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ORIGINAL PAPER

High Bcl-2/Bax ratio in Walker tumor cells protects mitochondria but does not prevent H_2O_2-induced apoptosis via calcineurin pathways

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Abstract It has been previously shown that Walker 256 tumor cells express a high content of the anti-apoptotic protein Bcl-2 which protects mitochondria against the damaging effects of Ca^{2+}. In the present study, we analyze H_2O_2-induced apoptotic death in two different types of tumor cells: Walker 256 and SCC-25. Treatment with H_2O_2 (4 mM) increased reactive oxygen species generation and the concentration of cytosolic free Ca^{2+}. These alterations preceded apoptosis in both cell lines. In Walker cells, which show a high Bcl-2/Bax ratio, apoptosis was dependent on calcineurin activation and independent of changes in mitochondrial membrane potential ($\Delta\Psi_m$), as well as cytochrome *c* release. In contrast, in SCC-25 cells, which show a lower Bcl-2/Bax ratio, apoptosis was preceded by a decrease in $\Delta\Psi_m$, mitochondrial permeability transition, and cytochrome *c* release. Caspase-3 activation occurred in both cell lines. The data suggest that although the high Bcl-2/Bax ratio protected the mitochondria of Walker cells from oxidative stress, it was not sufficient to prevent apoptosis through calcineurin pathways.

Keywords Bcl-2/Bax ratio · Calcineurin · Calcium homeostasis · Oxidative stress · Apoptosis · Mitochondrial permeability transition

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Abbreviations BA: bongkrekic acid · BAPTA: 1,2-bis(2-aminophenoxy) ethane N, N, N', N'-tetraacetic acid · CCCP: carbonyl cyanide m-chloro phenyl hydrazone · $[Ca^{2+}]_{cyt}$ · cytosolic free calcium concentration · CsA: cyclosporin A · DHE: dihydroethidium · $DioC_6(3)$: 3,3′-dihexyloxacarbocyanine iodide · ER: endoplasmatic reticulum · FITC: fluorescein-5-isothiocyanate · MOMP: mitochondrial outer membrane permeabilization · MPT: mitochondrial permeability transition · PI: propidium iodide · ROS: reactive oxygen species · SCC: squamous cell carcinoma · $\Delta\Psi_m$: mitochondrial electrical transmembrane potential

Introduction

Apoptosis is controlled by several genes, especially by the members of the Bcl-2 gene family. Bcl-2 itself is an anti-apoptotic gene, first identified in follicular non-Hodgkin lymphoma, and has the ability to block various apoptotic signals. Bax is another member of the Bcl-2 family, but exerts a pro-apoptotic effect (Gross et al., 1999; Vaskivuo et al., 2002; Gustafsson and Gottileb, 2006). Under death stimuli, Bax is translocated from cytosol to the mitochondria where this protein forms homodimers on the outer mitochondrial membrane, thus leading to its permeabilization (MOMP). MOMP is considered to be the "point of no return" in the cell death process since several important pro-apoptotic factors can be released through the pore formed, including cytochrome *c*, Smac/DIABLO, and the apoptosis inducing factor (AIF) (Kang, 2001; Alirol and Martinou, 2006). MOMP induction and apoptosis however are prevented by Bcl-2 overexpression (Gross et al., 1999; Garrido et al., 2006), since this protein forms heterodimers with Bax, thus impairing its

pro-apoptotic function (Green, 2005; Gustafsson and Gottileb, 2006). The Bcl-2/Bax ratio is a key factor in the regulation of apoptosis, since a low Bcl-2/Bax ratio can lead to apoptosis, whereas high ratios can render cells resistant to apoptotic stimuli (Korsmeyer et al., 1993; Vaskivuo et al., 2002).

Mitochondria play important roles during cell life, such as ATP production, Ca^{2+} transport, reactive oxygen species (ROS) generation, and apoptosis (Garrido et al., 2006; Alirol and Martinou, 2006). An increase in the cytosolic Ca^{2+} concentration, followed by an accumulation of mitochondrial Ca^{2+}, induces oxidative stress and mitochondrial permeability transition (MPT) (Kowaltowski et al., 2001). Cytochrome *c* released either via MOMP or MPT, mediates activation of apoptosis-protease activating factor 1 (Apaf-1), in the presence of ATP, to form the complex named apoptosome. This complex recruits and activates caspase-9 which triggers caspase-3 activation and subsequent cell death (Green, 2005; Garrido et al., 2006; Alirol and Martinou, 2006). Increased cytosolic Ca^{2+} can also activate the phosphatase 2B, known as calcineurin (Shibasaki and McKeon, 1995). The exact role of calcineurin in apoptosis is still unclear, but it can exhibit either anti or pro-apoptotic properties, depending on the stimulus, the cell type, and the phosphorylation status of the p38 mitogen-activated protein kinase (p38 MAPK) (Asai et al., 1999; Kakita et al., 2001; Kang, 2001; Iwai-Kanai and Hasegawa, 2004). This enzyme is one of a group of signalling enzymes which mediate an environmental stress response in various cell types and has been implicated in the induction of apoptosis (Lotem et al., 1999; Kang, 2001).

Several studies have shown that Bcl-2 overexpression protects cells against apoptosis (Vander Heiden and Thompson, 1999; Harris and Thompson, 2000). In a previous report, it was shown that Walker 256 tumor cells constitutively overexpress the Bcl-2 protein, which confers mitochondrial stabilization and resistance to Ca^{2+}-induced MPT (Milani et al., 2001). Therefore, in the present study, we analyzed the mechanisms involved in H_2O_2-induced apoptosis of both Walker 256 and SCC-25 cells. The results indicate that the high Bcl-2/Bax ratio of Walker 256 tumor cells protects the mitochondria but does not prevent H_2O_2-induced apoptosis through calcineurin pathways. In contrast, in SCC-25 tumor cells, which have a lower Bcl-2/Bax ratio, apoptosis is mediated by MPT.

