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Review Article

Redox Aspects of Bcl-2 Function

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ABSTRACT

The oncogene Bcl-2 has attracted recent research attention as recognition of the importance of Bcl-2 control over apoptosis commitment in disease development and clinical response to therapy has been targeted for pharmacological intervention. Much of the basic science research regarding Bcl-2 has focused on the role that Bcl-2 plays in directly regulating mitochondrial function. This has come about because of Bcl-2's localization to mitochondrial membranes and its reported interaction with the mitochondrial megachannel. During the time that the mitochondrial function of Bcl-2 was being investigated, a smaller, yet potentially as important, role for Bcl-2 was being pursued by investigators who were following up the initial study of Bcl-2 knockout mice. These mice expressed a phenotype consistent with that of mice exposed to chronic oxidative stress. This research into the redox aspects of Bcl-2 function has led to a hypothesis that Bcl-2-expressing cells have enhanced antioxidant capacities that suppress oxidative stress signals generated during the initiation phase of many apoptotic pathways. This review will further develop the idea of Bcl-2's role in regulating cellular redox pathways associated with apoptosis, as well as integrate recently reported evidence that ties the antioxidant effects of Bcl-2 to mitochondrial function, thereby unifying both mitochondrial and redox aspects of Bcl-2 function. Antiox. Redox Signal. 2, 537–550.

INTRODUCTION

In addition to its critical role in organism development, the process of cell deletion known as apoptosis is now believed to be abnormally regulated in a number of different human diseases such as cancer, certain autoimmune disorders, AIDS, and various neurodegenerative diseases including Alzheimer's (Thompson, 1995). The need to understand further the etiologies of these diseases has generated an intense effort to delineate further the biochemical and molecular pathways involved in the regulation of apoptosis. Some of the most important aspects of this regulation appear to be governed by the Bcl-2 family of genes (Adams and Cory, 1998). This family of genes has been especially implicated in cancer. Bcl-2 itself, the prototypic member of this family, was originally discovered as the gene abnormally expressed due to the t(14;18) translocation associated with follicular lymphoma. In such cases, where Bcl-2 is abnormally expressed, apoptosis is blocked, leading to a survival advantage for the tumor cell. In spite of extensive research, the mechanism by which Bcl-2 blocks apoptosis remains elusive. However, the fact that Bcl-2 is localized in the mitochondrial membrane has generated considerable scrutiny of the role mitochondria play in facilitating aspects of apoptosis and Bcl-2's ability to block them at that site in the cell (Zamzami *et al.*, 1998).

A seemingly independent aspect of Bcl-2's function was initially discovered several years ago but has not been actively pursued. In 1993,

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Hockenbery *et al.* (1993) reported that Bcl-2 functioned in an antioxidant pathway to prevent apoptosis. This finding forms the basis for a theory that has emerged subsequent to that report suggesting that reactive oxygen species (ROS) are generated during the process of apoptosis and mediate at least part of the overall process (Buttke and Sandstrom, 1995; Slater *et al.*, 1995; Jacobson, 1996). An antioxidant activity, presumably enhanced in Bcl-2-expressing cells, would act to block the ability of ROS to mediate apoptosis. Recently, new evidence to support this theory has been put forth. Moreover, this new data is not inconsistent with an important role for mitochondrial functions in the process of apoptosis and Bcl-2's ability to modulate these functions. The purpose of this article is to review the evidence supporting Bcl-2's role in regulating cellular redox pathways associated with apoptosis.

ROS AND ANTIOXIDANTS IN APOPTOSIS

The suggestion by Hockenbery *et al.* (1993) that Bcl-2 functioned in an antioxidant pathway was based on their observation that menadione- and hydrogen peroxide (H_2O_2)-induced apoptosis was blocked in cells with enforced Bcl-2 expression. Thus, the idea emerged that ROS might be produced during apoptosis and mediate downstream events associated with the apoptotic process. These ROS could be suppressed by antioxidants present in the cell and, if Bcl-2 functioned in an antioxidant pathway, Bcl-2's inhibition of apoptosis could be explained on this basis. Two other reports that came out at the same time as that of Hockenbery *et al.* also supported this idea. In the first, Veis *et al.* (1993) examined the phenotype of Bcl-2 knockout mice and found that they have pathologies associated with defects in antioxidant pathways. For example, these mice suffered from severe polycystic kidney disease and follicular hypopigmentation; both phenotypes are consistent with chronic oxidative stress. In the second, Kane *et al.* (1993) described the suppression of apoptosis in cultured neuronal cells that overexpress Bcl-2. In control cells, ROS were generated when the cells were stimulated to undergo apoptosis, but this effect was suppressed in the Bcl-2-expressing cells. Moreover, the Bcl-2-expressing cells had higher intracellular levels of the antioxidant glutathione (GSH). This finding of increased amounts of GSH in Bcl-2-expressing cells has been confirmed in a number of other cell systems (Ellerby *et al.*, 1996; Mirkovic *et al.*, 1997).

Other studies have also suggested a role for ROS as part of the regulation of apoptosis. These reports have been extensively reviewed (Buttke and Sandstrom, 1995; Slater *et al.*, 1995; Jacobson, 1996; Hampton and Orrenius, 1998). Generally, apoptosis can be induced by the addition of ROS or depletion of cellular antioxidants and inhibited by the addition of exogenous antioxidants. Moreover, in many cases, apoptosis is associated with a stimulated production of intracellular ROS. ROS can be generated in cells by a variety of mechanisms to induce apoptosis. Intracellular ROS can be generated in cells in response to apoptosis-inducing agents such as tumor necrosis factor (TNF) or ceramide. In addition, as mentioned above, apoptosis can be induced by exogenously added H_2O_2, an important ROS, but it can also be induced through oxidation of the endogenous cellular thiols, *i.e.*, GSH, that act to protect the cell from ROS produced during oxidative metabolism, *i.e.*, H_2O_2. Intracellular GSH can be oxidized or inactivated by agents such as menadione, diamide, or diethyl maleate (Hockenbery *et al.*, 1993; Marchetti *et al.*, 1997; Mirkovic *et al.*, 1997). Lowering intracellular levels of GSH with an inhibitor of its synthesis, buthionene sulfoximine (BSO), also drives cells into apoptosis due to the oxidative stress created by the absence of this critical thiol antioxidant (Kane *et al.*, 1993).

On the basis of the ability of ROS to stimulate apoptosis, it becomes evident that antioxidants may act to suppress this process. A number of antioxidants have been identified to have such activity (Buttke and Sandstrom, 1995; Slater *et al.*, 1995). These include naturally occurring intracellular antioxidants such as GSH, which has already been mentioned, but also thioredoxin (Baker *et al.*, 1997). Exogenously added thiol antioxidants such as *N*-acetylcysteine (NAC) are also potent inhibitors of apop-

tosis (Mayer and Noble, 1994). Indeed, NAC has been used to treat AIDS patients by suppressing the apoptosis of T cells infected with the virus (Herzenberg *et al.,* 1997). In addition to antioxidant agents, antioxidant enzymes may also act to suppress ROS-mediated apoptosis. The best studied of these is superoxide dismutase (SOD). Superoxide is one of the ROS produced during the apoptosis process (Cai and Jones, 1998), and cells that have been engineered to overexpress SOD are relatively resistant to apoptosis induction (Wong *et al.,* 1989). Bcl-2 overexpression (Merad-Saidoune *et al.,* 1999) mimics the effect of SOD overexpression, suggesting a relationship between these proteins, and transfection of SOD-deficient yeast stains with a Bcl-2 expression construct protects them from oxidative stress-induced cell death (Kane *et al.,* 1993). Additionally, the phenotype of SOD knockout mice is strikingly similar to Bcl-2 knockout mice (Huang *et al.,* 1997). Thus, whereas Bcl-2 protein would not be expected to have SOD-like activity, Bcl-2 must mimic SOD by either preventing superoxide production during apoptosis or scavenging superoxide directly by acting as an antioxidant.

BCL-2 AND MITOCHONDRIA

Bcl-2 is the prototypic member of a rather large and growing family of proteins that share significant homology, in spite of fact that some members of the family have anti-apoptotic activity, *e.g.,* Bcl-2 and Bcl-X_L, whereas others have pro-apoptotic function, *e.g.,* Bax and Bak (Adams and Cory, 1998). Although Bcl-2 and Bax may be capable of functioning as independent proteins, their activity is also regulated through dimerization (Korsmeyer *et al.,* 1993). The homology among the family members is conserved in four domains: BH1, BH2, BH3, and BH4.

The BH3 domain appears to be critical for the dimerization property. Sequence analysis has shown that many Bcl-2 family members have a hydophobic stretch of amino acids at the carboxy-terminal region that is responsible for their localization to cellular membranes, including the endoplasmic reticulum, nuclear envelope, and mitochrondrial membrane (Adams and Cory, 1998). At the expense of understanding the role of Bcl-2 localized in other membranes of the cell, the focus of research on apoptosis over the last few years has been on mitochondria. This focus has been driven by two independent discoveries demonstrating the participation of mitochondria in the process of apoptosis. These discoveries indicated that: (i) mitochondria undergo a membrane permeability transition (PT) during apoptosis (Zamzami *et al.,* 1995, 1998; Susin *et al.,* 1998) and (ii) a number of apoptogenic factors, *e.g.,* cytochrome-*c*, are released from the mitochondria of apoptotic cells (Liu *et al.,* 1996; Zou *et al.,* 1997).

