

Decrease in intracellular superoxide sensitizes Bcl-2-overexpressing tumor cells to receptor and drug-induced apoptosis independent of the mitochondria

M-V Clément¹, JL Hirpara² and S Pervaiz^{*2}

¹ Department of Biochemistry, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260, Singapore

² Department of Physiology, National University of Singapore, 2 Medical Drive, MD9 03-06, Singapore 117597, Singapore

* Corresponding author: S Pervaiz, Department of Physiology, National University of Singapore, 2 Medical Drive, MD9 #03-06, Singapore 117597, Singapore. Tel: +65-6874-6602; Fax: +65-6778-8161; E-mail: phssp@nus.edu.sg

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Abstract

At least two mechanisms of early cytosolic acidification during apoptotic signaling have been described, one that involves caspase 8 activation downstream of receptor ligation and another dependent on mitochondria-derived hydrogen peroxide during merocil-induced apoptosis. Here, we show that Bcl-2 inhibits both mechanisms of acidification. Moreover, Bcl-2 overexpression resulted in a slightly elevated constitutive level of superoxide anion and pH in CEM leukemia cells. Interestingly, decreasing intracellular superoxide concentration with an inhibitor of the β -nicotinamide adenine dinucleotide phosphate oxidase or by transient transfection with a dominant-negative form of the guanosine triphosphate-binding protein Rac1 resulted in a significant increase in the sensitivity of CEM/Bcl-2 cells to CD95- or merocil-induced apoptosis. This increase in sensitivity was a direct result of a significant increase in caspase 8 activation and caspase 8-dependent acidification in the absence of caspase 9 activity or cytochrome *c* release. These findings suggest a mechanism of switching from mitochondria-dependent to mitochondria-independent death signaling in the same cell, provided the intracellular milieu is permissive for upstream caspase 8 activation, and could have implications for favorably tailoring tumor cells for drug treatment even when the mitochondrial pathway is compromised by Bcl-2. *Cell Death and Differentiation* (2003) 10, 1273–1285, doi:10.1038/sj.cdd.4401302

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Abbreviations: DPI, diphenyleiiodonium; Cyt *c*, cytochrome C; BCECF-AM, 2',7'-bis(2-carboxyethyl)-5,6-carboxyfluorescein; DCFH-DA, 2',7'-dichlorofluorescein diacetate; O₂⁻, superoxide; H₂O₂, hydrogen peroxide

Introduction

Apoptosis is a genetically programmed death pathway triggered in response to both physiological signals and pathological insults.¹ The effector components of this execution pathway involve the activation of intracellular caspases and death amplification factors released from the mitochondria, such as cytochrome *c* (Cyt *c*), apoptosis-inducing factor, Smac/Diablo, and caspase proteases.² Whereas some death stimuli induce execution pathways that engage the mitochondria, there are others that function independent of the mitochondria. This phenomenon has been clearly demonstrated in cells triggered to undergo apoptosis through ligation of the cell surface receptor CD95 (Apo-1/Fas). Cells that readily recruit and robustly activate caspase 8 in response to CD95 ligation undergo death execution independent of the mitochondria (Type I pathway) via direct downstream activation of execution caspases. Alternatively, cells that fail to induce early caspase 8 activation strongly upon receptor ligation are dependent upon mitochondria-derived proapoptotic factors to trigger efficient caspase activation for the execution of the death command (Type II pathway).³ Overexpression of Bcl-2 prevents the mitochondrial release of proapoptotic factors and therefore inhibits death signaling in Type II cells by blocking caspase activation downstream of the mitochondria.

Despite the clearly established two pathways leading to activation of executioner caspases and cell death, one common event following induction of apoptosis is acidification of the intracellular milieu.⁴ Indeed, a number of recent reports have demonstrated that cytosolic acidification is a permissive environment for the execution of the death signal.^{5–10} At least two mechanism(s) for cytosolic acidification have been described, one that functions downstream of caspase activation and the other that involves mitochondria-derived hydrogen peroxide (H₂O₂). The former was demonstrated with CD95-mediated apoptosis¹¹ and more recently with somatostatin-induced receptor-mediated apoptosis,⁹ and the latter was shown by us during drug-induced apoptosis in HL60 leukemia cells with a novel anticancer agent merocil.⁸ Acidification upon receptor-mediated apoptosis is inhibited by Bcl-2;^{12,13} however, there is no evidence to suggest an inhibitory role for Bcl-2 on H₂O₂-mediated cytosolic acidification. Hence, in this report using the Type II leukemia cell line CEM, in which the mitochondrial pathway was shut down by Bcl-2 overexpression (CEM/Bcl-2), we first investigated if in addition to the inhibition of CD95-mediated caspase-dependent acidification, Bcl-2 could also inhibit drug-induced H₂O₂-dependent drop in cytosolic pH. Indeed, we show that Bcl-2 inhibits both caspase (specifically caspase 8)-dependent as well as H₂O₂-dependent cytosolic acidification. Intrigued by these findings, we further questioned whether the death

inhibitory effect of Bcl-2 was mediated by a decrease in mitochondrial production of H_2O_2 on the one hand and the generation of a nonpermissive intracellular milieu for caspase 8 activation on the other. In this regard, our recent observations have highlighted the regulatory role of intracellular superoxide anion (O_2^-) and cytosolic acidification on apoptotic signaling in tumor cells. Maintaining a slightly elevated intracellular concentration of O_2^- inhibited apoptotic signaling, irrespective of the trigger, and conversely a decrease in intracellular O_2^- resulted in a dramatic increase in the sensitivity of tumor cells to apoptosis by facilitating caspase activation.^{14–16} Therefore, in addition to establishing a link between the death-inhibitory activity of Bcl-2 and its ability to create an environment nonconducive for caspase 8 activation, we investigated if a decrease in intracellular O_2^- could override or bypass Bcl-2 protection during death signaling, and if this effect was mediated via facilitating caspase 8 activation. Here, we report that a decrease in intracellular O_2^- , achieved by pharmacological inhibition of the β -nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex or by transient transfection with a dominant-negative form of the guanosine triphosphate (GTP)-binding protein Rac1 enhances the sensitivity of CEM/Bcl-2 cells to apoptosis. This increase in sensitivity to CD95- or merocil-induced apoptosis is mediated by a significant increase in caspase 8 activity and caspase 8-dependent cytosolic acidification and results in the death of CEM/Bcl-2 cells independent of the mitochondrial pathway. These findings suggest a novel mechanism by which a switch from mitochondria-dependent to mitochondria-independent death signaling could occur in the same cell, provided the intracellular milieu is permissive for caspase 8-dependent acidification, and could have potential implications for favorably tailoring the response of tumor cells to drug treatment even when the mitochondrial death signaling is compromised by overexpression of Bcl-2.

Results

Bcl-2 blocks caspase and H_2O_2 -induced cytosolic acidification

In order to assess the effect of Bcl-2 overexpression on caspase and H_2O_2 -mediated acidification, CD95 ligation was used as a model for caspase-mediated acidification,¹¹ whereas merocil was used as a trigger for the mitochondrial

generation of H_2O_2 and H_2O_2 -dependent acidification.⁸ The leukemia cell line CEM stably transfected with the empty vector (CEM/Neo) or with Bcl-2 (CEM/Bcl-2) (Figure 1a) was triggered to undergo apoptosis by incubation with 50 μ g/ml merocil or 0.25 μ g/ml of anti-CD95 (clone CH11). Unlike CEM/Neo cells, caspase activation (Figure 1b) and apoptosis