Materials and methods

Walker 256 tumor

Nine-week-old male Wistar rats (*Rattus novergicus albinos*) were obtained from the UNICAMP Central Animal Breeding Center. The animals were kept under standard laboratory conditions (20–22°C and 12 h/12 h light/dark cycle) with free access to a standard diet (Labina/Purina, Campinas, SP, Brazil) and tap water. Animal experiments followed the University guidelines for the use of animals in experimental studies (protocol no. 487-1, approved by the UNICAMP Ethics Committee in 2002) and the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication no. 85–23, revised in 1996).

The rat Walker 256 tumor cell line (originally obtained from the Christ Hospital Line, National Cancer Institute Bank, Cambridge, MA, USA) is kept frozen in liquid nitrogen. For experimental procedures, Walker cells were maintained by consecutive intraperitoneal inoculation, each with 20×10^6 tumor cells in phosphate-buffered saline (PBS). Walker cells were isolated from ascitic fluid 4–5 days after inocululation (Degasperi et al., 2006), and used when the cell viability was >98%, as estimated by the trypan blue exclusion method. During the experiments, Walker cells were maintained in RPMI 1640 medium (Cultilab, Campinas, SP, Brazil) supplemented with 10% fetal bovine serum (FBS; Cultilab) at 37°C in a humidified atmosphere of 5% CO_2.

SCC-25 cell culture

The human cell line SCC-25 (CRL-1628, ATCC, Manassas, VA, USA) was maintained in DMEM/F12 medium (Cultilab) supplemented with 10% FBS, 400 ng/ml hydrocortisone (Sigma, St. Louis, MO, USA), 100 μg/ml gentamycin (Cultilab), 100 IU/ml penicillin (Cultilab), and 100 mg/ml streptomycin (Cultilab) at 37°C in a humidified atmosphere of 5% CO_2. The cell density of the cultures was routinely maintained below 80% confluence.

Determination of oxygen consumption

Basal oxygen consumption was measured using an Oroboros oxygraph-2 k (Oroboros Instruments, Innsbruck, Austria) with 2.5×10^6 cells/ml (at 37°C) in RPMI 1640 and DMEM/F12 medium, for Walker and SCC-25 tumor cells, respectively.

Oxidative stress induction

Different concentrations of H_2O_2 (0.1, 1, 3, 4, and 5 mM) were tested in normal fibroblasts, Walker and SCC-25 cells. The percentage of cell death was determined by flow cytometry as described below. Based on the aims of this work, we looked for a H_2O_2 concentration sufficient to cause about 50% of oxidative stress-induced cell death of Walker and SCC-25 cells in a short period of time. The H_2O_2 concentration chosen was 4 mM, 40-times higher than the concentration necessary to induce similar fibroblasts cell death (data

not shown). Walker and SCC-25 cells were exposed to 4 mM H_2O_2 for different periods of time in their respective media. H_2O_2 was diluted in PBS at the time of use, and the H_2O_2 concentration of the stock solution (~8.8 M) was measured periodically. Control cells received the same volume of PBS. In all experiments, cells were washed once in PBS after treatment with H_2O_2.

Flow cytometry analysis

The samples were analyzed in a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with an argon laser and CellQuest software (version 4.1). Seven to ten thousand events were acquired for each sample. Walker and SCC-25 populations were identified by their light-scattering characteristics, enclosed in electronic gates, and analyzed for the intensity of the fluorescent probes signal.

Analysis of cell viability by annexin V and PI staining

Walker and SCC-25 cells were labelled with annexin V following the manufacturer's instructions (Laboratory of Immunology, Universidade de Sao Paulo, Sao Paulo, Brazil). Briefly, 10^6 cells/ml were harvested at each point in time, washed with PBS and resuspended in a binding buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$) containing annexin V-FITC (1:500). After 20 min of incubation at room temperature in the dark, cells were also stained with propidium iodide (PI, 1:50, Sigma). Apoptosis was quantified by FACS analysis as the number of annexin V-FITC positive and PI negative cells divided by total number of cells, while necrosis was quantified as the number of PI positive and annexin V-FITC negative cells divided by the total number of cells.

Measurement of ROS levels

Walker and SCC-25 cells (10^6 cells/ml) were incubated with 2 μM dihydroethidium (DHE, Molecular Probes, Carlsbad, CA, USA) probe at 37°C in a humidified atmosphere of 5% CO_2 for 40 min and analyzed by flow cytometry (Degasperi et al., 2006).

Measurement of cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_{cyt}$)

Walker and SCC-25 cells (10^6 cells/ml) were loaded with 3 μM Fluo-3AM fluorescent probe (Molecular Probes) in the presence of 1 μM pluronic acid F-127 (Molecular Probes) and 30 μg/ml BSA (Sigma) at 37°C in a humidified atmosphere of 5% CO_2 for 40 min. Nonhydrolysed Fluo-3AM was removed by washing the cells in their respective medium just prior to acquisition of fluorescence by flow cytometry (Degasperi et al., 2006).