Zamzami *et al.* (1995) originally reported that in cells undergoing apoptosis, the mitochondrial membrane potential, $\Delta\Psi_m$, is lost and this disruption of $\Delta\Psi_m$ has been attributed to the opening of the inner mitochondrial PT pore. Opening of these pores or so-called "mega-channels" allows for the free distribution of solutes and equilibrium of ions within the mitochondrial matrix (Reed *et al.,* 1998). This, in turn, causes mitochondrial swelling and uncoupling of oxidative phosphorylation. This uncoupling of the respiratory chain causes cessation of ATP production, and electrons normally going to molecular oxygen are shunted into ROS production. The PT pore is thought to consist of multiprotein complex consisting of an inner mitochondrial transmembrane protein, the adenine nucleotide translocator (ANT), that associates with an outer transmembrane protein, the voltage-dependent anion channel (VDAC) (Susin *et al.,* 1998). Other proteins in the complex may include the peripheral benzodiazepine receptor and matrix cyclophilin D (Gross *et al.,* 1999). The PT pore regulates ADP/ATP exchange and disruption of this process during apoptosis leads to several consequences including acidification of the cytosol (Vander Heiden *et al.,* 1999).

In addition to the loss of $\Delta\Psi_m$, a number of factors are released from mitochondria that also play important roles in facilitating apoptosis. One of the factors that may be associated with PT is the apoptosis-inducing factor (AIF) (Susin *et al.,* 1996). AIF is targeted to the nucleus and may participate in the nuclear

changes associated with apoptosis. The other factors released from mitochondria participate in caspase-mediated apoptosis. These include cytochrome *c* and Apaf-1 (Liu *et al.*, 1996; Zou *et al.*, 1997). Together with caspase-9, cytochrome *c* and Apaf-1 form a complex that activates caspase-3 by proteolytic cleavage of its proform into its active form (Gross *et al.*, 1999). Caspase-3, once activated, is responsible for many of the downstream events associated with apoptosis including activation of DNA fragmentation factor (DFF) (Liu *et al.*, 1997). DFF activates endonucleases that cleave DNA in a manner characteristic of apoptosis. Certain caspases, including caspases 2 and 9, may also be normally sequestered in mitochondria and redistributed to the cytosol and activated on initiation of apoptosis (Susin *et al.*, 1999).

The exact mechanism by which cytochrome *c* and these other factors are released from mitochondria is not completely understood but it does not appear to be due to the loss of $\Delta\Psi_m$ because cytochrome *c* release precedes loss of $\Delta\Psi_m$ at least in some systems (Cai *et al.*, 1998). On the other hand, members of the Bcl-2 family are involved in regulating both of these features. For example, overexpression of Bcl-2 independently blocks both loss of $\Delta\Psi_m$ and release of cytochrome *c* (Zamzami *et al.*, 1996; Kluck *et al.*, 1997; Yang *et al.*, 1997). Conversely, enforced expression of Bax, a pro-apoptotic member of this family, results in altered $\Delta\Psi_m$ and release of cytochrome *c* (Jurgensmeier *et al.*, 1998; Reed *et al.*, 1998). Clues related to how Bcl-2 family members may act in membranes to regulate apoptosis have come through structural analysis of the proteins. The X-ray crystallographic structure of Bcl-X_L revealed a similarity to bacterial diphtheria toxin and suggested a pore-forming function (Muchmore *et al.*, 1996). The structures of Bcl-2 and Bax are similar with regard to this possibility. Subsequently, it has been shown that Bcl-2, Bcl-X_L, and Bax are able to form functional ion channels when added to synthetic membranes *in vitro* (Minn *et al.*, 1997; Reed, 1998). However, the properties of these channels vary considerably among the family members. Thus, during apoptosis Bax is translocated from the cytosol to mitochondria where it may form a large channel in the outer membrane, allowing cytochrome *c* to be released. Bcl-2 or Bcl-X_L may form small conductance channels in mitochondrial membranes that act to counterbalance the effect of Bax by maintaining electrical homeostasis and membrane integrity or, alternatively, their presence may prevent channel formation by Bax (Reed, 1998). Of course, Bcl-2 family members dimerize with themselves and each other and how this activity functions in the regulation of apoptosis at the level of the mitochondria is not clear.

ROS GENERATION BY MITOCHONDRIA DURING APOPTOSIS

Mitochondria are the primary source of ROS generated during apoptosis, although they may not be the only source. The loss of cytochrome *c* described above produces an interruption in the electron transport chain because cytochrome *c* is responsible for transferring electrons from respiratory complex III to IV (Cai *et al.*, 1998). This loss of cytochrome *c* is responsible for an inhibition of oxygen consumption in cells undergoing apoptosis (Ghafourifar *et al.*, 1999).

However, as demonstrated in an elegant study by Cai and Jones, superoxide is also generated as a result of this diversion or leakage of electrons (Cai and Jones, 1998). Moreover, a shift in the redox potential of the cell toward oxidation as measured by the GSH/GSSG ratio occurs due to the oxidation of GSH to GSSG by the ROS. Cai and Jones show that this shift in redox potential occurs 2 hr after the initiation of apoptosis, coinciding with superoxide production, whereas the loss of cytochrome *c* in their system was detected at 1 hr. Thus, superoxide production and the shift in redox potential are the result of cytochrome *c* release and not the cause. Since the loss of $\Delta\Psi_m$ occurs later, it is also a result of the ROS production and subsequent redox change and not the cause of these effects or of the release of cytochrome *c* (Cai and Jones, 1998).

On the basis of the report by Cai and Jones, it is now well established that mitochondria produce ROS during apoptosis (Cai and Jones, 1998). However, the idea that ROS played important roles in apoptosis was controversial for

a time because of an early report indicating that cells lacking mitochondria DNA, and hence unable to carry out respiration, were still able to undergo apoptosis (Jacobson *et al.*, 1993). In addition, other reports suggested that cells could still undergo apoptosis under conditions of virtual anaerobiosis, a condition where mitochondria were presumed to only produce very low levels of ROS (Jacobson and Raff, 1995; Shimizu *et al.*, 1995). An antioxidant role for Bcl-2 was also questioned in these reports because Bcl-2 expression blocked apoptosis in both situations. These issues have now been largely resolved based on three recent reports. In the first of these, Esposti and McLennan demonstrate that H_2O_2, the product of SOD's reaction with superoxide, is produced by mitochondria undergoing apoptosis in virtual anaerobiosis (Degli Esposti and McLennan, 1998). Nanomolar quantities of H_2O_2 were detected using very sensitive techniques in samples of cells and mitochondria that had been depleted of oxygen and exposed to ceramide. The authors provide two possible explanations for this finding that involve the presence of submicromolar concentrations of oxygen remaining in the medium under anaerobic conditions, which could catalyze superoxide and H_2O_2 production and that the abundant reduced iron present in mitochondria could catalyze self-sustaining Fenton reactions with the H_2O_2 that is produced. Esposti and McLennan deduce that the former conclusions that ROS produced by mitochondria do not play a role in apoptosis because cells in virtual anaerobiosis maintain an apoptotic response are based on flawed assumptions.

In the second paper, which is also from this same group, Esposti *et al.* (1999) have examined the effect of Bcl-2 expression on the intracellular distribution and production of H_2O_2 under basal conditions and after treatment of lymphoma cells with apoptosis-inducing agents. Interestingly, they found that Bcl-2-expressing cells had increased availability of mitochondrial NAD(P)H and this was correlated with increased constitutive mitochondrial production of H_2O_2. In response to ceramide, H_2O_2 production was increased in the non-Bcl-2-expressing cells that underwent apoptosis but not in the Bcl-2-expressing cells that were resistant to apoptosis. This finding is in fundamental agreement with the results of Cai and Jones (1998), who showed that Bcl-2 expression blocked superoxide production through its ability to block cytochrome *c* release. The finding by Esposti *et al.* that Bcl-2-expressing cells have higher basal levels of H_2O_2 production is in agreement with the results of Steinman (1995), who several years ago claimed that, in addition to its well-known antioxidant property, Bcl-2 could also act as a pro-oxidant. These apparently disparate activities ascribed to Bcl-2 are now explained by Esposti *et al.* on the basis of their suggestion that Bcl-2 expression allows lymphoma cells to adapt to conditions of increased oxidative stress by fortifying their antioxidant defenses against the overproduction of ROS generated in response to apoptotic stimuli. They point out in particular that the increase in NAD(P)H observed in their study would fortify the ROS-scavenging defense of mitochondrial function via the coupled activities of glutathione reductase and glutathione peroxidase.

