Bcl-2 family members and apoptosis, taken to heart

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Bcl-2 family members and apoptosis, taken to heart. Am J Physiol Cell Physiol 292: C45–C51, 2007. First published August 30, 2006; doi:10.1152/ajpcell.00229.2006.-—Loss of myocardial cells via apoptosis has been observed in many cardiovascular diseases and has been shown to contribute to the initiation and progression of heart failure. The Bcl-2 family members are important regulators of the mitochondrial pathway of apoptosis. These proteins decide whether the mitochondria should initiate the cell death program and release proapoptotic factors such as cytochrome c. The Bcl-2 proteins consist of anti- and proapoptotic members and play a key role in regulating apoptosis in the myocardium. The antiapoptotic proteins have been demonstrated to protect against various cardiac pathologies, whereas the antiapoptotic proteins have been reported to contribute to heart disease. This review summarizes the current understanding of the role of Bcl-2 proteins in the heart.

Heart failure is one of the leading causes of morbidity and mortality in the developed world. Loss of cardiac myocytes via apoptosis is believed to contribute to the continuous decline of ventricular function in heart failure. Cardiac myocytes are terminally differentiated and not replaced after they are lost. With fewer myocytes, the ability of the myocardium to sustain contractile function is reduced. Apoptosis has been implicated in the death of cardiac myocytes in cardiomyopathy (65, 79), myocardial ischemia-reperfusion (28, 68, 74), and congestive heart failure (30, 64). Although there has been tremendous progress in our understanding of cell death, the physiological and biochemical factors that lead to loss of cardiac myocytes in various heart disorders remain unclear. However, a better understanding of the apoptotic process in the myocardium is clearly important, as it may lead to the identification of novel therapeutic strategies.

APOPTOTIC PATHWAYS

Apoptosis is a highly regulated, energy-dependent suicide program, whereby the cell activates a signaling cascade that leads to cell death without triggering an inflammatory response. Apoptosis is mediated by two distinct evolutionarily conserved pathways: the extrinsic and intrinsic cell death pathways (20). The extrinsic pathway is activated when death ligands, such as Fas ligand or TNF-α, bind to their cognate receptors at the plasma membrane. This causes homotrimerization of the receptor and recruitment of specific adaptor proteins, such as Fas-associated death domain protein and procaspase-8, into a death-inducing signaling complex. This, in turn, leads to activation of initiator caspase-8, which subsequently activates effector caspases (3, 60). In contrast, in the intrinsic pathway, the mitochondria play a central role in the integration and execution of a wide variety of apoptotic signals, including loss of growth factors, hypoxia, oxidative stress, and DNA damage. The mitochondria provide the energy required for execution of the apoptotic program and release of proapoptotic proteins such as cytochrome c, endonuclease G, and apoptosis-inducing factor. Release of cytochrome c leads to apoptotic protease-activating factor (Apaf-1)-mediated activation of initiator caspase-9, which in turn activates effector caspases (99). Thus the extrinsic and intrinsic pathways have different initiator caspases but converge at the level of the effector caspases. Endonuclease G and apoptosis-inducing factor translocate from the mitochondria to the nucleus during apoptosis and are capable of inducing DNA fragmentation independent of caspases (53, 80).

BCL-2 FAMILY MEMBERS

The intrinsic pathway of apoptosis is regulated by members of the Bcl-2 family (Fig. 1). This family is composed of pro- and antiapoptotic proteins that share up to four conserved regions known as Bcl-2 homology (BH) domains (1). Antiapoptotic members such as Bcl-2 and Bcl-XL contain all four subtypes of BH domains and promote cell survival by inhibiting the function of the proapoptotic Bcl-2 proteins. Antiapoptotic Bcl-2 proteins have been reported to protect cells from many different apoptotic stimuli and are important for cell survival (58, 62, 69, 86). Interestingly, in some circumstances, Bcl-2 and Bcl-XL are targets of caspases, and cleavage of these proteins converts them from prosurvival to proapoptotic molecules that are able to induce cytochrome c release from the mitochondria (13, 16).