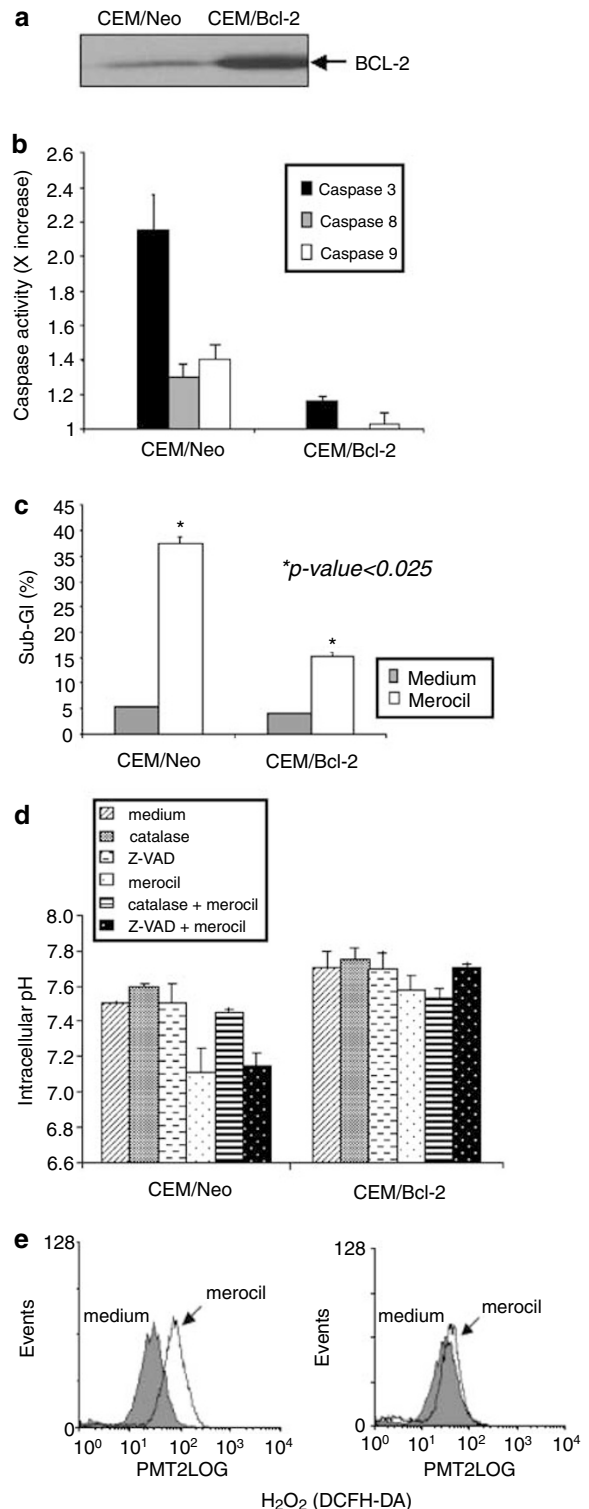


Figure 1 Bcl-2 inhibits drug-induced acidification. (a) Western blot analysis of Bcl-2 expression was performed in CEM/Neo and CEM/Bcl-2 cells using a primary anti-Bcl2 antibody (clone Bcl-2/100; BD Pharmingen, San Diego, CA, USA) as described in Materials and Methods. (b) In all, 1×10^6 CEM/Neo and CEM/Bcl-2 were exposed to 50 μ g/ml of merocil for 12 h and enzymatic activities of caspases 3, 8, and 9 were assessed using specific fluorimetric substrates. Data are shown as fold increase (\times increase in enzymatic activity). $1 \times$ represents the activity in control cells without the apoptotic trigger. (c) Cells were exposed to merocil for 18 h and apoptosis was assessed by PI staining and shown as sub-G1 (%). (d) In all, 1×10^6 CEM/Neo and CEM/Bcl-2 were exposed to 50 μ g/ml of merocil in the presence of catalase (1000 U/ml) or caspase inhibitor (50 μ M ZVAD-fmk) for 4 h (pHi). pHi was measured by using BCECF-AM as described in Materials and Methods. (e) In all, 1×10^6 cells (CEM/Neo or CEM/Bcl-2) were incubated with 50 μ g/ml of merocil for 4 h and intracellular H_2O_2 was determined by DCHF-DA staining and analyzed by flow cytometry as described in Materials and Methods

(sub-G1 fraction) were significantly inhibited in CEM/Bcl-2 cells (Figure 1c) upon exposure to merocil. In agreement with the data supporting an early drop in intracellular pH (pHi) during apoptotic signaling, the pHi of CEM/Neo cells dropped significantly ($\Delta\text{pH}=0.4$ (from 7.5 to 7.1) for merocil or $\Delta\text{pH}=0.3$ for anti-CD95) within 4 h of exposure to either apoptotic stimulus as shown in Figures 1d and 2a. Moreover, as shown previously with HL60 cells,⁸ merocil-induced acidification occurred independent of caspase activation (no effect of the general caspase inhibitor ZVAD-fmk), but was completely inhibited by preincubation with the H₂O₂ scavenger catalase (Figure 1d). On the contrary, preincubation of CEM/Neo cells with catalase had no effect on cytosolic acidification triggered by ligation of the CD95 death receptor (Figure 2a). However, CD95-induced acidification was downstream of caspase activation as evidenced by the inhibitory effect of the general caspase inhibitor ZVAD-fmk (data not shown). Furthermore, similar to the results obtained with somatostatin,⁹ our results showed that the specific caspase involved in the induction of acidification during CD95 ligation was caspase 8, as caspase 8-specific inhibitor LETD-fmk completely inhibited the drop in pHi (Figure 2a); inhibitors of caspase 3 (DEVD-fmk) or caspase 9 (LEHD-fmk) did not significantly prevent CD95-induced cytosolic acidification (data not shown). These findings confirm the existence of distinct mechanism(s) for cytosolic acidification in response to receptor- and drug-induced apoptosis.^{8,13,17} Interestingly, overexpression of Bcl-2 resulted in the inhibition of both H₂O₂ and caspase-mediated acidification (Figures 1d and 2a). These data, together with our observation that Bcl-2 inhibited H₂O₂ production upon merocil-induced apoptosis (Figure 1e), corroborate our recent findings demonstrating the role of mitochondrial H₂O₂ in merocil-induced cytosolic acidification.⁸ In this study and in our previous work, we used the probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) for the flow cytometric detection of intracellular H₂O₂. Although it has recently been suggested that the presence of Cyt *c* could cast doubt *vis-à-vis* the specificity of DCFH-DA, two observations presented here clearly demonstrate that in our system the change in fluorescence was indeed a measure of intracellular H₂O₂. First, we did not detect any Cyt *c* release at the time of detection of H₂O₂ (by DCFH-DA) in CEM/Neo cells incubated with merocil and second, Cyt *c* release upon CD95 ligation of CEM/Neo cells occurred without any

dichlorofluorescein (DCF) fluorescence, as these cells did not produce intracellular H₂O₂ (data not shown).

Decrease in intracellular O₂⁻ restores caspase 8 activity and acidification upon CD95-induced apoptosis in CEM/Bcl-2 cells

The inhibitory effect of Bcl-2 on mitochondrial generation of H₂O₂ as shown here with merocil is rather easily argued as

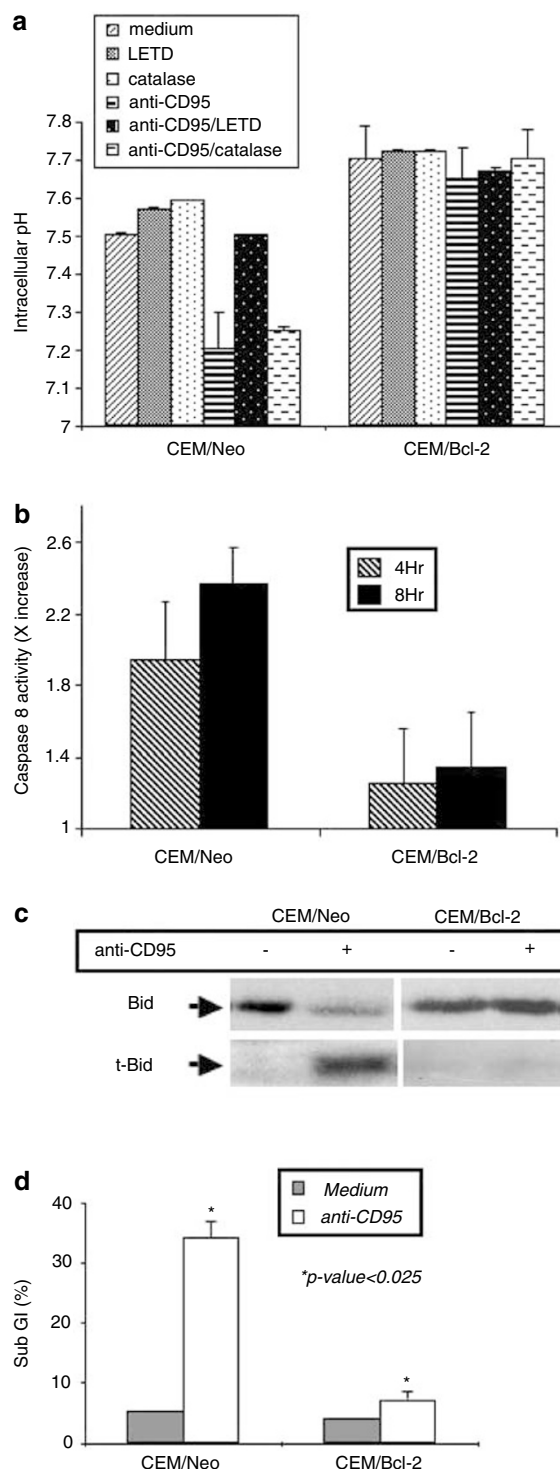


Figure 2 Bcl-2 inhibits CD95-induced acidification. **(a)** In all, 1×10^6 CEM/Neo and CEM/Bcl-2 were exposed to $0.25 \mu\text{g/ml}$ of anti-CD95 for 4 h (pHi) in the presence or absence of catalase (1000 U/ml) or caspase 8 inhibitor ($50 \mu\text{M}$ LETD-fmk). pHi was measured by using BCECF-AM as described in Materials and Methods. **(b)** A total of 2×10^6 CEM/Neo or CEM/Bcl-2 cells were incubated with $0.25 \mu\text{g/ml}$ of anti-CD95 for 4 or 8 h and caspase 8 activity was determined in lysates using a fluorimetric assay with caspase 8-specific substrate as described in Materials and Methods. Results are shown as fold increase (\times increase) in enzyme activity over the untreated control cells ($1 \times$). Data are means \pm S.D. of at least three independent experiments. **(c)** Lysates obtained from 5×10^6 cells (CEM/Neo and CEM/Bcl-2) following incubation with $0.25 \mu\text{g/ml}$ of anti-CD95 for 12 h were subjected to Western blot analysis for Bid cleavage using a polyclonal anti-Bid that recognizes the full-length Bid or an antibody that picks up the t-Bid as described in Materials and Methods. **(d)** Apoptosis was assessed following incubation of cells with anti-CD95 for 18 h by PI staining and data are shown as sub-G1 %

this would lead to not only an inhibition of cytosolic acidification but also block apoptotic signaling downstream of the mitochondria. This could also explain the inhibition of caspase 8 activity in CEM/Bcl-2 cells upon exposure to merocil (data not shown) as unlike receptor-induced caspase 8 activation, recent evidence places drug-induced caspase 8 activity downstream of the mitochondria.^{18,19} Earlier reports with CD95-induced apoptosis also argue in favor of a mechanism of caspase 8 activation downstream of the mitochondria in Type II cells, and hence the ability of Bcl-2 to block caspase 8 activation in this system.³ As receptor-induced cytosolic acidification has been attributed to early caspase 8 activation,⁹ the inhibition of CD95-induced cytosolic acidification in CEM/Bcl-2 cells suggests a role for Bcl-2 in inhibiting a step upstream of caspase 8 activation and acidification. Indeed, caspase 8 activity upon CD95-induced apoptosis is significantly inhibited in CEM/Bcl-2 cells (Figure 2b), which is accompanied by inhibition of proteolytic cleavage of the proapoptotic Bcl-2 protein Bid, a substrate of caspase 8 (Figure 2c), and as expected, ligation of the CD95 receptor significantly (P -value <0.025) blocked apoptotic death (sub-G1 fraction) in CEM/Bcl-2 cells (Figure 2d). In light of these data, we argue that the inhibition of CD95-mediated apoptosis by Bcl-2 could be due to an intracellular environment (of CEM/Bcl-2 cells) nonconducive for the activation or activity of caspase 8.