In the third paper, Jiang *et al.* (1999) tested whether cytochrome *c* release and loss of $\Delta\Psi_m$ occur in cells lacking mitochondrial DNA. Their results show that, in osteosarcoma cells treated with ethidium bromide to deplete mitochondrial DNA, cytochrome *c* release and disruption of $\Delta\Psi_m$ are preserved during staurosporine-induced apoptosis. However, the ROS-mediated oxidation of thiols, detected through assessment of the redox potential as indicated by the GSH/GSSG ratio, was greatly attenuated in this setting. This result suggests that the release of cytochrome *c* and loss of $\Delta\Psi_m$ are not due to the oxidation of thiols. Thus, the earlier conclusion of Jacobson *et al.* (1993) that mitochondrial oxidative phosphorylation is not required for apoptosis was confirmed in this recent study. Moreover, the work of Jiang *et al.* indicates that mitochondrial generation of ROS is not essential for the induction of apoptosis by staurosporine. These authors suggest, however, that mitochondrial generation of ROS may be important for parallel or potentiating events in apoptosis other than, or in addition to, cytochrome *c* release and caspase activation. Indeed, this otherwise supplemental signal mediated by ROS may be re-

quired in some systems such as TNF or Fas-induced apoptosis. One other important point that was made in the discussion of their paper deserves mention. They stated that the cells lacking mitochondrial DNA were more oxidized even in the untreated condition compared to the cells with normal mitochondria. They explain this on the basis of cellular sources of ROS other than mitochondria such as NADPH oxidase, lipooxygenases, xanthine oxidase, *etc*. The role of these processes in apoptosis is not known, but it seems possible that ROS could be generated during apoptosis by mechanisms not involving mitochondria that participate in the apoptosis cascade of events. If so, Bcl-2 would block these ROS through the up-regulation of antioxidant properties of the cell, *e.g.,* GSH, similar to what was shown above in the study by Esposti *et al* (1999).

Bcl-2 and GSH

Observations of increased levels of intracellular GSH in cells expressing Bcl-2 have been reported for many different systems. Previously, we reported that LYar cells, as well as other cells containing expression constructs for Bcl-2, had significantly higher levels of intracellular GSH, particularly in the nuclei of these cells (Mirkovic *et al.,* 1997; Voehringer *et al.,* 1998). The mechanism of how this is achieved, however, has remained elusive. This raises the question: Is GSH synthesis increased by increased activity or level of the cytosolic rate limiting enzyme γ-glutamyl cysteine synthesis (GCS), or is exogeneous GSH imported into cells? In our hands, there are no apparent differences in the enzymes responsible for GSH biosynthesis in Bcl-2-expressing cells (unpublished results). Therefore, one of the mechanisms a cell can employ to raise its GSH levels is by importing more GSH. GSH itself cannot freely cross membranes and therefore some transport mechanism must be invoked. The idea of GSH transport into cells through the amino acid cycle was first proposed by Miester *et al.* (1995) where extracellular GSH catabolism, and amino acids, especially cysteine, are imported (Meister, 1995). To facilitate GSH uptake into cells, γ-glutamyl tranfersase (GGT) hydrolyses GSH to glutamic acid and cysteinylglycine, which is then cleaved by a membrane-bound constitutive dipeptidase into the constituent amino acids of GSH (glutamate, cysteine, and glycine). The free amino acids can then enter the cell and contribute to the GSH pool or cysteine pool (Meister, 1995). GSH can then be synthesized from its amino acid components and transported to intracellular organelles (such as the nucleus), thereby raising total intracellular GSH levels without inhibiting GCS activity.

Recent findings using DNA microarrays support the GSH import hypothesis, where it was observed that significant quantitative differences at the level of mRNA correctly predicted an elevated protein level of surface CD53 when we compared the Bcl-2 $+/-$ cell model system (Voehringer *et al.,* 2000). CD53 (along with CD9, CD37, CD81, and CD82) is a member of the membrane-spanning tetraspanin 4 (TM4) family of surface proteins that facilitate the interaction between surface proteins; however, their binding partners are just now being identified (Maecker *et al.,* 1997). Recently, Nichols *et al.* (1998) showed that TM-4 proteins are involved in the formation of a complex with CD19, CD23, and GGT that regulates intracellular redox potential by recycling of extracellular GSH. Association of TM4 proteins and GGT was not specific only to B cells, because the authors have also reported these associations in T cells. In support of these findings, in Bcl-2-expressing cells that express high levels of CD53, GGT activity is significantly increased (in preparation).

ADAPTATION OF BCL-2-EXPRESSING CELLS THROUGH INDUCED TRANSCRIPTIONAL CONTROL OF METABOLIC PATHWAYS

Gene expression is regulated by intracellular homeostasis, with shifts in pro- and antioxidant conditions playing an important role in fine-tuning expression programs of a cell in response to its redox state. Because ROS are constantly generated during normal biological reactions, the cell is in a constant state of altering transcription in response to the oxidative load on it. Redox regulation of transcriptional

activation can occur on multiple levels. First, many transcription factors exist in the cytosol in their pro- form, where alterations in cytosolic redox can affect their activation and trigger nuclear localization. A well-studied example of this model is NF-κB (Staal *et al.,* 1990). Second, most transcription factors have cysteine residues in their DNA-binding domain forming zinc fingers that bind the DNA major groove through coordinate interactions between cysteines and zinc. These cysteines are redox sensitive, and the oxidation states of the cysteines regulate the ability of the transcription factor to bind the recognition sequence. Thioredoxin and Ref-1 are two known facilitators of DNA binding of these transcription factors via their ability to modify the redox state of the DNA-binding domain cysteines. Last, the redox environment of the nucleus as a whole most likely has an effect on accessibility of transcription factors to their targets. This may explain how some transcription factors, which have a plethora of transcriptional targets, only turn on specific genes. Therefore, the redox state of a cell and GSH subcellular compartmentalization has the potential to affect transcription at multiple levels, allowing fine-tuning of transcriptional responses to different stimuli.

Many models of apoptosis are known to have signal transduction pathways mediated by ROS. In a report investigating adenovirus P53-mediated apoptosis in a colon carcinoma cell line, the authors reported the induction of genes during apoptosis. Notably, they observed expression of p53-induced redox-related genes in pathways that lead to the generation of reactive oxygen species and oxidative damage to mitochondria (Polyak *et al.,* 1997). Presumably, in cells that do not undergo apoptosis, these pathways are attenuated.

Reports of nuclear pools of GSH and regulation of nuclear GSH localization suggest that Bcl-2 may function to modify transcriptional programs (Voehringer *et al.,* 1998; Voehringer, 1999). To this end, in addition to the above-mentioned differences in gene expression for surface markers, DNA array analysis of apoptosis-sensitive and -resistant (Bcl-2 − and +) cell lines revealed several novel apoptosis regulatory genes (Voehringer *et al.,* 2000). Striking among these were alterations in genes controlling intracellular redox through mitochondrial and nonmitochondrial pathways (the aforementioned CD53, fructose-1,6-bisphosphatase, fatty acid binding proteins, uncoupling protein, and voltage-dependent anion channel). Although some of these genes are known to modify redox directly, others alter redox through indirect signaling pathways.

In addition to the direct effect of CD53 on intracellular redox, fructose 1-6 biphosphatase, involved in gluconeogenesis, generates glucose-6-phosphate, suggesting that glucose-6-phosphate might be shunted into the pentose phosphate pathway to generate reducing equivalents (Stryer, 1999). Enhanced activity of the pentose phosphate pathway generates NADPH via the oxidation of glucose-6-phosphate to fructose-6-phosphate and glyceraldehyde-3-phosphate (Stryer, 1999). The reducing equivalents provided by NADPH, in turn, contribute to the reduction of oxidized glutathione by glutathione reductase.

This model supports our previous findings that apoptosis-resistant Bcl-2 overexpressing cells have elevated pools of glutathione primarily in the reduced form (Mirkovic *et al.,* 1997). Additionally, reports (mentioned above), specifically by Esposti *et al.*, describe NAD(P)H pools are elevated in Bcl-2-expressing cells (Esposti *et al.,* 1999). Thus, Bcl-2 may alter transcription of TM4 proteins and enzymes involved in NAD(P)H elevations to maintain an elevated, reduced pool of GSH within cells.

REDOX ALTERATIONS IN SIGNAL TRANSDUCTION

Redox status of the cell alters not only how it responds to exogeneous stimuli, but also defines the internal state of the cell. This leads to suppressed spontaneous apoptosis and prevents the ability of apoptosis-inducing agents to trigger apoptosis. Some of this suppression may be due to direct interactions of the inducing agents with a reducing environment (as many proapoptotic compounds are also oxidants), but also indirectly by mediating the response of signaling and transcriptional

pathways. Down-regulation of three mitochondrial-targeted response pathways in response to apoptotic stimuli has been reported in gene array results, as stated above.

Although fatty acid binding proteins (FABPs) have not previously been investigated with respect to apoptosis, studies describing FABPs in the nematode suggest these proteins may act as molecular scavengers, binding toxic oxidized fatty acids generated during oxidative stress (Mei *et al.,* 1997). From these reports it can be suggested that FABPs may play an important role in terminating a signal transduction cascade of lipid peroxidation, observed as an early step in the commitment phase of many apoptotic systems. It is interesting that increased lipid peroxidation was observed in Bcl-2 knockout mice (Hockenbery *et al.,* 1993). The increased lipid peroxidation observed by Hockenbery *et al.* led them to speculate that Bcl-2 functioned in some direct manner to inhibit lipid peroxidation. Studies showing elevated FABPs in Bcl-2-expressing cells, however, suggest that Bcl-2 may function, through nuclear GSH compartmentalization, as a transcriptional regulator upstream of lipid peroxidation (Voehringer *et al.,* 1998; Voehringer, 1999), thereby enhancing a cell's capacity to buffer and terminate these destructive pathways.

