Regulation of Bcl-2 Family Proteins During Development and in Response to Oxidative Stress in Cardiac Myocytes

Association With Changes in Mitochondrial Membrane Potential

Stuart A. Cook, Peter H. Sugden, Angela Clerk

Abstract—Cardiac myocyte apoptosis is potentially important in many cardiac disorders. In other cells, Bcl-2 family proteins and mitochondrial dysfunction are probably key regulators of the apoptotic response. In the present study, we characterized the regulation of antiapoptotic (Bcl-2, Bcl-xL) and proapoptotic (Bad, Bax) Bcl-2 family proteins in the rat heart during development and in oxidative stress–induced apoptosis. Bcl-2 and Bcl-xL were expressed at high levels in the neonate, and their expression was sustained during development. In contrast, although Bad and Bax were present at high levels in neonatal hearts, they were barely detectable in adult hearts. We confirmed that H2 O2 induced cardiac myocyte cell death, stimulating poly(ADP-ribose) polymerase proteolysis (from 2 hours), caspase-3 proteolysis (from 2 hours), and DNA fragmentation (from 8 hours). In unstimulated neonatal cardiac myocytes, Bcl-2 and Bcl-xL were associated with the mitochondria, but Bad and Bax were predominantly present in a crude cytosolic fraction. Exposure of myocytes to H2 O2 stimulated rapid translocation of Bad (5 minutes) to the mitochondria. This was followed by the subsequent degradation of Bad and Bcl-2 (from 30 minutes). The levels of the mitochondrial membrane marker cytochrome oxidase remained unchanged. H2 O2 also induced translocation of cytochrome c from the mitochondria to the cytosol within 15 to 30 minutes, which was indicative of mitochondrial dysfunction. Myocytes exposed to H2 O2 showed an early loss of mitochondrial membrane potential (assessed by fluorescence-activated cell sorter analysis) from 15 to 30 minutes, which was partially restored by 1 hour. However, a subsequent irreversible loss of mitochondrial membrane potential occurred that correlated with cell death. These data suggest that the regulation of Bcl-2 and mitochondrial function are important factors in oxidative stress–induced cardiac myocyte apoptosis. (Circ Res. 1999;85:940-949.)

Key Words: myocyte n mitochondrial membrane potential n apoptosis n oxidative stress n Bcl-2

Programmed cell death, or apoptosis, is an essential component of normal development and a response to pathological disease states. In the heart, cardiac myocyte apoptosis is a feature of many pathological disorders; it occurs, for example, in myocardial infarction and congestive heart failure (reviewed in Reference 1). Apoptosis allows surplus or damaged cells to be removed in the absence of an inflammatory response. The mechanisms involved are not fully understood. A key component is the activation of a family of proteases, the caspases, which participate in a cascade and ultimately cleave a set of proteins, causing disassembly of the cell (reviewed in Reference 2). Although the caspase cascade may be pivotal to the apoptotic response, caspase activation is regulated by a variety of other factors. These include established intracellular signaling pathways, such as the mitogen-activated protein kinases and protein kinase B (reviewed in Reference 1). The Bcl-2 family, whose functions are less well-defined, also regulate the apoptotic response (reviewed in References 3 and 4).

Bcl-2 family proteins may be either pro- or antiapoptotic. Thus, Bcl-2 and Bcl-xL protect cells from apoptosis, whereas Bax and Bad promote the response.3 At least 15 members of this family have been identified; they each share homology within ±4 regions (BH1 to 4). Although Bcl-2 and Bcl-xL contain all 4 regions of homology, Bax lacks the BH4 domain, and others (eg, Bad) contain only the BH3 “death” domain. The function of these proteins is not clear, but Bcl-2, Bcl-xL, and Bax can form ion-conductive pores in lipid bilayers, probably acting as dimers/oligomers.5,6 Because Bcl-2 and Bcl-xL are predominantly localized to the outer mitochondrial membrane and Bax translocates to the mitochondria on activation, they may function to regulate ion fluxes within the cell (reviewed in References 3, 4, and 7). Alternatively, they may act directly to regulate caspase...
activation. Structural studies indicate that BH3 domains may fit into a hydrophobic pocket in Bcl-2, Bcl-xL, or Bax, allowing dimerization and presumably modulating the function of these proteins. The activities of Bcl-2 proteins are, therefore, regulated by their subcellular localization (i.e., cytosolic versus mitochondrial) and their dimerization/oligomerization. Indeed, dimerization of Bax triggers its translocation to the mitochondria and induces apoptosis.8