The proapoptotic members can be separated into two structurally distinct subfamilies. J) The “multidomain” proteins (Bax and Bak) share three BH regions and lack the BH4 domain. They are structurally similar to the antiapoptotic proteins (1, 81). 2) “BH3-only” proteins, which include Bnip3, Nix/Bnip3L, Bid, Noxa, Puma, and Bad, share only the BH3 domain and are structurally diverse (39). Most of the Bcl-2 family members contain a transmembrane domain at their COOH terminus, which is important for their targeting to intracellular membranes. Anti- and proapoptotic Bcl-2 proteins...
can be found in the cytosol, endoplasmic reticulum, mitochondria, and nuclear envelope (24, 26, 54, 97).

The BH3-only proteins function as death signal sensors in the cell and play a major role in transducing signals from the cytosol to the mitochondria. In mammals, ≥10 different BH3-only proteins, which differ in their expression pattern and mode of activation, have been identified. Their proapoptotic activity is regulated by transcription and/or posttranslational modification, and they selectively respond to specific death signals in the pathways they monitor (Fig. 2). For example, Noxa and Puma are under p53-mediated transcriptional control and are upregulated in response to DNA damage (61, 67). Bnip3 is upregulated in response to hypoxia via hypoxia-inducible factor-1α-dependent transcription (6, 71) and has been reported to be activated under acidic conditions (48).

Phosphorylated Bad is sequestered by 14-3-3 proteins under normal conditions, and growth factor deprivation leads to Bad dephosphorylation and activation (88, 94). Bim has been reported to monitor cytoskeletal integrity in some cell types and associates with the microtubule network. Disruption of microtubule function results in the release of Bim and activation of apoptosis (70). Bid is subjected to proteolytic cleavage by caspase-8, granzyme B, or calpain, and truncated Bid (tBid) translocates to the mitochondria, resulting in energetic failure and release of proapoptotic factors (10, 52, 55, 56).

The BH3-only proteins initiate cell death through the activation of Bax and Bak. Studies using cells derived from knockout mice lacking both Bax and Bak have demonstrated that Bax and Bak are essential for initiation of cell death through the intrinsic pathway. For instance, cells lacking both Bax and Bak are completely resistant to apoptotic stimuli that activate the intrinsic pathway, including staurosporine, UV light, and growth factor deprivation (90). Cells lacking only Bax or Bak were not resistant to these stimuli, suggesting a functional redundancy between Bax and Bak. Moreover, these cells were completely resistant to tBid-induced cytochrome c release and apoptosis (90). Similarly, overexpression of Bim or Bad failed to induce apoptosis in these cells, whereas expression of Bax restored susceptibility of the cells to Bim and Bad (98). Under normal conditions, Bax is localized to the cytosol, but, in response to death stimuli, Bax undergoes a conformational change that triggers its translocation to and insertion into the outer mitochondrial membrane. This leads to permeabilization of the outer mitochondrial membrane and release of proapoptotic proteins. It is not clear how Bax is kept in an inactive state in the cytosol and what controls the conformational change in Bax in response to stress. However, Bax function has been reported to be directly inhibited by Bcl-XL and Mcl-1 (77), as well as other antiapoptotic proteins such as apoptosis receptor with caspase association recruitment domain (33, 63), humanin (31), Ku70 (75), and heat shock protein 60 (32). In contrast, Bak is always localized to the mitochondria as an integral membrane protein and has been reported to be maintained in an inactive conformation by antiapoptotic Bcl-2 family proteins (92) or type 2 voltage-dependent anion channel (VDAC) (14).

Mitochondria are dynamic organelles that are constantly undergoing fission and fusion to adapt to changing conditions of the cell. Several recent studies have reported that mitochondrial morphology changes during apoptosis, resulting in small round mitochondrial fragments (23, 43, 46). The proapoptotic Bcl-2 proteins have been reported to mediate apoptosis through the mitochondrial fission pathway. Karbowski et al. (46) reported that Bak colocalized with dynamin-related protein-1 (Drp-1), which is involved in mitochondrial scission, at defined foci on the mitochondrial membrane at the onset of apoptosis. More importantly, a dominant-negative form of Drp-1 inhibited fragmentation and apoptosis, but not Bax translocation to the foci, in response to staurosporine treatment. In Caenorhabditis elegans, Drp-1-mediated mitochondrial fragmentation was induced by the BH3-only protein EGL-1 during developmental apoptosis (43), and overexpression of the BH3-only proteins Bnip3 and Bik has been reported to induce fragmentation in mammalian cells (26, 34).

