

Potential involvement of F_0F_1 -ATP(synth)ase and reactive oxygen species in apoptosis induction by the antineoplastic agent erucylphosphohomocholine in glioblastoma cell lines

A mechanism for induction of apoptosis via the 18 kDa mitochondrial translocator protein

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Abstract Erucylphosphohomocholine (ErPC3, ErufosineTM) was reported previously to induce apoptosis in otherwise highly apoptosis-resistant malignant glioma cell lines while sparing their non-tumorigenic counterparts. We also previously found that the mitochondrial 18 kDa Translocator Protein (TSPO) is required for apoptosis induction by ErPC3. These previous studies also suggested involvement of reactive oxygen species (ROS). In the present study we further investigated the potential involvement of ROS generation, the participation of the mitochondrial respiration chain, and the role of the mitochondrial F_0F_1 -ATP(synth)ase in the pro-apoptotic effects of ErPC3 on U87MG and U118MG human glioblastoma cell lines. For this purpose, cells were treated with the ROS chelator butylated hydroxyanisole (BHA), the mitochondrial respiration chain inhibitors rotenone, antimycin A, myxothiazol, and the uncoupler CCCP. Also oligomycin and piceatannol were studied as inhibitors of the F_0 and F_1 subunits of the mitochondrial F_0F_1 -ATP(synth)ase, respectively. BHA was able to attenuate apoptosis

induction by ErPC3, including mitochondrial ROS generation as determined with cardiolipin oxidation, as well as collapse of the mitochondrial membrane potential ($\Delta\psi_m$). Similarly, we found that oligomycin attenuated apoptosis and collapse of the $\Delta\psi_m$, normally induced by ErPC3, including the accompanying reductions in cellular ATP levels. Other inhibitors of the mitochondrial respiration chain, as well as piceatannol, did not show such effects. Consequently, our findings strongly point to a role for the F_0 subunit of the mitochondrial F_0F_1 -ATP(synth)ase in ErPC3-induced apoptosis and dissipation of $\Delta\psi_m$ as well as ROS generation by ErPC3 and TSPO.

Keywords Erucylphosphohomocholine · TSPO · F_0F_1 -ATP(synth)ase · Oligomycin · Mitochondrial membrane potential · Glioblastoma

Introduction

Glioblastomas are the most common brain tumors. They present an unfavorable prognosis due to their aggressiveness and likelihood of recurrence [1]. Thus, the development of novel anti-glioblastoma treatments is mandatory, including an understanding of the underlying mechanisms of potential new treatments. Alkylphosphocholines (APC) comprise a class of anticancer agents targeting plasma membranes [2, 3]. Previously, we discovered that the APC derivatives erucylphosphocholine (ErPC) and its congener erucylphosphohomocholine (ErPC3, ErufosineTM) induce apoptosis in otherwise highly apoptosis-resistant malignant glioma cell lines while sparing their non-tumorigenic counterparts [4–6]. We also found that the mitochondrial 18 kDa Translocator Protein (TSPO) is required for apoptosis induction by ErPC3 [7–9]. In animal experiments,

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ErPC was shown to cross the blood–brain barrier after repeated intravenous injections. Hence, it constitutes a promising therapeutic candidate for brain tumors, including glioblastoma [10] and currently is undergoing a clinical phase I trial. Furthermore, we analyzed the APC-induced cell death process and found that ErPC as well as ErPC3 promote tumor cell apoptosis independent of p53 function and death receptor/death ligand interactions [6, 11].

We recently investigated the mechanisms by which ErPC and ErPC3 induce cell death. Mitochondria, as well as Apaf-1 and caspase-3, play major roles in ErPC- and ErPC3-induced apoptosis [7, 12, 13]. ErPC3 treatment was also found to result in mitochondrial membrane depolarization and cytochrome *c* release in intact glioma cells [7]. Indeed, we were able to show that ErPC3 acts directly on mitochondria. In particular, ErPC3 induced swelling of isolated rat liver mitochondria and decreased ATP synthesis in a concentration-dependent manner via permeabilization of the inner mitochondrial membrane [13]. Since ErPC3 displays direct toxic effects on mitochondria, we concluded that ErPC3 (and its congener ErPC) target these organelles. In support of this statement, cyclosporin A (CsA), an inhibitor of the mitochondrial permeability transition pore (MPTP) complex, significantly reduced ErPC3-induced apoptotic responses (7,13). Moreover, using genetic manipulation of a rat glioma cell line (C6) to induce knockdown of the 18 kDa TSPO, we demonstrated that ErPC and ErPC3 indeed activate the mitochondrial apoptotic pathway via the TSPO [9]. The TSPO, also known as the peripheral-type benzodiazepine receptor (PBR), can be found in the outer membrane of mitochondria in close association with the voltage-dependent anion channel, VDAC, and the adenosine nucleotide transporter (ANT) [14–16]. It was further found that TSPO activation by ErPC3 resulted in reactive oxygen species (ROS) generation leading to oxidation of cardiolipins at mitochondrial levels [8]. This mitochondrial ROS generation causes activation of the MPTP and consequently dissipation of the mitochondrial membrane potential, release of cytochrome *c*, and subsequent activation of the mitochondrial apoptosis cascade, including caspase-9 and -3 activation, and DNA fragmentation [7, 8, 17, 18]. Thus, ErPC3 appears to be able to activate the mitochondrial apoptosis pathway, apparently by ROS generation as a consequence of TSPO activation.