Interestingly, Bcl-2 expression has been linked to a change in the redox status of the cell, with reports linking its antioxidant activity to an indirect effect on the cells' antioxidant defenses by creating a slightly pro-oxidant intracellular milieu.^{20–22} Indeed, our results showing an increase in the steady-state levels of intracellular O_2^- in the stably transfected CEM/Bcl-2 cells, compared to the CEM/Neo cells (Figure 3), provide impetus to this hypothesis and is in line with our published reports that an increase in steady-state levels of intracellular O_2^- inhibits apoptosis triggered via CD95 ligation,¹⁶ and contrarily a decrease in intracellular O_2^- enhances the sensitivity of tumor cells to a variety of apoptotic triggers.^{14–16,23} In order to confirm a role for the higher steady-state intracellular concentration of O_2^- of CEM/Bcl-2

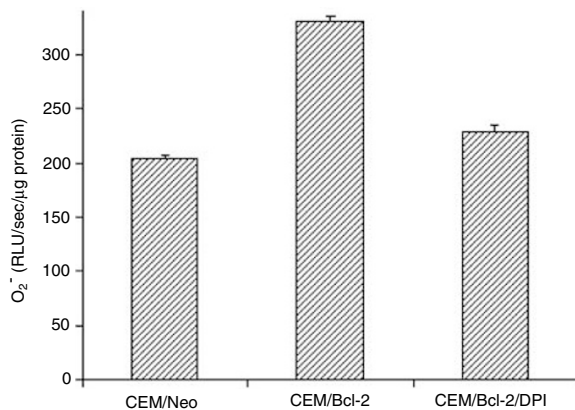


Figure 3 DPI decreases intracellular O_2^- in CEM/Bcl-2 cells. Steady-state levels of intracellular O_2^- were determined in 1×10^6 CEM/Neo or CEM/Bcl-2 or CEM/Bcl-2 cells treated for 1 h with 2.5μ M DPI (CEM/Bcl-2/DPI) by the lucigenin-based chemiluminescence assay described in Materials and Methods. Means \pm S.D. of three independent measurements are shown

cells in the inhibition of caspase 8-mediated acidification upon CD95 triggering, we investigated whether a decrease in intracellular O_2^- could restore caspase 8 activity and cytosolic acidification upon CD95 ligation. In order to accomplish this, we exploited the NADPH oxidase blocking activity of the widely used inhibitor diphenyleneiodonium (DPI) to decrease intracellular concentration of O_2^- .¹⁶ Incubation of CEM/Bcl-2 cells with 2.5μ M DPI for 2 h (CEM/Bcl-2/DPI) resulted in a significant decrease in intracellular O_2^- as compared to the CEM/Bcl-2 cells (Figure 3). CEM/Bcl-2 and CEM/Bcl-2/DPI cells were then triggered to undergo apoptosis by incubation with 0.25μ g/ml of anti-CD95, followed by measurements of pH_i (4 h), caspase 8 and 3 activities (4–8 h), and cell death (18 h). Unlike CEM/Bcl-2 cells (Figure 2a), CEM/Bcl-2/DPI cells underwent cytosolic acidification (Δ pH=0.4) as shown in Figure 4a, significant increases in caspases 8 and 3 activities (Figure 4b), cleavage of the caspase 8 substrate Bid (Figure 4c), and cell death (47 *versus* 3% in CEM/Bcl-2 cells) upon CD95 ligation (Figure 4b). In addition, consistent with the role of caspase 8 in cytosolic acidification, blocking caspase 8 activity (50μ M LETD-fmk) inhibited cytosolic acidification in response to anti-CD95 in CEM/Bcl-2/DPI cells (Figure 4a). Similar to the results obtained in CEM/Neo cells, inhibitors of caspase 3 (DEVD-fmk) or caspase 9 (LEHD-fmk) did not significantly prevent CD95-induced cytosolic acidification in CEM/Bcl-2/DPI cells (data not shown).

CD95-mediated apoptosis in CEM/Bcl-2/DPI cells is independent of Cyt c release and caspase 9 activity

There could be two possible explanations for the sensitivity of CEM/Bcl-2/DPI cells to CD95-mediated apoptosis. One simple argument could be that the inhibitory activity of Bcl-2 was compromised²⁴ and that the increase in caspase 8 activity observed upon preincubation with DPI was simply a function of breakdown of mitochondrial protective effect of Bcl-2, thereby restoring mitochondrial death signaling in these cells. An alternative and more intriguing possibility could be that CEM/Bcl-2/DPI cells are using a mitochondria-independent pathway for caspase activation and cell death. Our results showed that despite the increase in the overall sensitivity of CEM/Bcl-2/DPI cells to cell death, there was no detectable Cyt c release in CEM/Bcl-2/DPI cells upon incubation with anti-CD95 as compared to the CEM/Neo cells (Figure 5a). Consistent with our data shown in Figure 4b, the sensitivity of CEM/Bcl-2/DPI cells to CD95 receptor-induced cell death was significantly increased (P -value <0.05) than that of the CEM/Bcl-2 cells (Figure 5b). In addition, unlike caspase 8 inhibitor (LETD-fmk), preincubation of cells with the caspase 9 inhibitor (LEHD-fmk) had no effect on the increased sensitivity of CEM/Bcl-2/DPI cells to CD95-mediated apoptosis (Figure 5b). This is in stark contrast to the CEM/Neo cells, where both inhibitors were equally effective in rescuing cells from anti-CD95-induced cell death (Figure 5b). In order to confirm the complete absence of mitochondrial involvement in the increased sensitivity of CEM/Bcl-2/DPI cells to anti-CD95 treatment, we also assessed the change in the mitochondrial transmembrane potential ($\Delta\psi_m$). Indeed, we show that the

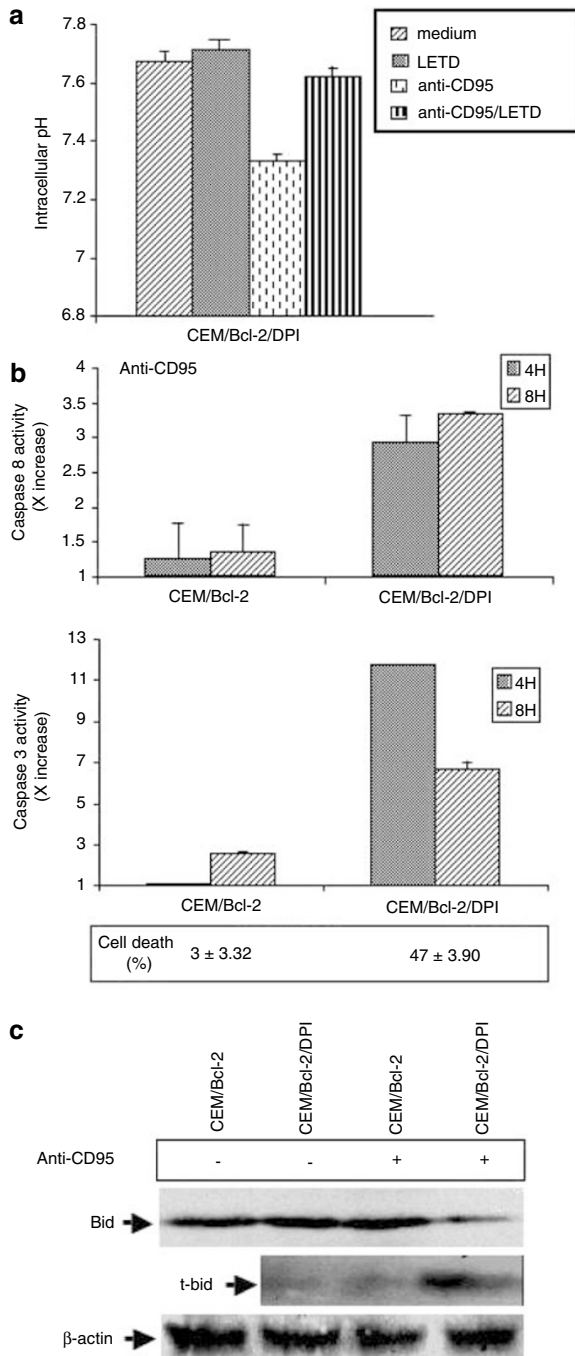


Figure 4 Decrease in intracellular O_2^- facilitates cytosolic acidification via increased caspase 8 activity. (a) CEM/Bcl2/DPI cells (1×10^6) were treated with 0.25 μ g/ml of anti-CD95 in the presence or absence of caspase 8 inhibitor LETD-fmk (50 μ M) for 4 h and pH_i was measured as described in Materials and Methods. Data shown are means \pm S.D. of three independent experiments. (b) Sensitivity to apoptosis is enhanced in CEM/Bcl-2/DPI cells. 2×10^6 CEM/Bcl-2 or CEM/Bcl-2/DPI cells were treated with 0.25 μ g/ml of anti-CD95 for 4 or 8 h and caspases 8- or 3-specific substrates as described in Materials and Methods. Results are shown as fold increase (\times increase) in enzyme activities over the untreated control cells ($1 \times$). Cell death was determined by the MTT assay following incubation with anti-CD95. Data are means \pm S.D. of at least three independent experiments. (c) Bid cleavage in lysates from CEM/Bcl-2 and CEM/Bcl-2/DPI cells following exposure to anti-CD95 (0.25 μ g/ml for 12 h) was detected by Western blotting as described in Materials and Methods. Membrane was probed with anti- β actin as an internal control for equal loading

