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Forum Review

Redox Mechanisms of Cytoprotection by Bcl-2

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ABSTRACT

Bcl-2 is a multifunctional protein that protects against cell death induced by a wide variety of stimuli. The best characterized antiapoptotic Bcl-2 mechanism of action involves direct binding to proapoptotic proteins, *e.g.,* Bax, inhibiting their ability to oligomerize and form pores in the mitochondrial outer membrane, through which soluble mitochondrial proapoptotic proteins, *e.g.,* cytochrome *c,* are released into the cytosol. Bcl-2 also exerts antiapoptotic and antinecrotic effects that are mediated by its influence on cellular redox state and apparently independent of its interaction with proapoptotic proteins. Bcl-2 expression increases cell resistance to oxidants, augments the expression of intracellular defenses against reactive oxygen species, and may affect mitochondrial generation of superoxide radicals and hydrogen peroxide. This review focuses on the protective effects of Bcl-2 related to changes in mitochondrial redox capacity. *Antioxid. Redox Signal.* 7, 508–514.

INTRODUCTION

THE *bcl-2* oncogene was first described in a lymphoblastic leukemia cell line (42, 53) and found to promote cell proliferation, tumor generation, and resistance against cell death (45, 54). The product of this gene, Bcl-2, is an integral membrane protein targeted to the outer mitochondrial membrane (41), although it may also associate with other cellular membranes (16, 19, 38). Overexpression of this protein protects against both apoptotic and necrotic cell death induced by a variety of agents, including chemotherapeutic drugs, irradiation, oxidants, and glutathione depletion (17, 21, 29, 52; see Table 1). The range of cell death protocols in which Bcl-2 is found to be protective is indicative of the multifunctional character of this protein. Indeed, Bcl-2 has been shown to regulate transcription (36, 56), interact with proapoptotic members of the Bcl-2 family, *e.g.,* Bax (31, 43), regulate caspase activation (11, 20), have pore-forming properties (47), alter intracellular Ca^{2+} homeostasis (28, 33, 39), and increase cellular resistance to oxidative stress (10, 17, 22, 25, 40). This review focuses on the redox mechanisms through which Bcl-2 protects against cell death.

Bcl-2 PROTECTS AGAINST OXIDANT-INDUCED CELL DEATH

The concept that Bcl-2 increases cellular redox capacity was first suggested by Hockenbery *et al.* (17), based on the observation that this protein is located at the mitochondrion, a primary intracellular site of reactive oxygen species (ROS) generation. These authors also observed that Bcl-2 protects against cell death induced by oxidants, *e.g.,* hydrogen peroxide (H_2O_2) and menadione, in a manner similar to antioxidant molecules and enzymes, *e.g., N*-acetylcysteine and glutathione peroxidase. Finally, they found that classical apoptotic signals increased cellular lipid peroxidation in a manner prevented by Bcl-2. Their suggestion that Bcl-2 protects against oxidative stress was supported by the finding that Bcl-2 knockout mice displayed a 43% greater level of oxidized brain proteins, 27% fewer cerebellar neurons, and defective melanin synthesis and polycystic kidney disease, phenotypes consistent with chronic oxidative stress (15, 55).

Following these initial findings, many groups demonstrated that Bcl-2 overexpression protects cells against oxidant-mediated

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Table 1. Protection Against Oxidative Cell Death by Bcl-2 Family Members

Cell/animal type	*Form of cell death*	*References*
T cells from *bcl-2* transgenic mice	γ radiation, H_2O_2, menadione	17, 48, 52
Burkitt's lymphoma cell line transfected with *bcl-2*	C_6-ceramide, TNF-α, resulting in cellular oxidative stress	12
Saccharomyces cerevisiae expressing *bcl-2, ced-9,* or *bcl-xl*	Menadione, H_2O_2	6
SY5Y neuroblastoma cell line overexpressing Bcl-xL	H_2O_2	30
T cells transfected with *bcl-2*	*tert*-Butyl hydroperoxide	59
GT1-7 and PC12 cell lines overexpressing Bcl-2	Glutathione depletion, menadione, *tert*-butyl hydroperoxide, cyanide/aglycemia	21, 40, 61
HeLa, MCF-7, and mouse lymphoma cell lines overexpressing Bcl-2	Glutathione depletion, γ radiation	35, 36, 46

TNF-α, tumor necrosis factor-α.

damage promoted by γ-irradiation, H_2O_2, *tert*-butyl hydroperoxide, cyanide plus glucose deprivation, and ischemia/reperfusion (17, 21, 29, 40, 49, 52, 59; see Table 1). Overexpression of ced-9, the nematode homologue of Bcl-2, or the antiapoptotic protein Bcl-xL is also protective against oxidative damage and cell death (6), suggesting that this effect is a general role of the antiapoptotic members of the Bcl-2 family.