Determination of the mitochondrial electrical transmembrane potential ($\Delta\Psi_m$) in intact cells

Walker and SCC-25 cells (10^6 cells/ml) were incubated with 0.2 and 0.5 nM $DioC_6(3)$ (Sigma), respectively, in 400 μl of medium at 37°C in a humidified atmosphere of 5% CO_2 for 30 min. One half of each cell sample (200 μl) was separated into a new tube, with the addition of 50 μM CCCP (Sigma), a protonophore that dissipates $\Delta\Psi_m$ (Campos et al., 2004). Both samples were incubated for an additional 30 minutes. In non-saturated concentrations, $DioC_6(3)$ binds preferentially to mitochondria, since the $\Delta\Psi_m$ is much higher (−180 mV) than the plasma membrane potential (−60 mV). Results were normalized using the F/F_{CCCP} ratio, where F is the mean intensity of fluorescence of $DioC_6(3)$ (maximum fluorescence) and F_{CCCP} is mean fluorescence in the presence of CCCP (minimum fluorescence).

Determination of $\Delta\Psi_m$ in digitonin-permeabilized cells

$\Delta\Psi_m$ in digitonin-permeabilized cells was estimated as changes in the fluorescence of safranine O (Sigma) (Holden and Sze, 1989), recorded using a spectrofluorometer (Hitachi, model F4500, Tokyo, Japan) operating at excitation and emission wavelengths of 495 and 586 nm, respectively, with slit widths of 5 nm. After treatment with H_2O_2, both cell lines (10^6 cells/ml) were permeabilized with 20 μM digitonin (Sigma) (Campos et al., 2004) in standard reaction medium (125 mM sucrose, 65 mM KCl, 10 mM Tris-HCl (pH 7.2), 2.5 mM Na_2HPO_4, 0.33 mM EGTA, 5 mM succinate, and 5 μM safranine O) under constant stirring at 37°C.

Detection of Bcl-2 and Bax levels by Western blotting

Protein was extracted from Walker and SCC-25 cells as previously described (Zecchin et al., 2005) and protein concentrations were determined using the Bradford method (Bradford, 1976). Fifty micrograms of each protein lysate were electrophoresed on 12% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes (Bio-Rad, Hercules, USA), and stained with Ponceau S (Sigma) to verify transfer efficiency and confirm equality of sample loading. The membranes were blocked with 5% non-fat dry milk in 20 mM Tris-HCl (pH 7.6) containing 150 mM NaCl and 0.1% Tween-20 (TBST) for 16 h and probed for 2 h at room temperature with the antibodies anti-Bcl-2 (1:200, C-2, Santa Cruz, Biotechnology, CA, USA), anti-Bax (1:500, B-9, Santa Cruz) and anti-beta-actin (1:30000, AC-15, Sigma). After washing with TBST, membranes were incubated for 1 h with anti-mouse IgG-HRP (horseradish peroxidase) conjugated

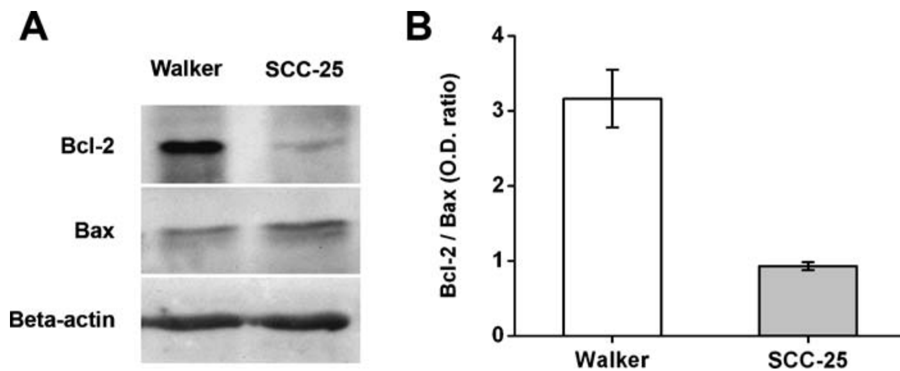

Fig. 1 *Bcl-2 and Bax detection in Walker and SCC-25 cells:* (**A**) The levels of Bcl-2 and Bax were determined by Western blotting using equal amounts of protein lysates resolved in 12% SDS-PAGE and stained with anti-Bcl-2 (∼26 kDa), and anti-Bax (∼23 kDa), as described in *Materials and Methods*; (**B**) the Bcl-2/Bax ratio was determined by densitometric analysis of the Western blottings and the results normalized for beta-actin values

secondary antibody (1:1000, Bio-Rad, Hercules, CA, USA). Reactions of the membranes were identified using an enhanced chemiluminescence detection system (ECL detection kit, Amersham Pharmacia Biotech, GE Healthcare UK Ltd, Little Chalfont, England), the membranes exposed to XOmat AR films (Eastman Kodak Company, Rochester, NY, USA), and the results quantified by densitometric analysis using a GS-700 Densitometer and the Molecular Analyst Software (BioRad). The results were normalized for beta-actin values.

Detection of mitochondrial cytochrome *c* release by Western blotting

Cells were resuspended in 0.2 ml of medium containing 300 mM sucrose, 10 mM HEPES buffer (pH 7.2), 20 μM EDTA supplemented with a 1% protease inhibitor mix (Calbiochem, San Diego, CA, USA) and 1 mM phenylmethanesulfonyl fluoride (PMSF) (Campos et al., 2004). Cells were permeabilized for 60 s under vigorous vortexing with 0.01% digitonin and centrifuged for 8 min at $12000 \times g$. Pellets were resuspended in the same medium and an equal volume of the lysis medium (62.5 mM Tris-HCl (pH 6.8), 3% SDS, 5% 2-mercaptoethanol, 10% glycerol), and protein concentrations were determined using the Bradford method (Bradford, 1976). Fifty micrograms of each protein lysate were electrophoresed on 12% SDS-polyacrylamide gel, transferred onto nitrocellulose membranes and blocked as described for Bcl-2 detection. Membranes were probed with anti-cytochrome *c* (1:500, 6 H2.B4, Promega, Madison, WI, USA) for 2 h and, after washing with TBST, incubated for 1 h with anti-mouse IgG-HRP conjugated secondary antibody (Bio-Rad, 1:1000), they were then developed as described for Bcl-2.