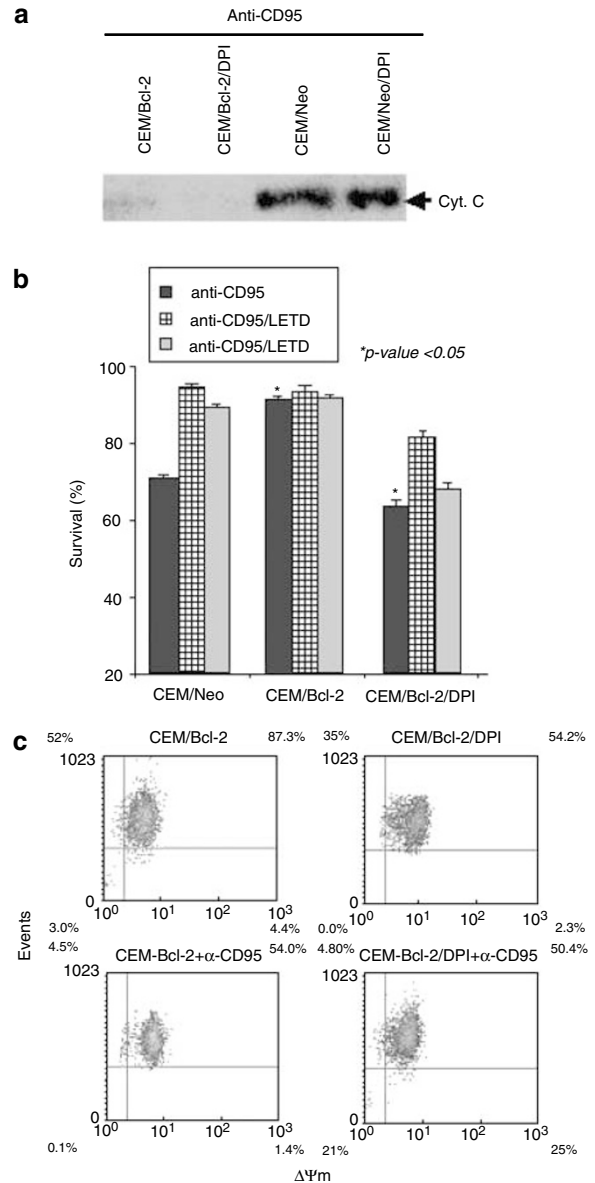


Figure 5 Decrease in intracellular O_2^- bypasses Bcl-2 protection. (a) Absence of Cyt *c* release in CEM/Bcl-2/DPI cells upon anti-CD95-induced apoptosis. Release of Cyt *c* was determined by Western blotting with anti-Cyt *c* in cytosolic extracts of 20×10^6 cells following exposure to 0.25 μ g/ml anti-CD95 for 6 h as described in Materials and Methods. (b) Increased sensitivity of CEM/Bcl-2/DPI cells to apoptosis is independent of caspase 9 activity. A total of 1×10^6 CEM/Bcl-2 or CEM/Bcl-2/DPI or CEM/Neo cells were exposed to 0.25 μ g/ml of anti-CD95 for 18 h in the presence or absence of either caspase 8 inhibitor (50 μ M LETD-fmk) or caspase 9 inhibitor (50 μ M LEHD-fmk) and cell viability was determined by the MTT assay as described elsewhere.³⁵ Data shown are means \pm S.D. of at least three independent experiments. (c) Mitochondrial $\Delta\psi_m$ was measured in CEM/Bcl-2 or CEM/Bcl-2/DPI cells after 6 h incubation with anti-CD95 (0.25 μ g/ml) followed by loading with the fluorescent probe DiOC₆ (40 nM) and analyzed by flow cytometry. At least 20 000 events were analyzed by WinMDI software

increase in sensitivity of CEM/Bcl-2/DPI cells to anti-CD95 (Figure 5c), further providing evidence that the mitochondrial pathway was not involved in this death signaling. It is therefore plausible that the small percentage of cells that underwent

apoptosis in CEM/Bcl-2 cells could be due to mitochondrial-independent activation of effector caspases. Our results indicate that the increase in sensitivity of CEM/Bcl-2/DPI cells (47% cell death) over the CEM/Bcl-2 cells (<5% cell death) to receptor-induced apoptosis is due to an increase in caspase 8 activity and caspase 8-dependent cytosolic acidification, which drives the death signal in the absence of Cyt *c* release and caspase 9 activity. These findings suggest a switch from mitochondria-dependent to mitochondria-independent death signaling in the same cell.

Merocil-induced apoptosis in CEM/Bcl-2/DPI cells is also independent of the mitochondrial pathway

Interestingly, similar to anti-CD95-induced apoptosis, exposure of CEM/Bcl-2/DPI cells to merocil also resulted in significantly increased activities of caspases 8 and 3 (but not caspase 9) and cell death (59% in CEM/Bcl-2/DPI cells *versus* 18% in CEM/Bcl-2 cells) as shown in Figure 6a. However, despite the fact that CEM/Bcl-2/DPI cells acidified upon exposure to merocil ($\Delta\text{pH}=0.47$), the mechanism of cytosolic acidification did not involve H_2O_2 production as preincubation with catalase had no effect on the drop in cytosolic pH (Figure 6b). Interestingly, and unlike CEM/Neo cells (shown in Figure 1d), merocil-induced acidification upon decreasing intracellular O_2^- (CEM/Bcl-2/DPI cells) was inhibited by blocking caspase 8 activation (Figure 6b). The fact that this decrease in cytosolic acidification was independent of intracellular H_2O_2 production was further corroborated by our results demonstrating the complete absence of an intracellular increase in H_2O_2 production (similar to CEM/Bcl-2 cells) in merocil-treated CEM/Bcl-2/DPI cells (Figure 6c). These results indicate that the inhibitory effect of Bcl-2 on merocil-induced intracellular production of H_2O_2 was not compromised in CEM/Bcl-2/DPI cells, and support our data on CD95-induced apoptosis that the mitochondrial protective effect of Bcl-2 was not over-ridden in CEM/Bcl-2/DPI cells. Moreover, similar to CD95-mediated apoptosis (Figure 5b), preincubation of cells with the caspase 9 inhibitor (LEHD-fmk) had no

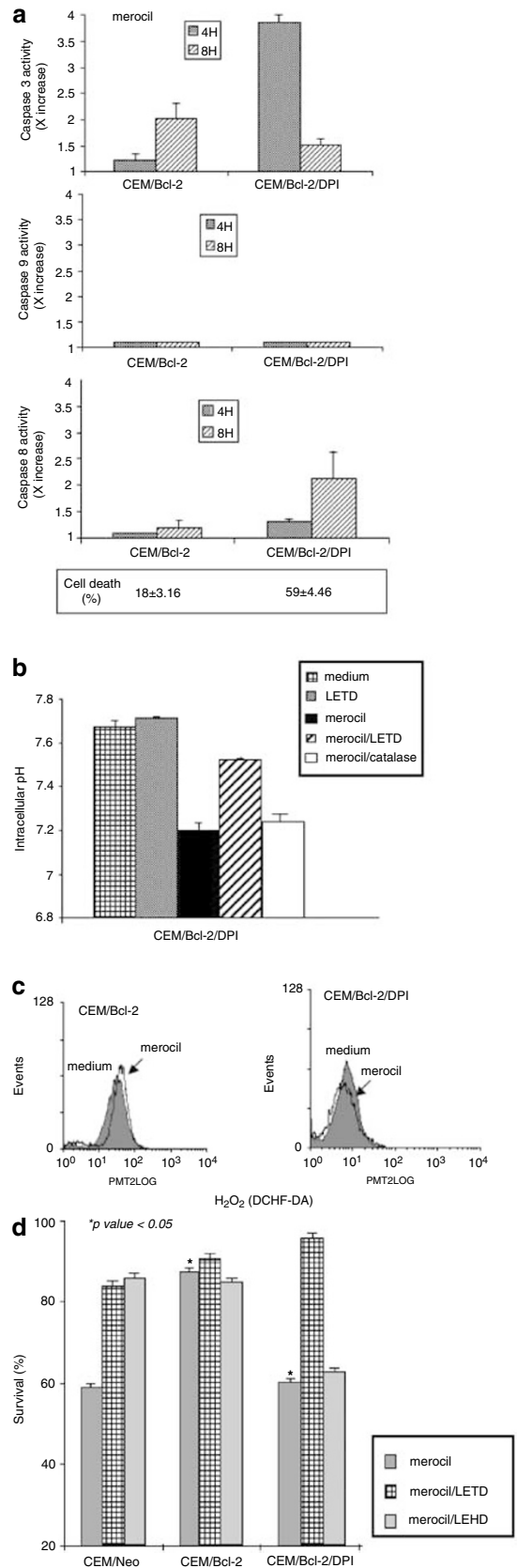


Figure 6 Decrease in intracellular O_2^- sensitizes CEM/Bcl2 cells to merocil-induced apoptosis via increased caspase 8 activation. **(a)** A total of 2×10^6 CEM/Bcl-2 or CEM/Bcl-2/DPI cells were treated with $50 \mu\text{g/ml}$ of merocil for 4 or 8 h and enzymatic activities of caspases 8, 9, and 3 were determined in lysates using a fluorescent assay with a caspase 8-, 9-, or 3-specific substrate as described in Materials and Methods. Results are shown as fold increase (\times increase) in enzyme activities over the untreated control cells ($1 \times$). Cell death was determined by the MTT assay following 18 h incubation with merocil. Data are means \pm S.D. of at least three independent experiments. **(b)** CEM/Bcl2/DPI cells (1×10^6) were treated with $50 \mu\text{g/ml}$ of merocil in the presence or absence of caspase 8 inhibitor LETD-fmk ($50 \mu\text{M}$) for 4 h and pH was measured as described in Materials and Methods. Data shown are means \pm S.D. of three independent experiments. **(c)** A total of 1×10^6 CEM/Bcl-2 or CEM/Bcl-2/DPI cells were incubated with $50 \mu\text{g/ml}$ of merocil for 4 h and intracellular H_2O_2 was determined by DCHF-DA staining and analyzed by flow cytometry as described in Materials and Methods. **(d)** A total of 1×10^6 CEM/Bcl-2 or CEM/Bcl-2/DPI or CEM/Neo cells were exposed to $50 \mu\text{g/ml}$ of merocil for 18 h in the presence or absence of either caspase 8 inhibitor ($50 \mu\text{M}$ LETD-fmk) or caspase 9 inhibitor ($50 \mu\text{M}$ LEHD-fmk) and cell viability was determined by the MTT assay as described in Materials and Methods. Data shown are means \pm S.D. of at least three independent experiments