Mitochondria exert a dual function in the cell. On the one hand, these organelles play a central role in determining the “point-of-no-return” in the apoptotic process and on the other hand are pivotal for cellular bioenergetics [19]. Mitochondrial oxidative phosphorylation plays a key role in energy production, the generation of free radicals, and apoptosis [20]. However, despite extensive study the requirement of oxidative phosphorylation for the efficient

execution of cell death is still incompletely understood and controversial. For example, it was shown that ρ^0 cells devoid of mitochondrial DNA, which lack a functional electron transport chain and are incapable of generating ATP from mitochondria, undergo apoptosis as efficiently as their parental cells [21–23]. Other authors demonstrated the opposite, i.e. other cells devoid of mitochondrial DNA (such as SK-Hep1 cells or C2C12 myocytes) are resistant to apoptosis [24–26]. Similarly, oligomycin, a specific inhibitor of the mitochondrial F_0F_1 -ATP(synth)ase (thereby an inhibitor of the mitochondrial electron transport chain and ATP generation), showed pro-apoptotic effects in several studies [27–29] but also anti-apoptotic effects have been reported in other studies [30–32]. Interestingly, down-regulation of the catalytic subunit (F_1) of the mitochondrial F_0F_1 -ATP(synth)ase is a hallmark of a variety of different human tumors, including breast, liver and lung tumors [33]. Thus, studies elucidating the role of the F_0F_1 -ATP(synth)ase in carcinogenesis are needed.

The aim of this study was to further investigate the involvement of ROS generation in ErPC3-mediated apoptosis and to characterize the participation of the mitochondrial respiration chain and the mitochondrial F_0F_1 -ATP(synth)ase in the execution of ErPC3-induced cell death. Butylated hydroxyanisole (BHA) was one ROS scavenger used in this study in combination with assays of cardiolipin oxidation to determine ROS generation by ErPC3. To determine involvement of the mitochondrial respiration chain, we used the mitochondrial respiration chain inhibitors rotenone, antimycin A, myxothiazol, the uncoupler CCCP, and oligomycin as an inhibitor of the F_0 subunit of the mitochondrial F_0F_1 -ATP(synth)ase, as well as piceatannol, an inhibitor of the F_1 subunit of this enzyme. The results obtained suggest a role for the F_0 subunit of the mitochondrial F_0F_1 -ATP(synth)ase in ErPC3-induced apoptosis, dissipation of the mitochondrial membrane potential ($\Delta\Psi_m$), the generation of ROS, and ATP depletion. Parts of this study have been presented in abstract form [34–36].

Materials and methods

Cell culture

The human glioblastoma cell lines U87MG and U118MG were generously provided by Prof. G. Bernhardt, University of Regensburg, Germany. Cells were cultured at 37°C in an atmosphere of 5% CO₂ and 90% relative humidity, as described previously [7]. Briefly, U87MG cells were maintained in EMEM and U118MG cells in DMEM supplemented with 10% (U87MG) or 5% (U118MG) heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin,

100 µg/ml streptomycin, 1% sodium pyruvate and 1% non-essential amino acids.

Drugs

Erucylphosphohomocholine (ErPC3, Erufosine™) was generously provided by Genzyme Pharmaceuticals (Liestal, Switzerland). Antimycin A, butylated hydroxyanisole (BHA), carbonyl cyanide 3-chlorophenyl-hydrazone (CCCP), cyclosporin A, myxothiazol, oligomycin, ouabain, piceatannol, and rotenone were obtained from Sigma–Aldrich (St. Louis, MO).

Assay material

WST-1 reagent, Cell Death Detection ELISA^{PLUS} Kit (Cell Death Kit), and ATP Bioluminescence Assay Kit CLS II were from Roche Applied Science (Mannheim, Germany). Bicinchoninic acid (BCA) protein assay reagents kit was from Pierce (Rockford, IL, USA). The fluorescent dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was from Calbiochem (Merck, Darmstadt, Germany). Standard chemicals for the assays described below were from various commercial sources.

Drug treatments

To induce apoptosis and collapse of the mitochondrial membrane potential, human glioblastoma cells were treated with ErPC3 as described previously [7–9, 12, 13]. The involvement of ROS generation in ErPC3-induced cell death was studied by applying the ROS scavenger BHA as described previously [34]. We measured ROS generation by ErPC3 and its potential inhibition by BHA by assays of cardiolipin oxidation, using 10-*N*-Nonyl-Acridine Orange (NAO), according to methods applied previously [8, 18]. To study the involvement of the mitochondrial respiration chain in these events, we treated cells with the following inhibitors: 2 µM rotenone, 2 µg/ml antimycin A, 1 µM myxothiazol, and 5 µM CCCP. The inhibitor concentrations were selected according to procedures described previously [31, 37]. In addition, we did viability tests and found that at these concentrations effects on viability did not appear to be a major factor (see “Results”; Fig. 4). An exception may be rotenone, which showed moderate lethal effects at all concentrations higher than 50 nM applied to the U118MG cell line. For these reasons, we decided to apply the concentrations previously used by others [31, 37], to remain in line with these studies. In the case of co-incubation with ErPC3, these inhibitors were added to the culture medium 1 h prior to ErPC3 treatment, for all experiments. The role of the mitochondrial F₀F₁-ATP(synth)ase in this system was assayed by using the F₀F₁-ATP(synth)ase inhibitors oligomycin and

piceatannol, as described before [13, 31]. The concentrations of oligomycin (5 µg/ml) and piceatannol (5 µM) used for the present study were effective as described in the “Results” and “Discussion”. The drugs were dissolved in their appropriate vehicles: DMSO (BHA, CCCP, rotenone), ethanol (antimycin A, cyclosporin A, oligomycin, piceatannol), methanol (myxothiazol), or double distilled water (ouabain).

Measurement of cell viability

Cell viability was determined by the WST-1 assay according to the manufacturer’s instructions as described previously [7]. Briefly, human glioblastoma cells were seeded in flat-bottom 96-well microtiter plates (1 × 10⁴/100 µl/well), allowed to attach overnight and treated with different concentrations of respiration chain inhibitors and uncoupler for 24 h. Control cells were treated with the appropriate vehicles. WST-1 reactivity was measured with an ELISA reader (TECAN, Zurich, Switzerland) at 450 nm wavelength (reference 690 nm).

Apoptosis assays

Morphology

Morphological changes characteristic for apoptosis such as rounding and membrane blebbing were documented by phase-contrast microscopy with an inverted microscope (Leica DM IRB, Leica Microsystems, Wetzlar, Germany).

