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Original Contribution

BCL-2 FAMILY PROTEINS REGULATE MITOCHONDRIAL REACTIVE OXYGEN PRODUCTION AND PROTECT AGAINST OXIDATIVE STRESS

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Abstract—Bcl-2 family proteins protect against a variety of forms of cell death, including acute oxidative stress. Previous studies have shown that overexpression of the antiapoptotic protein Bcl-2 increases cellular redox capacity. Here we report that cell lines transfected with Bcl-2 paradoxically exhibit increased rates of mitochondrial H_2O_2 generation. Using isolated mitochondria, we determined that increased H_2O_2 release results from the oxidation of reduced nicotinamide adenine dinucleotide-linked substrates. Antiapoptotic Bcl-2 family proteins Bcl-xL and Mcl-1 also increase mitochondrial H_2O_2 release when overexpressed. Chronic exposure of cells to low levels of the mitochondrial uncoupler carbonyl cyanide 4-(triflouromethoxy)phenylhydrazone reduced the rate of H_2O_2 production by Bcl-xL overexpressing cells, resulting in a decreased ability to remove exogenous H_2O_2 and enhanced cell death under conditions of acute oxidative stress. Our results indicate that chronic and mild elevations in H_2O_2 release from Bcl-2, Bcl-xL, and Mcl-1 overexpressing mitochondria lead to enhanced cellular antioxidant defense and protection against death caused by acute oxidative stress.

Keywords—Free radicals, Hydrogen peroxide, Digitonin, Apoptosis, Necrosis, Complex I, Antioxidant

INTRODUCTION

The Bcl-2 protein inhibits cell death promoted by a wide variety of stimuli when overexpressed (for reviews see [1–3]). This protein is located predominantly in intracellular membranes, and most of its protective effects against cell death have been attributed to its mitochondrial location [1–3]. Mitochondria play essential roles in the regulation of key steps in both apoptotic and necrotic cell death by affecting energy metabolism, participating in intracellular Ca^{2+} homeostasis, regulating the activation of caspases, and releasing reactive oxygen species (ROS) [1,4–6]. Bcl-2 overexpression has been previously shown to act at multiple steps of mitochondrially regulated cell death such as increasing maximal mitochondrial Ca^{2+} uptake capacity [7], preventing the release of proapoptotic mitochondrial intermembrane proteins [8–10], and preventing oxidative stress following deadly stimuli [4,11–13].

The prevention of oxidative stress following cell-death-initiating stimuli is associated with an increase in the total antioxidant capacity of Bcl-2 overexpressing cells [13–15]. It has been hypothesized [12] that this enhanced antioxidant capacity is related to a chronic increase in cellular ROS under physiological conditions secondary to Bcl-2 overexpression. Indeed, many redox-related genes are activated by increased H_2O_2 levels [16,17] and can be determinant in the apoptotic process [13]. Data using a single Bcl-2-transfected cell line support the idea that Bcl-2 chronically increases ROS [12]. However, other groups [11,18] have not found changes in mitochondrial ROS release in Bcl-2 overexpressing cells under physiological conditions but have uncovered a protection against oxidative stress by Bcl-2 following apoptotic stimuli. It has been argued that the lack of detection of a Bcl-2 effect under physiological conditions is due to the use of less sensitive ROS probes [12].

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If an effect of Bcl-2 on mitochondrial ROS release under physiological conditions is confirmed, the mechanism through which such a change could occur would be of great interest. We demonstrated [7,19] that Bcl-2 does not alter mitochondrial functional parameters such as respiration and the inner membrane potential, suggesting that these are not direct causes for any possible changes in ROS release by this organelle. Furthermore, a link between chronic increases in ROS release by Bcl-2 and protection against acute oxidative cell death has not been established.

In this study we evaluated the effects of Bcl-2 and antiapoptotic Bcl-2 family proteins Bcl-xL and Mcl-1 on mitochondrial ROS release using a H_2O_2 detection system that is more sensitive than techniques applied previously to similar comparisons. We additionally tested the relationship of endogenous mitochondrial ROS production with sensitivity to acute cell death caused by oxidative stress. Our findings support the hypothesis that these Bcl-2 family proteins protect against acute cell death by increasing cellular antioxidant capacity due to chronic changes in mitochondrial ROS release.

EXPERIMENTAL PROCEDURES

Cell cultures

PC12 pheochromocytoma and immortalized hypothalamic GT1-7 neuronal cell lines transfected with the human *bcl-2* gene (Bcl-2+) or with a control retroviral construct (Bcl-2−) were maintained as described previously [20]. Western blot analysis indicated that Bcl-2 levels were undetectable in Bcl-2- cells and that the protein was present at high levels in the mitochondrial fraction of Bcl-2+ preparations (not shown). Parental MM 8226 cells (control cells) were transfected with *bcl-xl* (Bcl-xL cells) and *mcl-1* (Mcl-1 cells) and cultured as previously described [21]. Western blots of control cells did not exhibit any detectable Bcl-xL or Mcl-1. Prior to experiments, the cells were suspended in growth medium supplemented with 10 mM Hepes, pH 7.0. Suspended cells were kept at room temperature for up to 5 h. Cell viability, as assessed by a cell count in the presence of trypan blue, was above 95% even after 5 h at room temperature. The suspended cells were centrifuged and resuspended in the medium used in the experiment just prior to each determination. Cell protein content was determined using the Biuret method. The protein/cell count ratio was not affected by the level of Bcl-2 family protein expression. All experiments were conducted at 37°C.

