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Ibuprofen-induced Walker 256 tumor cell death: cytochrome *c* release from functional mitochondria and enhancement by calcineurin inhibition

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Abstract

The participation of mitochondria in the mechanism of tumor cell death induced by non-steroid anti-inflammatory drugs is uncertain. Here we show that ibuprofen induces death of Walker 256 tumor cells independently on mitochondrial depolarization as estimated by flow cytometry using $DioC_6(3)$. Oligomycin increased mitochondrial transmembrane potential in both ibuprofen-treated and non-treated cells, indicating that ATP synthesis was sustained during cell death. Cyclosporin A, but not bongkrekic acid, both mitochondrial permeability transition inhibitors, increased the percentage of cell death in the presence of ibuprofen. FK506, a calcineurin inhibitor like cyclosporin A, also increased ibuprofen-induced cell death. Moreover, we showed that cytochrome *c* was released during ibuprofen-induced cell death. In conclusion, death of Walker 256 tumor cells induced by ibuprofen does not impair mitochondrial function, involves cytochrome *c* release and is accompanied by a rescue pathway via calcineurin activation.

Keywords: Apoptosis; Ibuprofen; Mitochondrion; Electrical transmembrane potential; Cytochrome *c*; Calcineurin

1. Introduction

Apoptotic signals may converge to mitochondria leading to the release of intermembrane factors, such as cytochrome *c*, procaspase 9, and apoptosis inducing factor (AIF), which induce cell death (for review [1–6]). At least two mechanisms have been proposed to explain mitochondrial outer membrane permeabilization and release of apoptogenic factors. One involves regulation of the Bcl-2 family of proteins, Bax, Bak and Bid, that form pores in the mitochondrial outer membrane [7–10]. The other involves opening of the mitochondrial permeability transition (MPT) pore that results in matrix swelling, leading to rupture of the outer membrane and release of intermembrane components. MPT is rapidly followed by dissipation of electrical transmembrane potential ($\Delta\Psi_m$) and loss of mitochondrial function [11,12].

Ibuprofen (IBU), a non-steroid anti-inflammatory drug (NSAID) known to have analgesic, anti-pyretic and anti-inflammatory properties due to its inhibitory effect on cyclooxygenases-1 and -2 (COX-1 and COX-2), has been shown to induce cell death in many different tumor cells [13–16]. Cell killing by ibuprofen and other NSAIDs can be unrelated to inhibition of prostaglandin synthesis and may involve downregulation of Bcl-2 expression [15,17], blockade of AKT/PKB activation [18], and reactive oxygen species production [19]. Indeed, Bax translocation to the outer mitochondrial membrane during apoptosis induced by aspirin in different tumor cell lines precedes

Abbreviations: BA, bongkrekic acid; CCCP, carbonyl cyanide *m*-chloro phenyl hydrazone; COX, cyclooxygenase; CsA, cyclosporin A; $DioC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; FITC, fluorescein-5-isothiocyanate; IBU, ibuprofen; MEM, minimum essential medium; MPT, mitochondrial permeability transition; NSAID, non-steroid anti-inflammatory drug; PI, propidium iodide; W256, Walker 256 tumor; $\Delta\Psi_m$, mitochondrial electrical transmembrane potential

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dissipation of mitochondrial membrane potential, processing of caspases and release of cytochrome *c* and AIF [20,21]. In contrast, it has been shown that cyclosporin A (CsA), a classical MPT inhibitor, prevents NSAIDs-induced mitochondrial depolarization in isolated liver mitochondria and cell death in cultured cells [22–24], suggesting the participation of MPT in this mechanism.

In this work, we assessed how mitochondria respond during the process of cell death induced by ibuprofen in cultured Walker tumor (W256) cells of hematopoietic origin. These cells naturally overexpress Bcl-2 protein, which confer mitochondrial stabilization and is related to the high resistance to intracellular Ca^{2+} load [25]. We have chosen these cells in order to evaluate if and how resistant mitochondria respond to high ibuprofen concentration. Our data indicate that cell killing by ibuprofen leads to cytochrome *c* release but does not impair mitochondrial function. Also, this cell death follows a concomitant participation of a rescue pathway via calcineurin activation.

2. Materials and methods

2.1. Materials

Minimum essential medium (MEM), fetal bovine serum (FBS), L-glutamine, sodium bicarbonate solution and antibiotics were purchased from GIBCO BRL. Ibuprofen sodium salt, staurosporine and cyclosporin A were purchased from Sigma. Bongkrekic acid (BA) was from Biomol. FK506 (tacrolimus) was from Janssen Labs. Annexin V-FITC apoptosis detection kit was from Oncogene. Monoclonal antibody against cytochrome *c* was from eBioscience. 3,3′-Dihexyloxacarbocyanine iodide ($DioC_6(3)$) was purchased from Molecular Probes. Opti-4CN was from Bio-Rad. All other materials were also of high-quality grades.

2.2. Animals

Wistar rats were kept under standard laboratory conditions at 20–22 °C and a natural day–night cycle, with free access to regular food and tap water. All experiments were carried out according to the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health.

2.3. Cell culture

W256 cells of monocytoid origin [26] were maintained intraperitoneally through the injection of 2×10^7 cells every 3–5 days in 2–3-month-old Wistar rats. Tumor cells present in the ascitic fluid were purified in Hystopack gradient (Sigma), counted using a Neubauer chamber and used when >98% of cells exhibited Trypan blue exclusion. For culture, purified W256 cells were seeded in MEM supplemented with 10% FBS, 2 mM L-glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin in a humidified atmosphere, 5% CO_2 at 37 °C in an incubator at an initial density of 1×10^5 cells/ml [27].