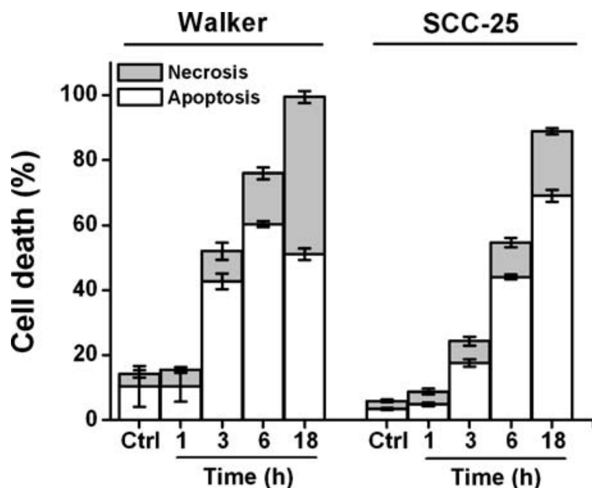

Fig. 2 *H_2O_2-induced cell death*. Walker and SCC-25 cells were treated with 4 mM H_2O_2 for different periods of time, washed in PBS, preincubated with annexin V or PI, and analyzed by flow cytometry, as described in *Materials and Methods*; control cells (Ctrl) were incubated in PBS for 18 h. Values are mean ± SEM of at least 3 independent experiments

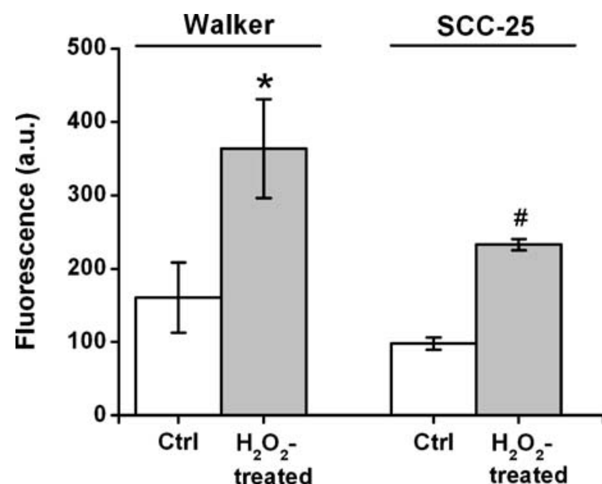

Fig. 3 *H_2O_2-induced ROS production by tumor cells*. Walker and SCC-25 cells were treated with 4 mM H_2O_2 for up to 1 h, washed in PBS and probed with 2 μM dihydroethidium (DHE), as described in *Materials and Methods*; Values are mean ± SEM of 7 independent experiments. *Significantly different from control at $p < 0.05$ level; #significantly different from control at $p < 0.001$ level

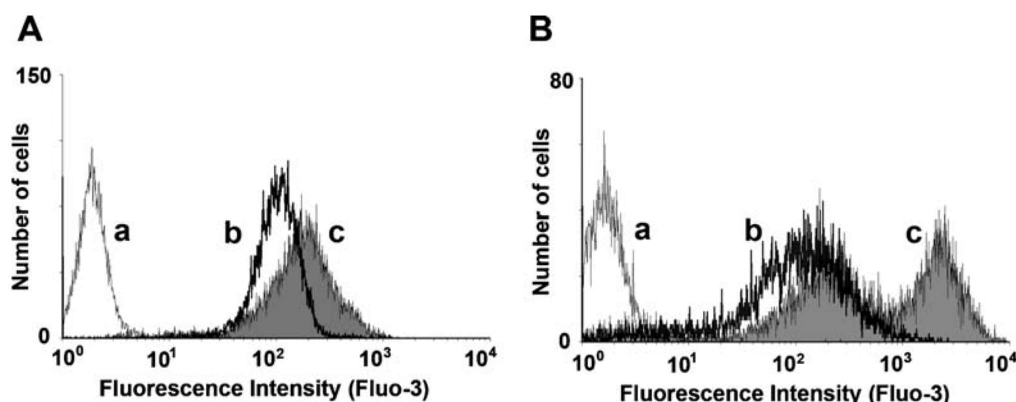

Fig. 4 *Changes in [Ca^{2+}]$_{cyt}$ after H_2O_2 treatment*. Walker (**A**) and SCC-25 (**B**) cells were treated with 4 mM H_2O_2 for up to 1 h, washed in PBS and loaded with 3 μM Fluo-3AM, as described in *Materials and Methods*; representative histograms are presented for unlabeled control cells (**a**), control cells (**b**) and cells treated with H_2O_2 (**c**)

Detection of cytochrome *c* release by immunocytochemistry

After H_2O_2 treatment, cell culture samples were washed and fixed in 3.7% paraformaldehyde in PBS for 1 h. Walker cells were fixed in suspension, while SCC-25 cells were fixed on chamber slides (Lab-Tek). Cells were then washed in PBS with 0.1% polyvinyl-pyrrolidone (PVP, Sigma) (PBS+PVP), permeabilized in 0.1% sodium citrate in PBS + PVP containing 0.5% Triton X-100 for 5 min at 4°C and blocked in 10% goat serum diluted in PBS+PVP for 40 min at room temperature. Cells were then incubated with anti-cytochrome *c* (1:1000, 6H2.B4, Promega), overnight at 4°C, washed in PBS+PVP, and incubated with 1:800-diluted Cy3-conjugated affinipure goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA, USA) for 45 min at room temperature. Mitochondrion localization was determined using 50 nM MitoTracker green FM (Molecular Probes) in PBS+PVP at room temperature for 20 min. Finally, cells were washed, mounted onto microscope slides with coverslips and analyzed on an epifluorescence microscope (Axioplan, Carl Zeiss, NY, USA).