Mitochondrial isolation

Mitochondria were isolated from digitonin-permeabilized cells as described by Moreadith and Fiskum [22] in isolation buffer containing 210 mM mannitol, 75 mM sucrose, 1 mg/mL BSA, 5 mM Hepes, and 1 mM EGTA, pH 7.2 (KOH). Mitochondria isolated in this manner typically displayed respiratory control ratios between 3 and 6, when respiring on NADH-linked substrates.

H_2O_2 release

H_2O_2 was measured by following the oxidation of 50 μM amplex red (Molecular Probes A12222) in the presence of 1 U/mL horseradish peroxidase (HRP) [23,24] recorded on a temperature-controlled fluorescence spectrophotometer equipped with continuous stirring and operating at excitation and emission wavelengths of 563 and 587 nm, respectively. Because amplex red presents a slow rate of spontaneous oxidation in the presence of HRP (<2% of rates in the presence of cells or mitochondria), all traces were subtracted from a baseline trace recorded in the same media devoid of cells or mitochondria. Data were calibrated by adding known quantities of a freshly prepared H_2O_2 stock quantified by its absorbance at 240 nm ($E = 43.6\ M \cdot cm^{-1}$).

H_2O_2 removal

H_2O_2 removal was measured as described previously [25] from freshly isolated cell homogenates suspended in phosphate-buffered saline. Briefly, 0.5 mg/mL of total cell protein was incubated for 1 min with 1.0 μM H_2O_2 and a 1-μL aliquot was taken and diluted in 2 mL of suspension buffer with 50 μM amplex red and 1.0 U/mL horseradish peroxidase. A single fluorescence reading was taken at 563 nm emission and 587 nm excitation. Amplex red readings in samples untreated with H_2O_2 were negligible, indicating that oxidation of the dye by cytosolic components, e.g., NADH, did not significantly affect measurements under these conditions.

Lactate dehydrogenase (LDH) activity

LDH activity was measured using Sigma Diagnostics LDH kit No. 500 in 10 μL undiluted growth media from cells plated 2 h previously at 1 mg protein/mL in the presence of varying H_2O_2 concentrations. Total releasable LDH was measured by treating cells with 0.05% digitonin.

Reagents

Amplex red was purchased from Molecular Probes. Horseradish peroxidase (P8125), EGTA, digitonin, malate, glutamate, pyruvate, succinate, BSA, rotenone, antimycin A, and alamethicin were from Sigma–Aldrich. All other reagents were of analytical purity grades.

Data analysis

Traces are representative of data collected from at least three similar repetitions. Averages represented in scatter and bar graphs were calculated from data collected from three to nine repetitions using different preparations. Error bars indicate standard errors (SE) and significance was calculated using pairwise Tukey tests, conducted by SigmaStat.

RESULTS

Figure 1 shows H_2O_2 release from two distinct cell lines (GT1-7 hypothalamic tumor and PC12 pheochromocytoma cells), comparing the effects of Bcl-2 expression on cellular H_2O_2 production by following the time-dependent oxidation of amplex red in the presence of HRP [25]. This method is sensitive to physiological levels of H_2O_2 release and detects between 0.025 and 0.05 nmol $H_2O_2 \cdot min^{-1} \cdot mg^{-1}$ cell protein under normal growth conditions. In intact cells, no difference in H_2O_2 release between Bcl-2− and Bcl-2+ cells was apparent (left panels), a result consistent with previous findings [11,18].

Based on previous work showing that Bcl-2 overexpression increases mitochondrial H_2O_2 release in Burkitt's lymphoma and promyelocytic leukemia cell lines [12] and the finding that Bcl-2 overexpression enhances cytosolic antioxidant levels in the cell lines used in our study [14], we reevaluated H_2O_2 release in digitonin-permeabilized cells respiring in state 4 (non-phosphorylating) conditions. Low digitonin concentrations selectively permeabilize the plasma membrane, promoting a large dilution of cytosolic components (including intracellular antioxidants) while maintaining cell architecture and mitochondrial function unaltered [26]. This is the preferred method to study the effects of Bcl-2 in mitochondria from transfected cell lines, since mitochondrial isolation may promote damage to the organelle in a Bcl-2-inhibited manner [7,27]. Under these