2.4. Determination of mitochondrial transmembrane potential in intact cells with $DioC_6(3)$ using flow cytometry

To avoid interference from the plasma membrane potential and more accurately detect changes in mitochondrial function, we first found the best concentration of $DioC_6(3)$, a cationic lipophilic fluorescent probe, for measuring mitochondrial electrical transmembrane potential ($\Delta\Psi_m$) by flow cytometry [28]. In non-saturating concentrations, $DioC_6(3)$ binds preferentially to mitochondria, since the magnitude of their electrical membrane potential is much higher (−180 mV) than plasma membrane potential (−60 mV). The F/F_{CCCP} ratio was used to normalize the results from the triplicate experiments, where F is the mean fluorescence intensity of $DioC_6(3)$ (maximum fluorescence) and F_{CCCP} is the mean fluorescence in the presence of carbonyl cyanide *m*-chloro phenyl hydrazone (CCCP) (minimum fluorescence), a protonophore that dissipates the electric membrane potential from both plasma and mitochondrial membranes. For $DioC_6(3)$ probe titration, 4×10^4 cells were incubated with increasing concentrations of $DioC_6(3)$ in 400 μl of MEM supplemented with 2% FBS, 2 mM L-glutamine at 37 °C in a humidified CO_2 incubator (5% CO_2) for 20 min. The cell sample was split into two tubes and 40 μM CCCP was added to one of them. After further incubation in identical conditions for 20 min, the cells were analyzed in a FACSCalibur flow cytometer (Becton Dickinson). For CCCP titration, 0.2 nM $DioC_6(3)$ probe was added to 5 ml of medium containing 5×10^5 cells. The cells were then separated into various tubes, each containing 200 μl and incubated in identical conditions as described for $DioC_6(3)$ titration for 20 min. Increasing concentrations of CCCP were added to tubes that were further incubated at 37 °C for 20 min and analyzed by flow cytometry. Alternatively, 4×10^4 cells were incubated with 0.2 nM $DioC_6(3)$ in 400 μl of MEM supplemented with 2% FBS, 2 mM L-glutamine at 37 °C for 40 min. A volume of 200 μl of cells was separated into new tubes, each containing 1 μg/ml oligomycin, 1 μM cyclosporin A, 1 μM bongkrekic acid or without any compound, which were incubated at the same condition for the last 20 min. KCN (10 mM) was added to the cells after 40 min immediately before analysis.

2.5. Determination of mitochondrial transmembrane potential in permeabilized W256 cells

$\Delta\Psi_m$ was estimated using safranin O. Fluorescence changes of safranin O [29] were recorded on a model F-4010 Hitachi spectrofluorometer operating at 495/586 nm

excitation/emission wavelength pair, with slit widths of 5 nm. W256 cells (5×10^6 total) permeabilized with 0.001% digitonin, as described by Vercesi et al. [30], were incubated in 1.3 ml of standard reaction medium (125 mM sucrose, 65 mM KCl, 10 mM HEPES buffer pH 7.2, 1 mM $MgCl_2$, 2 mM potassium phosphate, 0.2% BSA) with 5 mM succinate plus 4 μM rotenone under constant stirring at 37 °C. Safranin O was used at 10 μM. The results are representative of at least three independent experiments.

2.6. Analysis of cell viability by annexin-V and propidium iodide staining

Cells were labeled with annexin-V following manufacturer instructions. Briefly, approximately 5×10^4 cells were harvested at each time point, washed twice with PBS, resuspended in 80 μl binding buffer (BB) containing annexin V-FITC 1:300. After 15 min incubation at room temperature, the cells were centrifuged at $1000 \times g$ for 5 min and resuspended in 200 μl BB containing propidium iodide (PI) at 1:50. Apoptosis was quantified as the number of annexin V-FITC-positive and PI-negative cells over the total number of cells.

2.7. Mitochondrial fraction isolation and immunoblot

W256 cells were collected at different time points, washed twice in ice cold phosphate-buffered saline (PBS) and resuspended in 0.2 ml of medium containing 300 mM sucrose; 10 mM HEPES buffer, pH 7.2; 20 μM EDTA supplemented with 1% protease inhibitors mix and 1 mM phenylmethanesulfonyl fluoride (PMSF). Cells were permeabilized for 60 s under vigorous vortexing with 0.01% digitonin and centrifuged for 8 min at $12{,}000 \times g$. Mitochondrial fractions 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) was added. Approximately 15 μg of total protein lysates were boiled for 5 min, separated in 10% SDS-PAGE, and transferred to nitrocellulose membranes. The membrane was blocked with 5% non-fat dry milk plus 5% bovine serum albumine (BSA) in TBS-T (100 mM NaCl, 10 mM Tris–HCl (pH 7.5), 0.1% Tween 20) for 2 h at room temperature. Blot was incubated with the antibody for cytochrome *c* at 1:1000 in TBS-T/5% BSA at room temperature for 2 h, followed by reaction with an anti-mouse secondary antibody (1:2000) for 1 h at room temperature and developed using Opti-4CN reagent (Bio-Rad).

2.8. Statistical analysis

Experiments were performed in triplicate using tumor cells from one rat and each experiment was repeated at least three times. The mean value of each of these conditions was used as one data point. Significance was determined by ANOVA, and post hoc Bonferroni test, with $p < 0.05$, using SSPS version 11 software.

