC and ATP/ADP transport across the mitochondrial membrane.

There are conflicting data regarding the relationship of Bcl-2 to VDAC and ATP/ADP transport across the mitochondrial membrane favors interaction between Bcl-2 and VDAC. In further support of this hypothesis, we find greater association of Bcl-2 and VDAC in Bcl-2 hearts during ischemia. However, the precise mechanism by which Bcl-2 reduces consumption of glycolytically generated ATP will require further study.

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Mitochondria regulate tightly the transport of ATP and ADP. If ATP generated in the mitochondria cannot get to the cytosol because of the inhibition of VDAC under normal aerobic conditions, cell function will be impaired. Alternatively, if oxygen is limiting, it would be advantageous to block mitochondrial consumption of glycolytically generated ATP. Bcl-2 mediated inhibition of adenine nucleotide transport or inhibition of the F$_{1}$F$_{0}$-ATPase would only be protective during ischemia. Under normal aerobic conditions, inhibition of these processes would impair the transport of ATP and/or the generation of ATP by oxidative phosphorylation. Interestingly, it has been suggested that mitochondrial ATP/ADP transport is inhibited during ischemia. Our data indicate that overexpression of Bcl-2 does not block adenine nucleotide transport into mitochondria under normal aerobic conditions; however, on addition of an uncoupler, mitochondria from Bcl-2 hearts exhibit a reduced rate of ATP hydrolysis, consistent with reduced entry of ATP into the mitochondria. Bcl-2 insertion into the mitochondrial membrane is enhanced by decreased pH, which occurs during ischemia. The finding that acidic conditions are required to induce cell death by a Bcl-2 family protein-dependent pathway is consistent with a functional role of Bcl-2 during ischemia.

In summary, we find that Bcl-2, in addition to reducing ischemic injury, reduces acidification and the rate of decline in ATP during ischemia by reducing the consumption of glycolytically generated ATP. This may be involved as an early step in Bcl-2–induced cardioprotection.

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Expanded Materials and Methods

Heart Perfusion and Nuclear magnetic resonance spectroscopy

The heart was placed in a 10 mm diameter nuclear magnetic resonance (NMR) tube, and coronary effluent was evacuated from the overflow outlet above the heart. Temperature was maintained at 37.0 ± 0.5°C by the variable temperature unit of the NMR spectrometer.

Figure 1 shows typical $^{31}$P NMR spectra before and during ischemia. Relative changes in the concentration of high-energy phosphate metabolites were measured during the ischemia/reperfusion protocol by acquiring consecutive 5-minute $^{31}$P NMR spectra using a Varian 500 MHz spectrometer with an 11.7-Tesla superconducting magnet at the $^{31}$P resonance frequency of 202.47MHz. The areas under each spectral peak were measured and expressed as a percentage of the initial peak areas, i.e., preischemic control spectrum from each heart. Intracellular pH was estimated from the chemical shift between inorganic phosphate (Pi) and phosphocreatine (PCr) as described previously (1).

In some hearts, phenylphosphonic acid (PPA, 15mM, Fluka) was added to the perfusate to measure extracellular pH (2). The pH of the buffer containing PPA was adjusted to 7.4. Extracellular pH was determined by the shift difference between PPA and PCr (Figure 1). The formula used to determine extracellular pH was $pH_e = pK + \log\left(\frac{\delta_{\text{max}} - \delta_e}{\delta_e - \delta_{\text{min}}\right)$, where $\delta_{\text{max}} = 16.24$ and $\delta_{\text{min}} = 14.27$ are the chemical shift differences between the PCr and PPA resonances in the presence of excess acid and base, respectively; $\delta_e$ is the measured chemical shift difference, and pK is the acid dissociation constant. As reported previously, we used a pK of 6.99 (2). For these studies, hearts were not bathed in perfusate.

Heart and Mitochondria Preparation for Western Blot Analysis
For whole cell samples, the frozen heart was homogenized in ice-cold lysis buffer containing (in mmol/L): NaCl 70, HEPES 20 (pH 7.7), MgCl₂ 2.5, EDTA 0.1, 1% Triton X-100, and protease inhibitors. The heart homogenate was centrifuged at 10,000g for 30 minutes (4°C) and the supernatant was used for western blot analysis. For isolation of the mitochondria-enriched fraction, frozen hearts were homogenized in buffer containing (in mmol/L): sucrose 250, Tris-HCl 10 (pH 7.4), EDTA 1, Na₃VO₄ 1, NaF 1, and protease inhibitors. The homogenate was centrifuged at 1,000g for 10 minutes (4°C) and the supernatant was then centrifuged at 10,000g for 30 minutes (4°C). After washing and re-centrifugation the pellet was re-suspended in lysis buffer. The supernatant was further centrifuged at 100,000g for 45 minutes (4°C) and saved as the cytosolic fraction.