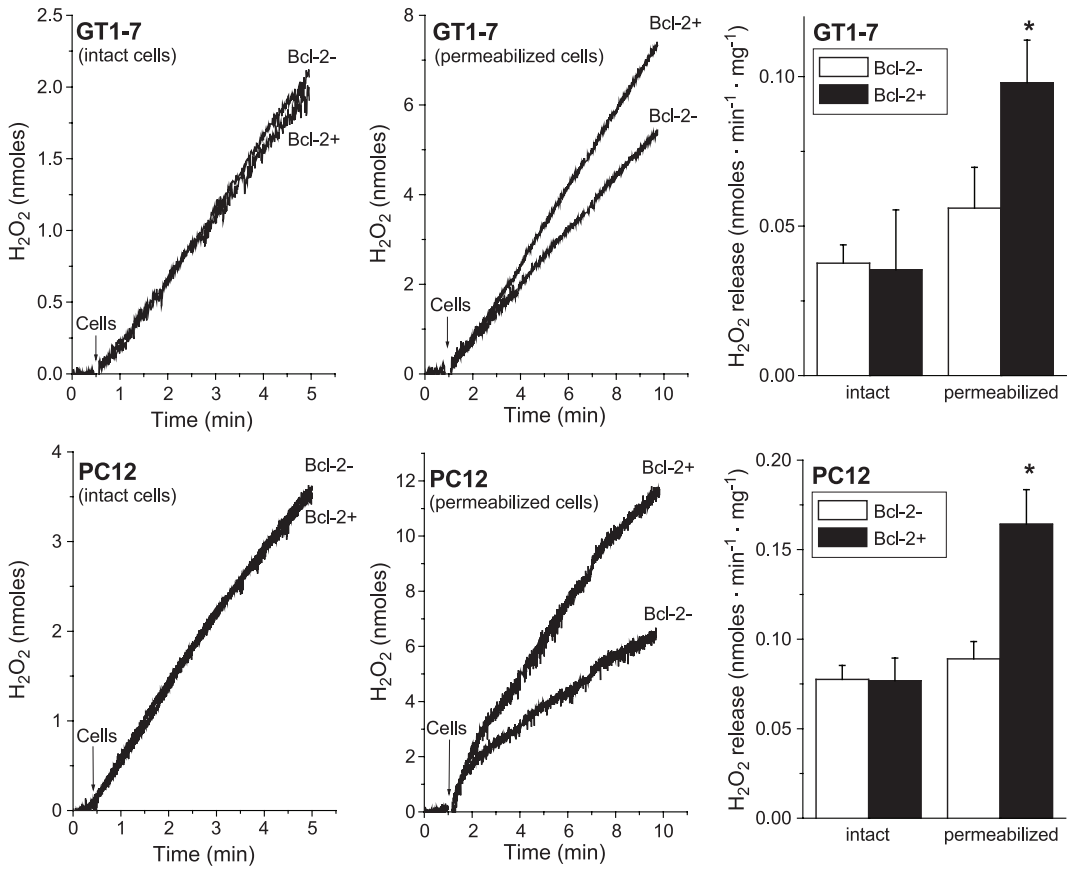

Fig. 1. Bcl-2 overexpression increases H_2O_2 generation in digitonin-permeabilized cells. GT1-7 or PC12 Bcl-2- and Bcl-2+ cells (as indicated) were added at a concentration of 10 mg protein/mL to 37°C media containing 1 U/mL HRP and 50 μM amplex red to measure H_2O_2 release (as described under Experimental Procedures). Intact cells were incubated in phenol red-free modified Eagle's medium supplemented with 10 mM Hepes, pH 7.2. Permeabilized cells were incubated at 37°C in 250 mM sucrose, 5 mM pyruvate, 5 mM malate, 5 mM glutamate, 100 μM EGTA, 1 mg/mL BSA, 0.001 or 0.004% digitonin (GT1-7 and PC12 cells, respectively), 1 μg/mL oligomycin, and 10 mM K^+-Hepes, pH 7.2. Leftmost and center panels depict representative tracings of intact and permeabilized cell measurements, respectively. The panels on the right show averages of at least three repetitions. *$p < 0.05$ compared to Bcl-2- cells.

conditions and in the presence of NADH-linked substrates, we found that both lines of Bcl-2+ cells generated significantly higher H_2O_2 levels than their respective control transfectants (Fig. 1, right panels).

To determine whether mitochondria are responsible for the Bcl-2-dependent differences in H_2O_2 release, we incubated permeabilized GT1-7 and PC12 cells in the absence of respiratory substrates (Fig. 2). Under these conditions, endogenous redox sources are rapidly depleted (1–2 min after the addition of the cells). The H_2O_2 detection rates after this period were similar in Bcl-2- and Bcl-2+ cells, but the increased H_2O_2 release rates observed in Bcl-2+ cells could be recovered by adding the mitochondrial NADH-linked substrate pyruvate, indicating that the difference in H_2O_2 release in Bcl-2+ cells is of mitochondrial origin.