3. Results and discussion

3.1. W256 cell death induced by ibuprofen

In Fig. 1A, it is shown that ibuprofen (500 μM), impairs the growth of cultured W256 cells (open circles). This is correlated with massive cell death as estimated by Trypan blue exclusion method (Fig. 1B, open bars). This result is in apparent contrast with Tang et al. [31,32], who observed that W256 cells were not sensitive to inhibition of cyclooxygenases, but of lipoxygenases. These authors used ibuprofen up to 100 μM, which is about the maximum dose still specific for COX 2 inhibition. We have chosen, however, a concentration known to cause other effects independent on COX 2 inhibition, but still below the one that produce toxic symptoms in the plasma (704 mg/l or 3 mM) [33]. Even though 100 μM is the dose that produces the anti-inflammatory effects, higher plasma concentrations of ibuprofen may be necessary to promote anti-tumor activity.

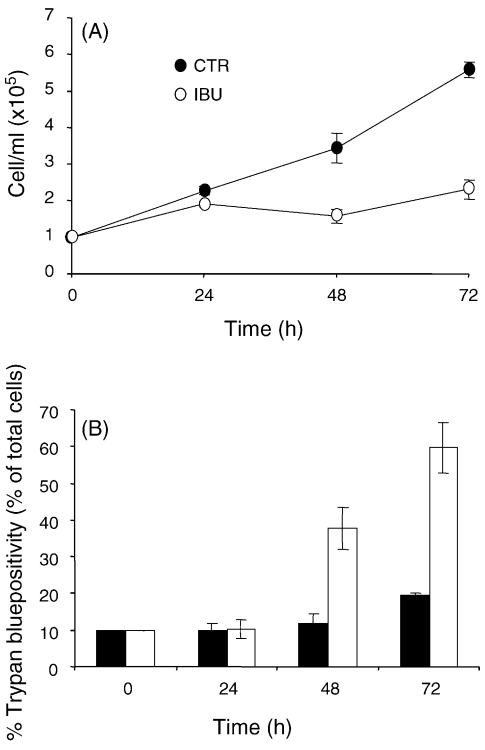

Fig. 1. Ibuprofen induces Walker 256 tumor (W256) cell death. (A) W256 cells were cultured for 72 h as described in Section 2 in the absence (filled circles) or in the presence of 500 μM ibuprofen (open circles) and the number of cells per ml was estimated. (B) W256 cells were collected every 24 h and the loss of viability was estimated by Trypan blue exclusion method and expressed as percentage of Trypan blue positivity. Control cells: filled bars; ibuprofen-treated cells: open bars.

3.2. Stimulation of MPT in permeabilized W256 cells by ibuprofen

It has been reported that NSAIDs promote MPT when directly added to isolated liver mitochondria incubated in the presence of Ca^{2+} and phosphate [24]. To ascertain whether ibuprofen induces MPT in W256 cells permeabilized with digitonin, safranin was used as a probe for $\Delta\Psi_m$. Fig. 2 shows that in the absence of Ca^{2+}, 500 μM ibuprofen did not significantly affect $\Delta\Psi_m$ formation and its response to ADP and oligomycin, an ATPsynthase inhibitor, additions (compare lines a and b). However, in the presence of 500 μM ibuprofen and 10 μM Ca^{2+}, the shift in safranin fluorescence associated with $\Delta\Psi_m$ generation was much faster in the presence of CsA that prevents opening of the MPT pore (lines c and d, with or without CsA, respectively). The subsequent addition of 50 μM Ca^{2+} was followed by a transient decrease of $\Delta\Psi_m$ in the presence of CsA (line c) and by a larger $\Delta\Psi_m$ decrease that was not completely recovered in the absence of CsA (line d). These findings are in agreement with previous studies showing that ibuprofen plus Ca^{2+} induce MPT in isolated rat liver mitochondria [24,34].

3.3. Ibuprofen-induced W256 cell death is not preceded by disruption of mitochondrial membrane potential and is potentiated by CsA

The ability of ibuprofen to induce MPT in permeabilized W256 cells mitochondria prompted us to investigate

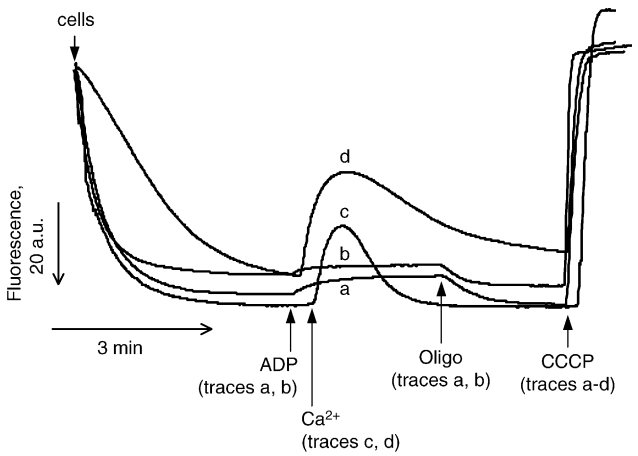

Fig. 2. Ibuprofen stimulates MPT induced by Ca^{2+} in permeabilized W256 cells. To test the direct ibuprofen action on mitochondria, 5×10^6 digitonin-permeabilized W256 cells were incubated under constant agitation at 37 °C in medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES buffer pH 7.2, 1 mM $MgCl_2$, 2 mM potassium phosphate, 0.2% BSA, 5 mM succinate plus 4 μM rotenone and 10 μM safranin O. Traces were recorded in the absence of ibuprofen (control: trace a) or in the presence of 500 μM ibuprofen (trace b) or 500 μM ibuprofen and 10 μM $CaCl_2$ without CsA (trace d) or with 1 μM CsA (trace c). Additions of 20 μM ADP and 1 μg/ml oligomycin (traces a and b), 50 μM $CaCl_2$ (traces c and d), or 8 μM CCCP are indicated by arrows. Traces are representative of three independent experiments. a.u. = arbitrary units.