Detection of caspase-3 activation

Caspase-3 activation was measured as recommended by the manufacturer (Calbiochem). Walker and SCC-25 cells (10^6 cells/ml) were incubated with FITC-DEVD-FMK (1:300) in non-supplemented medium for 40 min at 37°C in a humidified atmosphere of 5% CO_2. Some experiments also included 30 min of pre-incubation with 20 μM z-VAD-FMK (BD Biosciences), an effective inhibitor of a broad spectrum of caspases, prior to H_2O_2 addition. Cells were then washed, resuspended in non-supplemented medium and analyzed by flow cytometry.

Statistical analysis

The results from at least 3 independent experiments, each performed in duplicate or triplicate, are displayed as means ±S.E.M. Comparisons between groups were performed using a one-way Analysis of Variance (ANOVA) with Tukey's *post-hoc* analysis. The level of significance was set at $p < 0.05$.

Results

H_2O_2 treatment-induced apoptosis of Walker and SCC-25 cells despite differences in Bcl-2/Bax ratio

Figure 1 shows that the Bcl-2/Bax ratio is 3.4-fold higher in Walker than in SCC-25 cells. Nevertheless, no differences

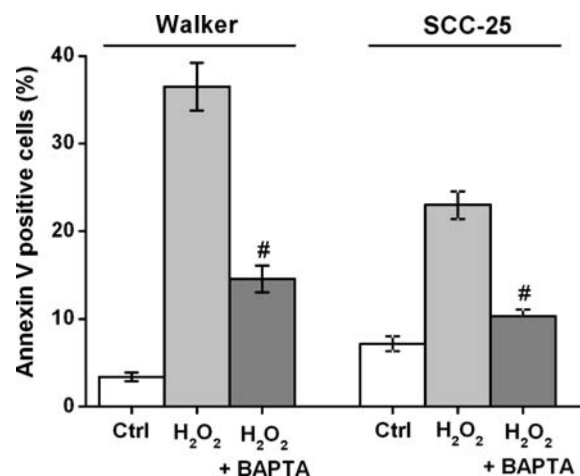

Fig. 5 *Inhibition by BAPTA of H_2O_2-induced cell death*. Walker and SCC-25 cells were pre-incubated with 10 μM BAPTA-AM for 40 min and treated with 4 mM H_2O_2 for 3 h; cells were then washed and the percentage of apoptosis determined after incubation with annexin V by flow cytometry, as described in *Materials and Methods*. Values are mean ± SEM of 7 independent experiments. #Significantly different from control at $p < 0.001$ level