The Bcl-2 family is also regulated by proteolytic cleavage9,10 and by phosphorylation. Bad has ≥ 2 phosphorylation sites (Ser-112 and Ser-136) and, in some cell types, phosphorylation of Ser-136 by protein kinase B11 or through activation of mitogen-activated protein kinase cascades12 results in binding to 14–3-3 proteins.13 This sequesters Bad in the cytosol, preventing interaction with Bcl-2 and inhibiting the proapoptotic function of Bad. Bad may also be phosphorylated on Ser-112 at the mitochondria by protein kinase A, causing it to dissociate from the mitochondria and preventing its proapoptotic function.14 Conversely, apoptotic stimuli may induce dephosphorylation of Bad, which would then translocate to the mitochondria. Bcl-2 is phosphorylated on multiple sites within an N-terminal unstructured loop.15–17 Although phosphorylation of Ser-70 is activating,17 phosphorylation of other sites may be inactivating and lead to apoptosis.16

The principal site of action of the Bcl-2 family proteins seems to be the mitochondria, and it is increasingly apparent that most, if not all, apoptotic responses involve mitochondrial dysfunction and a loss of the mitochondrial membrane potential (ΔΨ).7 Indeed, this step may be an irreversible commitment to cell death. Recent studies indicate that apoptosis is associated with the loss of cytochrome c (and potentially other apoptotic factors18) from the intermembrane space of the mitochondria and subsequent release into the cytosol. Cytochrome c binds to apoptosis-activating factor 1 to form a complex with caspase-9, which becomes activated.19 Caspase-9 triggers a proteolytic cascade, activating other caspases (including caspase-3) and resulting in cell death.20 The trigger for cytochrome c release is not known. Some studies indicate that the mitochondrial permeability transition pore (MPTP) may open and cause loss of ΔΨ. This may result in the swelling of the mitochondria, rupturing the outer membrane and releasing cytochrome c into the cytosol.21 However, in other studies, release of cytochrome c is not associated with the loss of ΔΨ.7 Bcl-2 and/or Bcl-xL may function in the mitochondrial membrane to prevent loss of cytochrome c and thus inhibit apoptosis.22,23 an effect that may be mediated by direct interaction with apoptosis-activating factor 1,24,25

The Bcl-2 proteins are becoming increasingly recognized as important modulators of cardiac myocyte apoptosis. Bcl-2 mRNA is expressed in both developing and adult hearts,26 and the protein is upregulated after coronary occlusion in rat hearts27 or myocardial infarction in human hearts.28 As in other cells, overexpression of Bcl-2 protects cardiac myocytes from apoptosis.29 Proapoptotic Bax is upregulated in the rat heart after coronary occlusion,27 and its overexpression in the ventricles of spontaneously hypertensive rat hearts may contribute to apoptosis.30 Bcl-x and proapoptotic Bax are also expressed in cardiac myocytes and are upregulated during cytokine-induced cardiac myocyte apoptosis.31

In the heart, ischemia/reperfusion injury results in cardiac myocyte cell death by both necrotic and apoptotic mechanisms.1 One of the key features of this disorder is the generation of reactive oxygen species (ROS) during both the ischemic and reperfusion phases.32 Oxidative stress induces cardiac myocyte apoptosis in vitro33,33–35 and may be a major contributing factor in the apoptosis observed in ischemia/reperfusion injury and congestive heart failure. In the present study, we studied the regulation of members of the Bcl-2 family that are recognized as either antiapoptotic (Bcl-2, Bcl-xL) or proapoptotic (Bad, Bax). We characterized the developmental expression of these proteins in the rat heart and determined their subcellular localization in cardiac myocytes. We also examined the effects of oxidative stress on Bcl-2 proteins and on mitochondrial function. The role(s) of the Bcl-2 proteins in relation to mitochondrial dysfunction in cardiac myocyte apoptosis is discussed.