For determination of Bcl-2, VDAC, Bak, Bcl-xL, and apoptosis-inducing factor (AIF), 50µg of homogenate protein was separated by electrophoresis on a 14% Tris/glycine gel. After transfer to a nitrocellulose membrane, the membrane was incubated with anti-Bcl-2 antibody (Transduction Laboratories, KY, USA), anti-VDAC antibody (Calbiochem, CA, USA), anti-Bak antibody (Upstate Biotechnology, NY, USA), anti-Bcl-xL antibody (Cell Signaling, MA, USA), or anti-AIF antibody (BD PharMingen, CA, USA) in TBS-T with 5% BSA/milk at 4°C overnight. To confirm equal protein loading, membranes were stained with Ponceau S solution. Membranes were incubated with the secondary antibody, appropriate horseradish peroxidase-conjugated IgG in TBS-T with 5% dry fat milk for 1 hour at room temperature. Immunoreactive protein was visualized using an enhanced chemi-luminescence analysis kit (Amersham Pharmacia Biotech Inc. NJ, USA).

**Isolated Mitochondria Study**

Freshly isolated mitochondria were prepared from the ventricles of two hearts by differential centrifugation in ice cold buffer containing (in mmol/L): mannitol 225, sucrose 75, MOPS 5, EGTA 0.5, taurine 2, 0.2% BSA (pH 7.25). The mitochondria (25mg/mL) were then incubated in buffer containing
(in mmol/L): Mannitol 225, Sucrose 25, MOPS 5, EGTA 1, KH$_2$PO$_4$ 5, and taurine 2 supplemented with 0.2% BSA (pH = 7.4 at 25°C). The isolated mitochondria were placed on ice and used within 3 hours. Measurement of mitochondrial respiration was performed at 25°C in a chamber (600µL) connected with Clark-type O$_2$ electrode (Instech, PA, USA) and O$_2$ monitor (Model 5300, YSI, Inc. OH, USA). The mitochondria (~300µg) was incubated in a chamber containing respiration buffer (in mmol/L): KCl 120, MOPS 5, EGTA 1, KH$_2$PO$_4$ 5, 0.2% BSA. After addition of glutamate/malate (10mmol/L), state 3 respiration was measured by addition of ADP (0.125 – 0.5mmol/L). Upon depletion of ADP, state 4 respiration was determined. Uncoupled respiration was determined by adding an uncoupler, 2,4,-dinitrophenol (DNP, 50µM). The maximum ADP-stimulated respiration (V$_{max}$) was estimated from a linear regression of double-reciprocal plots.

ATP uptake into mitochondria and hydrolysis was measured by adding ATP (875µM) to mitochondria (~150µg) followed by addition of the mitochondrial uncoupler DNP (50µM). After the indicated time, oligomycin (1µg/mL) was added to block ATP hydrolysis and the supernatant was obtained by brief centrifugation (1min). ATP remaining in the supernatant was measured. To confirm that ATP hydrolysis was dependent on ATP transport into the mitochondria, an ANT blocker, atractyloside (ATR, 50µM), was treated prior to DNP addition. To assess the contribution of VDAC, 4’-diisothiocyanato-2,2’-disulfonic acid stilbene, (DIDS, 100µM), was used.

To examine the direct effect of Bcl-2 on mitochondrial ATP uptake and hydrolysis, we incubated recombinant Bcl-2 protein (rBcl-2, Full length, HA-tag (7kDa), Amprox, USA) with freshly isolated mitochondria for 5 min at room temperature. To confirm that rBcl-2 was incorporated with mitochondria, the sample was centrifuged and the mitochondrial pellet was obtained then washed twice. Then the mitochondria were stored in lysis buffer for western blot. For the ATP uptake experiment, the mitochondria were incubated with or without rBcl-2 at room temperature for 5 min prior to the experiments.
References


**Figure 1.** 31P-NMR spectra before and during ischemia. In some hearts, the extracellular pH indicator PPA was added to the buffer.