DNA fragmentation

Cytoplasmic histone-associated DNA fragments indicating ongoing apoptosis were quantitatively measured using the Cell Death Kit, following manufacturer’s instructions, as described previously [7]. ErPC3 was applied at the concentrations which are known to result in pronounced apoptosis (U87MG: 45 µM; U118MG: 15 µM) [7, 8]. As mentioned above, uncoupler and inhibitors of the mitochondrial respiratory chain and oxidative phosphorylation were added to the culture medium 1 h prior to ErPC3 treatment. Apoptosis was assayed at the time points indicated in the legends to Figs. 5, 6, and 8. Substrate absorption indicative of apoptosis was measured at 405 nm (reference 490 nm).

Caspase cleavage

U87MG and U118MG cells seeded in culture flasks (75 cm²) were co-treated for 12 h with oligomycin or piceatannol and ErPC3 (45 µM for U87MG, and 15 µM for U118MG). Control cells were treated with vehicle only. Following treatment, cells were harvested, washed,

transferred to microvials, and resuspended in 200 μ l lysis buffer (0.02% digitonin, 250 mM sucrose, 80 mM KCl, 5 mM EDTA). After 5 min on ice, cell lysates were centrifuged (700 \times g, 10 min, 4°C). Supernatants were transferred to fresh microvials and centrifuged for 25 min at 10,000 \times g, 4°C, with the final supernatants containing the cytosolic fractions. The protein content of the cytosolic fractions was quantified using bicinchoninic acid (BCA) protein assay reagents, according to the manufacturer's instructions (Pierce). Equal protein amounts of the cytosolic fractions were used to detect caspase-3 processing by SDS-PAGE and Western blot analysis with polyclonal rabbit anti-cleaved caspase-3 (1:2000, Cell Signaling, Beverly, MA) [7]. Labeling with COX IV (monoclonal mouse anti-COX-IV, 1:2000, Molecular Probes, Eugene, OR) was used as quality control for cell fractionation. Membrane-bound antibodies were visualized using Immobilon Western detection reagents (Millipore, Billerica, MA). Loading and transfer of even amounts of protein was confirmed by Ponceau S staining.

Mitochondrial membrane potential assay

Collapse of the mitochondrial membrane potential ($\Delta\Psi_m$) was analyzed by fluorescence assisted cell sorting (FACS) using the fluorescent lipophilic dye JC-1 as previously described [7, 18, 38, 39]. Cells were seeded in 6-well plates (2 \times 10⁵ cells/well). After achieving confluence, culture medium was changed to culture medium with or without drugs (ErPC3, BHA, CsA, oligomycin, piceatannol) as indicated in the “Results”. Cells treated with drug vehicle, are referred to as “control”. As positive control for mitochondrial membrane potential depolarization we treated the cells with the proton ionophor CCCP (50 μ M). After drug treatment, spontaneous free floating and trypsinized cells were collected for $\Delta\Psi_m$ determination by flow cytometry using JC-1 dye according to the manufacturer's instructions (Calbiochem). Briefly, cell suspensions were centrifuged at 500 \times g and the cell pellets were resuspended in 500 μ l of phosphate-buffered saline (PBS) at 4°C and transferred to FACS tubes (Falcon, Bedford, MA). Subsequently, 10,000 events were recorded per sample with a FACScan flow cytometer (Becton–Dickinson, Franklin Lakes, NJ) and analyzed using CellQuest software (Becton–Dickinson, Franklin Lakes, NJ).

ATP determination

Cellular ATP concentrations were determined based on the luciferin-luciferase reaction using the ATP Bioluminescence Assay Kit CLS II (Roche Applied Science), according to procedures described previously [40]. U87MG and U118MG cells seeded in culture flasks (25 cm²) were

co-treated with oligomycin or piceatannol and ErPC3 (45 μ M for U87MG; 15 μ M for U118MG) for 12 h. Cells were trypsinized and counted using Trypan blue solution to calculate the amount of living cells. 1 \times 10⁵ living cells were transferred to microvials, centrifuged at 800 \times g for 7 min at 4°C, and the supernatant removed carefully. The cell pellet was resuspended in 1 ml deionized water and ATP was extracted by boiling for 10 min and subsequent centrifugation at 12,000 \times g for 5 min at 4°C [40]. The resulting supernatant was used for bioluminescence measurement of ATP levels according to the instructions provided by the manufacturer (Roche Applied Science). Briefly, 50 μ l supernatant was mixed with 50 μ l luciferase reagent in triplicate in a white microtiter plate (clear bottom) on a microplate luminometer (GloMaxTM 96, Promega, Madison, WI). The light signal was integrated for 10 s after a delay of 1 s. Using this assay, luminescence (relative light units, RLU) is directly proportional to the ATP concentration. Data of ATP levels were normalized as a percentage of the value of the corresponding untreated cells.

Data analysis

Data are expressed as means \pm SD, $n > 5$ (or $n = 4$ in Fig. 3). To determine statistically significant differences, Kruskal–Wallis non-parametric, one-way analysis of variance (ANOVA) was carried out, since standard deviations typically differed significantly between groups, as indicated by Bartlett's test for homogeneity of variance. As the sample sizes were relatively small, Mann–Whitney was used as a post-hoc test, and $P < 0.05$ was considered to indicate statistically significant differences. The program used for statistical analysis was InStatGraphPadTM (GraphPad Software, San Diego, CA).

Results

The ROS scavenger butylated hydroxyanisole and the MPTP inhibitor cyclosporin A inhibit ErPC3-induced dissipation of $\Delta\Psi_m$

Previously we had found that ErPC3 treatment activates the TSPO, causing ROS generation at mitochondrial levels as well as collapse of the $\Delta\Psi_m$, indicative of MPTP opening, and mitochondrial apoptosis pathway induction [7, 8]. In the present study we wanted to determine whether ROS generation by ErPC3 may actually contribute to MPTP opening. To this end, the non-specific ROS scavenger 3-tert-butyl-4-hydroxyanisole (BHA; 200 μ M) [34] was applied prior to treatment with ErPC3 to counteract ROS generation and the subsequent effects on $\Delta\Psi_m$ collapse.