A new role for the Bcl-2 family proteins is emerging as regulators of mitochondrial energetics. For instance, during ischemia, when mitochondrial electron transport and mitochondrial ATP generation are inhibited because of lack of oxygen, the F1F0-ATPase runs in reverse and pumps protons out of the matrix while glycolytic ATP is consumed in an
attempt to restore the mitochondrial membrane potential (73). Bcl-2 has been demonstrated to reduce the rate of ATP consumption during ischemia by inhibiting the F$_1$F$_0$-ATPase (42). Moreover, the BH3-only protein Bad has been reported to exist in a mitochondrial complex that includes glucokinase. Liver cells from Bad-knockout mice lack this complex and exhibit decreased mitochondrial respiration with glucose as a substrate, suggesting that Bad plays a role in regulating glucokinase activity (19). Adenine nucleotide transport across the mitochondrial membranes is an essential part of the process of mitochondrial energetics. The VDAC, together with adenine nucleotide translocator (ANT), transports ADP to the mitochondria and ATP to the cytosol, and the Bcl-2 family proteins have been reported to modulate activities of VDAC and ANT. Bcl-2 or Bcl-X$_L$ overexpression was found to maintain ATP/ADP exchange and mitochondrial respiration during growth factor deprivation (84), suggesting that Bcl-2 and Bcl-X$_L$ function to promote an open conformation of VDAC. In contrast, it was reported that Bcl-2 inhibited ANT activity and promoted VDAC closure, whereas Bax enhanced VDAC opening (4, 78). Another study reported that tBid induced VDAC closure, whereas Bax had no effect (72). Clearly, the function of the Bcl-2 proteins in ATP/ADP transport is controversial, and further studies are needed to define the role of the Bcl-2 proteins in mitochondrial energetics.

REGULATION OF APOPTOSIS

Exactly how the Bcl-2 family proteins regulate apoptosis is still unclear, but at least three different models of regulation have emerged from the literature. In the first model, the proapoptotic Bax and Bak are maintained in an inactive conformation through direct interactions with one or two different antiapoptotic Bcl-2 proteins. In response to an apoptotic stimulus, BH3-only proteins bind to and neutralize the antiapoptotic Bcl-2 proteins, thereby releasing Bax and Bak (Fig. 3A). Overexpression of Bcl-2 or Bcl-X$_L$ has been reported to prevent Bax translocation and activation (22, 85, 95), and Bak has been demonstrated to be sequestered by Bcl-X$_L$ and Mcl-1 in the mitochondria under normal conditions (92). In addition, it has been reported that certain BH3-only proteins display selective binding to specific antiapoptotic Bcl-2 family members. For instance, it has been reported that Bad interacts with Bcl-2 and Bcl-X$_L$, but not with Mcl-1, whereas Noxa binds to Mcl-1, but not to Bcl-2 and Bcl-X$_L$ (9). This indicates that, depending on the death stimulus, activation of more than one BH3-only protein may be required to release a proapoptotic protein to initiate apoptosis, allowing for tight control over apoptosis. This has been demonstrated by Willis et al. (92), who found that release of Bak from Bcl-X$_L$ and Mcl-1 and its subsequent activation required overexpression of Bad and Noxa; Bad and Noxa were without effect when either was expressed alone. In contrast, overexpression of Puma, which has been reported to have equal high affinity for all antiapoptotic Bcl-2 proteins, was sufficient to induce Bax-mediated cell death.

Alternatively, it has been shown that certain BH3-only proteins can interact with the proapoptotic proteins and trigger apoptosis by binding directly to Bax and Bak (Fig. 3B). It has been reported that tBid and Bim can directly interact with Bax (8, 36). Moreover, tBid, as well as peptides corresponding to the BH3 domains of Bim or Bid, directly activated Bax in cell-free assays (49, 50). A mutated Bax, unable to bind to tBid, did not mediate cell death induced by tBid (8), and