Materials and Methods

Preparation of Heart and Myocyte Extracts

Hearts from Sprague-Dawley rats were extracted in buffer A (in mmol/L: β-glycerophosphate 20 [pH 7.5], NaF 50, microcystin LR 0.002, EDTA 2, NaVO₃ 0.2, benzamidine 10, leupeptin 0.2, trans-e epoxy succinyl-L-leucylamido-(4-guanidino)butane 0.01, dithiothreitol 5, phenylmethylsulfonyl fluoride 0.3, 1% [vol/vol] Triton X-100), and samples were prepared as previously described.36 Myocytes were prepared by an adaptation37 of the method of Iwaki et al38 and plated on gelatin-coated dishes (1.4 × 10⁵ cells/mm²). In all experiments, serum was withdrawn for 24 hours before treatment. For immunoblotting, myocytes were washed and scrapped into buffer A. Samples were centrifuged, and the supernatants were spiked with sample buffer. Protein content was measured by the BioRad Bradford method.39

Terminal Deoxynucleotidyl dUTP Nick-End Labeling Analysis of Cardiac Myocytes

Myocytes were plated in 8-well Laboratory-Tek tissue-culture chamber slides precoated with laminin and gelatin (600 cells/mm²). After treatment, myocytes were fixed, permeabilized, and labeled by deoxyribonucleotidyl transferase (0.1 U/µL) incorporation of biotinylated dUTP (2 nmol/L) in terminal transferase buffer (Boehringer-Mannheim). Biotinylated dUTP was detected with extravidin-FITC. Myocytes were counterstained with antibodies to β-myosin heavy chain (Vector Laboratories) and streptavidin-Texas Red anti-mouse IgG, and they were examined by confocal microscopy (Leica TCS-4D laser scanning confocal microscope). For each of 3 experiment, 3 fields were examined for each treatment performed in duplicate. Cells were identified as apoptotic if they showed unequivocal terminal deoxynucleotidyl dUTP nick-end labeling (TUNEL) staining in the nucleus. Necrotic cells were not identified because these cells become nonadherent and are removed when the medium is decanted and the cell layer is washed in PBS.

Subcellular Fractionation

Cells were scraped into buffer B (in mmol/L: HEPES 10 [pH 7.5], mannitol 200, and sucrose 70), which contained protease and phosphatase inhibitors. Samples were centrifuged (500g) to pellet nuclei, unbroken cells, and plasma membrane debris (nuclear fraction). The supernatants were recentrifuged (10 000g) to separate the mitochondrial fraction from the cytosolic fraction. The mitochondrial fraction was resuspended in buffer B containing 1% (vol/vol) Triton X-100. The protein content of each fraction was determined.
by the BioRad Bradford assay. Samples were boiled with sample buffer.

Western Blot Analysis
Proteins were separated by SDS-PAGE and transferred to nitrocellulose, as previously described. Nonspecific binding was blocked, and the blots were incubated with primary antibodies and then horseradish peroxidase–conjugated secondary antibodies (Amerham, 1/5000 dilution). Bands were detected by enhanced chemiluminescence, and blots were exposed to Hyperfilm MP for 30 s to 2 minutes. Laser scanning densitometry was used for semiquantitative analysis of the data.

Analysis of ΔΨ
Loss of ΔΨ was assessed by fluorescence-activated cell sorter (FACS) analysis of cells stained with Rhodamine 123 (R123) or 5,5′,6,6′-tetrachloro-1′,3′,3′,6-tetraethylbenzimidazolocarbocyanide iodine (JC-1). Cells were incubated with 5 μmol/L R123 (Calbiochem) or 10 μg/mL JC-1 (Molecular Probes), harvested by trypsinization, and analyzed by FACS (10 000 cells/sample). The excitation wavelength was 488 nm. The emission fluorescence for R123 was monitored at 582 nm, and JC-1 was monitored at 530 and 582 nm. The data were analyzed using Cell Quest (Becton Dickinson, Immunocytometry Systems).