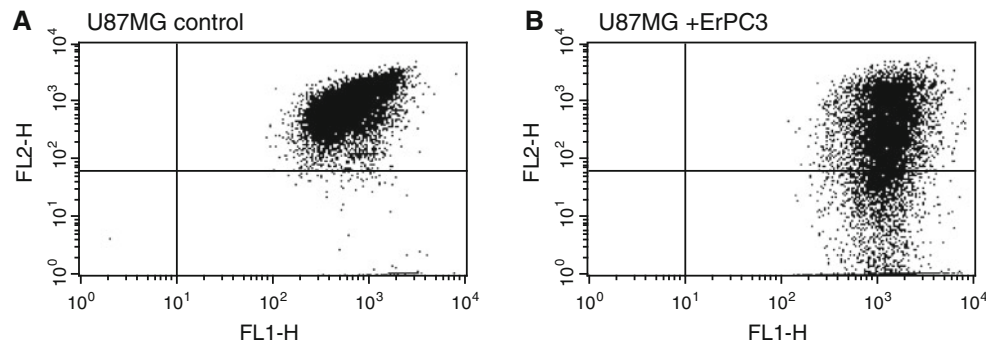


Fig. 1 Effects of ErPC3 on the mitochondrial membrane potential ($\Delta\Psi_m$) in the glioblastoma cell lines U87MG using the dye JC-1. The FACS patterns of U87MG cells labeled with JC-1 in the absence (a) or presence of ErPC3 (b) are shown. **a** Cells with polarized mitochondria are found in the *upper right quadrant* of the plots, corresponding to high emission of fluorescence at both 590 nm (FL2-H, *orange-red*) and 527 nm (FL1-H, *green*). **b** After ErPC3 treatment for 6 h (U87MG), membrane depolarization becomes evident as a decrease

in the 590 nm signal and an increase in the 527 nm signal, in the *lower right quadrant*. Lines defining quadrants were arbitrarily chosen to highlight changes in the fluorescence of cell populations. On the y-axis, *red fluorescence* (emissions at 590 nm) indicates the mitochondrial uptake of the JC-1 dye and the formation of J-aggregates within the matrix. On the x-axis, *green fluorescence* (emissions at 527 nm) indicates cytosolic retention of the JC-1 monomers

Furthermore, the $\Delta\Psi_m$ was measured in U87MG and U118MG cells that were treated with ErPC3 in the presence of the specific MPTP inhibitor cyclosporin A (CsA; 10 μ M). Cyclosporin A is reported to inhibit the VDAC component of the MPTP [41]. ErPC3 treatment consistently caused robust and very significant dissipation of $\Delta\Psi_m$ in U87MG and U118MG glioblastoma cells, in comparison to untreated controls (Fig. 1), as described previously [7, 8]. This dissipation of $\Delta\Psi_m$ by ErPC3 treatment was prevented by co-treatment with BHA of U87MG as well as U118MG cells i.e. BHA significantly reduced the number of cells undergoing $\Delta\Psi_m$ collapse otherwise induced by ErPC3. In particular, both U118MG and U87MG cells treated with BHA plus ErPC3 showed 5 times less cells displaying collapse of the $\Delta\Psi_m$ than cells treated with ErPC3 only (Fig. 2). We also found that ErPC3 induced cardiolipin oxidation, indicative of ROS generation at mitochondrial levels (Fig. 3). The ROS scavenger BHA, as expected, prevented ROS generation by ErPC3 (Fig. 3). We also have found that BHA prevented induction of apoptosis via the mitochondrial apoptosis pathway [34], as also demonstrated previously by others in different cell lines [42, 43]. Moreover, co-treatment of cells with ErPC3 and the VDAC inhibitor CsA also significantly reduced the number of cells displaying collapse of $\Delta\Psi_m$ compared to treatment with ErPC3 alone (in U87MG to less than half; in U118MG to less than one-third; Fig. 2). Consistent with previous studies, this indicates that ROS generation by ErPC3 is required to cause opening of the MPTP and in particular the VDAC component [8, 18, 44]. Depending on the cell line investigated, BHA and CsA alone appear to have either no effect on $\Delta\Psi_m$ or slightly depolarizing effects in U87MG and U118MG cells

(Fig. 2). Previously, as published as an abstract [34], we had also assayed several traditional ROS scavengers to establish the mechanism of ROS generation involved (in addition to BHA described above). These data were at best equivocal (see “Discussion”), suggesting that ROS generation due to ErPC3, other than causing cardiolipin oxidation at mitochondrial levels [8, and the present study], does not play a major role in apoptosis induction by ErPC3 [34]. Thus, our present data indicate that BHA in our experiment prevents ROS generation at mitochondrial levels together with collapse of the $\Delta\Psi_m$ and induction of apoptosis that otherwise are caused by ErPC3 and TSPO activation [8, 18].

Effects of inhibitors of respiration and uncouplers on ErPC3-induced apoptosis

As we elucidated in the previous section that ErPC3-induced ROS generation leads to MPTP opening and apoptosis, we wanted to further investigate whether the mitochondrial respiration chain is involved in these processes induced by ErPC3. It has been suggested before that the mitochondrial respiration chain may be involved in ROS generation by ErPC3 and the TSPO [44–47]. We chose the following uncoupler and inhibitors of various aspects of the mitochondrial respiration chain system: antimycin A, an inhibitor acting on center Q_i of mitochondrial complex III and having the highest affinity for the bc_1 complex; CCCP, one of the most frequently used uncouplers of oxidative phosphorylation; myxothiazol, the most tightly binding and potent inhibitor of complex III, which also inhibits complex I; oligomycin, the classical