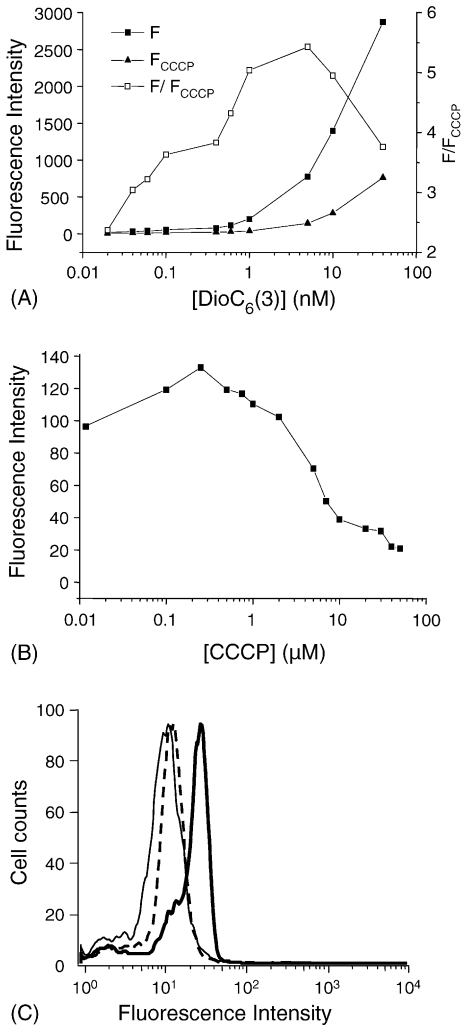

Fig. 3. Determination of the optimal conditions for analysis of the mitochondrial $\Delta\Psi$ in intact cells using flow cytometry. (A) Determination of $DioC_6(3)$ concentration. W256 cells were incubated in the presence of increasing concentrations of the probe for 40 min. Mean fluorescence values for each concentration of $DioC_6(3)$ in the absence (F—filled squares) or in the presence (F_{CCCP}—filled triangles) of 40 μM CCCP for the last 20 min are shown at the left vertical axis; the F/F_{CCCP} ratios (open squares) are shown at right vertical axis. (B) Determination of CCCP concentration. For membrane depolarization, W256 cells were incubated in the presence of 0.2 nM of $DioC_6(3)$ for 40 min and increasing concentration of CCCP for the last 20 min. Plot shows the values of the mean fluorescence. (C) Effect of mitochondrial inhibitors on $\Delta\Psi_m$. Histograms show W256 cells incubated for the same period of time in the presence of 0.2 nM of $DioC_6(3)$ (thick line), plus 1 μg/ml oligomycin and 10 mM KCN (dashed line) or plus 40 μM CCCP (thin line). Results are representative of three independent experiments.

whether these mitochondrial alterations would mediate cell death induced by this drug. $DioC_6(3)$ was used to evaluate $\Delta\Psi_m$ by flow cytometry in intact cells [35,36]. To minimize plasma membrane potential contribution to the overall fluorescence, cells were labeled with increasing concentrations of $DioC_6(3)$ in the absence (F: Fig. 3A, filled squares) or in the presence of 40 μM CCCP (F_{CCCP}: Fig. 3A, filled triangles). The resulting F/F_{CCCP} ratio was plotted as open squares. In the concentration range of 0.1–

0.2 nM $DioC_6(3)$, low variations of both F and the F/F_{CCCP} ratio indicated an appropriate concentration of $DioC_6(3)$ to be used. The ratio F/F_{CCCP} decreased at $DioC_6(3)$ concentrations above 10 nM, probably due to probe quenching. Under our experimental conditions, 40 μM CCCP was able to disrupt total (plasma plus mitochondrial) membrane potential of the cells (Fig. 3B). To confirm that at the concentration of 0.2 nM $DioC_6(3)$ only $\Delta\Psi_m$ was detected, mitochondrial respiration and ATP hydrolysis were inhibited by potassium cyanide plus oligomycin (Fig. 3C, dashed line) to eliminate $\Delta\Psi_m$. Under these conditions, the remaining fluorescence was similar to the basal fluorescence obtained with CCCP (thin line), confirming that in this range of $DioC_6(3)$ (0.1–0.2 nM) only the $\Delta\Psi_m$ was detected.