Statistical Analysis
Results are expressed as mean±SEM. Differences between means were evaluated by unpaired 2-tailed Student’s t test.
An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results
Developmental Regulation of Bcl-2 Family Proteins
The expression of antiapoptotic (Bcl-2, Bcl-xL) and proapoptotic (Bad, Bax) members of the Bcl-2 family was assessed in rat heart ventricles by Western blot analysis (Figure 1). In neonatal rat ventricles, the Bcl-2 antibody detected 2 bands of 26 and 32 kDa (Figure 1A), which may represent different isoforms. Although not apparent in Figure 1A, in some blots, both the 26- and 32-kDa forms appeared as multiple bands (see below), and these may correspond to differentially phosphorylated forms. Bcl-xL was detected as a 31-kDa band (Figure 1B), Bad was detected as a doublet of 25 kDa (Figure 1C), and Bax was detected as a doublet of 21 kDa (Figure 1D). The levels of Bcl-2 and Bcl-xL were sustained during development (Figures 1A and 1B). In contrast, the expression of Bad (Figures 1C and 2A) and Bax (Figures 1D and 2B) declined substantially during development.

Subcellular Localization of Bcl-2 Family Proteins
Bcl-2 and Bcl-xL are predominantly localized to the mitochondria in nonmyocytic cell types, whereas Bax and Bad are primarily cytosolic (reviewed in References 3 and 7). We examined the subcellular distribution of these proteins in primary cultures of neonatal myocytes by differential centrifugation and Western blot analysis. Bcl-2 and Bcl-xL were detected in the mitochondrial fraction (Figure 3A and 3B), along with the mitochondrial marker cytochrome oxidase (Figure 3E). Only a small fraction of 32-kDa Bcl-2 immunoreactivity was detected in the cytosolic fraction (Figure 3D). The levels of Bcl-2 and Bcl-xL were sustained during development (Figures 1A and 1B). In contrast, the expression of Bad (Figures 1C and 2A) and Bax (Figures 1D and 2B) declined substantially during development.

Stimulation of Cardiac Myocyte Apoptosis by Oxidative Stress
Oxidative stress induces apoptosis in cardiac myocytes. We confirmed that apoptosis occurs in myocytes exposed to H$_2$O$_2$ (0.5 mmol/L) by examining the cleavage of poly(ADP-ribose) polymerase (PARP) and caspase-3 and by TUNEL analysis. PARP is cleaved by caspase-3 to generate a 25-kDa fragment. However, the levels of PARP cleavage were not significantly different between control and treated myocytes.
fragment from the N-terminus and an 85-kDa fragment from the C-terminus. We studied PARP using a monoclonal antibody, C2-10, which detects an epitope at the N-terminus of the 85-kDa cleavage product. In unstimulated cells, the PARP holoenzyme was detected as a 117-kDa band on Western blot analysis. A, Bcl-2 (30 μg of protein/lane) was detected in nuclear and mitochondrial fractions, with a small fraction of the 32-kDa isoform in cytosol. B, Bcl-xL (30 μg of protein/lane) was detected in nuclear and mitochondrial fractions, with proportionally greater amounts in cytosol. C, Bad (50 μg of protein/lane) was detected in all 3 fractions, but with proportionally greater amounts in cytosol. D, Bax (60 μg protein/lane) was detected in all 3 fractions, but with proportionally greater amounts in cytosol. E, Cytochrome oxidase (10 μg protein/lane) was detected in nuclear and mitochondrial fractions. Positions of relative molecular mass markers are shown on left of each blot. Experiment was repeated with similar results.

Figure 3. Subcellular fractionation of Bcl-2 family proteins in primary cultures of neonatal ventricular myocytes. Myocyte extracts were separated by differential centrifugation. Low-speed spin pellet (nuclear fraction; N) contained nuclei and unbroken cells; high-speed spin pellet (mitochondrial fraction; M) contained mitochondria and heavy membranes, and supernatant (cytosolic fraction; Cy) was a crude cytosolic extract. Proteins were separated by SDS-PAGE and assessed by Western blot analysis. A, Bcl-2 (30 μg of protein/lane) was detected in nuclear and mitochondrial fractions, with a small fraction of the 32-kDa isoform in cytosol. B, Bcl-xL (30 μg of protein/lane) was detected in nuclear and mitochondrial fractions, with proportionally greater amounts in cytosol. C, Bad (50 μg of protein/lane) was detected in all 3 fractions, but with proportionally greater amounts in cytosol. D, Bax (60 μg protein/lane) was detected in all 3 fractions, but with proportionally greater amounts in cytosol. E, Cytochrome oxidase (10 μg protein/lane) was detected in nuclear and mitochondrial fractions. Positions of relative molecular mass markers are shown on left of each blot. Experiment was repeated with similar results.