Bcl-2 expression also correlates with protection against the depletion of cellular glutathione (21, 35, 36, 57), a peptide whose sulfhydryl groups serve as the major source of antioxidant reducing power (34). Removing glutathione in Bcl-2-overexpressing cells restores sensitivity to cell death without affecting Bcl-2 levels (1, 36), suggesting that Bcl-2 protects against oxidants indirectly by increasing redox capacity. Some Bcl-2-overexpressing cell lines do, in fact, exhibit elevated levels of H_2O_2-removing enzymes, *e.g.,* glutathione and thioredoxin peroxidase (10). Moreover, overexpression of these antioxidant systems protects against cell death, independent of Bcl-2 expression levels (14, 60) (See Fig. 1).

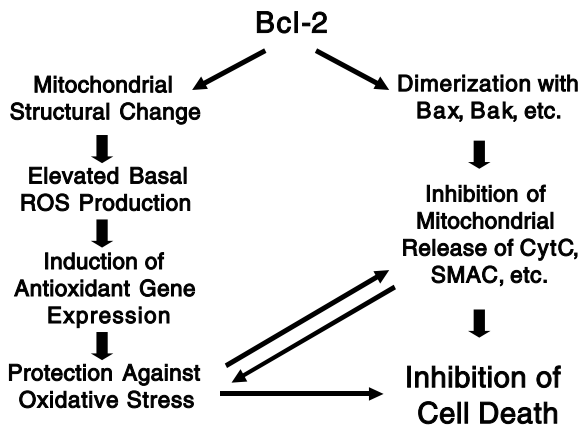

FIG. 1. Protection by Bcl-2 against cell death mediated by both anti-Bax and antioxidant mechanisms. CytC, cytochrome *c*.

Bcl-2 INCREASES CELLULAR REDOX CAPACITY

The initial observation that Bcl-2 protects against lipid oxidation and cell death promoted by oxidants, but does not inhibit the generation of ROS, suggested an increased ability to remove ROS in Bcl-2-overexpressing cells (17). Subsequent work revealed that Bcl-2-overexpression increased the antioxidant capacity of neural cell lines through elevation of either catalase, glutathione peroxidase, glutathione reductase, or reduced glutathione and NAD(P)H (10; see Table 2). Mirkovic *et al.* (36) found that depleting intracellular glutathione reversed the protection conferred by Bcl-2 against radiation-induced apoptosis, suggesting this protection was independent of the presence of the protein itself. The same effect was observed with cells overexpressing Bcl-xL, a protein with antiapoptotic and molecular characteristics similar to Bcl-2 (2). The correlation between Bcl-2, glutathione, and protection against cell death was subsequently well established in many cell death protocols and different Bcl-2-overexpressing cell lines (1, 35, 57; for review, see 56).

We and others have also found that Bcl-2 overexpression results in increased intracellular and mitochondrial NAD(P)H (10, 12, 22), another important redox source, responsible for the regeneration of reduced glutathione and thioredoxin (see below and 18). In mitochondria, increased levels of NAD(P)H prevent oxidation of inner mitochondrial membrane proteins that modulate the mitochondrial permeability transition (PT) (for review, see 23). The PT causes mitochondrial inner membrane depolarization and uncoupling of oxidative phosphorylation. Moreover, the net influx of solutes into the mitochondrial matrix through the PT pore causes large amplitude osmotic swelling, rupture of the mitochondrial outer membrane, and release of proapoptotic proteins, *e.g.,* cytochrome *c*, from their normal exclusive location within the space between the inner and outer membranes (5, 7, 32, 63). Consequently, PT may trigger necrosis or "accidental apoptosis," such as that which occurs when a necrotic event is insufficiently powerful to lead to immediate cell death, but sufficient to activate apoptotic pathways, *e.g.,* release of proapoptotic proteins from mitochondria (8). In Bcl-2-overexpressing cells, PT is inhibited