effect on cell sensitivity to merocil-mediated apoptosis in CEM/Bcl-2/DPI, while a significant inhibition was detected in CEM/Neo cells (Figure 6d). Finally, as with anti-CD95-induced apoptosis, there was a significant increase in the sensitivity (P -value < 0.05) of CEM/Bcl-2/DPI cells to merocil-induced cell death over the CEM/Bcl-2 cells (Figure 6d). These data demonstrate that a decrease in intracellular O_2^- bypasses resistance to apoptosis conferred by Bcl-2 over-expression by facilitating caspase 8-dependent cytosolic acidification independent of the trigger (anti-CD95 or merocil), and are strongly suggestive of a diversion from mitochondria-dependent pathway to mitochondria-independent death signaling in the same cell line.

Transient expression of RacN17, a dominant-negative form of the small GTP-binding protein Rac1 in CEM/Bcl-2 cells diverts CD95- and merocil-mediated apoptosis from mitochondria dependent to mitochondria independent

Metabolic pathways leading to the generation of ROS are best characterized in phagocytic cells. In these cells, O_2^- is generated by the NADPH oxidase complex.^{25,26} In nonphagocytic cells including tumor cells, the proteins responsible for producing O_2^- and other ROS are less well defined, but could well function in a manner similar to the phagocyte NADPH-oxidase complex. Indeed, it has recently been shown that similar to the phagocyte oxidant-generating system, the production of ROS in nonphagocytic cells is regulated by the small GTP-binding protein Rac1.²⁷⁻³⁰ To this effect, we recently demonstrated that the expression of a constitutively activated form of Rac1 leads to an increase in intracellular O_2^- and inhibition of the apoptotic pathway in M14 human melanoma cell line. Conversely, inhibition of Rac1 activation in a significant increase in the sensitivity of melanoma cells to apoptosis.¹⁴ RacN17-mediated increase in tumor cell sensitivity to apoptosis was directly linked to a decrease in

intracellular O_2^- . Therefore, in order to provide a physiological basis for the data presented with DPI and to dispel any doubts *vis-à-vis* the use of a chemical, we questioned if similar to our results with DPI, transfection of CEM/Bcl-2 cells with RacN17 could lead to caspase 8-dependent cytosolic acidification upon CD95 ligation or merocil treatment. Indeed, the expression of RacN17 in CEM/Bcl-2 cells (CEM/Bcl-2/RacN17; Figure 7a) resulted in a significant decrease in the intracellular concentration of O_2^- as compared to the cells transfected with the vector alone (CEM/Bcl-2/pIRES)

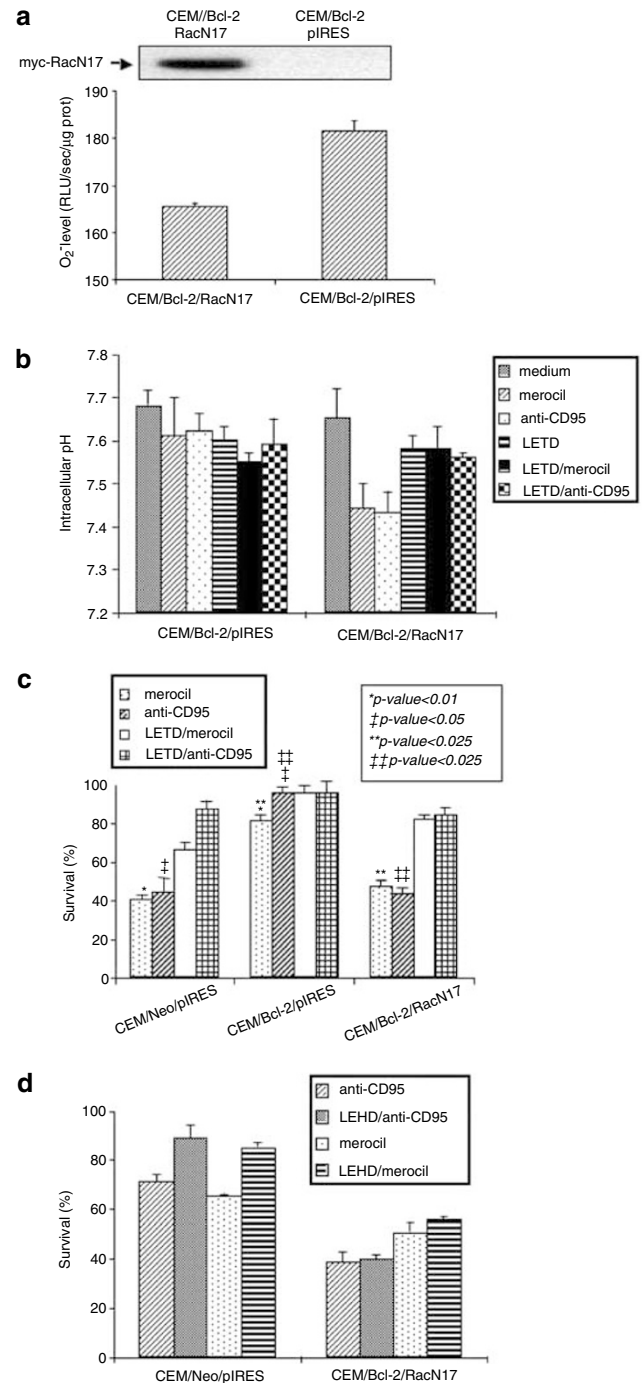


Figure 7 Expression of a dominant-negative form of Rac 1 enhances sensitivity of CEM/Bcl-2 cells independent of the mitochondria. **(a)** Transient transfection of CEM/Bcl-2 cells with either the empty pIRES vector or pIRESRacN17 was performed as described in Materials and Methods. At 48 hours post transfection, the expression of transiently expressed RacN17 protein was detected by Western blotting as described in Materials and Methods. Intracellular O_2^- was measured in lysates of 1×10^6 cells by the lucigenin-based chemiluminescence assay and is shown as RLU/s/g protein. **(b)** CEM/Bcl-2/pIRES or CEM/Bcl-2/RacN17 cells (1×10^6) were exposed to 50 μ g/ml of merocil or 0.25 μ g/ml of anti-CD95 for 4 h in the presence or absence of caspase 8 inhibitor (50 μ M LETD-fmk) and pH was measured by BCECF as described in Materials and Methods. **(c)** Increased sensitivity of CEM/Bcl-2/RacN17 cells to apoptosis is inhibited by blocking caspase 8 activity. Cells were treated as in (b) and survival was assessed 18 h after incubation with either anti-CD95 or merocil by the β -gal survival assay described in Material and Methods. **(d)** Increased sensitivity of CEM/Bcl-2/RacN17 cells to merocil or anti-CD95 is independent of caspase 9 activity. CEM/Neo/pIRES or CEM/Bcl-2/RacN17 cells were treated with merocil (50 μ g/ml) or anti-CD95 (0.25 μ g/ml) in the presence or absence of caspase 9-specific inhibitor (50 μ M LEHD-fmk). Survival was assessed 18 h following incubation with anti-CD95 or merocil by the β -gal survival assay described Material and Methods. All data shown are mean \pm S.D. of at least three independent experiments

(Figure 7a). In addition, whereas CEM/Bcl-2/pIRES cells did not undergo cytosolic acidification in response to either CD95 ligation or merocil treatment, CEM/Bcl-2/RacN17 cells facilitated cytosolic acidification following induction of apoptosis that was significantly inhibited by the caspase 8 tetrapeptide inhibitor (Figure 7b). It has to be pointed out that the decrease in intracellular O_2^- (11%) and ΔpH (-0.2) obtained upon transient transfection with RacN17 was the result of measurements performed in the total cell population in which routinely 35–40% of the cells were transfected. Hence, the changes observed in O_2^- and pH were understandably lower than those obtained upon DPI treatment of CEM/Bcl-2 cells (32% and -0.4 , respectively). In addition, the sensitivity to receptor- or drug-induced apoptosis was increased (P -value <0.025) in CEM/Bcl-2/RacN17 cells (compared to CEM/Bcl-2/pIRES cells), which could be reversed upon blocking caspase 8 activation as shown in Figure 7c. It should be pointed out that transfection with the empty vector pIRES did not affect the ability of Bcl-2 to inhibit death signaling as shown by the relative insensitivity of CEM/Bcl-2/pIRES cells to merocil (*P -value <0.01) or anti-CD95 ($^{\ddagger}P$ -value <0.05) treatment, compared to the CEM/Neo/pIRES cells (Figure 7c). Furthermore, similar to the absence of mitochondrial involvement in the enhanced sensitivity of CEM/Bcl-2/DPI cells in receptor- or drug-induced apoptosis, there was no Cyt *c* release (data not shown), and blocking caspase 9 activity with the LEHD-fmk tetrapeptide did not inhibit apoptosis in CEM/Bcl-2/RacN17 cells, unlike CEM/Neo/pIRES cells (Figure 7d). In these experiments, survival of CEM/Bcl-2/pIRES, CEM/Bcl-2/RacN17, and CEM/Neo/pIRES cells was assessed by the β -galactosidase (β -Gal) survival assay that allows for the specific measurement of survival of pIRES- or pIRES/RacN17-transfected cells¹⁴ (see Materials and Methods). These results further consolidate our finding that the increase in apoptotic sensitivity of CEM/Bcl-2 cells in a reduced intracellular O_2^- milieu is not due to compromise of the mitochondrial protective ability of Bcl-2, but indeed due to the induction of caspase-dependent cytosolic acidification and activation of the caspase cascade, independent of the mitochondria.