Response of Bcl-2 and Bad to H2O2 Stimulation

The Bcl-2 family is a key regulator of the apoptotic response (reviewed in References 3 and 4). We examined the response of antiapoptotic Bcl-2 and proapoptotic Bad to H2O2-induced cardiac myocyte apoptosis. Both the 26- and 32-kDa immunoreactive Bcl-2 bands were lost from the mitochondrial fraction from 5 to 15 minutes, with a substantial loss (~50%) by 30 to 60 minutes (Figure 6A). A progressive increase in the 32-kDa band in the cytosolic fraction occurred over this time course, but the 26-kDa isoform remained undetectable (Figure 6B). To confirm that the loss of Bcl-2 from the mitochondrial fraction did not reflect gross mitochondrial disruption, we determined the levels of cytochrome oxidase in these samples. No change in cytochrome oxidase immunoreactivity occurred after H2O2 treatment for ≤120 minutes (Figure 6C).

Bad was detected as a single band in the mitochondrial fraction (Figure 7A) but as a doublet in the cytosolic fraction (Figure 7B). H2O2 stimulated an increase in Bad immunoreactivity in the mitochondrial fraction between 5 and 30 minutes, which declined thereafter to below basal levels (Figure 7A). In the cytosol, the lower band decreased in intensity from 5 to 15 minutes, and it was essentially lost by
60 minutes (Figure 7B). The intensity of the upper band was maintained for ≤60 minutes, but it was lost by 120 minutes. To identify the Bad species appearing in the mitochondrial fraction, we directly compared the relative molecular masses of the immunoreactive bands in the cytosolic and mitochondrial fractions (Figure 7C). The band in the mitochondrial fraction from unstimulated myocytes (lane 2) and myocytes exposed to H$_2$O$_2$ (0.5 mmol/L, 5 minutes, lane 3) corresponded to the upper band in the cytosolic fraction (lanes 1 and 4) and may represent a phosphorylated form.

**Cytochrome c Redistribution and Loss of ΔΨ in Myocytes Subjected to Oxidative Stress**

Redistribution of cytochrome c from the mitochondria to the cytosol is a critical event in the progression of apoptosis in many systems (reviewed in Reference 18). In unstimulated myocytes, as expected, cytochrome c was predominantly present in the mitochondrial fraction (Figure 8A and 8B). H$_2$O$_2$ (0.5 mmol/L) induced a loss of cytochrome c from the mitochondrial fraction within 15 to 30 minutes (Figure 8A) and a corresponding increase in the cytosolic fraction (Figure 8B).

The redistribution of cytochrome c has been linked with the loss of ΔΨ and the opening of the MPTP. However, this linkage is contentious and may depend on cell type and stimulus. We assessed ΔΨ in H$_2$O$_2$-stimulated myocytes using R123 or JC-1. The fluorescence intensity of these dyes in the mitochondria of a given cell is dependent on ΔΨ. Maintenance of ΔΨ results in a high intensity of fluorescence with R123 (population M1). As ΔΨ is lost, the fluorescence intensity of R123 decreases (population M2). In unstimulated myocytes, the M1 and M2 populations were approximately equal (Figure 9A and 9F). Exposure of myocytes to H$_2$O$_2$ (0.5 mmol/L) induced a shift in fluorescence from M1 to M2 at 15 and 30 minutes (Figure 9B and 9F). However, at 45 and 60 minutes, evidence of repolarization existed, as shown by a redistribution from the M2 to the M1 population, such that the 60-minute values of M1 were significantly greater than (and the values of M2 were significantly less than) the values at 30 minutes (Figure 9C and 9F). At later times (>3 hours), the proportion of cells in the M1 population again declined (Figure 9D and 9F). As a positive control, myocytes were treated with the F$_1$-ATPase inhibitor oligomycin (10 μg/mL) and the mitochondrial uncoupler carbonyl cyanide 3-chlorophenylhydrazine (50 μmol/L) for 4 hours. As expected, virtually all cells were present in the M2 population (Figure 9E and 9F).