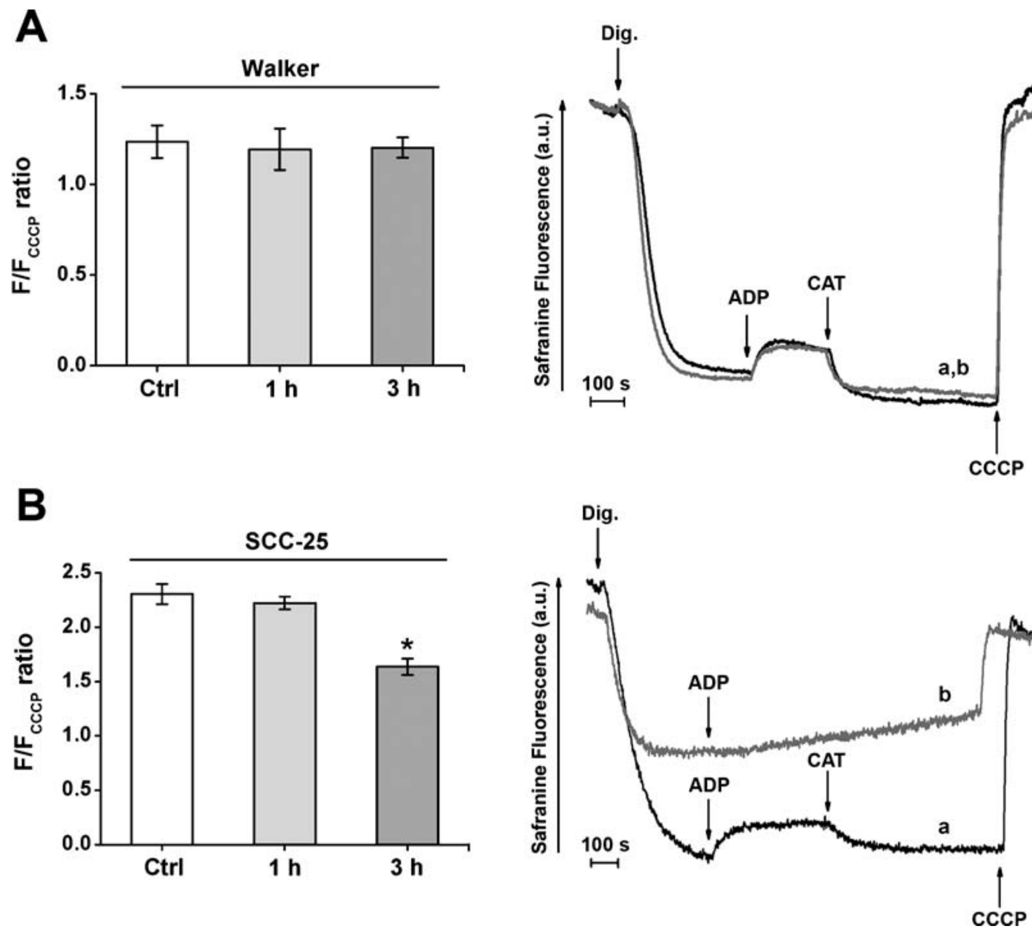

Fig. 6 $\Delta\Psi_m$ *analysis before and after tumor cells treatment with* H_2O_2. Walker (**A**) and SCC-25 (**B**) cells were treated with 4 mM H_2O_2 for up to 3 h and washed in PBS; $\Delta\Psi_m$ was analyzed by flow cytometry using DioC6(3), as described in *Materials and Methods (left panels)*. The ratio of DioC6(3) fluorescence in the absence of CCCP and in its presence was measured to estimate changes in $\Delta\Psi_m$. Values are mean ± SEM of 7 independent experiments. *Significantly different from control at $p < 0.05$ level. $\Delta\Psi_m$ was also analyzed by changes in safranine O fluorescence *(right panels)*. After treatment with 4 mM H_2O_2 for 3 h, Walker and SCC-25 cells were washed and permeabilized with digitonin (Dig.) in standard reaction medium, as described in *Materials and Methods*. Traces **a** and **b** represent control and H_2O_2-treated cells, respectively, with arrows indicating the addition of 20 μM digitonin, 30 μM ADP, 4 μM CAT, and 1 μM CCCP. Traces are representative of at least 3 independent experiments

were observed in the rates of basal oxygen consumption (data not shown), suggesting that both cell lines had a comparable number of mitochondria. Treatment with 4 mM H_2O_2 killed approximately 50% of the Walker cells after 3 h and 50% of the SCC-25 cells after 6 h, mainly by apoptosis (Fig. 2).

Increased ROS production and elevation of $[Ca^{2+}]_{cyt}$ preceding H_2O_2-induced cell death in Walker and SCC-25 cells

Increases in both ROS production (Fig. 3) and $[Ca^{2+}]_{cyt}$ (Fig. 4) after H_2O_2 treatment preceded cell death in both cell lines (Fig. 2). BAPTA, an intracellular Ca^{2+} chelator, decreased the apoptosis in these cells at least 2.0-fold (Fig. 5), strongly suggesting the participation of $[Ca^{2+}]_{cyt}$ in the process.

H_2O_2-induced cell death preceded by $\Delta\Psi_m$ disruption in SCC-25 cells

Flow cytometry in intact cells and the safranine method in permeabilized cells revealed that H_2O_2 did not affect $\Delta\Psi_m$ in Walker cells, in contrast to what happened in SCC-25 cells (Fig. 6). Furthermore, the safranine experiments demonstrated that Walker cell mitochondria maintained the ability to phosphorylate additional ADP even after pre-incubation with H_2O_2 (Fig. 6A). ADP addition was followed by an increase in safranine fluorescence that could be reversed by carboxyatractyloside (CAT), an inhibitor of the ADP-ATP

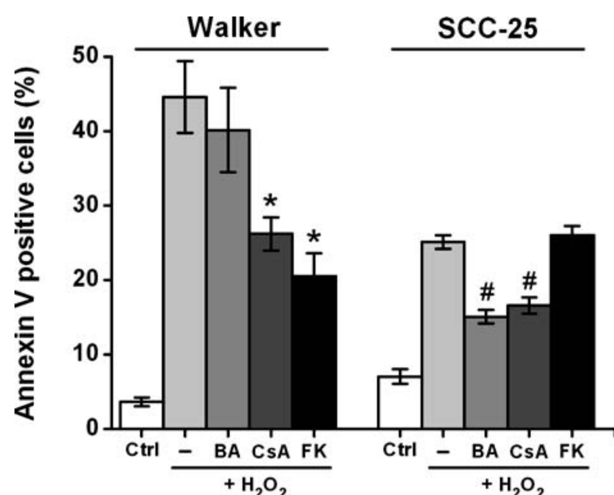

Fig. 7 *Effect of calcineurin and MPT inhibitors on H_2O_2-induced Walker and SCC-25 cell death*. Walker and SCC-25 cells were treated with 4 mM H_2O_2 for 3 h in the absence (or presence) of bongkrekic acid (BA, 0.5 μM), cyclosporin A (CsA, 1 μM) and FK-506 (FK, 0.5 μM), as indicated in the figure; cells were washed, and the percentage of apoptosis was determined after incubation with annexin V by flow cytometry, as described in *Materials and Methods*. Values are mean ± SEM of 7 independent experiments. *Significantly different from H_2O_2 at $p < 0.05$ level; #significantly different from H_2O_2 at $p < 0.001$ level

carrier. Similar results were observed for the SCC-25 control cells, but not after pre-incubation with H_2O_2 (Fig. 6B).

H_2O_2-induced cell death mediation by calcineurin in Walker cells and by MPT in SCC-25

Since elevations in $[Ca^{2+}]_{cyt}$ and in ROS production may lead to MPT (Kowaltowski et al., 2001), the occurrence of this process was investigated in H_2O_2-induced apoptosis using the MPT inhibitors bongkrekic acid (BA) and cyclosporin A (CsA). While both BA and CsA decreased apoptosis 2.0-fold in SCC-25 cells (Fig. 7), only CsA prevented death of Walker cells. In order to ascertain the participation of calcineurin in the process of Walker cell death, we tested the effect of the immunesupressor FK-506, which specifically inhibits calcineurin without any effect on MPT (Griffiths and Halestrap, 1991). FK-506 caused a significant decrease in the number of apoptotic Walker cells, although that of the SCC-25 remain unchanged, strongly suggesting that calcineurin activation, but not mitochondrial MPT, participates in the process of Walker cell death.