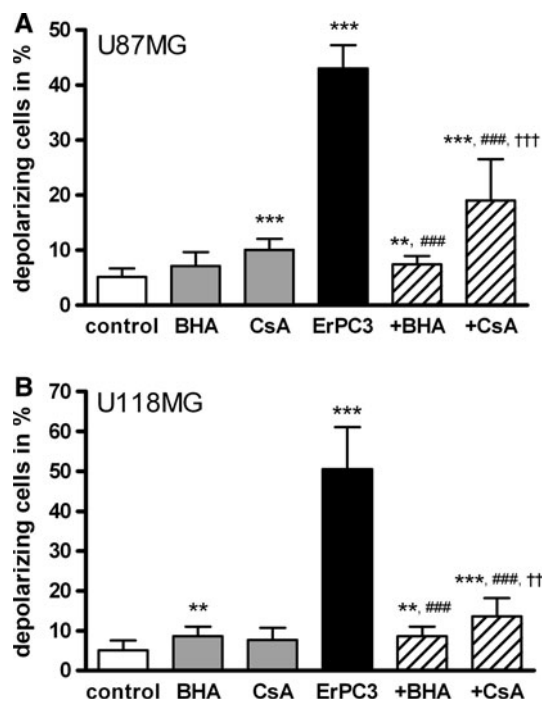


Fig. 2 Effects of BHA and CsA on ErPC3-induced breakdown of the inner mitochondrial membrane potential ($\Delta\Psi_m$) in the glioblastoma cell lines U87MG and U118MG using the dye JC-1. U87MG (a) and U118MG (b) cells were treated with ErPC3 (45 and 15 μM , respectively) and/or BHA (200 μM) or CsA (10 μM) for 6 h (U87MG) and 8 h (U118MG) and $\Delta\Psi_m$ was evaluated as illustrated in this figure and in Fig. 1. The percentages displayed in each column refer to the cell numbers in the *lower right quadrant* of the $\Delta\Psi_m$ analysis plot as a fraction of the total numbers of cells analyzed (example displayed in Fig. 1). These average percentages are derived from each time 9 independent experiments. In both cell lines co-treatment with BHA or CsA reduced the incidence of $\Delta\Psi_m$ collapse otherwise induced by ErPC3 itself. *** $P < 0.001$ versus the appropriate control; ** $P < 0.01$ versus the appropriate control; ### $P < 0.001$ versus ErPC3; †† $P < 0.01$ versus CsA; ††† $P < 0.001$ versus CsA

inhibitor of mitochondrial F_0F_1 -ATP(synth)ase; and rotenone, the classic inhibitor of complex I of the respiratory chain [48]. For our study, we applied concentrations of these uncouplers and inhibitors as reported in previous studies [31, 37]. We also used the WST-1 test to determine viability of the cell lines U87MG and U118MG after treatments with increasing concentrations of the different inhibitors of the mitochondrial respiration chain (ranging from 0.5 till 10 μM , or from 0.5 till 10 $\mu\text{g/ml}$) for 24 h (Fig. 4). The WST-1 tests indicated that antimycin A did not have any effects on cell viability in both glioma cell lines and the same holds true for myxothiazol. Oligomycin reduced cell viability of U118MG cells in a dose-dependent manner, but not of U87MG cells. In this latter cell line, rotenone exhibited a slight dose-dependent toxic effect. In U118MG cells, rotenone displayed moderate toxic effects at the same concentrations, as presented in

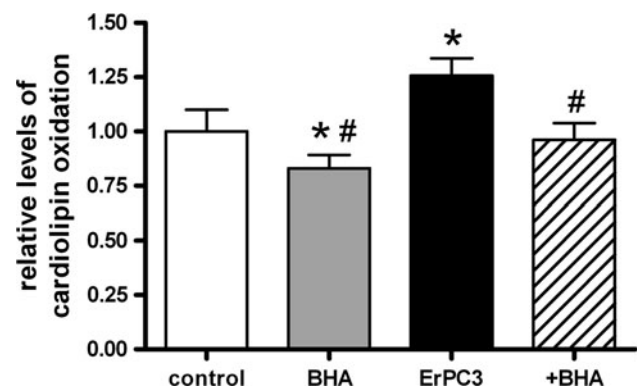
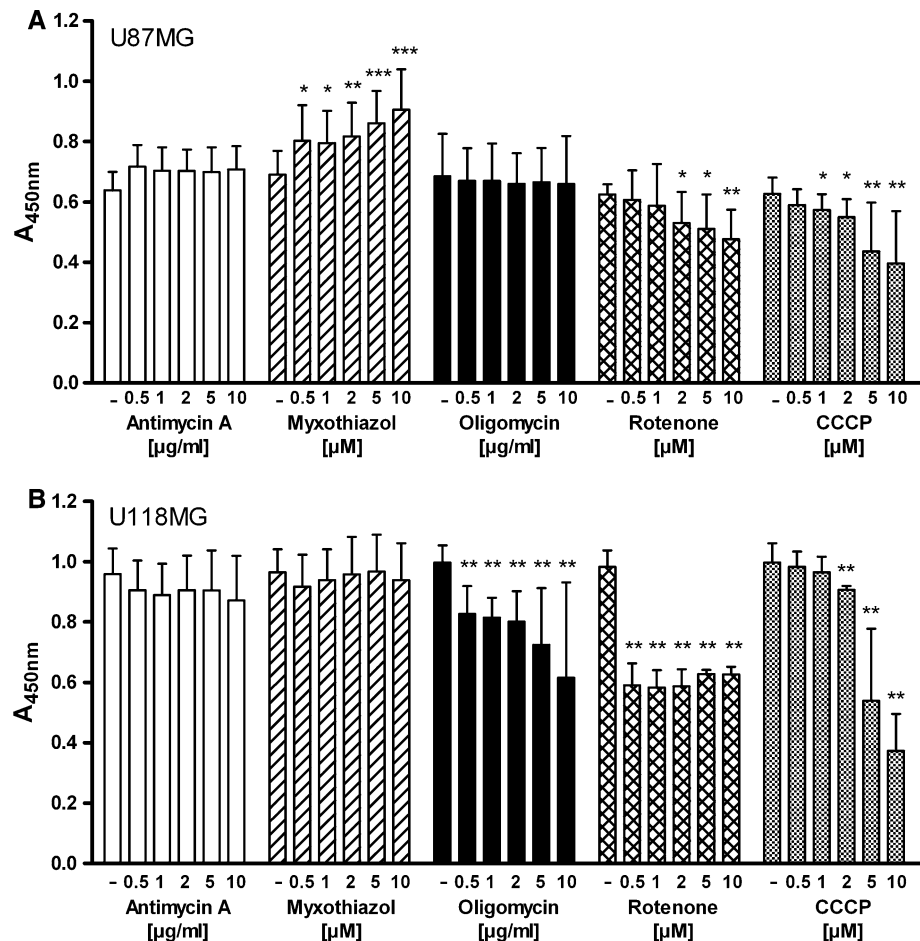