There are several sites of mitochondrial ROS production including Complexes I and III of the electron transport chain and possibly several tricarboxylic acid cycle dehydrogenases [28,29]. Furthermore, mitochondrial ROS release is altered by changes in respiratory rates and the use of different respiratory inhibitors. While increasing respiration with uncouplers generally reduces ROS release, inhibiting specific respiratory complexes enhances electron leakage from sites upstream of the inhibition [30,31]. ROS production by Bcl-2+ and Bcl-2− mitochondria was compared using different respiratory substrates and inhibitors in an attempt to further identify the molecular basis for the effects of Bcl-2 on ROS generation. In these experiments, we used isolated GT1-7 and PC12 mitochondria to avoid amplex red oxidation by nonmitochondrial cellular components. Using isolated mitochondria, amplex red oxidation in the absence of respiratory substrates was negligible (results not shown). H_2O_2 release from isolated Bcl-2+ mitochondria was enhanced relative to Bcl-2- preparations in the presence

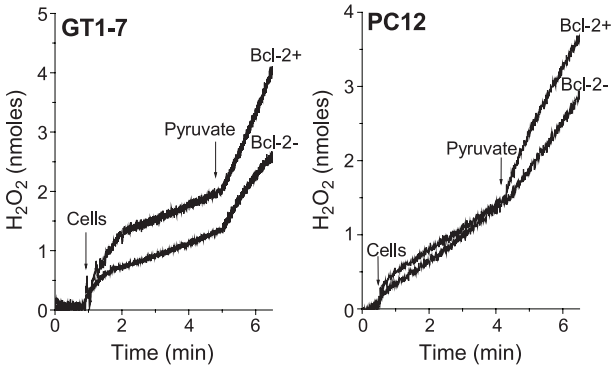

Fig. 2. Increased H_2O_2 release in Bcl-2+ cells is of mitochondrial origin. Permeabilized GT1-7 and PC12 cells were incubated under the conditions described in the legend to Fig. 1, except that the mitochondrial respiratory substrates pyruvate, malate, and glutamate were not present in the incubation buffer. Pyruvate (5 mM) was added where indicated.

of NADH-linked substrates malate plus glutamate (Mal + Glu) or pyruvate (Pyr). Rotenone (Rot), a Complex I inhibitor, enhanced H_2O_2 release in both Bcl-2+ and Bcl-2- preparations (Fig. 3), suggesting that the difference in ROS generation originates from an electron source upstream of rotenone inhibition, such as iron–sulfur centers in Complex I [28,32].

Support for a Complex-I-associated effect of Bcl-2 on mitochondrial ROS production came from experiments performed with the Complex II substrate succinate (Succ). GT1-7 Bcl-2+ and Bcl-2- mitochondria generated equal amounts of H_2O_2 when energized with succinate (Fig. 3). In PC12 mitochondria, H_2O_2 release rates supported by succinate were significantly higher in Bcl-2 transfectants unless antimycin A (AA), which increases ROS release at the level of coenzyme Q [33], was present. As these results argue against an involvement of coenzyme Q/Complex III in Bcl-2-regulated ROS production in the presence of succinate, we hypothesized that Bcl-2 affects ROS production by NADH-linked substrates, e.g., malate, that can be generated from succinate via matrix tricarboxylic acid cycle enzymes. To eliminate this potential contribution to succinate-driven ROS generation, we incubated mitochondria with alamethicin (Ala), which forms large pores in mitochondrial membranes resulting in organellar swelling, rupture, and release of soluble matrix components. Under these conditions, succinate oxidation via membrane-bound succinate dehydrogenase (Complex II) resulted in H_2O_2 release rates that were equal for Bcl-2- and Bcl-2+ mitochondria. Thus, increases in ROS release in Bcl-2+ mitochondria occur due to changes in NADH metabolism, and electron leakage leading to ROS release between Complexes II and IV is equal in cells with different levels of Bcl-2 expression. H_2O_2 release in alamethicin-permeabilized mitochondria was measured using only succinate as a substrate due to the interference of added NADH with the amplex red detection system.

Previous experiments by our group using the same cell lines have determined that Bcl-2 does not significantly change mitochondrial respiratory rates or membrane potential [7,19]. This suggests that changes in respiratory rates are not responsible for increases in ROS release in Bcl-2+ mitochondria. We have also found previously that Bcl-2+ mitochondria contain larger quantities of NAD(H) and an increased ability to maintain NAD(H) in its reduced state [34]. This effect could be related to the enhanced H_2O_2 generation promoted by Bcl-2 as mitochondrial ROS production is very sensitive to changes in NAD(H) redox state [35,36]. To investigate this possibility, we measured H_2O_2 release in the presence of different respiratory conditions that maintain $NADH/NAD^+$ ratios at either very high or low levels by, respectively, decreasing or maximizing elec-