Finally, to link the increase in sensitivity observed upon transfection with RacN17 (CEM/Bcl-2/RacN17 cells) to a drop in intracellular O_2^- , we used a pharmacological inhibitor of Cu/Zn superoxide dismutase, diethyldithiocarbamate (DDC),^{14,16} to maintain increased intracellular steady-state levels of O_2^- prior to triggering apoptosis with anti-CD95 (Table 1). Our results showed that preincubation with DDC completely inhibited the drop in cytosolic pH observed upon

Table 1 Incubation with DDC maintains steady-state increase in intracellular O_2^- levels in CEM/Bcl-2/pIRES and CEM/Bcl-2/RacN17 cells

Cells	Medium	DDC
CEM/Bcl-2/ pIRES	181.5 \pm 2.12	677.5 \pm 7.77
CEM/Bcl-2/ RacN17	165.5 \pm 0.7	619 \pm 12.72

Cells (1×10^6) were exposed to 500 μ M DDC for 4 h and intracellular O_2^- was determined by a lucigenin-based chemiluminescence assay as described in Materials and Methods. Data shown are RLU/s/ μ g of protein and are means \pm S.D. of three independent experiments

ligation of the CD95 receptor in CEM/Bcl-2/RacN17 cells (Figure 8a). Furthermore, the increase in sensitivity of the cells to anti-CD95 treatment obtained in CEM/Bcl-2/RacN17 cells was also reverted by DDC exposure (Figure 8b). These data provide further support to our findings that the increase in sensitivity to apoptosis observed upon RacN17 transfection was linked to intracellular O_2^- concentration.

Transient transfection with Bcl-2 increases intracellular O_2^- and pH and decreases cell sensitivity to apoptosis

In order to provide further evidence that the increase in intracellular O_2^- and cytosolic pH was linked to Bcl-2 expression, and that elevated level observed in the CEM cells stably transfected with Bcl-2 (CEM/Bcl-2) was not a coincidental finding, we transiently transfected CEM cells with a vector containing Bcl-2 (pcDNA3-Bcl-2). At 48 h post-transfection, the intracellular levels of O_2^- , cytosolic pH, and sensitivity to anti-CD95- and drug-induced apoptosis were assessed. Indeed, our results showed that transient transfection of Bcl-2 resulted in significant increases in the constitutive

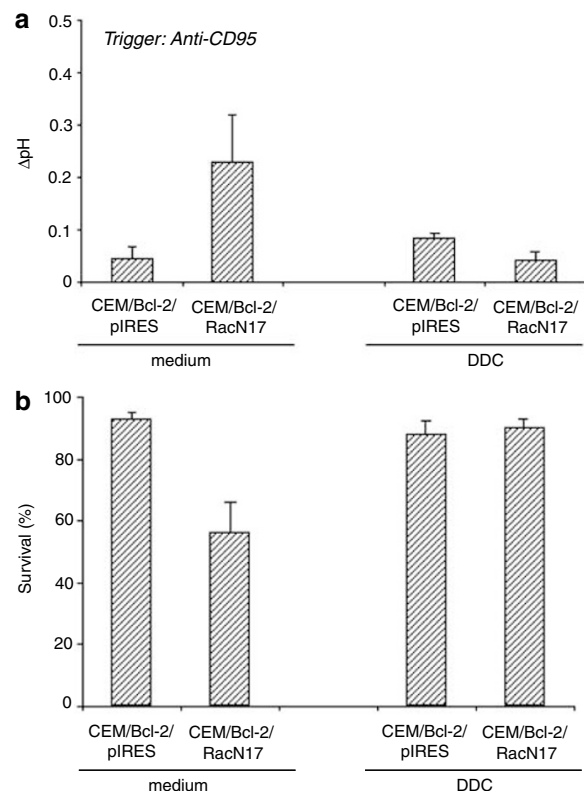


Figure 8 Maintaining increased intracellular concentration of O_2^- prevents the drop in pH and the increase in sensitivity of CEM/Bcl-2/RacN17 cells to anti-CD95 treatment. CEM/Bcl-2 cells transiently transfected with either the empty pIRES vector (CEM/Bcl-2/pIRES) or pIRES RacN17 (CEM/Bcl-2/RacN17) were exposed to 500 μ M DDC for 1 h prior to incubation with anti-CD95 (0.25 μ g/ml) for 4 h for measurement of pH (a) or 18 h for determination of cell survival (b). pH was measured by loading with BCECF-AM and is shown as the change in pH (ΔpH) and cell survival was determined by the β -Gal survival assay, as described in Materials and Methods. Data shown are means \pm S.D. of three independent observations

level of intracellular O_2^- (Figure 9a) and cytosolic pH (Figure 9b). Whereas cells transfected with the empty vector (CEM/vector) underwent cytosolic acidification upon receptor or drug-induced apoptosis, cells transiently transfected with Bcl-2 (pcDNA3-Bcl-2) resisted the drop in pH triggered by anti-CD95 or merocil (Figure 9b). In addition, and as expected, the sensitivity of pcDNA3-Bcl-2 cells was significantly decreased to apoptosis triggered either by ligation of the CD95 receptor or by exposure to merocil (Figure 9c). The absence of a complete inhibition of pH and cell death upon transfection with Bcl-2 could be attributed to the transfection efficiency of 35–40% routinely obtained in these cells. These data corroborate our findings with the stably transfected CEM cells used in this study, and suggest that Bcl-2 overexpression results in an increase in the intracellular constitutive level of O_2^- that could, in part, be responsible for the death-inhibiting activity of Bcl-2.

Discussion

The functional characterization of apoptotic signaling into mitochondria dependent or mitochondria independent is basically derived from the inhibitory effect of Bcl-2 on the former, but not the latter. Irrespective of the nature of the death signaling circuitry, there is a growing body of evidence to suggest that an early drop in cytosolic pH precedes apoptotic execution. Indeed, a number of recent reports have demonstrated that cytosolic acidification is a permissive environment for the execution of the death signal.^{5–10} At least two mechanism(s) for the drop in pH following apoptotic triggering have been described, one that functions downstream of caspase activation^{9,11} and the other that involves mitochondria-derived H_2O_2 .⁸ Whereas acidification upon receptor-mediated apoptosis has been directly linked to upstream caspase 8 activation and is inhibited by overexpression of Bcl-2,^{9,12,13} we recently demonstrated the direct involvement of mitochondrial H_2O_2 in the early drop of cytosolic pH during merocil-induced apoptosis in HL60 leukemia cells. The present results confirm the involvement of caspase 8 in CD95 signaling-induced acidification in CEM leukemia cells, which is blocked upon overexpression of Bcl-2 (Figure 2). In addition, similar to our results obtained with HL60 cells, merocil-induced cytosolic acidification in CEM cells was blocked by H_2O_2 scavenger catalase or the membrane-permeable catalase mimetic EUK-8 (data not shown), but not by the caspase inhibitors (Figures 1 and 2). These data were corroborated by experimental evidence demonstrating a significant intracellular increase in H_2O_2 production in CEM cells treated with merocil (Figure 1). Interestingly, we also present data to demonstrate that overexpression of Bcl-2 inhibits both CD95 receptor-induced acidification, as well as H_2O_2 -mediated cytosolic acidification triggered upon exposure to the anticancer agent merocil.