A second interesting pathway observed during apoptosis, but suppressed in Bcl-2-expressing cells, involves mitochondrial uncoupling protein (UCP). UCP-1, originally identified in brown adipose tissue and implicated in regulating thermogenesis (Cassard *et al.,* 1990), has since been detected in skeletal muscle and appears to regulate fatty acid metabolism (Boss *et al.,* 1997; Fleury *et al.,* 1997). Mitochondria are the targets of UCP function as they are involved in uncoupling electron transfer from oxidative phosphorylation, an observation seen frequently in mitochondria-mediated apoptosis. Free fatty acids are also known uncouplers of electron transport, and release of apoptotic factors from the mitochondria has been attributed to the effect of free fatty acids (FFAs) (ceramide and arachidonic acid) on mitochondria (Quillet-Mary *et al.,* 1997). Because mitochondria are the source of cytochrome *c*, apoptosis-initiating factor, and ROS generated during apoptosis, specific up-regulation of message for one of the UCP family members (UCP-2) suggests that there may be another mechanism through which mitochondrial function is altered prior to any observable mitochondrial deregulation. Whether UCP-2 can function to activate mitochondrial release of apoptotic factors (increased levels of UCP-2 have been shown to induce peroxide generation from mitochondria; Negre-Salvayre *et al.,* 1997, or whether free fatty acids are also required, needs to be determined; however, this pathway may prove to be significant because it indicates a transcriptional level of control of mitochondrial function.

VDAC-1 is also elevated at the mRNA level to a greater extent in non-Bcl-2-expressing cells when compared to Bcl-2-positive cells in response to radiation. VDAC-1 is part of a PT complex that includes the adenine nucleotide translocator and cyclophilin D. Opening of the PT, with release of these factors, may be the ultimate checkpoint before commitment to apoptosis occurs, and has been shown to be under the regulation of Bcl-2 family members (Hirsch *et al.,* 1997; Shimizu *et al.,* 1999). Indeed, Tsujimoto *et al.* recently reported that both pro- and anti-apoptotic members of the Bcl-2 family are capable of binding the PT pore though interactions with VDAC-1 (Shimizu *et al.,* 1999). Linkage between UCP, VDAC, Bcl-2, and Tom70, a mitochondrial translocase, has been previously reported, where the cytosolic domain of the mitochondrial translocase interacts and organizes these proteins (Schleiff *et al.,* 1997). How all these players function in regulating mitochondrial ROS production and apoptosis is yet to be determined; however, the regulation of genes that encode mitochondrial proteins appears to be important.

MITOCHONDRIAL GSH

The role of mitochondria in apoptosis signaling and control has been investigated rigorously over the past 5 years. Although a role for Bcl-2 regulation of mitochondrial GSH pools has yet to be shown, it has long been known that one of the sites of GSH subcellular localization is mitochondria. Because of the generation of ROS during electron transport, mito-

chondria require additional mechanisms for dealing with a localized oxidative environment. Examples of mitochondrial oxidative defense pathways include elevated GSH, increased activity of GSH peroxidases, and Cu/Zn SOD.

Nomura *et al.* (1999) recently showed that mitochondrial phospholipid hydroperoxide glutathione peroxidase (PHGPx) suppresses mitochondrial-mediated apoptosis. PHGPx protects membranes from oxidative stress, but only the mitochondrial, as opposed to the nonmitochondrial PHGPx form, appears to do this. Additionally, as mentioned previously, Merad-Saidoune has shown that when brain GSH was depleted using the GCS inhibitor BSO, (which depletes cytosolic, but not nuclear GSH), overproduction of Cu/Zn-SOD or Bcl-2 prevented brain mitochondrial respiratory dysfunction in a Parkinson's model (Merad-Saidoune *et al.*, 1999). These results emphasize the importance of mitochondrial control of endogenous ROS generation, and that disruption of these processes in disease can lead to inappropriate cell death.

GSH EFFLUX

An intriguing area of Bcl-2/GSH research that has recently been gaining recognition is GSH efflux from cells selected to undergo apoptosis. Although the observation of GSH efflux is rapid in many situations, as in Fas-mediated apoptosis (van den Dobbelsteen *et al.*, 1996), the degree and timing of this process is still under investigation. Nevertheless, multiple reports have highlighted the possibility that GSH efflux may be one of the steps in mediating apoptosis and that Bcl-2 may suppress this pathway. Ghibelli *et al.* (1998) have shown that by blocking carrier-mediated GSH efflux with methionine or cystathionine, cells were rescued, and, once the apoptotic stimulus was removed, could proliferate. Many agents can block apoptosis, but few are able to mimic the ability of Bcl-2 to confer enhanced clonogenicity of cells that would have undergone apoptosis.

Furthering these findings, Meredith *et al.* (1998) reported that in HeLa expressing a conditional Bcl-2 construct, GSH efflux through the RsGshT or sinusoidal transporter was suppressed when Bcl-2 levels were elevated. The impact of these observations at the cellular level are becoming realized in a number of recent reports focusing on GSH efflux and apoptosis (Benard *et al.*, 1999; Gao *et al.*, 1999; Oda *et al.*, 1999). In the first two reports, apoptosis was studied in small intestinal epithelium and the basolateral membrane of polarized MDCK cells. In the first study, it was reported that GSH levels decreased and GSH efflux was increased during differentiation of intestinal epithelial cells from the crypt to the villius (Benard *et al.*, 1999). In the latter study, cellular protein toxins such as modeccin, *Pseudomonas* toxin, diphtheria toxin, and ricin all triggered basolateral efflux of reduced GSH. However, the authors show that this effect is blocked by caspase inhibitors, suggesting efflux of GSH in this case occurs following the commitment phase of apoptosis and is not directly responsible for triggering apoptosis (Oda *et al.*, 1999).

A recent report by Gao *et al.* (1999) carries the concept of GSH efflux to the pathological level, where they described potential abnormal transport of GSH in the airway epithelium of cystic fibrosis (CF) patients. Using cell lines lacking the CF transmembrane conductance regulator (CFTR), they found that GSH content was reduced by 55% in apical fluids of CFTR cultures when compared to congenic cells where CFTR had been restored. Because CF patients have pathologic inflammation where oxidative stress plays a role, regulation of GSH efflux is an important parameter that should be closely investigated. What role Bcl-2 may play in this scenario has yet to be determined, however, it is intriguing possibility that Bcl-2 may be interfering with the normal function of the CFTR.

An important point to make in the discussion of GSH efflux and intracellular GSH in general is the requirement of a reduced active site in caspases for their appropriate functioning. Orrennius and colleagues have addressed this issue and shown that there is a critical widow where caspase activation and apoptosis is favored (Hampton and Orrenius, 1998). If GSH levels drop too low, oxidation of the active site cysteine can occur rendering the caspase cascade impotent.

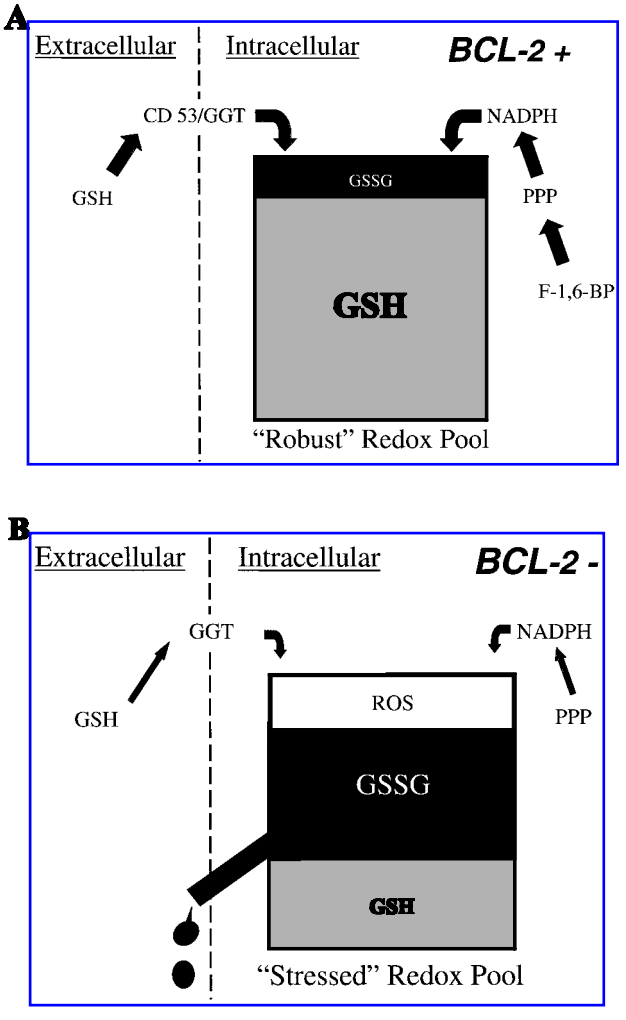

FIG. 1. Effect of Bcl-2 on altering cellular redox status via distinct metabolic pathways. (A) In Bcl-2-expressing cells, enhanced expression of CD53 has been reported. CD53, a member of the TM4 family of surface protein, interacts with γ-glutamyl transferase (GGT), enhancing breakdown and import of GSH to contribute to intracellular GSH pools. Additionally, fuctose-1,6-bisphosphatase (F-1,6-BP) contributes substrate for the pentose phosphate pathway (PPP) generating the reducing equivalents in the form of NADPH to maintain intracellular GSH mainly in its reduced form. **(B)** In Bcl-2 negative cells CD53 levels are virtually undetectable, GGT activity is diminished, and NADPH pools are smaller due to lack of substrate. This in combination with extrusion of oxidized GSH (GSSG) leads to increased ROS generation and oxidative stress. Thus, Bcl-2-expressing cells have higher levels of overall reducing equivalents (in the form of GSH and NADPH) and are more capable of handling exogeneous and endogenous oxidative stresses placed upon them.