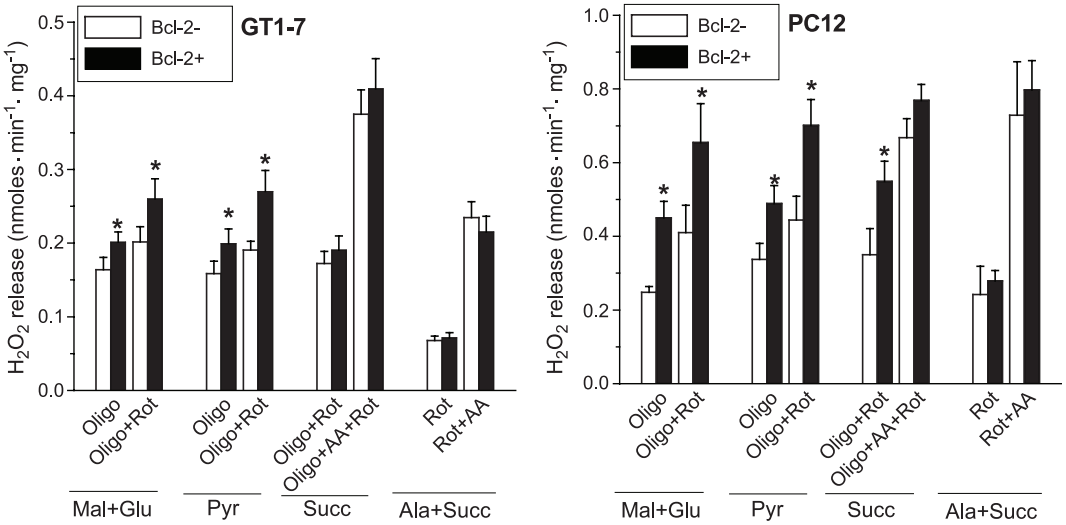

Fig. 3. NADH is the electron source for increased ROS release in Bcl-2+ cells. Isolated GT1-7 and PC12 cell mitochondria (0.5 mg/mL) were incubated at 37°C in 250 mM sucrose, 1 mM EGTA, 1 mg/mL BSA, 1 mM K^+-P_i, 5 mM Mg^{2+}, 2 mM ATP, and 10 mM K^+-Hepes, pH 7.2, containing 1 U/mL HRP and 50 μM amplex red. Malate plus glutamate (Mal + Glu, 5 mM each), 5 mM pyruvate (Pyr), 5 mM succinate (Succ), 100 nM alamethicin (Ala), 1 μg/mL oligomycin (Oligo), 100 nM rotenone (Rot), and/or 200 nM antimycin A (AA) were added where indicated. *$p < 0.05$ compared to Bcl-2-mitochondria.

tron transport (Fig. 4). We found that H_2O_2 release levels were higher in both Bcl-2+ cell types, regardless of whether respiration was maximized by oxidative phosphorylation (ADP) or uncoupler (FCCP), or decreased by the ATP synthetase inhibitor oligomycin (Oligo). These results demonstrate that the Bcl-2 effect on ROS production is independent of changes in NADH/NAD^+ ratios and is probably related to increased electron leakage at or prior to Complex I.

If the increase in mitochondrial H_2O_2 release promoted by Bcl-2 bears any relevance to ability to advance tumor generation or protect against cell death, similar effects should be observed with other antiapoptotic Bcl-2 family proteins. Thus, we worked with multiple myeloma cells (MM 8226) transfected with Bcl-xL and Mcl-1 antiapoptotic proteins. Bcl-xL is structurally similar to Bcl-2 , while Mcl-1 is a larger protein, lacking the BH4 homology region [2,21]. Both proteins are effective protectors against a variety of forms of cell death, including oxidative damage [2,21,37,38]. We found (Fig. 5) that Mcl-1 moderately increased cellular H_2O_2 release measured in intact cells, digitonin-permeabilized cells, or isolated mitochondria, while Bcl-xL presented a very strong H_2O_2-stimulating effect under these conditions. Again, increased H_2O_2 release was observed only using NADH-linked substrates. Parallel measure-