To confirm that an early loss of ΔΨ occurred, we used an alternative fluorophore, JC-1, that concentrates in the mitochondria, where it exists as monomers at a low membrane potential and as red fluorescent J-aggregates at a high membrane potential. In intact cells, JC-1 monomers accumulate as ΔΨ is lost. This is associated with an increase in fluorescence intensity at 530 nm (FL1) and a decrease in fluorescence at 585 nmol/L (FL2). In isolated mitochondria, the FL2 fluorescence or the FL2/FL1 ratio may correlate more closely with ΔΨ than the FL1 fluorescence. Consistent with the R123 data (Figure 9), H$_2$O$_2$ decreased the fluorescence of FL2 from 0 minutes (Figure 10A) to 30 minutes (Figure 10B), and this was reflected in a decrease in the FL2/FL1 ratio (Figure 10D). An increase in the fluorescence of FL1 also occurred (Figure 10A and 10B). At 60 minutes, an indication of a restoration of ΔΨ existed (Figure 10C and 10D).

**Discussion**

It is increasingly recognized that the Bcl-2 family and mitochondrial dysfunction are key components of the apo-
apoptotic process (reviewed in Reference 45). There have been few studies of either in relation to cardiac myocyte apoptosis. From the present study, it is clear that both anti- and proapoptotic Bcl-2 family proteins are expressed in the intact heart (Figure 1) and in isolated cardiac myocytes (Figure 3). However, although the proteins that are generally recognized as antiapoptotic (Bcl-2, Bcl-xL) are sustained during rat heart development, the proapoptotic proteins (Bad, Bax) seem to be downregulated. Recent evidence indicates that, in some situations, Bcl-2 may promote withdrawal from the cell cycle, whereas Bax induces cell cycle progression. It is, therefore, possible that the sustained expression of Bcl-2 and the downregulation of Bax may be associated with the withdrawal of myocytes from the cell cycle in the perinatal period. Reexpression of Bax in terminally differentiated myocytes, as is seen in human heart failure and in spontaneously hypertensive rat hearts, may promote apoptosis. Consistent with studies in other cell types, in unstimulated myocytes, Bcl-2 and Bcl-xL were essentially localized to the mitochondria, whereas Bad and Bax were predominantly present in the crude cytosolic fraction (Figure 3).

At least 2 alternatively spliced isoforms of Bcl-2 have been identified. Bcl-2α is composed of 236 amino acids, whereas Bcl-2β is lacking the C-terminal region and consists of 199 amino acids. The Bcl-2 antibody used in this study detected 2 immunoreactive species of 26 and 32 kDa (Figure 1A), both of which may exist as multiple bands (Figure 6A and 6B). It is generally accepted that Bcl-2α migrates at 26 kDa, and it has been reported that phosphorylation produces a band of 30 kDa, which may correspond to our 32-kDa Bcl-2 band(s). However, it is possible that this band may represent an alternative isoform expressed in the rat heart or may be a cross-reactive protein. Bcl-2 is phosphorylated on multiple sites within the N-terminal unstructured loop, which may account for the multiple bands within each immunoreactive species. Although Bcl-xL is structurally similar to Bcl-2 and possesses a similar N-terminal loop, we detected only a single band of 31 kDa (Figure 1B) and found no evidence of multiple bands. The Bad antibody detected 2 bands in the heart, which is consistent with a phosphorylated form (Figures 1C and 7); this is discussed in more detail below. Bax exists as multiple isoforms, which may account for the multiple bands detected in the heart (Figure 1D).
H$_2$O$_2$ was used at concentrations between 0.1 and 0.5 mmol/L, alone or in combination with FeSO$_4$. Our TUNEL data are consistent with these studies, and we found that the time course of this translocation (within 30 minutes) corresponded to a loss of cytochrome $c$ from the mitochondria, whereas von Harsdorf et al. transiently exposed myocytes to $H_2O_2/FeSO_4$ for 1 hour before incubating them in $H_2O_2$-free medium for a further 19 hours. The prolonged stress may activate additional pathways, resulting in further degradation of the 85-kDa cleavage product. In support of this, we detected an increase in a 25-kDa band (Figure 4B). This band has not been previously reported, but we suggest it may represent further degradation of the 85-kDa cleavage product.