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**Fig. 3.** Models of Bcl-2 protein regulation in apoptosis. A: BH3-only proteins bind to and neutralize antiapoptotic Bcl-2 proteins, allowing Bax/Bak to become activated and initiate apoptosis. B: BH3-only proteins directly activate proapoptotic Bax/Bak protein. C: antiapoptotic Bcl-2 proteins sequester BH3-only proteins and keep them inactive.
mutants of Bid that did not interact with Bax were inefficient at activating apoptosis (8, 89), suggesting that a direct interaction between tBid and Bax is required for activation of Bax and induction of apoptosis. Interestingly, imaging of fluorescently labeled Bax and tBid showed that these proteins did not colocalize during apoptosis. Bax associated into distinct clusters on the mitochondria during apoptosis, whereas tBid circumscribed the mitochondrial membrane (66). Thus these studies suggest that although a direct interaction between tBid and Bax may be required for Bax activation, this interaction occurs transiently, and, once Bax is activated, tBid is released.

Finally, recent data suggest that antiapoptotic Bcl-2 family members sequester BH3-only proteins, preventing the activation of proapoptotic Bax and Bak (Fig. 3C). Eventually, the activated BH3-only protein will overcome the antiapoptotic Bcl-2 protein, thereby triggering the death process by direct activation of Bax/Bak or, possibly, activation of some other unknown factor in the cytosol or mitochondria required for Bax/Bak activation. This model is supported by a study by Cheng et al. (15), who found that Bcl-2 or Bcl-XL could sequester arriving BH3-only proteins, such as tBid and Bad, in a stable complex at the mitochondria, preventing Bax and Bak activation. Moreover, Bim is found to be strongly associated with Mcl-1 in viable myeloma cells (27) and Mcl-1 effectively inhibits Bim-mediated release of mitochondrial cytochrome c (35). The interaction between Bim and Mcl-1 is disrupted when apoptosis is induced (27). Similarly, Mcl-1 was recently reported to interact with tBid to inhibit cytochrome c release (17). Clearly, these studies demonstrate that the Bcl-2 proteins are likely regulated by multiple mechanisms, which may differ across cell types or within the same cell responding to different stimuli.

**ROLE OF BCL-2 FAMILY PROTEINS IN THE MYOCARDIUM**

It is becoming evident that the Bcl-2 family proteins play a central role in regulating apoptosis in the cardiovascular system. Pro- and antiapoptotic Bcl-2 proteins are expressed in the myocardium during development and in adult hearts. In the human heart, the ratio of pro- to antiapoptotic Bcl-2 proteins has been shown to shift toward proapoptotic in various pathological processes such as myocardial infarction, dilated cardiomyopathy, and ischemic heart disease (2, 21, 51).

Antiapoptotic Bcl-2 proteins have therapeutic potential for heart disease, since they have been shown to protect myocardial cells from various stresses. Bcl-2 has been shown to block p53-mediated apoptosis in cardiac myocytes (47), increase the calcium threshold for permeability transition pore opening in heart mitochondria (96), and inhibit hypoxia-reoxygenation-induced apoptosis in isolated adult cardiac myocytes (45). Moreover, transgenic mice overexpressing Bcl-2 in the heart had fewer apoptotic cells, reduced infarct size, improved recovery of cardiac function after ischemia-reperfusion (5, 12, 42), and attenuated phenotype in an animal model of cardiomyopathy (91). A consequence of ischemia is inhibition of electron transport and mitochondrial generation of ATP and the F\textsubscript{1}F\textsubscript{0}-ATPase running in reverse to consume glycolytically generated ATP (73, 87). Interestingly, Imahashi et al. (41) reported that transgenic mice overexpressing Bcl-2 in the heart showed a decreased rate of ATP decline during ischemia as well as reduced acidification, suggesting that Bcl-2 might provide myocardial protection by inhibiting consumption of glycolytically generated ATP by the F\textsubscript{1}F\textsubscript{0}-ATPase. Elevated expression of Bcl-X\textsubscript{L} by adenoviral gene transfer or perfusion of the heart with the BH4 peptide derived from Bcl-X\textsubscript{L} linked to a protein transduction domain (TAT-BH4) reduced ischemia-reperfusion injury in rat hearts in vivo and ex vivo (11, 40). In addition, Bcl-2 has been reported to play an important role in preconditioning. Exposure of hearts to short cycles of ischemia-reperfusion led to significant induction of Bcl-2 expression (57), whereas reduction of Bcl-2 levels via antisense oligonucleotides eliminated delayed ischemic preconditioning (37).