Along these lines Hentze *et al.* (1999) have shown that by separating hepatotoxicity by type I depletion of GSH from the direct effects of GSH on liver cell apoptosis, intact GSH status is required for CD95-mediated apoptosis in mouse hepatocytes. By depleting intracellular GSH with phorone to inhibit GSH transferase, they showed that mice treated with CD95 were protected when GSH was depleted; however, with supplementation of GSH by administration of GSH-AM toxicity was restored. They suggest that the intracellular redox state is crucial for effective caspase activation (supported by previous reports from Hampton and Orrenius, 1998) and depletion of intracellular GSH prevents sufficient CD95-mediated caspase activation for apoptosis fulfillment. They further recognize that GSH compartmentalization, as in nuclear compartmentalization in the case of Bcl-2 overexpression (Voehringer *et al.*, 1998), may disrupt the localized GSH levels at the cell surface for CD95-mediated DISC formation and caspase 8 signaling. In support of this are the results showing that Bcl-2 inhibits CD95-induced hepatocyte apoptosis.

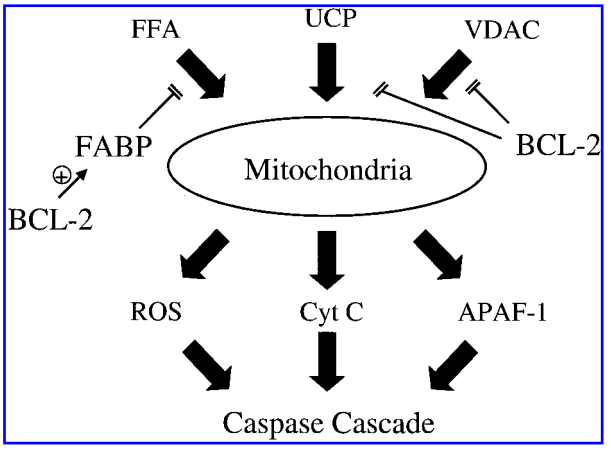

FIG. 2. Bcl-2 down-regulation of signaling pathways observed in apoptosis. Another possible level where Bcl-2 may play a role is through controlling the expression of certain genes targeted at protecting or disrupting mitochondria. By microarray analysis, it was observed that Bcl-2 cells expressed enhanced levels of fatty acid binding proteins (FABP), which might serve to buffer the generation of free fatty acids (FFA) during the early stages of apoptosis. Additionally, in cells lacking Bcl-2 protection from apoptosis, it was observed that the message for mitochondrial uncoupling protein (UCP) and voltage-dependent anion channel (VDAC) increased rapidly following an acute apoptotic stimulus. Both of these signals (which were suppressed in Bcl-2-expressing cells) could be deleterious to normal mitochondrial function, leading to cytochrome *c* release (Cyt C), generation of reactive oxygen species (ROS), and release of apoptogenic factors such as APAF-1. Such events would trigger the effector phase of apoptosis by activating the caspase cascade, leading ultimately to cell death.

SUMMARY AND CONCLUSIONS

Bcl-2 has been implicated in regulating apoptosis at many different levels from direct interactions between Bcl-2 and mitochondrial channels that release apoptogenic factors, to indirect alterations of cellular biochemistry though sequestration of divalent cations and GSH. One unifying theme that emerges, however, is the underlying connection between Bcl-2 and regulation of intracellular redox state of a cell. Because the redox state of the cell is crucial in regulating signaling and transcriptional pathways, some of the complexity of Bcl-2 function may be explained by its effect on cellular biochemistry at multiple levels though alterations in the cellular "redox pool" (Fig. 1). Additionally, as we have described above, down-regulation of three mitochondrial-targeted response pathways in response to apoptotic stimuli were observed in Bcl-2-expressing cells (Fig. 2), thereby regulating mitochondrial function indirectly though altered transcriptional responses. Therefore, although Bcl-2 may have a direct effect on mitochondrial PT, there are most likely other effects that are less direct, yet just as important in determining whether a cell undergoes apoptosis or not.

Since the evolution of cyanobacteria, cells have had to continually content with survival in an oxidizing environment even to this day (Ding *et al.,* 1998). During evolutionary selection, many systems have evolved to regulate and respond to a cell's redox state. Indeed, Bcl-2 and the Bcl-2 family members may have evolved as part of this complex mechanism controlling responses to cellular redox, survival, or death as outcomes. Further studies into the regulation of intracellular redox by the Bcl-2 family members will shed light on this hypothesis and further expand our knowledge into the interactions between oxidative stress, Bcl-2, apoptosis, and disease pathology.

ABBREVIATIONS

AIF, Apoptosis-Inducing factor; ANT, adenine nucleotide translocator; BSO, buthionene sulfoximine; CFTR, cystic fibrosis transmembrane conductance regulator; DFF, DNA fragmentation factor; F-1,6-BP, fructose 1,6 bisphosphatase; FABP, fatty acid binding proteins; FFA, free fatty acids; GCS, γ-glutamyl cysteine synthetase; GGT, γ-glutamyl transferase; GSH, glutathione; H_2O_2, hydrogen peroxide; NAC, ***N***-acetylcysteine; PHGPx, phospholipid hydroperoxide glutathione peroxidase; PPP, pentose phosphate pathway; PT, permeability transition; ROS, reactive oxygen species; SOD, superoxide dismutase; TM4, membrane tetraspanin 4; TNF, Tumor Necrosis Factor; UCP, uncoupling protein; VDAC, voltage-dependent anion channel.

REFERENCES

ADAMS, J.M., and CORY, S. (1998). The Bcl-2 protein family: arbiters of cell survival. Science **281,** 1322–1326.

BAKER, A., PAYNE, C.M., BRIEHL, M.M., and POWIS, G. (1997). Thioredoxin, a gene found overexpressed in human cancer, inhibits apoptosis in vitro and in vivo. Cancer Res. **57,** 5162–5167.

BENARD, O., MADESH, M., ANUP, R., and BALASUBRAMANIAN, K.A. (1999). Apoptotic process in the monkey small intestinal epithelium: I. Association with glutathione level and its efflux. Free Radic. Biol. Med. **26,** 245–252.

BOSS, O., SAMEC, S., PAOLONI-GIACOBINO, A., ROSSIER, C., DULLOO, A., SEYDOUX, J., MUZZIN, P., and GIACOBINO, J.P. (1997). Uncoupling protein-3: a new member of the mitochondrial carrier family with tissue-specific expression. FEBS Lett. **408,** 39–42.

BUTTKE, T.M., and SANDSTROM, P.A. (1995). Redox regulation of programmed cell death in lymphocytes. Free Radic. Res. **22,** 389–397.

CAI, J., and JONES, D.P. (1998). Superoxide in apoptosis. Mitochondrial generation triggered by cytochrome c loss. J. Biol. Chem. **273,** 11401–11404.

CAI, J., YANG, J., and JONES, D.P. (1998). Mitochondrial control of apoptosis: the role of cytochrome c. Biochim. Biophys. Acta **1366,** 139–149.

CASSARD, A.M., BOUILLAUD, F., MATTEI, M.G., HENTZ, E., RAIMBAULT, S., THOMAS, M., and RICQUIER, D. (1990). Human uncoupling protein gene: structure, comparison with rat gene, and assignment to the long arm of chromosome 4. J. Cell Biochem. **43,** 255–264.

DEGLI ESPOSTI, M., and MCLENNAN, H. (1998). Mitochondria and cells produce reactive oxygen species in virtual anaerobiosis: relevance to ceramide-induced apoptosis. FEBS Lett. **430,** 338–342.

DING, W.X., SHEN, H.M., ZHU, H.G., and ONG, C.N. (1998). Studies on oxidative damage induced by cyanobacteria extract in primary cultured rat hepatocytes. Environ. Res. **78,** 12–18.

ELLERBY, L.M., ELLERBY, H.M., PARK, S.M.,

HOLLERAN, A.L., MURPHY, A.N., FISKUM, G., KANE, D.J., TESTA, M.P., KAYALAR, C., and BREDESEN, D.E. (1996). Shift of the cellular oxidation-reduction potential in neural cells expressing Bcl-2. J. Neurochem. **67,** 1259–1267.

ESPOSTI, M.D., HATZINISIRIOU, I., MCLENNAN, H., and RALPH, S. (1999). Bcl-2 and mitochondrial oxygen radicals. New approaches with reactive oxygen species-sensitive probes. J. Biol. Chem. **274,** 29831–29837.

FLEURY, C., NEVEROVA, M., COLLINS, S., RAIMBAULT, S., CHAMPIGNY, O., LEVI-MEYRUEIS, C., BOUILLAUD, F., SELDIN, M.F., SURWIT, R.S., RICQUIER, D., and WARDEN, C.H. (1997). Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia. Nature Genet. **15,** 269–272.