Working under these experimental conditions it was possible to demonstrate that death of W256 cells was not preceded by a progressive decrease of $\Delta\Psi_m$ (Fig. 4A and B). Instead, there were two well-defined populations in the cell culture: one with high $DioC_6(3)$ fluorescence, representing living and normal-sized cells (upper right quadrant), and a population with basal fluorescence of dead small cells (lower left quadrant) more evident in ibuprofen-treated cells (Fig. 4B). Cells lacking or with intermediate $DioC_6(3)$ fluorescence were not observed in the population of normal-sized cells after 24 h of treatment with ibuprofen (Fig. 4B, upper left quadrants), or at any other time (results not shown), as observed with CCCP that disrupt $\Delta\Psi_m$ (Fig. 4C and D). This indicates that ibuprofen-induced cell death is not followed by gradual loss of $\Delta\Psi_m$ and that the loss of $\Delta\Psi_m$ is a very late event during cell death induced by ibuprofen. This conclusion is further supported by the lack of protective effect of the MPT inhibitors BA and CsA. As shown in Fig. 5A and B, the combination of ibuprofen and CsA increased both apoptotic (% annexin-V positive) and late apoptotic cells (% annexin-V plus propidium iodide positive cells—inset), as well as cells with condensed chromatin (not shown), when compared to cells treated only with ibuprofen. BA produced no effect on cells viability either alone or in combination with ibuprofen (Fig. 5A and B). The stimulation of ibuprofen effect by CsA rules out the involvement of MPT during death of W256 cells. CsA has other targets different than the pore component cyclophilin D, which can either induce or protect cells from apoptosis, depending on cell type and stimulus [37–39]. We propose that one or more of these targets could account for the CsA effects observed here.

Consistent with a lack of MPT involvement in cell death induced by ibuprofen, CsA (Fig. 5C) and BA (Fig. 5D) did not promote hyperpolarization of the mitochondria. The mean fluorescence of both control and ibuprofen-treated cells along the culture period generated F/F_{CsA} and F/F_{BA}

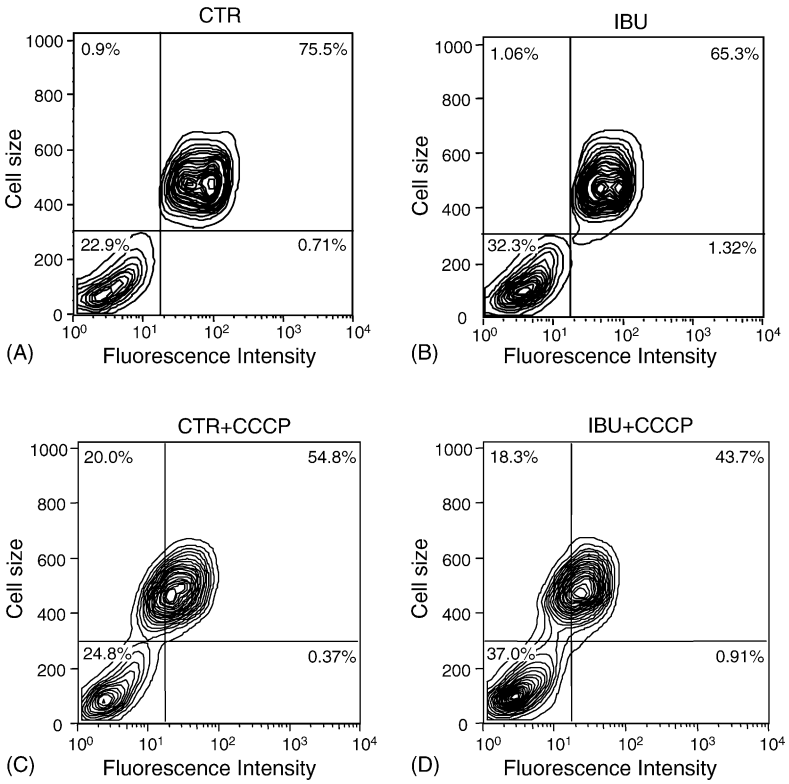

Fig. 4. Ibuprofen-induced W256 cell death is not preceded by a progressive loss of $\Delta\Psi_m$. (A–D) W256 cells were cultured for 24 h as described in Section 2 in the absence (CTR—A and C) or in the presence of 500 μM ibuprofen (IBU—B and D). Density plot shows W256 cells analyzed by flow cytometry after 24 h of culture and incubated for additional 40 min with $DioC_6(3)$ (A and B) or plus CCCP for the last 20 min (C and D). Results are representative of three independent experiments.