The ability of Bcl-2 to inhibit merocil-induced H_2O_2 production from the mitochondria supports the inhibitory effect of Bcl-2 on mitochondrial ROS generation, which could be linked to the well-documented mitochondrial protective activity of Bcl-2, such as inhibition of peroxide-mediated damage to mitochondrial membranes that allows the egress of proapoptotic

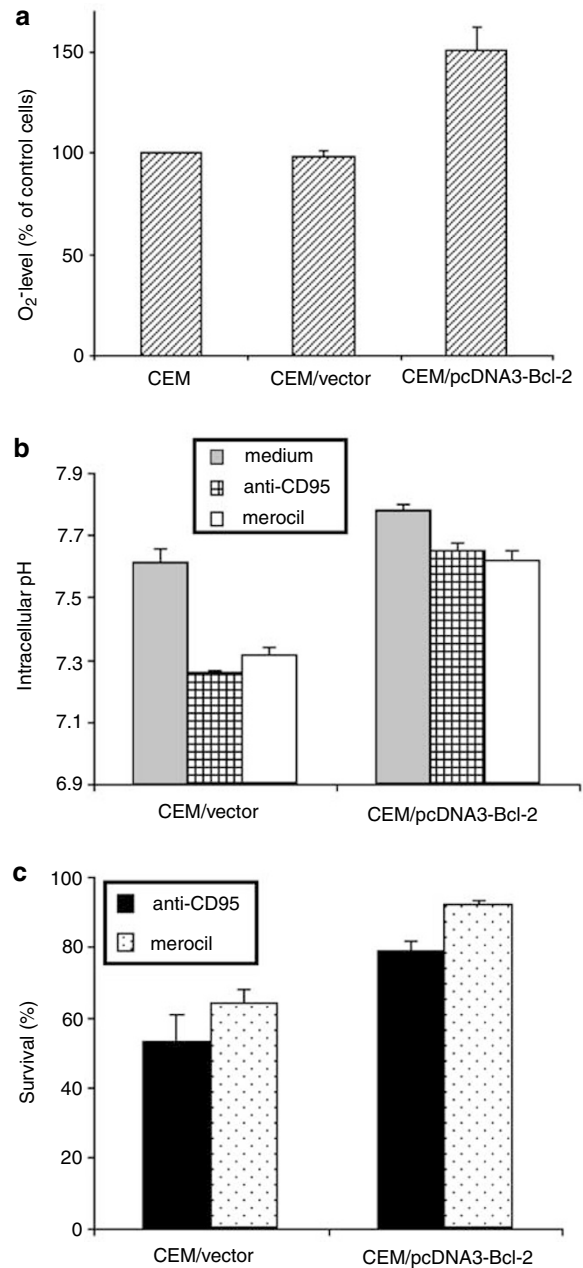


Figure 9 Transient transfection with pcDNA3-Bcl-2 increases intracellular O_2^- and inhibits apoptotic acidification and cell death. **(a)** Transient transfection of CEM cells with pcDNA3-Bcl-2 was performed as described Material and Methods. The intracellular level of O_2^- was determined in the CEM or CEM/vector or CEM/pcDNA3-Bcl-2 cells by the lucigenin-based chemiluminescence assay as described Material and Methods. Data are shown as percentage difference in intracellular O_2^- relative to the untransfected cells (set as 100%). Mean \pm S.D. of three independent observations is shown. **(b)** In all, 1×10^6 CEM/vector or CEM/pcDNA3-Bcl-2 cells were incubated with 50 μ g/ml of merocil or 0.25 μ g/ml of anti-CD95 for 4 h (cytosolic pH) or **(c)** 18 h for cell survival. Cytosolic pH was determined by BCECF-AM loading and cell survival was assayed by the β -Gal survival assay as described in Material and Methods

factors.^{31,32} On the contrary, the inhibitory effect of Bcl-2 on CD95-induced apoptosis appears to be mediated by its ability to inhibit caspase 8 activation and caspase 8-dependent cytosolic acidification, thereby creating a nonpermissive

intracellular milieu for death execution. In the context of Type II cells, such as CEM, this could simply be a function of Bcl-2-mediated inhibition of the release of mitochondrial proapoptotic factors required for the amplification of the caspase cascade. However, an alternative hypothesis could be that Bcl-2 has an inhibitory effect on caspase 8 activation, independent of the mitochondria. This would imply that cells that retain a completely functional Bcl-2 with respect to death signaling downstream of the mitochondria, could still be tailored to a conducive intracellular milieu for caspase activation in the complete absence of mitochondrial involvement. To this end, we have recently demonstrated that a permissive intracellular milieu is a function of a decrease in intracellular O_2^- concentration and cytosolic acidification.^{5,10,14,15} An increase in intracellular O_2^- inhibits apoptotic signaling irrespective of the trigger,^{15,16} and pharmacological or molecular inhibition of the intracellular O_2^- producing oxidase complex (NADPH oxidase) results in an increase in cell sensitivity to apoptosis via a direct or indirect effect on caspase activation pathways.^{14,15} Intrigued by these findings, here we set out to investigate if the death inhibitory activity of Bcl-2 could be a function of a pro-oxidant intracellular milieu (increase in the constitutive level of O_2^-), and if so could the sensitivity to apoptosis be restored by favorably tailoring the intracellular environment of CEM/Bcl-2 cells. Indeed, not only do we provide evidence that the intracellular O_2^- concentration is elevated in CEM/Bcl-2 cells, but in order to further link this to the inhibitory effect of Bcl-2 on caspase activation, we demonstrate here that decreasing intracellular O_2^- facilitates caspase 8 activity and caspase 8-dependent acidification during apoptosis in CEM/Bcl-2 cells. The increase in intracellular O_2^- in CEM/Bcl-2 cells compared to the CEM/Neo cells is in agreement with our earlier findings that an increase in intracellular O_2^- level is sufficient to induce inhibition of caspase activity and cell death.^{5,14–16,23} In order to be convinced that our results, indeed, were a function of targeted decrease in the intracellular level of O_2^- , we used a pharmacological inhibitor of the NADPH oxidase (DPI) and cells transfected with the dominant-negative form of Rac 1 (RacN17). DPI is a widely used inhibitor of O_2^- production, and we have recently shown that the expression of RacN17 in tumor cells results in the inhibition of O_2^- production and increase in sensitivity to apoptosis.¹⁴ Although the small GTP-binding protein Rac 1 is involved in functions other than the activation of NADPH oxidase, such as JNK activation and actin polymerization, in our recent report using targeted mutagenesis of Rac 1, we demonstrated that only the NADPH oxidase-interacting residues conferred resistance to apoptosis.¹⁴ In addition, our data with DDC, an inhibitor of Cu/Zn SOD, provide evidence to support that the increase in sensitivity of CEM/Bcl-2/RacN17 cells to apoptosis was linked to intracellular O_2^- concentration; maintaining a higher steady-state level of O_2^- in the presence of RacN17 completely reverted the increase in sensitivity and the drop in pH observed upon anti-CD95 ligation. In addition, a further proof linking Bcl-2 overexpression to the increase in the constitutive level of O_2^- is provided by our results, demonstrating a significant increase in intracellular steady-state level of O_2^- upon transient transfection of CEM cells with pcDNA3-Bcl-2 (Figure 9).

An easy explanation for the enhanced sensitivity of CEM/Bcl-2 cells upon decreasing intracellular O_2^- could be that the mitochondrial protective ability of Bcl-2 was compromised in CEM/Bcl-2/DPI or CEM/Bcl-2/RacN17 cells following anti-CD95 or drug-induced apoptosis. This would simply result in mitochondrial H_2O_2 production (upon merocil treatment), release of Cyt *c*, assembly of the apoptosome, and caspase 9-driven activation of the caspase cascade. Interestingly, the increase in sensitivity of CEM/Bcl-2 cells upon decreasing intracellular O_2^- did not involve the mitochondrial death pathway, as there was no detectable Cyt *c* release or drop in mitochondrial $\Delta\psi_m$ in CEM/Bcl-2/DPI cells upon CD95 ligation. Further evidence to support the absence of mitochondrial signaling in CEM/Bcl-2/DPI cells was the inability of caspase 9 inhibitor (LEHD-fmk) to block death signaling in CEM/Bcl-2/DPI cells, while the same inhibitor significantly blocked death in the mitochondria-dependent CEM/Neo cells (Figures 5b and 6d). Although one could question the absolute specificity of the tetrapeptide caspase inhibitor(s), data showing differences in the responses between CEM/Bcl-2/DPI or CEM/Bcl-2/RacN17 cells and CEM/Neo or CEM/Neo/piRES cells to the LEHD-fmk tetrapeptide are in favor of some specificity. Furthermore, the absence of caspase 9 involvement is also supported by the lack of LEHDase activity in CEM/Bcl-2/DPI cells upon merocil treatment, while DEV-Dase and LETDase activities were significantly increased. What is of interest is that the decrease in intracellular O_2^- -sensitized CEM/Bcl-2 cells to merocil-induced apoptosis by switching from H_2O_2 -dependent to caspase 8-dependent cytosolic acidification, independent of the mitochondria. These data clearly indicate that the increase in caspase 8 activation and sensitivity of CEM/Bcl-2/DPI cells to apoptosis was not a function of mitochondrial-dependent caspase activation and provides an alternative mechanism for the inhibitory effect of Bcl-2 on caspase 8 activation. Hence, in addition to its inhibitory effect on mitochondrial death pathway, overexpression of Bcl-2 could inhibit death signaling by creating an intracellular milieu nonpermissive for caspase 8 activation, that is, an increase in intracellular O_2^- .

Collectively, these findings provide further support to our earlier reports that a slight increase in intracellular O_2^- is inhibitory to apoptotic signaling, irrespective of the trigger.^{14–16,23} Thus, in the presence of a conducive intracellular milieu for caspase 8 activity (low intracellular O_2^-), a mitochondria-dependent death signal could be diverted to a mitochondria-independent signal in the same cell line. These data support the recent observation that existence of cells with restricted ability to engage mitochondria-dependent or mitochondria-independent pathways upon CD95-induced apoptosis may not be entirely true, and a switch from one to the other could occur in the same cell, provided the conditions are optimal.^{33,34} This bypass effect is a direct outcome of increased caspase 8 activity that can trigger cytosolic acidification and amplification of the caspase cascade. Furthermore, contrary to the general notion that the mitochondrial pathway is required for drug-induced apoptosis, our data demonstrate that an increase in caspase 8 activity can effectively drive the execution signal in the complete absence of mitochondrial involvement, thereby converting a mitochondrial death signal into a mitochondria-independent signal

during drug-induced apoptosis. These findings could have tremendous implications for designing therapies to enhance or divert apoptotic signaling in hematopoietic and other malignancies, where overexpression of the oncoprotein Bcl-2 limits the efficacy of chemotherapy regimens. Thus, the probable effector mechanism(s) for increasing the apoptotic sensitivity of cancer cells could be to manipulate intracellular O_2^- and facilitate cytosolic acidification, thereby creating an environment permissive for efficient apoptotic execution and enhancing the efficacy of chemotherapy. The exact mechanism involved in the inhibition of caspase 8 activity by the slightly raised intracellular level of O_2^- still remains to be elucidated and is the focus of our ongoing investigations.