GAO, L., KIM, K.J., YANKASKAS, J.R., and FORMAN, H.J. (1999). Abnormal glutathione transport in cystic fibrosis airway epithelia. Am. J. Physiol. **277,** L113–L118.

GHAFOURIFAR, P., KLEIN, S.D., SCHUCHT, O., SCHENK, U., PRUSCHY, M., ROCHA, S., and RICHTER, C. (1999). Ceramide induces cytochrome c release from isolated mitochondria. Importance of mitochondrial redox state. J. Biol. Chem. **274,** 6080–6084.

GHIBELLI, L., FANELLI, C., ROTILIO, G., LAFAVIA, E., COPPOLA, S., COLUSSI, C., CIVITAREALE, P., and CIRIOLO, M.R. (1998). Rescue of cells from apoptosis by inhibition of active GSH extrusion. FASEB J. **12,** 479–486.

GROSS, A., MCDONNELL, J.M., and KORSMEYER, S.J. (1999). BCL-2 family members and the mitochondria in apoptosis. Genes Dev. **13,** 1899–1911.

HAMPTON, M.B., and ORRENIUS, S. (1998). Redox regulation of apoptotic cell death in the immune system. Toxicol. Lett. **102–103,** 355–358.

HENTZE, H., KUNSTLE, G., VOLBRACHT, C., ERTEL, W., and WENDEL, A. (1999). CD95-Mediated murine hepatic apoptosis requires an intact glutathione status. Hepatology **30,** 177–185.

HERZENBERG, L.A., DE ROSA, S.C., DUBS, J.G., ROEDERER, M., ANDERSON, M.T., ELA, S.W., and DERESINSKI, S.C. (1997). Glutathione deficiency is associated with impaired survival in HIV disease. Proc. Natl. Acad. Sci. USA **94,** 1967–1972.

HIRSCH, T., MARZO, I., and KROEMER, G. (1997). Role of the mitochondrial permeability transition pore in apoptosis. Biosci. Rep. **17,** 67–76.

HOCKENBERY, D.M., OLTVAI, Z.N., YIN, X.M., MILLIMAN, C.L., and KORSMEYER, S.J. (1993). Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell **75,** 241–251.

HUANG, T.T., YASUNAMI, M., CARLSON, E.J., GILLESPIE, A.M., REAUME, A.G., HOFFMAN, E.K., CHAN, P.H., SCOTT, R.W., and EPSTEIN, C.J. (1997). Superoxide-mediated cytotoxicity in superoxide dismutase-deficient fetal fibroblasts. Arch. Biochem. Biophys. **344,** 424–432.

JACOBSON, M.D. (1996). Reactive oxygen species and programmed cell death. Trends Biochem. Sci. **21,** 83–86.

JACOBSON, M.D., and RAFF, M.C. (1995). Programmed cell death and Bcl-2 protection in very low oxygen. Nature **374,** 814–816.

JACOBSON, M.D., BURNE, J.F., KING, M.P., MIYASHITA, T., REED, J.C., and RAFF, M.C. (1993). Bcl-2 blocks apoptosis in cells lacking mitochondrial DNA. Nature **361,** 365–369.

JIANG, S., CAI, J., WALLACE, D.C., and JONES, D.P. (1999). Cytochrome c-mediated apoptosis in cells lacking mitochondrial DNA. Signaling pathway involving release and caspase 3 activation is conserved. J. Biol. Chem. **274,** 29905–29911.

JURGENSMEIER, J.M., XIE, Z., DEVERAUX, Q., ELLERBY, L., BREDESEN, D., and REED, J.C. (1998). Bax directly induces release of cytochrome c from isolated mitochondria. Proc. Natl. Acad. Sci. USA **95,** 4997–5002.

KANE, D.J., SARAFIAN, T.A., ANTON, R., HAHN, H., GRALLA, E.B., VALENTINE, J.S., ORD, T., and BREDESEN, D.E. (1993). Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. Science **262,** 1274–1277.

KLUCK, R.M., BOSSY-WETZEL, E., GREEN, D.R., and NEWMEYER, D.D. (1997). The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science **275,** 1132–1136.

KORSMEYER, S.J., SHUTTER, J.R., VEIS, D.J., MERRY, D.E., and OLTVAI, Z.N. (1993). Bcl-2/Bax: a rheostat that regulates an anti-oxidant pathway and cell death. Semin. Cancer Biol. **4,** 327–332.

LIU, X., KIM, C.N., YANG, J., JEMMERSON, R., and WANG, X. (1996). Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell **86,** 147–157.

LIU, X., ZOU, H., SLAUGHTER, C., and WANG, X. (1997). DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell **89,** 175–184.

MAECKER, H.T., TODD, S.C., and LEVY, S. (1997). The tetraspanin superfamily: molecular facilitators. FASeb J. **11,** 428–442.

MARCHETTI, P., DECAUDIN, D., MACHO, A., ZAMZAMI, N., HIRSCH, T., SUSIN, S.A., and KROEMER, G. (1997). Redox regulation of apoptosis: impact of thiol oxidation status on mitochondrial function. Eur. J. Immunol. **27,** 289–296.

MAYER, M., and NOBLE, M. (1994). *N*-acetyl-L-cysteine is a pluripotent protector against cell death and enhancer of trophic factor-mediated cell survival in vitro. Proc. Natl. Acad. Sci. USA **91,** 7496–7500.

MEI, B., KENNEDY, M.W., BEAUCHAMP, J., KOMUNIECKI, P.R., and KOMUNIECKI, R. (1997). Secretion of a novel, developmentally regulated fatty acid-binding protein into the perivitelline fluid of the parasitic nematode, *Ascaris suum*. J. Biol. Chem. **272,** 9933–9941.

MEISTER, A. (1995). Glutathione metabolism. Methods Enzymol. **251,** 3–7.

MERAD-SAIDOUNE, M., BOITIER, E., NICOLE, A., MARSAC, C., MARTINOU, J.C., SOLA, B., SINET, P.M., and CEBALLOS-PICOT, I. (1999). Overproduction of Cu/Zn-superoxide dismutase or Bcl-2 prevents the brain mitochondrial respiratory dysfunction induced by glutathione depletion. Exp. Neurol. **158,** 428–436.

MEREDITH, M.J., CUSICK, C.L., SOLTANINASSAB, S.,

SEKHAR, K.S., LU, S., and FREEMAN, M.L. (1998). Expression of Bcl-2 increases intracellular glutathione by inhibiting methionine-dependent GSH efflux. Biochem. Biophys. Res. Commun. **248,** 458–463.

MINN, A.J., VELEZ, P., SCHENDEL, S.L., LIANG, H., MUCHMORE, S.W., FESIK, S.W., FILL, M., and THOMPSON, C.B. (1997). Bcl-x(L) forms an ion channel in synthetic lipid membranes. Nature **385,** 353–357.

MIRKOVIC, N., VOEHRINGER, D.W., STORY, M.D., MCCONKEY, D.J., MCDONNELL, T.J., and MEYN, R.E. (1997). Resistance to radiation-induced apoptosis in Bcl-2-expressing cells is reversed by depleting cellular thiols. Oncogene **15,** 1461–1470.

MUCHMORE, S.W., SATTLER, M., LIANG, H., MEADOWS, R.P., HARLAN, J.E., YOON, H.S., NETTESHEIM, D., CHANG, B.S., THOMPSON, C.B., WONG, S.L., NG, S.L., and FESIK, S.W. (1996). X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. Nature **381,** 335–341.

NEGRE-SALVAYRE, A., HIRTZ, C., CARRERA, G., CAZENAVE, R., TROLY, M., SALVAYRE, R., PENICAUD, L., and CASTEILLA, L. (1997). A role for uncoupling protein-2 as a regulator of mitochondrial hydrogen peroxide generation. FASeb J. **11,** 809–815.

NICHOLS, T.C., GUTHRIDGE, J.M., KARP, D.R., MOLINA, H., FLETCHER, D.R., and HOLERS, V.M. (1998). Gamma-glutamyl transpeptidase, an ecto-enzyme regulator of intracellular redox potential, is a component of TM4 signal transduction complexes. Eur. J. Immunol. **28,** 4123–4129.

NOMURA, K., IMAI, H., KOUMURA, T., ARAI, M., and NAKAGAWA, Y. (1999). Mitochondrial phospholipid hydroperoxide glutathione peroxidase suppresses apoptosis mediated by a mitochondrial death pathway. J. Biol. Chem. **274,** 29294–29302.

ODA, T., SADAKATA, N., KOMATSU, N., and MURAMATSU, T. (1999). Specific efflux of glutathione from the basolateral membrane domain in polarized MDCK cells during ricin-induced apoptosis. J. Biochem. (Tokyo) **126,** 715–721.

POLYAK, K., XIA, Y., ZWEIER, J.L., KINZLER, K.W., and VOGELSTEIN, B. (1997). A model for p53-induced apoptosis. Nature **389,** 300–305.

QUILLET-MARY, A., JAFFREZOU, J.P., MANSAT, V., BORDIER, C., NAVAL, J., and LAURENT, G. (1997). Implication of mitochondrial hydrogen peroxide generation in ceramide- induced apoptosis. J. Biol. Chem. **272,** 21388–21395.

REED, J.C. (1998). Bcl-2 family proteins. Oncogene **17,** 3225–3236.