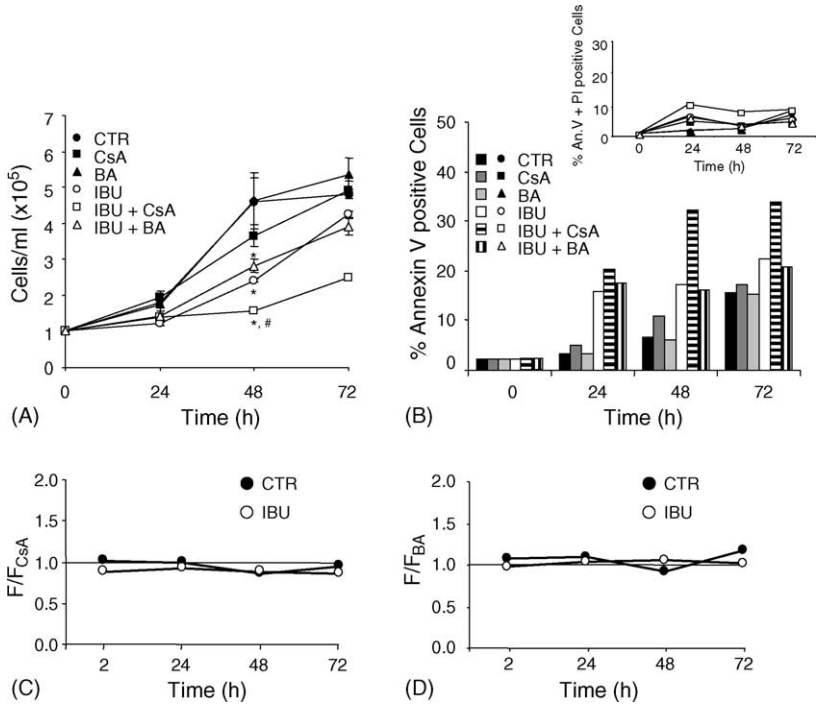

Fig. 5. Cell death induced by ibuprofen is independent on MPT and potentiated by cyclosporin A. (A) W256 cells were cultured for 72 h as described in Section 2 in the absence (CTR—filled circles) or in the presence of 1 μM cyclosporin A (CsA—filled squares), 1 μM bongkrekic acid (BA—filled triangles), 500 μM ibuprofen (IBU—open circles), 500 μM ibuprofen plus 1 μM cyclosporin A (open squares) or 500 μM ibuprofen plus 1 μM bongkrekic acid (open triangles). Number of cells per ml was quantified every 24 h by counting in Neubauer chamber under light microscope. * $p < 0.05$ compared to control, # $p < 0.05$ compared to IBU. (B) W256 cells were cultured as described in A. Every 24 h, 0.5×10^5 cells/ml were collected and processed for annexin V-FITC labeling and propidium iodide staining (inset) and immediately analyzed by flow cytometry. Bars represent percentage of annexin V-FITC positive cells. Control (CTR—black bars), 1 μM cyclosporin A (CsA—dark gray bars), 1 μM bongkrekic acid (BA—light gray bars), 500 μM ibuprofen (IBU—open bars), 500 μM ibuprofen plus 1 μM cyclosporin A (horizontal stripes) or 500 μM ibuprofen plus 1 μM bongkrekic acid (vertical stripes). Inset, lines represent percentage of annexin-V plus PI positive cells and are arranged as in A. Results are representative of three independent experiments. (C, D) W256 cells were cultured for 72 h as described in Section 2 in the absence or in the presence of 500 μM ibuprofen. 0.4×10^5 cells of each condition were further incubated with 0.2 nM $DioC_6(3)$ for 40 min and with or without 1 μM CsA or 1 μM BA for the last 20 min. The mean fluorescence ratios F/F_{CsA} (C) and F/F_{BA} (D) for control cells (filled circles) and ibuprofen-treated cells (open circles) were plotted for each time point. Results are representative of three independent experiments.

ratios not significantly different than one. These results rule out the MPT pore function either physiologically or during the death of W256 cells. A physiological role for MPT pore has been proposed by Kowaltowski and co-workers [40] that showed resting $\Delta\Psi_m$ was significantly increased in PC12 and SY5Y cells by CsA, but not significantly in Bcl-2-overexpressing PC12 cells. We have previously shown that W256 tumor cells naturally express high levels of Bcl-2 [25], which certainly contribute to prevent MPT pore function in these cells.

3.4. Mitochondrial membrane potential ($\Delta\Psi_m$) of dying cells is maintained by respiration, not by ATP hydrolysis

It has been reported that $\Delta\Psi_m$ in dying cells could also be maintained by ATP hydrolysis via ATP synthase, with a complete loss of $\Delta\Psi_m$ when this enzyme is inhibited by oligomycin [41,42]. In this regard, the fluorescence histogram profiles of Fig. 6A and B show that oligomycin (dashed line) induced a small increase of the mean fluorescence of $DioC_6(3)$ in both non-treated (Fig. 6A) and ibuprofen-treated cells (Fig. 6B). The value of the mean fluorescence found for each condition at 48 h of culture is shown in these figures. In the graph depicted in Fig. 6C, the mean fluorescence values of the cells cultured in the presence or absence of ibuprofen and incubated with oligomycin were used to generate the F/F_{oligo} ratio for each day, which was slightly below one along the 72 h of culture (Fig. 6C), as consequence of the persistent higher F_{oligo} mean. Considering that ATP synthase inhibition by oligomycin shifts respiration of intact mitochondria to state IV, the increase of $\Delta\Psi_m$ caused by oligomycin is consistent with a functional ATP generating system and a $\Delta\Psi_m$ sustained by respiration in ibuprofen-treated W256 tumor cells. Thus, ibuprofen alone does not affect mitochondrial function either in permeabilized (Fig. 2, line b) or in intact (Fig. 4B and Fig. 6C) cells, although it stimulates MPT induced by Ca^{2+} (Fig. 2, lines c and d).

3.5. Inhibition of calcineurin by FK506 increases ibuprofen-induced cell death

CsA, in addition of being a MPT inhibitor, is also an inhibitor of calcineurin, a Ca^{2+}/calmodulin-dependent pro-