TABLE 2. EFFECT OF BCL-2 ON CELLULAR REDOX STATUS

Cell type	*GSSG/(GSSG + GSH)*	*NAD^+/NADH*	*Catalase*	*SOD*
PC12 Bcl-2(−)	0.95 ± 0.10	9.8	17.0 ± 1.0	120 ± 12
PC12 Bcl-2(+)	0.25 ± 0.15	3.0	29.0 ± 1.4	220 ± 22
GT1-7 Bcl-2(−)	1.40 ± 0.25	34.0	34.0 ± 0.6	145 ± 10
GT1-7 Bcl-2(+)	0.70 ± 0.15	18.0	31.0 ± 0.3	164 ± 13

Ratios of oxidized over total glutathione [GSSG/(GSSG + GSH)], oxidized over reduced pyridine nucleotides (NAD^+/NADH), and catalase and superoxide dismutase (SOD) activities (in units/mg of protein) were measured in PC12 and GT1-7 neural cell lines overexpressing Bcl-2. Adapted from reference 10, with permission.

(22, 32, 49). The mechanism by which Bcl-2 inhibits the PT is indirect and mediated by a resistance of mitochondrial NAD(P)H to undergo oxidation in Bcl-2-overexpressing cells. Thus, we demonstrated that, in the presence of a relatively low concentration of *tert*-butyl hydroperoxide (0.2 m*M*), NAD(P)H is oxidized and PT occurs in wild-type GT1–7 neural cells, but neither event is observed in Bcl-2-overexpressing cells (Fig. 2) (22). However, when digitonin-permeabilized cells are exposed to a high concentration of *tert*-butyl hydroperoxide (0.8 m*M*), mitochondria within both normal and Bcl-2-overexpressing cells undergo the PT in response to extensive NAD(P)H oxidation. The sensitivity of wild-type cell mitochondria to PT is decreased and therefore similar to that of Bcl-2 mitochondria when exogenous reducing power is used to minimize the oxidation of NAD(P)H caused by the peroxide. These findings are in agreement with the observation that although Bcl-2 protects against PT and cell death promoted by *tert*-butyl hydroperoxide, a NAD(P)H oxidant, Bcl-2 is ineffective against thiol cross-linking agents, *e.g.,* diamide and phenylarsine oxide, that directly oxidize thiol groups responsible for PT opening in a manner independent of NAD(P)H redox state (22, 59).

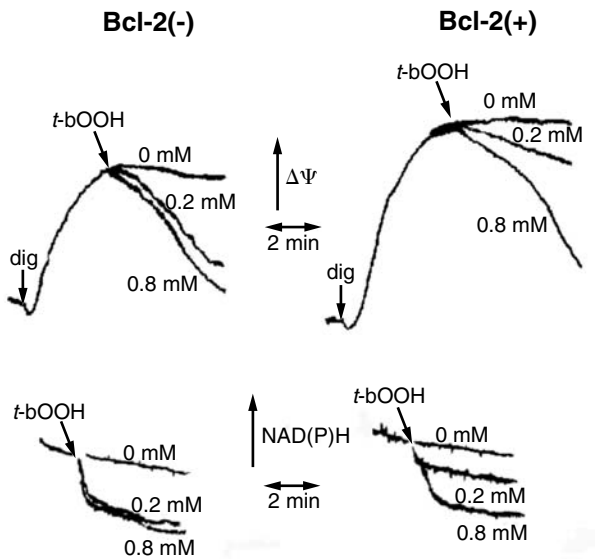

FIG. 2. Bcl-2(+) mitochondria in digitonin (dig)-permeabilized PC12 cells are more resistant to membrane potential (Δψ) decreases (upper panels) and NAD(P)H oxidation (lower panels) promoted by *tert*-butyl hydroperoxide (*t*-bOOH), added at the concentrations indicated. Adapted from reference 22, with permission.

The molecular mechanism by which Bcl-2 increases mitochondrial and cellular redox capacity remains unknown. However, the observation that different cell lines overexpressing Bcl-2 exhibit different patterns of elevated antioxidant defense systems (10) suggests that these phenotypes are a general response to effects of Bcl-2 on the normal intracellular environment, rather than a direct regulation of the transcription of these proteins by Bcl-2.

HOW DOES Bcl-2 INCREASE REDOX CAPACITY?