REED, J.C., JURGENSMEIER, J.M., and MATSUYAMA, S. (1998). Bcl-2 family proteins and mitochondria. Biochim. Biophys. Acta **1366,** 127–137.

SCHLEIFF, E., SHORE, G.C., and GOPING, I.S. (1997). Human mitochondrial import receptor, Tom20p. Use of glutathione to reveal specific interactions between Tom20-glutathione S-transferase and mitochondrial precursor proteins. FEBS Lett. **404,** 314–318.

SHIMIZU, S., EGUCHI, Y., KOSAKA, H., KAMIIKE, W., MATSUDA, H., and TSUJIMOTO, Y. (1995). Prevention of hypoxia-induced cell death by Bcl-2 and Bcl-xL. Nature **374,** 811–813.

SHIMIZU, S., NARITA, M., and TSUJIMOTO, Y. (1999). Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature **399,** 483–487.

SLATER, A.F., NOBEL, C.S., and ORRENIUS, S. (1995). The role of intracellular oxidants in apoptosis. Biochim. Biophys. Acta **1271,** 59–62.

STAAL, F.J., ROEDERER, M., and HERZENBERG, L.A. (1990). Intracellular thiols regulate activation of nuclear factor kappa B and transcription of human immunodeficiency virus. Proc. Natl. Acad. Sci. USA **87,** 9943–9947.

STEINMAN, H.M. (1995). The Bcl-2 oncoprotein functions as a pro-oxidant. J. Biol. Chem. **270,** 3487–3490.

STRYER, L. (1999). *Biochemistry.* (W.H. Freeman and Company, New York).

SUSIN, S.A., ZAMZAMI, N., CASTEDO, M., HIRSCH, T., MARCHETTI, P., MACHO, A., DAUGAS, E., GEUSKENS, M., and KROEMER, G. (1996). Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. J. Exp. Med. **184,** 1331–1341.

SUSIN, S.A., ZAMZAMI, N., and KROEMER, G. (1998). Mitochondria as regulators of apoptosis: doubt no more. Biochim. Biophys. Acta **1366,** 151–165.

SUSIN, S.A., LORENZO, H.K., ZAMZAMI, N., MARZO, I., BRENNER, C., LAROCHETTE, N., PREVOST, M.C., ALZARI, P.M., and KROEMER, G. (1999). Mitochondrial release of caspase-2 and -9 during the apoptotic process. J. Exp. Med. **189,** 381–394.

THOMPSON, C.B. (1995). Apoptosis in the pathogenesis and treatment of disease. Science **267,** 1456–1462.

VAN DEN DOBBELSTEEN, D.J., NOBEL, C.S.I., SCHLEGEL, J., COTGREAVE, I.A., ORRENIUS, S., and SLATER, A.F. (1996). Rapid and specific efflux of reduced glutathione during apoptosis induced by anti-Fas/APO-1 antibody. J. Biol. Chem. **271,** 15420–15427.

VANDER HEIDEN, M.G., CHANDEL, N.S., SCHUMACKER, P.T., and THOMPSON, C.B. (1999). Bcl-xL prevents cell death following growth factor withdrawal by facilitating mitochondrial ATP/ADP exchange. Mol. Cell **3,** 159–167.

VEIS, D.J., SORENSON, C.M., SHUTTER, J.R., and KORSMEYER, S.J. (1993). Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell **75,** 229–240.

VOEHRINGER, D.W. (1999). BCL-2 and glutathione: alterations in cellular redox state that regulate apoptosis sensitivity. Free Radic. Biol. Med. **27,** 945–950.

VOEHRINGER, D.W., HIRSCHBERG, D., HERZENBERG, L.A., STEINMAN, L., and HERZENBERG, L.A. (2000). Gene microarray identification of redox and mitochondrial elements that control resistance or sensitivity to apoptosis. Proc. Natl. Acad. Sci. USA **97,** 2680–2685.

VOEHRINGER, D.W., MCCONKEY, D.J., MCDONNELL, T.J., BRISBAY, S., and MEYN, R.E. (1998). Bcl-2 expression causes redistribution of glutathione to the nucleus. Proc. Natl. Acad. Sci. USA **95,** 2956–2960.

WONG, G.H., ELWELL, J.H., OBERLEY, L.W., and

GOEDDEL, D.V. (1989). Manganous superoxide dismutase is essential for cellular resistance to cytotoxicity of tumor necrosis factor. Cell **58,** 923–931.

YANG, J., LIU, X., BHALLA, K., KIM, C.N., IBRADO, A.M., CAI, J., PENG, T.L., JONES, D.P., and WANG, X. (1997). Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science **275,** 1129–1132.

ZAMZAMI, N., MARCHETTI, P., CASTEDO, M., DECAUDIN, D., MACHO, A., HIRSCH, T., SUSIN, S.A., PETIT, P.X., MIGNOTTE, B., and KROEMER, G. (1995). Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. J. Exp. Med. **182,** 367–377.

ZAMZAMI, N., SUSIN, S.A., MARCHETTI, P., HIRSCH, T., GOMEZ-MONTERREY, I., CASTEDO, M., and KROEMER, G. (1996). Mitochondrial control of nuclear apoptosis. J. Exp. Med. **183,** 1533–1544.

ZAMZAMI, N., BRENNER, C., MARZO, I., SUSIN, S.A., and KROEMER, G. (1998). Subcellular and submitochondrial mode of action of Bcl-2-like oncoproteins. Oncogene **16,** 2265–2282.

ZOU, H., HENZEL, W.J., LIU, X., LUTSCHG, A., and WANG, X. (1997). Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell **90,** 405–413.

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3. Heather M. Wilkins, Kristin Marquardt, Lawrence H. Lash, Daniel A. Linseman. 2011. Bcl-2 is a novel interacting partner for the 2-oxoglutarate carrier and a key regulator of mitochondrial glutathione. *Free Radical Biology and Medicine* . [CrossRef]
4. Navtej Kaur, Osama S. Naga, Håkan Norell, Amir A. Al-Khami, Matthew J. Scheffel, Nitya G. Chakraborty, Christina Voelkel-Johnson, Bijay Mukherji, Shikhar Mehrotra. 2011. T cells expanded in presence of IL-15 exhibit increased antioxidant capacity and innate effector molecules. *Cytokine* **55**:2, 307-317. [CrossRef]
5. Timothy S. Kern, Yunpeng Du, Casey M. Miller, Denise A. Hatala, Leonard A. Levin. 2010. Overexpression of Bcl-2 in Vascular Endothelium Inhibits the Microvascular Lesions of Diabetic Retinopathy. *The American Journal of Pathology* **176**:5, 2550-2558. [CrossRef]
6. Yang Ou, Xue Dong, Xin-Yong Liu, Xian-Chao Cheng, Yan-Na Cheng, Lu-Gang Yu, Xiu-Li Guo. 2010. Mechanism of Tetramethylpyrazine Analogue CXC195 Inhibition of Hydrogen Peroxide-Induced Apoptosis in Human Endothelial Cells. *Biological & Pharmaceutical Bulletin* **33**:3, 432-438. [CrossRef]
7. Adrienne N. Howard, Kathleen A. Bridges, Raymond E. Meyn, Joya Chandra. 2009. ABT-737, a BH3 mimetic, induces glutathione depletion and oxidative stress. *Cancer Chemotherapy and Pharmacology* **65**:1, 41-54. [CrossRef]
8. Andrea Lisa Holme, Shazib PervaizNeurohormetic Properties of the Phytochemical Resveratrol **20091218**, . [CrossRef]
9. R Franco, J A Cidlowski. 2009. Apoptosis and glutathione: beyond an antioxidant. *Cell Death and Differentiation* **16**:10, 1303-1314. [CrossRef]
10. Catherine Cantrel, Alain Zachowski, Blandine Geny. 2009. Over-expression of the Anti-apoptotic Protein Bcl-2 Affects Membrane Lipid Composition in HL-60 Cells. *Lipids* **44**:6, 499-509. [CrossRef]
11. Joya Chandra . 2009. Oxidative Stress by Targeted Agents Promotes Cytotoxicity in Hematologic Malignancies. *Antioxidants & Redox Signaling* **11**:5, 1123-1137. [Abstract] [Full Text PDF] [Full Text PDF with Links]
12. Dunyaporn Trachootham , Weiqin Lu , Marcia A. Ogasawara , Nilsa Rivera-Del Valle , Peng Huang . 2008. Redox Regulation of Cell Survival. *Antioxidants & Redox Signaling* **10**:8, 1343-1374. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
13. Devika S. Manickam, Aiko Hirata, David A. Putt, Lawrence H. Lash, Fusao Hirata, David Oupický. 2008. Overexpression of Bcl-2 as a proxy redox stimulus to enhance activity of non-viral redox-responsive delivery vectors. *Biomaterials* **29**:17, 2680-2688. [CrossRef]
14. J. L. Borowitz, G. E. Isom. 2008. Nicotine and Type 2 Diabetes. *Toxicological Sciences* **103**:2, 225-227. [CrossRef]
15. Tranum Kaur, Rajesh Gupta, Kim Vaiphei, Rakesh Kapoor, N.M. Gupta, K.L. Khanduja. 2008. Interplay Between Oncoproteins and Antioxidant Enzymes in Esophageal Carcinoma Treated Without and With Chemoradiotherapy: A Prospective Study. *International Journal of Radiation Oncology*Biology*Physics* **70**:2, 563-571. [CrossRef]
16. Xiong Ma, Jing Hua, Abdiaziz R. Mohamood, Abdel Rahmin A. Hamad, Rajani Ravi, Zhiping Li. 2007. A high-fat diet and regulatory T cells influence susceptibility to endotoxin-induced liver injury. *Hepatology* **46**:5, 1519-1529. [CrossRef]