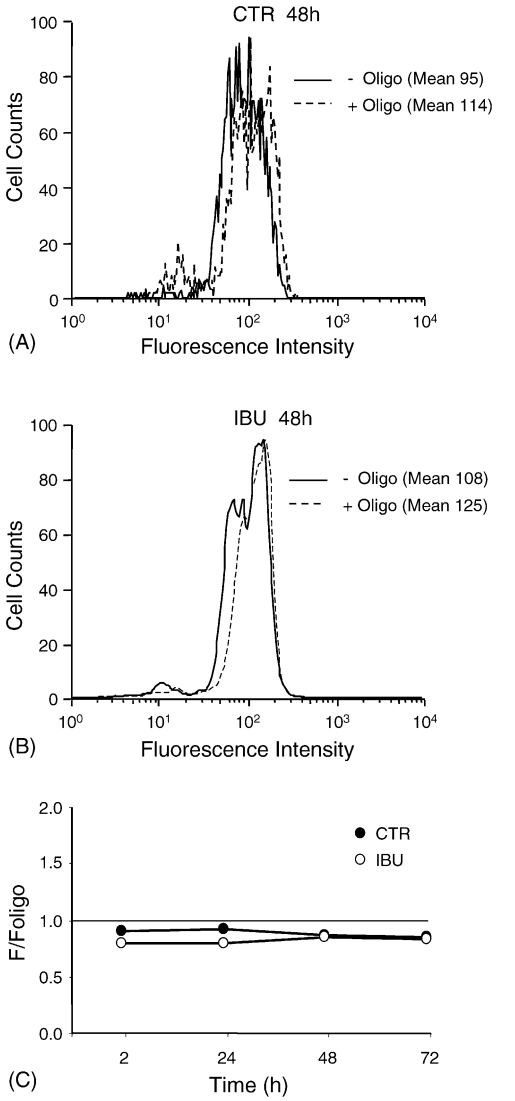

Fig. 6. Mitochondrial membrane potential ($\Delta\Psi_m$) of dying cells is maintained by respiration, not by ATP hydrolysis. (A and B) W256 cells were cultured as described in Section 2 in the absence (CTR—A) or in the presence of 500 μM ibuprofen (IBU—B). Histograms show W256 cells analyzed by flow cytometry after being cultured for 48 h and incubated for additional 40 min with $DioC_6(3)$ (continuous line) or with 1 μg/ml oligomycin (dashed line) for the last 20 min. Results are representative of three independent experiments. (C) W256 cells were cultured for 72 h as described in Section 2, in the absence (CTR—filled circles) or in the presence of 500 μM ibuprofen (IBU—open circles). 0.4×10^5 cells of each condition were further incubated with 0.2 nM $DioC_6(3)$ for 40 min and with or without 1 μg/ml oligomycin for the last 20 min. The mean fluorescence ratio F/F_{oligo} was plotted for each time point. Results are representative of three independent experiments.

tein phosphatase that may exert either pro- or anti-apoptotic effects, depending on the cell type [43–48]. We investigated whether inhibition of calcineurin could account for the CsA stimulation of cell death induced by ibuprofen by treating the W256 cells with FK506, an immunosuppressor that inhibits calcineurin, but does not affect cyclophilin D or MPT. FK506 alone did not have any effect on cell growth or viability (Fig. 7A and B, respectively), and did not significantly decrease ibuprofen effect on cell growth (Fig. 7A). FK506 stimulated apoptosis induced by ibuprofen, as shown by the increased percentage of annexin-V (Fig. 7B) and annexin-V plus propidium iodide (Fig. 7B, inset) positive dead cells. W256 cells growth in vivo is fast and kills the animal in 4–5 days. Although we have always begun the experiments with cells over 98% viable, the time between the onset of the ascitic fluid formation and the death of the host animal is narrow, and a few hours in difference for the tumor cells withdrawal can produce variations in cell death behavior in vitro. Indeed, two waves of cell death are sometimes observed in our experiments. The first, short-lived and less intense wave, represented by annexin-V plus propidium iodide positive cells (Fig. 7B, inset), is mostly necrotic and not always present (Fig. 5B, inset). The second wave, represented by annexin-V positive cells, is persistent and apoptotic. Our results suggest that calcineurin inhibition mediates CsA and FK506 effect on apoptosis induced by ibuprofen. CsA and FK506 are also inhibitors of peptidyl prolyl *cis*/*trans* isomerases or immunophilins. However, since both inhibit different groups of immunophilins [49], calcineurin likely is their common target. These results also show that calcineurin has an anti-apoptotic function in this system. This conclusion is based on the fact that inhibition of calcineurin by CsA or FK506 increased cell death only in the presence of ibuprofen, which shows that calcineurin may be specifically activated when cells are challenged with an insult, such as that induced by ibuprofen. A proposed mechanism by which calcineurin may rescue cells from death is through its guidance by Bcl-2 to the IP_3 receptor at the endoplasmic reticulum (ER), resulting in dephosphorylation of the IP_3 receptor and inhibition of Ca^{2+} release from this organelle [50], preventing Ca^{2+} toxicity. Calcineurin/Bcl-2 interaction may also account for the anti-apoptotic effects of Bcl-2 since targeting of Bcl-2 to the ER can also regulate Ca^{2+} homeostasis and inhibit apoptosis [51]. In a previous work [25], we showed that W256 tumor cells naturally express high levels of the anti-apoptotic protein Bcl-2, which was related to a high resistance to cytotoxic concentrations of intracellular Ca^{2+} and to oxidative stress. A putative interaction between calcineurin and Bcl-2 in W256 cells challenged with ibuprofen may ameliorate mitochondrial function through Bcl-2 and calcineurin targeting to either the ER, mitochondria or both [52,53] and then maintain the mitochondrial functions observed during ibuprofen-induced cell death.