Fig. 3 Effects of BHA on ErPC3-induced cardiolipin oxidation, indicative of mitochondrial ROS generation, as determined with 10-N-Nonyl-Acridine Orange (NAO) by FACS. U118MG glioblastoma cells were treated with ErPC3 (7.5 μM) and/or BHA (200 μM) for 24 h. Control was treated with vehicle. The level of measured cardiolipin oxidation in the control is referred to as 1. The levels of cardiolipin oxidation in the experimental groups (BHA; ErPC3; (ErPC3) + BHA) are represented as relative to the control. These averages are derived from each time 4 independent experiments. ErPC3 enhanced cardiolipin oxidation. Addition of BHA to ErPC3 treatment reduced NAO labelling to control levels. BHA by itself reduced NAO labelling to below control levels. * $P < 0.05$ versus control; # $P < 0.05$ versus ErPC3

Fig. 4. With an additional assay, applying concentrations lower than 0.5 μM , we found that 10–50 nM of rotenone was virtually non-toxic for U118MG cells (data not shown). CCCP also dose-dependently reduced survival of both glioma cell lines as compared to untreated controls (Fig. 4). With these assays, we also observed differences in the basal absorbance entity between U87MG and U118MG cells obtained. This could indicate a difference in either the amount of viable cells (due to differences in proliferation) or in the mitochondrial metabolic activity (e.g. due to reduced number of mitochondria or modified mitochondria [49]). Furthermore these data show that in terms of cell death induction, U118MG is more sensitive to inhibitors and uncouplers of the mitochondrial respiration chain than U87MG, in particular regarding uncoupling of oxidative phosphorylation and inhibition of mitochondrial F_0F_1 -ATP(synth)ase. Additionally, it was noticed that the viability values presented in Fig. 4 show both positive and negative changes for different substances, but this aspect was not investigated further as it was not part of the present project. Thus, in accord with previous studies [31, 37], our data indicate that the following concentrations of the respiratory chain inhibitors and uncouplers (2 μM rotenone, 2 $\mu\text{g/ml}$ antimycin A, 1 μM myxothiazol, 5 μM CCCP, and 5 $\mu\text{g/ml}$ oligomycin) are appropriate to study their effects of co-administration with ErPC3.

Using the Cell Death Kit we could show in a quantitative manner that most of the inhibitors and uncouplers of the mitochondrial respiration chain tested did not influence

Fig. 4 Effects of mitochondrial inhibitors on viability of human glioma cell lines. Viability of U87MG (a) and U118MG (b) cells was measured by the WST-1 test after incubation for 24 h with increasing concentrations of the following mitochondrial inhibitors: antimycin A, myxothiazol, oligomycin, rotenone, and CCCP, as indicated on the x-axes. Cells treated with vehicle only served as control (-). Absorption of 450 nm (A_{450nm}) was used as a positive measure for cell viability. For U87MG $n = 12$; for U118MG $n = 6$. * $P < 0.05$ versus control (-); ** $P < 0.01$ versus control (-); *** $P < 0.001$ versus control (-)



ErPC3-induced apoptosis in U87MG and U118MG cells. An exception was oligomycin (5 $\mu\text{g}/\text{ml}$), which is a specific inhibitor of the membranous proton channel (F_0) of mitochondrial F_0F_1 -ATP(synth)ase and consequently an inhibitor of oxidative phosphorylation and of the mitochondrial electron transport chain [27–32, 50]. This inhibitor of complex V was found to robustly and significantly reduce apoptotic levels induced by ErPC3 in U87MG and U118MG cells. Compared to treatment of U87MG cells with ErPC3 alone, co-treatment of U87MG cells with oligomycin caused significant reductions by 58% of apoptotic levels otherwise induced by ErPC3 (Fig. 5a). Also co-treatment of U118MG with this drug caused very significant reductions by 87% of apoptotic levels otherwise induced by ErPC3 (Fig. 5a).

For our study, we took into consideration that in mammalian cells oligomycin can be highly toxic [48]. In particular, depending on the cell line used, treatment with oligomycin can induce cell death after 12–13 h. Therefore, in all experiments oligomycin by itself was applied during 1 h before treatment and subsequently in combination with ErPC3 for 12 h in all experiments. Subsequent experiments were performed within the first 12 h after ErPC3 treatment

and we confirmed that oligomycin did not cause cell death during this time period (>95% trypan blue dye exclusion in control cultures). As mentioned above, inhibitors of respiration and uncouplers *per se* typically did not promote the induction of apoptosis in both cell lines even after 24 h of treatment (Fig. 5b). Thus our data suggest that in particular the F_0 subunit of the ATP(synth)ase is important for apoptosis induction by ErPC3.

At higher concentrations [IC_{50} 2.2 μM] oligomycin has also been reported to interfere with a non-mitochondrial target, the plasma membrane Na^+/K^+ ATPase [51]. We therefore tested the effect of ouabain, a specific Na^+/K^+ ATPase inhibitor [52], in both glioma cell lines. The reported IC_{50} for ouabain is 50 nM in vascular smooth muscle sarcolemma [53]. To ensure the effectivity of ouabain in our models, we selected the relatively high concentration of 100 nM of ouabain. This concentration of ouabain moderately affected viability of both the U118MG and U87MG cells (data not shown). Higher concentrations could be toxic in our models. Thus, ours and studies of others showed that ouabain is active at 100 nM. We found that, in contrast to oligomycin, ouabain did not suppress ErPC3-induced apoptosis (Fig. 5a). These results suggest that down-