The mechanisms that lead to caspase activation are not clear. In other cells, apoptosis is usually associated with the translocation of cytochrome $c$ into the cytosol, often accompanied by a loss of $\Delta \Psi$. Whether loss of $\Delta \Psi$ precedes or is a consequence of cytochrome $c$ translocation remains controversial. Although some studies infer that the MPTP and loss of $\Delta \Psi$ are essential for cytochrome $c$ release, others indicate that alternative mechanisms are involved. From this study, it is clear that oxidative stress stimulates cytochrome $c$ translocation in cardiac myocytes (Figure 8). We found that the time course of this translocation (within 30 minutes) corresponded to a loss of $\Delta \Psi$ (Figures 9 and 10), but cyclosporin A (which inhibits MPTP opening) did not inhibit cytochrome $c$ translocation (results not shown). Unexpectedly, we observed a partial restoration of $\Delta \Psi$ between 45 and 60 minutes (Figures 9 and 10), suggesting that cardiac myocytes may have some capacity for recovery. Similar recoveries of both $\Delta \Psi$ and energy levels have been demonstrated in other cell types. However, it is also possible that the early loss in $\Delta \Psi$ may be indicative of a subpopulation of myocytes undergoing more rapid cell death. These cells would fragment and be lost from the population subjected to FACS analysis at later time points. Because the remaining population of cells may be less damaged, the FACS data may suggest a partial recovery, when a change in the gated population is the true cause of the observed differences. The distinction between these 2 possibilities is extremely important, because in the first scenario, loss of $\Delta \Psi$ precedes or parallels the loss of cytochrome $c$ from the mitochondria, whereas in the second, loss of cytochrome $c$ clearly precedes the reduction in $\Delta \Psi$. Further studies of isolated cardiac myocyte mitochondria are necessary to clarify this issue. In myocytes, a subsequent progressive loss of $\Delta \Psi$ occurred over 6 hours (Figure 9), the time course of which correlates with PARP proteolysis (Figure 4A and 4B). We suggest that the loss of cytochrome $c$ from the mitochondria disrupts the electron transport chain, generating ROS that...
propagate cellular injury and may induce this phase of mitochondrial dysfunction. In support of this, it has been shown that mitochondrial electron transport is a significant source of oxidative stress in cardiac myocytes and other cells.60,61

The function of Bcl-2 in the mitochondria is not understood, although it has ion-conducting properties in lipid bilayers6 and appears to regulate proton flux.62 Bcl-2 also prevents the release of cytochrome c6,61 and subsequent activation of caspase-9.63 We observed that H2O2 induced the loss of Bcl-2 from the mitochondria (Figure 6), with a similar time course as the initial loss of ΔΨ (Figures 9 and 10) and cytochrome c release (Figure 8). It is tempting to speculate that the loss of Bcl-2 may trigger the loss of ΔΨ and result in release of cytochrome c. The mechanisms by which Bcl-2 is lost are unknown. We were unable to inhibit its loss by the caspase inhibitor ZVAD-fmk, suggesting that its degradation is not mediated by caspases (results not shown).