3.6. Apoptosis induced by ibuprofen involves cytochrome c release

Here we show that apoptosis induced by ibuprofen occurs independently on $\Delta\Psi_m$ collapse and MPT. Since mitochondrial depolarization is not a universal marker for apoptosis and cytochrome *c* release can occur in cells that preserve $\Delta\Psi_m$ during the process of death [54], we inves-

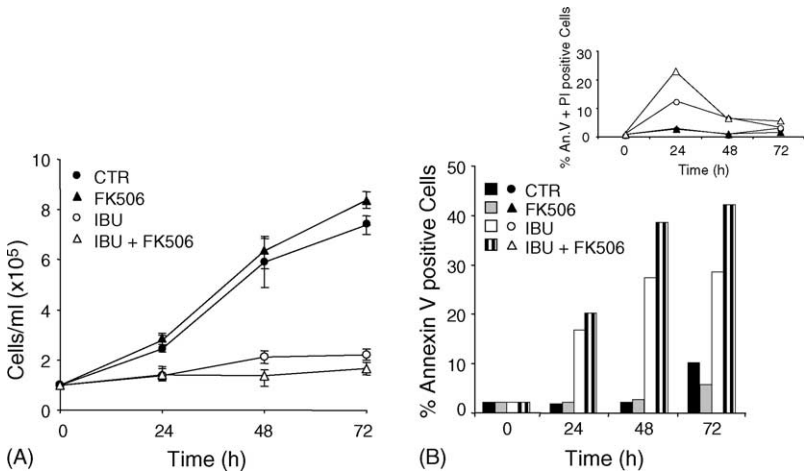

Fig. 7. FK506 increases ibuprofen-induced cell death. (A) W256 cells were cultured for 72 h as described in Section 2, in the absence (CTR—filled circles) or in the presence of 1 μM FK506 (filled triangles), 500 μM ibuprofen (IBU—open circles), or 500 μM ibuprofen plus 1 μM FK506 (open triangles). Number of cells per ml were quantified every 24 h in a Neubauer counting chamber under light microscope. (B) Every 24 h, 0.5×10^5 cells/ml were collected and processed for annexin V-FITC labeling and propidium iodide staining (inset) and immediately analyzed by flow cytometry. Bars represent percentage of annexin V-FITC positive. Control (CTR—black bars), 1 μM FK506 (light gray bars), 500 μM ibuprofen (IBU—open bars), or 500 μM ibuprofen plus 1 μM FK506 (vertical stripes). Inset, lines represent percentage of Annexin-V plus PI positive cells and are arranged as in A. Results are representative of three independent experiments.

tigated whether this apoptosis is dependent or not on cytochrome *c* release. In the immunoblot for cytochrome *c* using mitochondrial fraction, we demonstrated that cytochrome *c* is restricted to mitochondria under control conditions at 0 h or after 48 h (Fig. 8A), but is released from the organelle in ibuprofen-treated cells when about 50% of cells were dead after 48 h of culture (Fig. 8B). Staurosporine for 8 h in cells, which also results in about 50% of cell death (not shown), induced comparable level of cytochrome *c* release (Fig. 8C). Our results indicate that the high Bcl-2 expression in W256 may be necessary to stabilize functionally and structurally mitochondria and to counteract fortuitous Bax, Bak or Bid action. However, this high Bcl-2 expression in W256 may not be sufficient to avoid cytochrome *c* release and cell killing by ibuprofen.

In conclusion, W256 cell death induced by ibuprofen displays apoptotic features such as chromatin condensation, translocation of phosphatidylserine to the outer membrane leaflet and cytochrome *c* release with no loss of mitochondrial function or MPT. We propose that calcineurin activation plays an important role as a rescue pathway under these experimental conditions (Fig. 9).

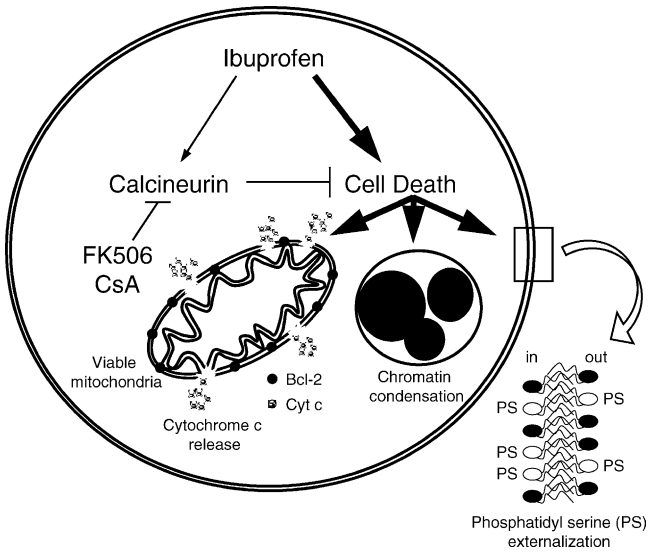

Fig. 9. Ibuprofen induces death of W256 cells that contain mitochondria with a high level of Bcl-2 expression. W256 cells death is accompanied by chromatin condensation, phosphatidylserine (PS) externalization and cytochrome *c* (Cyt *c*) release. Ibuprofen also activates a parallel rescue pathway via calcineurin, a phosphatase inhibited by either cyclosporin A or FK506.

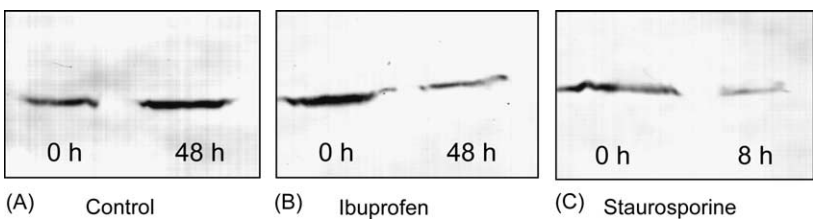

Fig. 8. Cell death induced by ibuprofen involves cytochrome *c* release. Mitochondrial fractions (15 μg) from W256 cells incubated in the absence (control: panel A) or in the presence of either 500 μM ibuprofen (panel B) for 48 h or 2 μM staurosporine (panel C) for 8 h were immunobloted for cytochrome *c*.

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