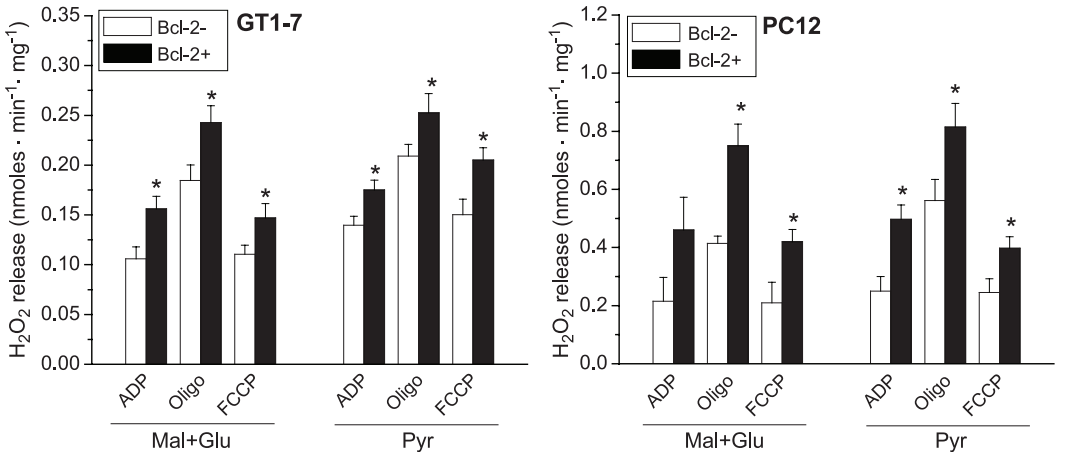

Fig. 4. Increased ROS release in Bcl-2+ cells is independent of respiratory energy coupling. Isolated GT1-7 and PC12 cell mitochondria (0.5 mg/mL) were incubated at 37°C in 250 mM sucrose, 1 mM EGTA, 1 mg/mL BSA, 1 mM K^+-P_i, 5 mM Mg^{2+}, and 10 mM K^+-Hepes, pH 7.2, containing 1 U/mL HRP and 50 μM amplex red. Malate plus glutamate (Mal + Glu, 5 mM each) or 5 mM pyruvate (Pyr). 200 μM ADP, 1 μg/mL oligomycin (Oligo), and 0.5 μM FCCP were added sequentially where indicated. *$p < 0.05$ compared to Bcl-2-mitochondria.

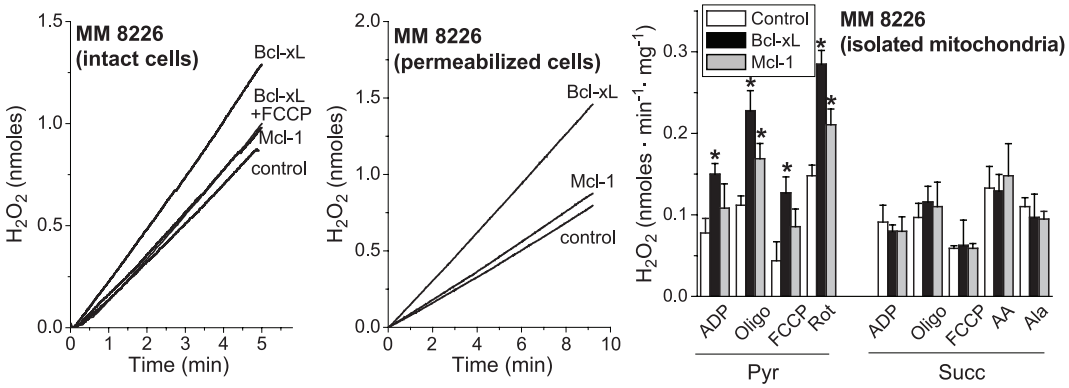

Fig. 5. Bcl-xL and Mcl-1 overexpression increases H_2O_2 generation. 10 mg/mL control, Bcl-xL, or Mcl-1 overexpressing MM 8226 cells (0.5 mg/mL) isolated MM 8226 mitochondria (as indicated) were added to 37°C media containing 1 U/ml HRP and 50 μM amplex red to measure H_2O_2 release (as described under Experimental Procedures). Intact cells were incubated in RPMI media supplemented with 5 U superoxide dismutase/mL and 10 mM Hepes, pH 7.2. FCCP (100 nM) was added where shown 15 min before the measurement. Permeabilized cells were incubated in 250 mM sucrose, 5 mM pyruvate, 5 mM malate, 5 mM glutamate, 100 μM EGTA, 1 mg/mL BSA, 0.005% digitonin, 1 μg/mL oligomycin, and 10 mM K^+-Hepes, pH 7.2. Experiments using isolated MM 8226 mitochondria were conducted under the conditions described in the legend to Fig. 3. *$p < 0.05$ compared to control mitochondria.

ments of respiratory rates and respiratory control ratios did not show any significant differences between transfected and untransfected cells (results not shown).

Since Bcl-xL-dependent differences in H_2O_2 release were detectable in intact MM 8226 cells, we used these cells to test the hypothesis that chronic increases in H_2O_2 generation are paradoxically related to protection against acute oxidative cell death. We found that H_2O_2 release in Bcl-xL cells could be reduced by mildly uncoupling mitochondria with nanomolar concentrations of FCCP (Fig. 5, far left). We cultured the cells in the presence of these concentrations of FCCP for 48 h, measured their ability to remove H_2O_2, and determined their resistance to H_2O_2-promoted cell death (Fig. 6). We found (Fig. 6, left) that Bcl-xL cell homogenates had a greater ability to remove H_2O_2 relative to controls, possibly reflecting enhanced catalase and peroxidase activity. Incubation with FCCP for 48 h reduced the H_2O_2 removal activity of Bcl-xL cells to levels more similar to those of controls. This effect was not due to a toxic effect of FCCP per se, since Bcl-xL cells treated with FCCP just prior to homogenization did not exhibit lower H_2O_2 removal activity. Thus, the decrease in H_2O_2 removal promoted by FCCP is dependent on long-term mild uncoupling and is associated with depressed mitochondrial ROS release.