Although the increased redox capacity of Bcl-2-overexpressing cells is well established, the cause of this increased antioxidant expression is still poorly understood. One approach to this problem is to assess the regulatory mechanisms responsible for determining the expression of redox-related genes, and to determine what relationship they may have to Bcl-2 expression.

The p53 tumor-suppressing gene is a well-known regulator of redox-related genes (44) and promotes cellular formation of ROS and cell death. Moreover, p53 acts upstream of Bcl-2 expression (37), and therefore it is highly unlikely that antioxidants are increased in Bcl-2-overexpressing cells due to p53 down-regulation. There is also no evidence that Bcl-2 regulates gene transcription by any mechanism other than its effects on glutathione levels and distribution (56). Therefore, it is probable that Bcl-2 alters some other cellular parameter, which then affects glutathione synthesis and redox-related gene expression.

Another known regulator of cellular redox capacity is local oxygen tension (3, 27, 62). As Bcl-2 is a mitochondrial protein, it could potentially affect respiration and therefore intracellular oxygen tension, resulting in changes in antioxidant levels. However, experiments conducted by our group and others have not observed any significant differences in the quantity of mitochondria or rates of respiration in Bcl-2-overexpressing cells (39, 49).

Antioxidant proteins are also expressed in response to increased production of intracellular H_2O_2 (9, 13). Although the increase in ROS generation that occurs in response to apoptotic stimuli is blunted in Bcl-2-overexpressing cells (5, 17,

21, 58), the effects of Bcl-2 on steady-state mitochondrial H_2O_2 release under physiological conditions are not well characterized. Hockenbery *et al.* (17) did not find any differences in ROS release in Bcl-2-overexpressing cells. However, other publications reported that Bcl-2-overexpressing cells generate more ROS than Bcl-2(−) controls under physiological conditions (1, 12). Indeed, we have also found that mitochondria isolated from Bcl-2 and Bcl-xL-overexpressing cells generate higher rates of H_2O_2 (Fig. 3). Esposti *et al.* (12) suggested that the lack of previous detection of higher levels of ROS release in Bcl-2-overexpressing cells was due to the use of less sensitive probes. We have also found (25) that the presence of higher intracellular antioxidant levels in Bcl-2(+) cells can compensate for higher mitochondrial ROS release, resulting in the detection of similar ROS levels in intact cells.

The presence of chronically higher levels of mitochondrially generated ROS could certainly account for the larger expression of antioxidants in Bcl-2(+) cells. As a result, these cells are protected against acute oxidative insults, and exhibit lower ROS accumulation when subjected to conditions that normally lead to oxidative stress (5, 17, 21, 58). However, the mechanism through which Bcl-2 increases mitochondrial ROS release is undetermined.

POSSIBLE MECHANISMS BY WHICH Bcl-2 INCREASES MITOCHONDRIAL ROS PRODUCTION

Esposti *et al.* (12) correlated the increase in ROS measured in Bcl-2(+) cells with increased NAD(P)H levels, a finding compatible with data from our group indicating that Bcl-2(+) cells and mitochondria contain larger quantities of NAD(P)H (10, 22). Armstrong and Jones (1) found that rotenone increased ROS release levels, a result also compatible with a significant role of NADH. Rotenone leads to the accumulation of electrons removed from NADH in the iron-sulfur centers of the mitochondrial electron transport chain Complex I, increasing superoxide radical formation at or prior to this site (4). Recent work performed with highly sensitive fluorescent probes for H_2O_2 indicates that even in the absence of Complex I inhibition, NADH-dependent respiration supports significant mitochondrial ROS production regulated by both NADH redox state and mitochondrial membrane potential (26, 50). Based on these findings, we hypothesize that elevated mitochondrial NAD(P)H redox state in Bcl-2-overexpressing cells is caused by altered electron transport chain dynamics.

Mitochondrial NADH redox state is intimately related to the inner membrane potential and respiratory rates. No differences in respiratory rates between Bcl-2(+) and Bcl-2(−) mitochondria are apparent. However, Bcl-2(+) mitochondria accumulate greater quantities of membrane-potential probes, a finding initially interpreted as an indication of larger inner membrane potentials (22, 49). We recently reported that the membrane potential is identical in Bcl-2(+) and Bcl-2(−) mitochondria, but that these mitochondria respond differently to membrane potential probes (24). Flow cytometry measurements indicate that Bcl-2 expression results in increased mitochondrial size and membrane structural complexity, possibly reflecting larger membrane content. These structural differences explain the altered response these mitochondria exhibit in response to membrane potential probes (24).