Phosphorylation of Bad at Ser-136 targets it to 14–3–3 proteins, which sequester Bad away from the mitochondria.58 Dephosphorylation of this site results in translocation to and association with Bcl-2, promoting apoptosis (reviewed in Reference 64). In cardiac myocytes, H2O2 induced the translocation of Bad to the mitochondria within 5 minutes (Figure 7A). In contrast to the accepted dogma,58 we found that the form of Bad present in the mitochondria comigrated with the upper band detected in the cytosol; it may represent a phosphorylated form (Figure 7C). A recent study suggested that Bad is phosphorylated on Ser-112 by protein kinase A at the mitochondria.14 Our data would fit with a model of Bad phosphorylation occurring at the mitochondria and with subsequent release into the cytosol. Subsequent to Bad translocation, it is lost from both the cytosolic and mitochondrial fractions (Figures 7A and 7B). The time course parallels the loss of Bcl-2 from the mitochondria (Figure 6A), suggesting that they may be associated. This is currently under investigation. Our data, showing that H2O2 induces the loss of both Bcl-2 and Bad (Figures 6 and 7), contrasts with the recent study by von Harsdorf et al.35 which showed no loss of either protein during oxidative stress–induced cardiac myocyte apoptosis.35 However, as mentioned above, we subjected our cells to a more prolonged cellular stress. It is now generally recognized that cardiac myocyte apoptosis is an important contributing factor to many disease states in the heart, although only limited studies exist on the mechanisms that may be involved. It is clear, however, that

Figure 9. Loss of ΔΨ induced by H2O2 (R123 FACS analysis). Cardiac myocytes were exposed to 0.5 mmol/L H2O2, stained with R123, and analyzed as described in Materials and Methods. M1 population represents myocytes with high R123 fluorescence (i.e., high ΔΨ); M2 population represents myocytes with low R123 fluorescence (i.e., low ΔΨ). A, Untreated myocytes; B, myocytes after 30 minutes of treatment with H2O2; C, myocytes after 1 hour of treatment with H2O2; D, myocytes after 6 hours of treatment with H2O2; E, myocytes exposed to oligomycin (10 μg/mL) and carbonyl cyanide 3-chlorophenylhydrazine (50 μmol/L) for 4 hours; and F, quantification of M1 and M2 populations in cells exposed to H2O2 for times indicated (n=6 to 9) or to oligomycin/carbonyl cyanide 3-chlorophenylhydrazine (P) (n=3). *P<0.002 vs unstimulated cells; †P<0.005 vs myocytes exposed to H2O2 for 0.5 hours.
overexpression of Bcl-2 can protect cardiac myocytes and that ischemia/reperfusion injury results in the opening of the MPTP, which is associated with mitochondrial dysfunction. On the basis of the present study, we propose that Bcl-2 maintains the homeostasis of the mitochondria. Translocation of Bad in response to H$_2$O$_2$ induces loss of Bcl-2 from the mitochondria, causing transient membrane depolarization and translocation of cytochrome c. Although damaged myocytes may recover to an extent, as illustrated by partial restoration of $\Delta\Psi$, cytochrome c cannot be recovered, resulting in irreversible mitochondrial dysfunction. Cell death then ensues. Further studies will clarify whether such mechanisms occur in vivo.

**Acknowledgments**

This work was funded by the British Heart Foundation. We thank Steve Rothery for assistance with the confocal microscopy.

**References**


**Figure 10. Early loss of $\Delta\Psi$ induced by H$_2$O$_2$ (JC-1 FACS analysis).** Cardiac myocytes were exposed to 0.5 mmol/L H$_2$O$_2$, stained with JC-1, and analyzed as described in Materials and Methods. A, Unstimulated myocytes; B, myocytes after 30 minutes of treatment with H$_2$O$_2$; C, myocytes after 60 minutes of treatment with H$_2$O$_2$. Early loss of $\Delta\Psi$ is associated with increase in FL1 fluorescence, as shown by shift to right (A and B, left), and decrease in FL2 fluorescence, as shown by shift to left (A and B, right). C, Indication of partial restoration of membrane potential. D, FL2/FL1 ratio. Data are expressed as mean±SEM (n=3).
Cook et al


49. Vanden Hoek TL, Shao Z, Li C, Schumacker PT, Becker LB. Mito-...
Regulation of Bcl-2 Family Proteins During Development and in Response to Oxidative Stress in Cardiac Myocytes: Association With Changes in Mitochondrial Membrane Potential

Stuart A. Cook, Peter H. Sugden and Angela Clerk

*Circ Res.* 1999;85:940-949
doi: 10.1161/01.RES.85.10.940

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/85/10/940

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/1999/11/05/85.10.940.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation Research* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation Research* is online at:
http://circres.ahajournals.org//subscriptions/