In parallel to H_2O_2 removal experiments, we measured resistance to acute oxidative stress in these cell types. Intact cells were incubated in the presence of mM concentrations of exogenous H_2O_2 for 2 h, and cell damage was measured by determining lactate dehydro-

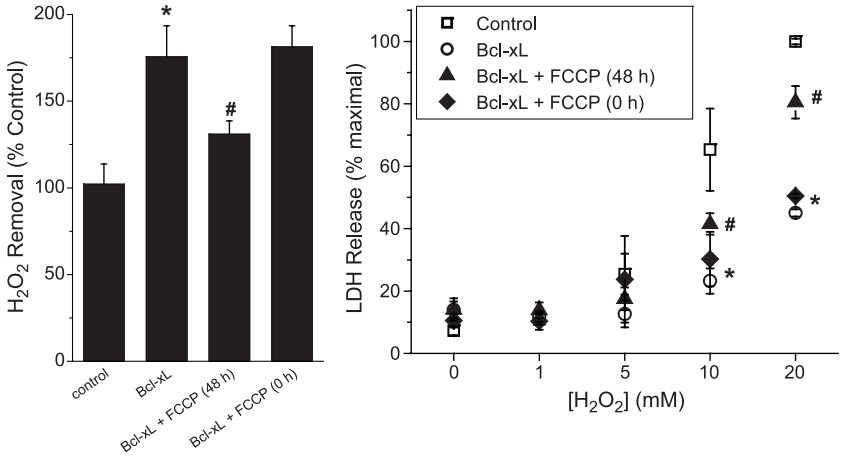

Fig. 6. Mild uncoupling decreases the effect of Bcl-xL overexpression on cellular H_2O_2 removal capacity and resistance to oxidative stress. Control or Bcl-xL cells were grown under control conditions or in the presence of 100 nM FCCP, as shown. H_2O_2 removal capacity (left) was determined in fresh cell homogenates as described under Experimental Procedures. Cell death, as indicated by LDH release (right), was determined in the growth medium after 2-h incubation in varying H_2O_2 concentrations, as shown. *$p < 0.05$ compared to control cells; $^{\#}p < 0.05$ compared to Bcl-xL cells.

genase activity in the growth media. We found (Fig. 6, right) that Bcl-xL cells were strongly resistant to damage under these conditions. However, Bcl-xL cells treated with FCCP for 48 h lost most of their resistance to H_2O_2-induced cell death. This increased sensitivity to H_2O_2 was not related to acute FCCP poisoning, since Bcl-xL cells treated with FCCP just prior to the addition of H_2O_2 exhibited injury similar to that of untreated cells. In addition, control cells treated with FCCP for 48 h or just prior to H_2O_2 exposure behaved similarly to untreated control cells (results not shown). Thus, increased mitochondrial ROS release, increased ability to remove H_2O_2, and resistance to oxidative stress are closely associated in Bcl-xL overexpressing cells.

DISCUSSION

One of the most intriguing properties of Bcl-2 family proteins is their ability to prevent both apoptotic and necrotic cell death. While the antiapoptotic effects of these proteins have been shown to be mediated by binding to proapoptotic proteins such as Bax, inhibiting their ability to oligomerize and form pores in the mitochondrial outer membrane through which soluble mitochondrial proapoptotic proteins are released into the cytosol [2,39,40], the mechanisms related to antinecrotic effects of Bcl-2 [41,42] are more poorly understood.

Several lines of evidence suggest that one of the mechanisms through which Bcl-2 family proteins protect against necrotic cell death involves enhanced antioxidant defenses in these cells: (i) these proteins protect against cell death induced by exogenous oxidants [11,37,38], (ii) depletion of intracellular antioxidants without affecting Bcl-2 levels can eliminate the protective effect of Bcl-2 or Bcl-xL [13,15,43,44], and (iii) Bcl-2 overexpressing cells contain higher levels of antioxidants [14,15]. However, the mechanism through which cells expressing Bcl-2 family proteins develop this increased resistance against oxidative stress was not established to date.