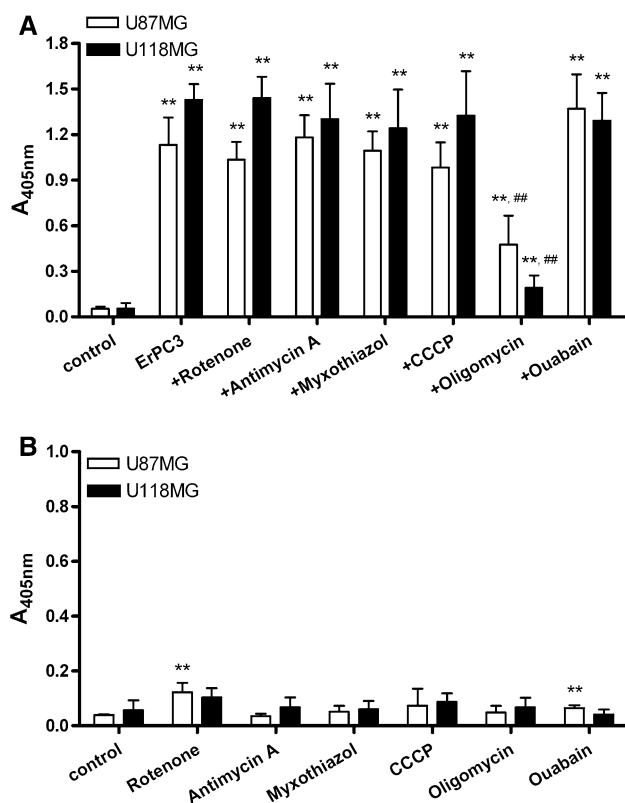


Fig. 5 Effects of inhibitors of respiration and uncouplers on ErPC3-induced apoptosis. **a** U87MG and U118MG cells were co-incubated with ErPC3 plus different respiratory chain inhibitors and uncouplers for 12 h and apoptotic levels were determined using the Cell Death kit. Cells treated with ErPC3 alone or not treated at all were used as controls. ErPC3 induced apoptosis in both cell lines, and oligomycin reduced this significantly in both cell lines. The other respiratory inhibitors and uncouplers did not counteract apoptosis induction by ErPC3. **b** In the U87MG cell line, rotenone and ouabain by themselves could induce apoptosis. Otherwise, the respiratory inhibitors and uncouplers *per se* typically did not promote the induction of apoptosis in the two cell lines. Absorbance of the 405 nm wavelength is indicative for the level of apoptosis. The results are the means \pm SD ($n = 6$). ** $P < 0.01$ versus the appropriate control; ### $P < 0.001$ versus the appropriate ErPC3

regulation of ErPC3-mediated apoptosis by oligomycin is not related to Na^+/K^+ ATPase suppression. These results further support our conclusion that the mitochondrial F_0F_1 -ATP(synth)ase is required for ErPC3-induced apoptosis.

It has been reported that cell death triggered by oligomycin can be delayed by culturing cells in high glucose-containing medium, which helps to maintain ATP levels via glycolysis [54]. We routinely cultured the U87MG and U118MG glioma cells in media containing 6 mmol/l glucose, which is close to reported physiological levels (5 mmol/l), suggesting that our conditions would not cause a delay of cell death potentially triggered by oligomycin. Nonetheless, we wanted to answer the question whether such a confounding temporal element is involved in the reduction of ErPC3-induced apoptosis by oligomycin. Therefore, we

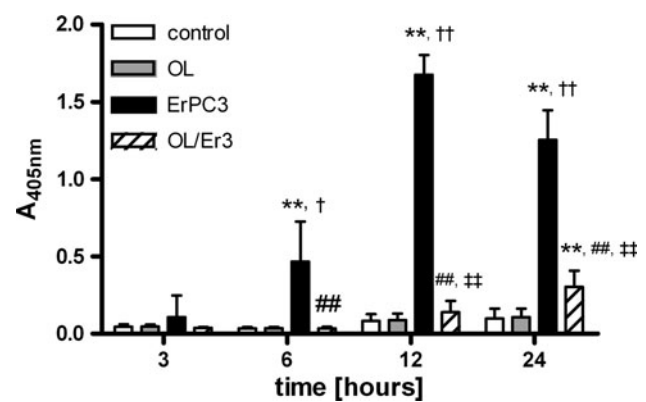


Fig. 6 Time course of ErPC3-induced apoptosis, with and without co-treatment with oligomycin. Apoptotic rates were followed by assessment of cytoplasmic histone-associated DNA fragments using the Cell Death Kit at various times after ErPC3 plus oligomycin co-treatment of U118MG cells. Open bars, vehicle-treated cells; grey bars, 5 $\mu\text{g}/\text{ml}$ oligomycin (OL); closed bars, 15 μM ErPC3; and hatched bars, oligomycin + ErPC3 (OL/Er3). Absorbance of the 405 nm wavelength is indicative for the level of apoptosis. The results shown are the means \pm SD. ($n = 6$) ** $P < 0.01$ versus control of the same time period; ## $P < 0.01$ versus ErPC3 of the same time period; † $P < 0.05$ versus ErPC3 of 3 h; †† $P < 0.01$ versus ErPC3 of 3 h; ††† $P < 0.01$ versus OL/Er3 of 3 h

assessed cytoplasmic histone-associated DNA fragments indicative of ongoing apoptosis in U118MG cells after 3, 6, 12, and 24 h of co-treatment with oligomycin (Fig. 6). Within the time frame of the experiment 5 $\mu\text{g}/\text{ml}$ oligomycin by itself did not affect apoptosis levels under all conditions tested (Fig. 6). Furthermore, at all time points after application (6, 12, and 24 h) oligomycin robustly reduced apoptosis levels induced by ErPC3 (by 90, 91, and 76%, respectively). This indicates that the results obtained were not adversely affected by time-dependent effects of oligomycin on modulation of apoptosis induction. Apart from the measured DNA fragmentation, effects of ErPC3 and oligomycin on apoptosis were also studied microscopically. Compared to untreated cells (Fig. 7a), ErPC3 by itself caused apoptotic morphological changes such as rounding and blebbing of our human glioblastoma cells (Fig. 7d), while oligomycin showed no effects by itself (Fig. 7b). Co-treatment of ErPC3 with oligomycin reduced the apoptotic effects of ErPC3 (Fig. 7e).