17. Yongjun Zhang, Xuefan Gu, Xiaobing Yuan. 2007. Phenylalanine activates the mitochondria-mediated apoptosis through the RhoA/Rho-associated kinase pathway in cortical neurons. *European Journal of Neuroscience* **25**:5, 1341-1348. [CrossRef]

18. Giuseppe Filomeni , Maria R. Ciriolo . 2006. Redox Control of Apoptosis: An Update. *Antioxidants & Redox Signaling* **8**:11-12, 2187-2192. [Abstract] [Full Text PDF] [Full Text PDF with Links]

19. Sudharsana Rao Ande, Phaneeswara Rao Kommoju, Sigrid Draxl, Michael Murkovic, Peter Macheroux, Sandro Ghisla, Elisa Ferrando-May. 2006. Mechanisms of cell death induction by L-amino acid oxidase, a major component of ophidian venom. *Apoptosis* **11**:8, 1439-1451. [CrossRef]

20. Gladys Tapia, Virginia Fernández, Cesar Pino, Rodrigo Ardiles, Luis A. Videla. 2006. The acute-phase response of the liver in relation to thyroid hormone-induced redox signaling. *Free Radical Biology and Medicine* **40**:9, 1628-1635. [CrossRef]

21. Susan Tamiji, Jean-Claude Beauvillain, Laurent Mortier, Nathalie Jouy, Martine Tual, Emmanuel Delaporte, Pierre Formstecher, Philippe Marchetti, Renata Polakowska. 2005. Induction of Apoptosis-Like Mitochondrial Impairment Triggers Antioxidant and Bcl-2-Dependent Keratinocyte Differentiation. *Journal of Investigative Dermatology* **125**:4, 647-658. [CrossRef]

22. Archana Jaiswal McEligot, Sun Yang, Frank L. Meyskens, Jr.. 2005. REDOX REGULATION BY INTRINSIC SPECIES AND EXTRINSIC NUTRIENTS IN NORMAL AND CANCER CELLS. *Annual Review of Nutrition* **25**:1, 261-295. [CrossRef]

23. David Oupicky, Harender S Bisht, Devika Soundara Manickam, Qing-hui Zhou. 2005. Stimulus-controlled delivery of drugs and genes. *Expert Opinion on Drug Delivery* **2**:4, 653-665. [CrossRef]

24. Angela K. Zimmermann, F. Alexandra Loucks, Shoshona S. Le, Brent D. Butts, Maria L. Florez-McClure, Ron J. Bouchard, Kim A. Heidenreich, Daniel A. Linseman. 2005. Distinct mechanisms of neuronal apoptosis are triggered by antagonism of Bcl-2/Bcl-x(L) versus induction of the BH3-only protein Bim. *Journal of Neurochemistry* **94**:1, 22-36. [CrossRef]

25. Yanhua Zheng, Hirohito Yamaguchi, Changhai Tian, Michael W Lee, Hong Tang, Hong-Gang Wang, Quan Chen. 2005. Arsenic trioxide (As2O3) induces apoptosis through activation of Bax in hematopoietic cells. *Oncogene* **24**:20, 3339-3347. [CrossRef]

26. Shinobu Yanada , Masao Sasaki , Shinichi Takayama , Yasufumi Kaneda , Nobuhiko Miwa . 2005. Hemagglutinating Virus of Japan–Artificial Viral Envelope Liposome-Mediated Cotransfer of bag-1 and bcl-2 Genes Protects Hepatic Cells Against Ischemic Injury Through BAG-1-Assisted Preferential Enhancement of Bcl-2 Protein Expression. *Human Gene Therapy* **16**:5, 627-633. [Abstract] [Full Text PDF] [Full Text PDF with Links]

27. Yu-Jin Jeong, Yean-Jung Choi, Hyang-Mi Kwon, Sang-Wook Kang, Hyoung-Sook Park, Myungsook Lee, Young-Hee Kang. 2005. Differential inhibition of oxidized LDL-induced apoptosis in human endothelial cells treated with different flavonoids. *British Journal of Nutrition* **93**:05, 581. [CrossRef]

28. Yean-Jung Choi , Myung-Kyu Lee , Yong-Jin Lee , Yu-Jin Jeong , Jung Han Yoon Park , Soon Sung Lim , Young-Hee Kang . 2004. Inhibition of Hydrogen Peroxide-Induced Endothelial Apoptosis by 2#,4#,7-Trihydroxyflavanone, a Flavonoid Form. *Journal of Medicinal Food* **7**:4, 408-416. [Abstract] [Full Text PDF] [Full Text PDF with Links]

29. John J. Haddad. 2004. On the antioxidant mechanisms of Bcl-2: a retrospective of NF-#B signaling and oxidative stress. *Biochemical and Biophysical Research Communications* **322**:2, 355-363. [CrossRef]

30. J NILSSON, O SODERBERG, K NILSSON, A ROSEN. 2004. Differentiation-associated redox-regulation in human B cell lines from stem cell/pro-B to plasma cell. *Immunology Letters* **94**:1-2, 83-89. [CrossRef]

31. David A. Hildeman. 2004. Regulation of T-cell apoptosis by reactive oxygen species. *Free Radical Biology and Medicine* **36**:12, 1496-1504. [CrossRef]

32. N Khanna. 2004. Benzamide riboside induced mitochondrial mediated apoptosis in human lung cancer H520 cells. *Life Sciences* **75**:2, 179-190. [CrossRef]

33. J Haddad. 2004. Redox and oxidant-mediated regulation of apoptosis signaling pathways: immuno-pharmaco-redox conception of oxidative siege versus cell death commitment. *International Immunopharmacology* **4**:4, 475-493. [CrossRef]

34. B Gabriel. 2003. Retroactive pathway involving mitochondria in electroloaded cytochrome c-induced apoptosis Protective properties of Bcl-2 and Bcl-XL. *Experimental Cell Research* **289**:2, 195-210. [CrossRef]

35. Jan Lewerenz, Julia Letz, Axel Methner. 2003. Activation of stimulatory heterotrimeric G proteins increases glutathione and protects neuronal cells against oxidative stress. *Journal of Neurochemistry* **87**:2, 522-531. [CrossRef]

36. Yong-Jin Lee, Il-Jun Kang, Rolf Bünger, Young-Hee Kang. 2003. Mechanisms of pyruvate inhibition of oxidant-induced apoptosis in human endothelial cells. *Microvascular Research* **66**:2, 91-101. [CrossRef]

37. A Meki. 2003. Dysregulation of apoptosis in scorpion envenomed children: its reflection on their outcome. *Toxicon* **42**:3, 229-237. [CrossRef]

38. Elisa Del Terra , Anna Francesconi , Debora Donnini , Francesco Curcio , Francesco Saverio Ambesi-Impiombato . 2003. Thyrotropin Effects on Ultraviolet Radiation-Dependent Apoptosis in FRTL-5 Cells. *Thyroid* **13**:8, 747-753. [Abstract] [Full Text PDF] [Full Text PDF with Links]

39. Jan Seyfried, Bernd O Evert, Cordelia S Schwarz, Matthias Schaupp, Jörg B Schulz, Thomas Klockgether, Ullrich Wüllner. 2003. Gene dosage-dependent effects of bcl-2 expression on cellular survival and redox status. *Free Radical Biology and Medicine* **34**:12, 1517-1530. [CrossRef]

40. Brian R. Gastman, J. William Futrell, and Ernest K. Manders. 2003. Apoptosis and Plastic Surgery. *Plastic and Reconstructive Surgery* **111**:4, 1481-1496. [CrossRef]

41. Quan Chen, Meredith Crosby, Alex Almasan. 2003. Redox regulation of apoptosis before and after cytochrome C release. *Korean Journal of Biological Sciences* **7**:1, 1-9. [CrossRef]

42. Y OKAMOTO, L OBEID, Y HANNUN. 2002. Bcl-xL interrupts oxidative activation of neutral sphingomyelinase. *FEBS Letters* **530**:1-3, 104-108. [CrossRef]

43. Hyun-Ock Pae, Hyuncheol Oh, Young-Gab Yun, Gi-Su Oh, Seon Il Jang, Kee-Myoung Hwang, Tae-Oh Kwon, Ho-Sub Lee, Hun-Taeg Chung. 2002. Imperatorin, a Furanocoumarin from Angelica dahurica (Umbelliferae), Induces Cytochrome c-Dependent Apoptosis in Human Promyelocytic Leukaemia, HL-60 Cells. *Pharmacology and Toxicology* **91**:1, 40-48. [CrossRef]

44. Didier Morin, Vassilios Papadopoulos, Jean-Paul Tillement. 2002. Prevention of cell damage in ischaemia: novel molecular targets in mitochondria. *Expert Opinion on Therapeutic Targets* **6**:3, 315-334. [CrossRef]

45. B Godley. 2002. Bcl-2 Overexpression Increases Survival in Human Retinal Pigment Epithelial Cells Exposed to H2O2. *Experimental Eye Research* **74**:6, 663-669. [CrossRef]