Redox-related genes are regulated by a variety of factors that include local oxygen tension and intracellular [H_2O_2] [16,17,45], parameters that could be altered by mitochondrial proteins such as Bcl-2. Since previous data from our group indicated that Bcl-2 does not alter normal mitochondrial respiration [7,19], it is unlikely that intracellular oxygen tension is affected by Bcl-2 levels. We therefore focused our attention on a possible effect of this protein on mitochondrial H_2O_2 release levels. We hypothesized that Bcl-2 could moderately increase mitochondrial ROS release under physiological conditions, leading to enhanced antioxidant expression levels and protection against acute oxidative stress-related cell death. Our hypothesis was supported by previous results suggesting higher mitochondrial H_2O_2 generation in Bcl-2-transfected cells [12,46] despite a lack of such a difference reported in other publications [11,18].

Using cell lines transfected with Bcl-2, Bcl-xL, and Mcl-1 and employing a highly sensitive H_2O_2 detection method, we found a significant 30–100% increase in mitochondrial H_2O_2 release relative to cells that express lower levels of these antiapoptotic proteins (Figs. 1–5). This effect is probably due to changes in electron leakage at the level of respiratory Complex I, since it could be observed only in the presence of NADH-linked substrates (Figs. 3 and 5). The promotion of ROS production by these Bcl-2 family proteins is not due to their effects on NAD(H) redox state, as indicated by the consistent results observed in the presence either of the uncoupler FCCP (oxidized redox state) or of the ATP synthetase inhibitor oligomycin (reduced redox state) (Figs. 4 and 5). We have not, at this juncture, identified the precise mechanism through which Bcl-2 family protein overexpression leads to enhanced ROS release from mitochondria. It is, however, possible that this effect of Bcl-2 proteins is due to changes in the composition or structure of Complex I, resulting in increased electron leakage but not enhanced electron transport. Alternatively, Bcl-2 family proteins may interact with Complex I, resulting in increased electron leakage. Furthermore, although our results suggest that Complex I is the main site for enhanced electron leakage in Bcl-2-family-protein-transfected mitochondria, they do not completely rule out a possibility for other electron leakage sites such as Complex III. Regardless of the site and mechanism through which the increase in ROS occurs, our results confirm and extend earlier reports suggesting elevated ROS generation with Bcl-2 overexpression [12] and are the first to demonstrate that three different anti-death Bcl-2 family proteins share the common characteristic of elevating basal rates of mitochondrial ROS production.

Despite the higher H_2O_2 release rate observed with Bcl-2 family protein expression and either isolated mitochondria or permeabilized cells, differences in H_2O_2 production measured with control vs overexpressing intact cells were either eliminated (Bcl-2, Fig. 1) or less evident (Bcl-xL, Fig. 5). These comparisons indicate that elevated mitochondrial ROS production is compensated by enhanced H_2O_2 removal capacities in the transfected cells. Our finding that the H_2O_2 removal rate of Bcl-xL cell homogenates is greater than that of control cells supports this interpretation (Fig. 6). Moreover, previous work with the same Bcl-2-transfected cells demonstrated elevated expression of catalase or glutathione reductase, two enzymes directly involved in the elimination of H_2O_2 [14]. These observations taken together suggest that the mild oxidative stress caused by elevated mitochondrial ROS production mediated by Bcl-2 family protein overexpression is responsible for inducing the expression of

one or more antioxidant systems. Our finding that 48 h exposure of Bcl-xL overexpressing cells to a level of a mitochondrial uncoupler that significantly lowers cellular H_2O_2 release also reduces the H_2O_2 removal capacity of cellular homogenates supports this hypothesis (Fig. 6).

Our results suggest that Bcl-2 family proteins may protect against oxidative cell death in a manner similar to that of ischemic preconditioning in which short nondamaging periods of ischemia protect against longer ischemic damage [47]. During preconditioning, mitochondrial ROS release is moderately increased, and this effect prevents large increases in ROS after ischemia [48,49]. The prevention of ROS release after ischemic preconditioning may be related to altered gene expression in delayed preconditioning [50] or, acutely, to redox activation of pathways capable of preventing mitochondrial ROS release such as ATP-sensitive K^+ channels [48,51].

Thus, while large increases in endogenous ROS accumulation are damaging to cells under many conditions, moderate increases in ROS such as those promoted by antiapoptotic Bcl-2 family members may be protective in a preconditioning-like manner, making cells better prepared to respond to acute oxidative stress. This effect explains the protective role of Bcl-2 family proteins against acute oxidative stress, in which the primary role of these proteins does not necessarily involve preventing the proapoptotic effects of proteins such as Bax or the prevention of caspase activation [37]. Indeed, protection against oxidative stress promoted by Bcl-2 family proteins may be vital to tumor cell survival since primary and metastatic tumors grow under a variety of oxygen tensions [52–54] and, presumably, intermittent periods of oxidative stress.

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ABBREVIATIONS

AA — antimycin A
Ala — alamethicin
BSA — bovine serum albumin
EGTA — ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
FCCP — carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
Glu — glutamate
HRP — horseradish peroxidase
LDH — lactate dehydrogenase
Mal — malate
Oligo — oligomycin
Pyr — pyruvate
ROS — reactive oxygen species
Rot — rotenone
Succ — succinate