A change in mitochondrial size and membrane content may also explain the increased NADH levels and ROS release in Bcl-2(+) cells. It is possible that Bcl-2 expression results in an increased ratio of mitochondrial matrix volume/membrane surface area, which could explain higher total matrix NAD(H) with equal respiratory activity. Under these conditions, the presence of higher levels of electron donors with equal electron transport rates could increase the probability of electron leakage at the respiratory chain or other mitochondrial redox sites, generating superoxide radicals and other ROS. A larger mitochondrial matrix volume could also support higher quantities of matrix enzymes, such as pyruvate, α-ketoglutarate, malate, glutamate, and isocitrate dehydrogenases, which could lead to more rapid NADH synthesis. Indeed, Bcl-2(+) mitochondria not only present increased total quantities of NADH and NAD^+, but also are more resistant to NADH oxidation (22). Based on these results and suppositions, we propose that increased NADH levels, possibly attributable to a larger matrix volume in Bcl-2(+) mitochondria, cause a subtoxic increase in mitochondrial ROS generation, ultimately increasing antioxidant capacity in Bcl-2(+) mitochondria and cells.

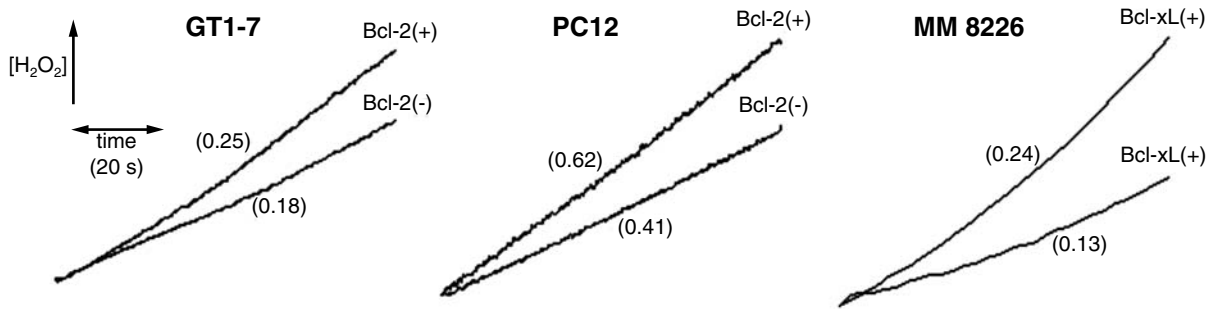

FIG. 3. Mitochondria isolated from GT1-7, PC12, and MM 8226 cells (as shown) were incubated in the presence of NADH-linked substrates and oligomycin, under experimental conditions similar to those described in references 24 and 25. H_2O_2 release was measured by following Amplex red oxidation in the presence of horseradish peroxidase, as described in references 25 and 50. Numbers in parentheses indicate H_2O_2 release rates, in nmol/min/mg of protein.

SUMMARY

The proposed effects of Bcl-2 on mitochondrial redox capacity, sensitivity to PT, release of cytochrome *c* caused by Bax or PT, and the relationship of these effects to cytoprotection are summarized in Fig. 1. Bcl-2 can inhibit the release of cytochrome *c* and other proapoptotic mitochondrial proteins by two mechanisms. One involves a direct interaction with proapoptotic proteins, *e.g.,* Bax and Bad, that localize or redistribute to the mitochondrial outer membrane. The other mechanism of inhibition is suppression of outer membrane disruption caused by the PT. Inhibition of PT by Bcl-2 is due to the increase in mitochondrial redox capacity afforded by Bcl-2 expression. In addition to decreasing the sensitivity of mitochondria to oxidant-induced PT, the increased redox capacity can protect against either necrotic or apoptotic cell death induced by oxidative stress through detoxification of ROS via glutathione reductase/peroxidase and other antioxidant systems. Finally, the dual mechanisms for inhibition of cytochrome *c* release by Bcl-2 also indirectly inhibit oxidative stress as extensive loss of cytochrome *c* results in a dramatic accumulation of electrons within mitochondrial redox components and stimulation of mitochondrial ROS generation (5, 26, 51).

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ABBREVIATIONS

H_2O_2, hydrogen peroxide; PT, permeability transition; ROS, reactive oxygen species.

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