The proapoptotic Bcl-2 proteins have been implicated in the pathogenesis of various cardiac diseases, including myocardial hypertrophy, myocardial infarction, and heart failure. For instance, chronic hypoxia, stretch, and chronic pressure overload caused significant apoptosis in rat hearts, which correlated with increased levels of Bax and decreased levels of Bcl-2 (18, 44). Moreover, Bax has been reported to be activated in cardiac cells in response to oxidative stress (33) and during ischemia (7). Capano and Crompton (7) showed that Bax translocation to the mitochondria during ischemia was dependent on AMP-activated protein kinase and p38 MAPK in neonatal cardiomyocytes. Mitochondrial damage was reduced and infarct size was decreased after ischemia-reperfusion in hearts from Bax-deficient mice compared with wild-type animals, implicating Bax as a major player in ischemia-reperfusion injury (38).

Among the BH3-only proteins, Bnip3, Nix/Bnip3L, Puma, Bid, and Bad have been implicated in cardiac myocyte death. For instance, Bid has been reported to be subjected to proteolytic cleavage during myocardial ischemia-reperfusion, leading to release of cytochrome c into the cytosol (10, 11, 76). Murriel et al. (59) reported that ischemia-reperfusion induced significant increases in proapoptotic Bad protein levels but a reduction in the levels of antiapoptotic Bcl-2 and Bcl-X\textsubscript{L} proteins. Puma was induced in cardiac myocytes subjected to hypoxia-reoxygenation, whereas deletion of Puma resulted in a decrease in infarct size and an improvement in cardiac function after ischemia-reperfusion (82).

Two important BH3-only proteins that have been associated with mitochondrial dysfunction and cell death in the myocardium are Bnip3 and its homolog Nix/Bnip3L. These two proteins localize to the mitochondria and are upregulated in response to various stresses. For instance, Bnip3 has been shown to contribute to ischemia-reperfusion injury and was found to be upregulated in failing hearts (29, 34, 71), whereas Nix/Bnip3L has been implicated in cardiac hypertrophy and development of cardiomyopathy (93). These two proteins have been demonstrated to be upregulated by two different pathways in the heart: Nix by G\textsubscript{q}\alpha-mediated stimuli, such as phenylephrine (25), and Bnip3 by hypoxia (6, 25, 48, 71).

Similar to other BH3-only proteins, overexpression of Nix leads to release of cytochrome c and activation of caspase-3 (93). In contrast, the mechanism by which Bnip3 promotes cell death is unclear and somewhat controversial. The cell death pathway mediated by Bnip3 is unusual in several ways: it can initiate apoptotic or necrotic cell death through opening of the mitochondrial permeability transition port, and it may cause caspase-dependent and -independent cell death (34, 48, 71, 83). In hypoxia/acidosis-induced cell death, ATP levels and plasma membrane integrity were retained in neonatal cardiac myo-
cytes, implying apoptotic death; however, pretreatment with broad-range caspase inhibitors did not block cell death, and poly(ADP-ribose) polymerase, a caspase-3 substrate, did not undergo detectable cleavage (48). In contrast, it has been reported that Bnip3-mediated cell death was reduced in the presence of a caspase inhibitor in cardiac myocytes (34, 71). Thus it is not clear whether these differences in Bnip3-mediated cell death are due to the differences in the systems used to study Bnip3 or whether they reflect cell-specific regulation of Bnip3. Recently, Bnip3 was reported to cause extensive frag-
ed cell death are due to the differences in the systems used to study Bnip3 or whether they reflect cell-specific regulation of Bnip3.

In conclusion, tremendous progress has been made in our understanding of cell death; however, there are still considerable gaps in knowledge regarding the specific processes causing myocyte cell death. A major contributing factor to the initiation and progression of many cardiovascular diseases is the death of myocardial cells via apoptosis. Thus it is of clinical interest to explore various strategies to prevent loss of myocardial cells (34, 71). In contrast, it has been reported that Bnip3-mediated cell death was reduced in the presence of a caspase inhibitor in cardiac myocytes (34, 71).

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