Decrease in Bcl-2/Bax ratio and mitochondrial cytochrome *c* release in SCC-25 cells but not in Walker upon H_2O_2 treatment

The Bcl-2/Bax ratio was not significantly affected by H_2O_2 treatment in Walker cells (3.09 ± 0.18 *versus* 3.17 ± 0.16 in control cells), while in SCC-25 treated cells, a significant decrease in Bcl-2/Bax ratio over that of the controls was noted (0.93 ± 0.03 *versus* 0.43 ± 0.07, $p < 0.001$ respectively). Such a decrease in Bcl-2/Bax ratio in SCC-25 cells was due mainly to an increase in the amount of Bax after H_2O_2 treatment. It should be emphasized that this decrease facilitates the occurrence of MOMP and cytochrome *c* release. Accordingly, the Western blotting of Fig. 8A shows that while Walker cells did not release cytochrome *c* after treatment with H_2O_2, the SCC-25 cells clearly released most of this protein. These results were confirmed by immunocytochemistry for cytochrome *c* (Fig. 8B). Cytochrome *c* release in SCC-25 cells was confirmed by a decrease in red fluorescence in comparison to the control after H_2O_2 treatment, although Walker cells maintained this fluorescence.

Caspase-3 mediation of H_2O_2-induced apoptosis in both tumor cell lines

The release of cytochrome *c* activates several apoptotic factors, including caspase-3. Treatment of both cell lines with H_2O_2 promoted a significant activation of caspase-3 (Fig. 9). The presence of 20 μM z-VAD-FMK, a pan caspase inhibitor, prevented the decrease in Walker cell viability from 36.5 ± 2.7 to 22.8 ± 2.4 and in that of SCC-25 cells from 23.0 ± 1.6 to 6.9 ± 0.6.

Discussion

The present results provide new insights into the role of the Bcl-2/Bax ratio and calcineurin pathways in oxidative stress-induced death of tumor cells. It was observed that constitutive Bcl-2 overexpression, with a high Bcl-2/Bax ratio, protects Walker cells from oxidative stress-induced MPT, MOMP and cytochrome *c* release. In contrast, in SCC-25 cells, which have lower Bcl-2 levels and lower Bcl-2/Bax ratio, H_2O_2 treatment was followed by an increase in $[Ca^{2+}]_{cyt}$ and in ROS generation that led to MPT, cytochrome *c* release and apoptosis, in agreement with data reported by Kowaltowski et al. (2001). MPT was evidenced in SCC-25 cells by sensitivity to cyclosporin A and bongkrekic acid, but not to the calcineurin inhibitor FK-506 (Fig. 7). Consistent with the occurrence of MPT in SCC-25 cells, caspase-3 was activated by the released of cytochrome *c*. Moreover a significant decrease in the Bcl-2/Bax ratio implicated MOMP in cytochrome *c* release and caspase-3 activation.

In contrast, in Walker cells, the high Bcl-2/Bax ratio prevented both MPT and MOMP induction under conditions of oxidative stress. Indeed, it was previously observed that digitonin-permeabilized Walker cells were resistant to Ca^{2+}-induced mitochondrial permeability transition (Milani et al., 2001). Although the high Bcl-2/Bax ratio confers mitochondrial protection, it does not prevent the calcineurin-activated