Materials and Methods

Cell lines

CEM human leukemia cells stably transfected with the control vector (CEM/Neo) or Bcl-2 (CEM/Bcl-2) were generously provided by Dr Roberta A Gottlieb (Scripps Cancer Center, La Jolla, CA, USA). Cells were maintained in RPMI 1640 supplemented with 5% fetal bovine serum and 20 μ g/ml of G418 in a 37°C incubator with 5% CO_2 . The expression of Bcl-2 was confirmed by Western blot analysis using a primary anti-Bcl2 antibody (clone Bcl-2/100 at 1 : 1000 dil.; BD Pharmingen, San Diego, CA, USA) and a secondary HRP-conjugated anti-mouse IgG (1 : 5000 dilution; Pierce, Rockford, IL, USA). Apoptosis was induced by exposure of cells (1×10^6 /ml) to 0.25 μ g/ml anti-CD95 IgM (clone CH11, Upstate Biotech., Lake Placid, NY, USA) or the anticancer agent merocil³⁵ (50 μ g/ml) for 4–18 h. Cell survival was determined by the MTT assay and apoptosis was assessed by propidium iodide (PI) staining for cell cycle (percentage of sub-G1 for apoptosis) as described previously.³⁵

Determination of caspases 3, 8, and 9 activities

Caspases 3, 8, and 9 activities were assayed by using AFC-conjugated substrates supplied by Biorad Laboratories (Hercules, CA, USA). Cells (1×10^6 cells/ml) were exposed to merocil (50 μ g/ml) or anti-CD95 (0.25 μ g/ml) for 4–18 h, washed twice with $1 \times$ PBS, resuspended in 50 μ l of chilled cell lysis buffer (provided by the supplier) and incubated on ice for 10 min. 50 μ l of $2 \times$ reaction buffer (10 mM HEPES, 2 mM EDTA, 10 mM KCl, 1.5 mM $MgCl_2$, 10 mM DTT) and 6 μ l of the fluorogenic caspase-specific substrates (DEVD-AFC for caspase 3, LETD-AFC for caspase 8, and LEHD-AFC for caspase 9) were added to each sample and incubated at 37°C for 30 min. Protease activity was determined by measuring the relative fluorescence intensity at 505 nm following excitation at 400 nm using a spectrofluorimeter (TECAN Spectrofluor Plus, Austria). Results are shown as fold increase (\times increase) in activity relative to the enzymatic activity obtained from untreated control cells ($1 \times$). Activation of caspase 8 was also assessed by Western blot analysis for Bid cleavage using a rabbit polyclonal anti-Bid IgG (Biovision Research Products, Paolo Alto, CA, USA) that recognizes the 22 kDa full-length Bid or a polyclonal anti-Bid (Cell Signaling, Beverly, MA, USA) that picks up the 15 kDa truncated Bid. To do so, lysates from 3×10^6 cells (CEM/Neo, CEM/Neo/DPI, CEM/Bcl-2, and CEM/Bcl-2/DPI) exposed to anti-CD95 for 6 h were subjected to 12% SDS-PAGE, transferred to polyvinylidene difluoride (PVDF), and incubated with anti-Bid (1 : 2000 dil.) followed by the anti-rabbit IgG-HRP. Membranes were then exposed to Super Signal Substrate Western Blotting Kit (Pierce, Rockford, IL, USA) for the detection of the 22 kDa Bid or the 15 kDa truncated Bid (t-Bid).

Measurement of pHi with BCECF

pHi was measured by loading cells with the fluorescent pH indicator 2',7'-bis(2-carboxyethyl)-5,6-carboxyfluorescein (BCECF-AM; Sigma, St Louis, MO, USA) as described elsewhere.^{8,36} Briefly, cells (1×10^6) were washed once with HBSS (Sigma, St Louis, MO, USA), resuspended in 0.1 ml of HBSS, and loaded with 10 μ l of 1 mM BCECF at 37°C for 30 min in the dark. The cell pellet was then washed once with HBSS and kept on ice. Immediately before flow cytometry analysis, the cell pellet was resuspended in 0.5 ml of HBSS and analyzed using a Coulter EPICS Elite ESP (Coulter, Hialeah, FL, USA) flow cytometer with the excitation set at 488 nm. A minimum of 10 000 events were analyzed and the ratio of BCECF fluorescence at 525 and 610 nm was used to obtain pHi from a pH calibration curve. In order to generate a pH calibration curve, cells were loaded with BCECF as above, washed once with HBSS, and then resuspended in high K^+ buffer (135 mM KH_2PO_4 , 20 mM NaCl, and 110 mM K_2HPO_4 , and 20 mM NaCl with a range of pH between 6.0 and 8.0). Immediately before flow cytometry, cells were loaded with 10 μ M nigericin (1 mM stock in absolute alcohol, Sigma, St Louis, MO, USA), and fluorescence ratio measurements (525 nm/610 nm) of cells in nigericin-containing buffers of a range of pH were then used to relate histogram channel numbers to pHi. To evaluate the effect of scavenging intracellular H_2O_2 on pHi, cells were incubated with catalase (1000 U/ml) for 1 h before treatment with merocil or anti-CD95.

Flow cytometric analysis of intracellular H_2O_2 concentration

Intracellular concentration of H_2O_2 was determined by staining with DCFH-DA (Molecular Probes, Eugene, OR, USA), which is oxidized to DCF by H_2O_2 . Cells were exposed to 0.25 μ g/ml of anti-CD95 or 50 μ g/ml of merocil for 2–8 h, loaded with 5 μ M DCFH-DA at 37°C for 15 min, and analyzed by flow cytometry (Coulter EPICS Elite ESP) using an excitation wavelength of 488 nm.

Transient transfection with RacN17 or Bcl-2

Transient transfections of CEM/Neo or CEM/Bcl-2 cells were performed using the SuperFect Transfection Reagents from QIAGEN GmbH (Germany). Briefly, 3 μ g of the pIRES (empty vector) or pIRESRacN17 or pcDNA3-Bcl-2 and 2 μ g of the pCMV β plasmid encoding for the β -gal protein were added to 20 μ l of the SuperFect Transfection Reagent and transfection was carried out as recommended by the vendor. At 48 h after transfection, cell lysates were collected and expressions of the transiently expressed RacN17 mutant protein or Bcl-2 protein were detected by Western blotting using 2 μ g/ml of a monoclonal anti-human myc epitope antibody or (Boehringer Mannheim, Indianapolis, IN, USA) or anti-Bcl-2 (clone Bcl-2/100; BD Pharmingen, San Diego, CA, USA).

β -Gal survival assay

Survival of transiently transfected cells was assessed by the β -gal survival assay as described elsewhere.¹⁴ Following triggering of apoptosis with anti-CD95 or merocil, % survival was calculated as: $((\beta\text{-gal activity}/\mu\text{g of protein of transfected cells incubated with the apoptotic trigger})/(\beta\text{-gal activity}/\mu\text{g of protein of transfected cells incubated without the trigger}))$. β -gal activity was measured using the Galacto-Star mammalian reporter Kit (TROPIX, Bedford, MA, USA). Protein concentration was determined using the Coomassie Plus protein assay reagent from Pierce (Pierce Chemical Company, Rockford, IL, USA).

Intracellular O_2^- measurement

A lucigenin-based chemiluminescence assay was used for measuring intracellular O_2^- as previously described.¹⁶ Chemiluminescence was monitored for 60 s using a Berthold Sirius Luminometer (Berthold detection systems GmbH, Bleichstrabe/Pforzheim/Germany). Lucigenin is a widely used chemiluminescent detector of intracellular O_2^- in biological systems.³⁷ Moreover, despite some reports questioning its validity,³⁸ the work of Li *et al.*³⁹ has elegantly demonstrated that lucigenin is a reliable assay for detecting O_2^- production by enzymatic and cellular sources. In order to assess the effect of NADPH oxidase inhibitor DPI on intracellular O_2^- levels, cells were preincubated with 2.5 μ M DPI for 1 h before assaying for intracellular O_2^- . Data are shown as relative light units/ μ g of protein (RLU/ μ g protein) \pm S.D. from three to six independent measurements. Protein concentration was determined using the Coomassie Plus protein assay reagent from Pierce (Pierce Chemical Company, Rockford, IL, USA).

Detection of cytosolic Cyt c

Cytosolic translocation of Cyt *c* was assessed by Western blot analysis of cytosolic extract from 30×10^6 cells as described previously.³⁵ In total, 60 μ g of protein was subjected to 12% SDS-PAGE followed by transfer to PVDF membrane using a Trans-blot SD semidry system (Bio-Rad Laboratories, Hercules, CA, USA). Membrane was then probed with 1 : 1000 dil. of anti-Cyt *c* (7H8.2C12; Pharmingen, San Diego, CA, USA) followed by the secondary anti-mouse IgG-HRP, and the signal was detected by the Super Signal Substrate Western Blotting Kit (Pierce, Rockford, IL, USA).

Determination of mitochondrial $\Delta\Psi_m$ by flow cytometry

Potential-sensitive probe 3, 3' dihexyloxycarbocyanine iodide (DiOC₆) was used to measure mitochondrial $\Delta\Psi_m$ as described elsewhere.³⁵ Briefly, 1×10^6 cells were incubated with 3, 3' DiOC₆ (40 nM) for 15 min at 37°C. Cells were washed twice with $1 \times$ PBS and immediately analyzed in Epic Profile flow cytometer with excitation set at 488 nm. Data were analyzed for 20 000 events using the WinMDI software.

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