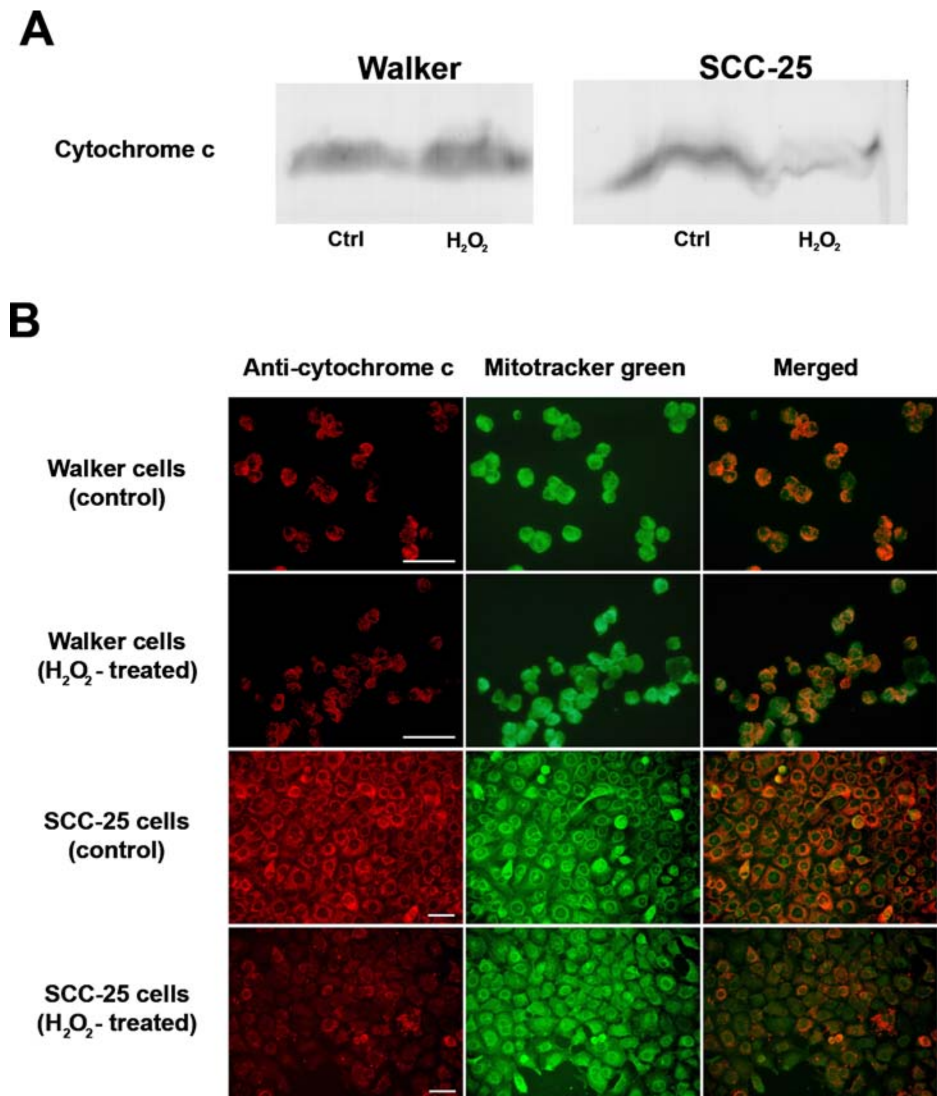

Fig. 8 *Detection of cytochrome c release after H_2O_2 treatment*. (**A**) Walker and SCC-25 cells were treated with 4 mM H_2O_2 for 3 and 6 h, respectively; protein lysates were then obtained, resolved in 12% SDS-PAGE, and stained with anti-cytochrome *c* (∼15 kDa), as described in *Materials and Methods*. (**B**) Double-labeling immunocytochemistry staining of untreated Walker and SCC-25 cells and those treated with 4 mM H_2O_2, respectively. Cells with punctate cythocrome *c* staining (red) overlapping mitochondrial staining (green) were considered to be cells with cytochrome *c* within mitochondria while those with diffuse cytochrome *c* staining showing lower fluorescence were considered to be cells having released cytochrome *c* from mitochondria. Bars represent 50 μm

apoptosis of Walker cells treated with H_2O_2, as demonstrated by FK-506 inhibition (Fig. 7). Calcineurin activation under these conditions can occur either via elevation of $[Ca^{2+}]_{cyt}$ or via the activation of calpain, a non-lysosomal Ca^{2+} dependent cysteine protease which has the endogenous calcineurin inhibitor CAIN/cabin 1 as substrate (Groenendyk et al., 2004; Fan et al., 2005; Wang et al., 2005). It is possible that activation of both calcineurin and calpain could be responsible for caspase-3 activation in Walker cells once caspase-12 and -9 have been activated, despite the absence of cytochrome *c* release (Fig. 8) (Morishima et al., 2002; Groenendyk et al., 2004).

The results observed for Walker cells are in apparent contrast with previous reports suggesting that (a) Bcl-2 overexpression inhibits calcineurin-activated cell death (Shibasaki and McKeon, 1995; Asai et al., 1999), and (b) calcineurin plays an antioxidant role when Walker tumor cells are treated with ibuprofen (Campos et al., 2004) or when cardiac myocytes are treated with H_2O_2 (Kakita et al., 2001). Altogether, these data suggest that calcineurin can act as an anti- and a pro-apoptotic protein. While the anti-apoptotic role of calcineurin is probably related to dephosphorylation of the nuclear factor of activated T-cell (NFAT) transcription factors (Asai et al., 1999; Kakita et al., 2001; Groenendyk et al., 2004), its apoptotic role is activated by different pathways: (1) complexation with Bcl-2, neutralizing its anti-apoptotic action (Shibasaki et al., 1997); (2) caspase-12 activation (Morishima et al., 2002); or (3) dephosphorylation of proteins such as Bax, caspase-9, and Bad (Groenendyk et al., 2004; Tantral et al., 2004). Dephosphorylated Bad can dimerize with Bcl-xL or other Bcl-2 family proteins, thus favoring the homodimerization of Bax, which induces MOMP, cytochrome *c* release and subsequent apoptosis (Wang et al., 1999). Considering the absence of cytochrome *c* release in

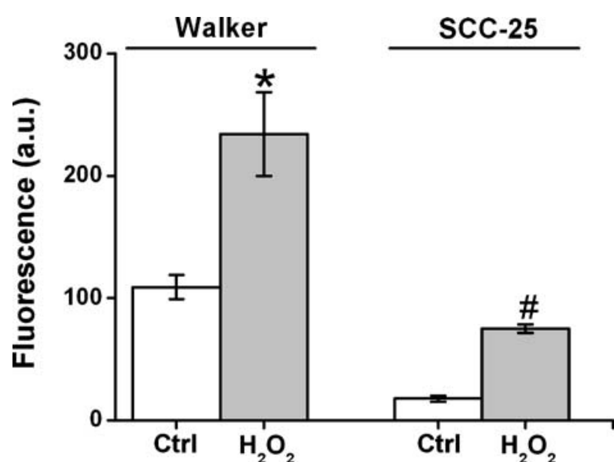

Fig. 9 *Caspase-3 activation by H_2O_2 treatment in both cell lines* Walker and SCC-25 cells were treated with 4 mM H_2O_2 for 3 h and analyzed by flow cytometry, as described in *Materials and Methods*. Values are mean ± SEM of 7 independent experiments. *Significantly different from control at $p < 0.05$ level; #significantly different from control at $p < 0.001$ level

H_2O_2-treated Walker tumor cells in this study (Fig. 8), the effects of calcineurin on the Bcl-2 protein family and consequent MPT and/or MOMP seem unlikely.

It can be concluded that although the high Bcl-2/Bax ratio protects Walker tumor cell mitochondria from oxidative stress, it is not sufficient to prevent apoptosis through calcineurin pathways. Since most cancer chemotherapies are apoptogenic, the present results may suggest new pathways for investigation.

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