Effect of the F_1 -ATP(synth)ase inhibitor piceatannol on ErPC3-induced apoptosis

To assess in more detail the potentially specific role of the mitochondrial F_0F_1 -ATP(synth)ase in the execution of ErPC3-induced cell death, we tested piceatannol, another specific inhibitor of this mitochondrial enzyme. In contrast to oligomycin, which targets the membrane (lipid phase)-directed F_0 , piceatannol targets specifically the matrix

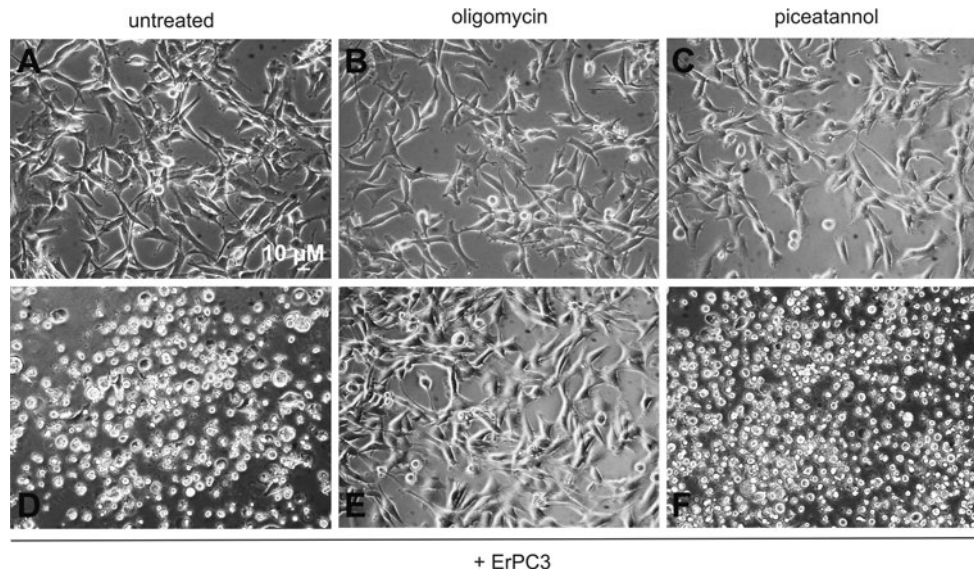


Fig. 7 U118MG cells were treated for 12 h with ErPC3, with or without oligomycin or with or without piceatannol, and apoptotic morphological changes such as rounding and blebbing were observed by phase-contrast microscopy. Code for the micrographs: **a** untreated; **b** oligomycin; **c** piceatannol; **d** ErPC3; **e** ErPC3 + oligomycin; **f** ErPC3 + piceatannol. Compared to untreated cells (**a**), ErPC3 by itself caused apoptotic morphological changes (**d**), while oligomycin

and piceatannol showed no effects by themselves (**b**, **c**). Co-treatment of ErPC3 with oligomycin reduced the apoptotic effects of ErPC3 (**e**), whereas co-treatment with piceatannol had no effect (**f**). Together, these results suggest that the activity of the F_0F_1 -ATP(synthase), specifically the F_0 component, is essential for ErPC3-induced apoptosis

(water phase)-exposed catalytic F_1 component of the enzyme [55] and therefore inhibits the ATP(synthase) by a different mechanism from that of oligomycin. We selected a concentration of 5 μM , which is slightly above the IC_{50} (4 μM) of piceatannol on ATPase activity of the purified F_1 complex [55]. We found that 5 μM of piceatannol was effective in decreasing ATP levels in both cell lines (see further below for details). With pilot studies we had found that concentrations of piceatannol lower than 20 μM (including the 5 μM concentration used for the present study) did not affect the viability of both cell lines (data not shown). Furthermore, in contrast to oligomycin, 5 μM piceatannol was not able to reduce apoptosis levels otherwise induced by ErPC3 (Figs. 7c, f, 8).

Effects of F_0F_1 -ATP(synthase) inhibition on dissipation of the $\Delta\Psi_m$ and caspase activation as components of the mitochondrial apoptosis pathway typically activated by ErPC3

As described previously [35], treatment of U87MG and U118MG cells with ErPC3 caused manifold increases in the number of cells displaying collapse of the $\Delta\Psi_m$ in comparison to untreated cells (Fig. 9). Compared to treatment of U87MG cells with ErPC3 alone, co-treatment of U87MG cells with oligomycin caused significant reductions by 80% of the number of cells undergoing collapse of $\Delta\Psi_m$ (Fig. 9b). Also co-treatment of U118MG with this

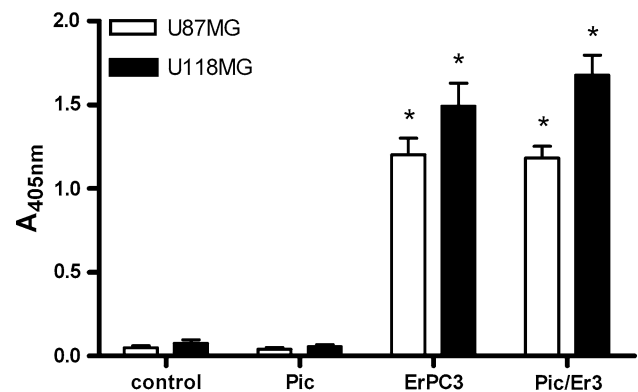


Fig. 8 Co-treatments with piceatannol showed no effect on ErPC3-mediated apoptosis as assayed with the Cell Death Kit. U87MG and U118MG cells were incubated for 12 h with piceatannol (Pic) or with ErPC3, or with ErPC3 in combination with Pic, or treated with vehicle (control). Absorbance of the 405 nm wavelength is indicative for the level of apoptosis. ($n = 4$) * $P < 0.05$ versus control

inhibitor caused significant reductions by 91% of the number of cells displaying collapse of $\Delta\Psi_m$, otherwise induced by ErPC3 (Fig. 9c). Oligomycin alone, at a concentration of 5 $\mu\text{g}/\text{ml}$, did not appear to have an effect on $\Delta\Psi_m$ in U87MG and U118MG cell lines (Fig. 9b, c). Piceatannol, however, does not modulate collapse of $\Delta\Psi_m$, neither alone nor in combination with ErPC3 (Fig. 9b, c). As another parameter of apoptosis induction, caspase-3 cleavage in response to ErPC3 was abrogated in the presence of oligomycin but not piceatannol (Fig. 10).

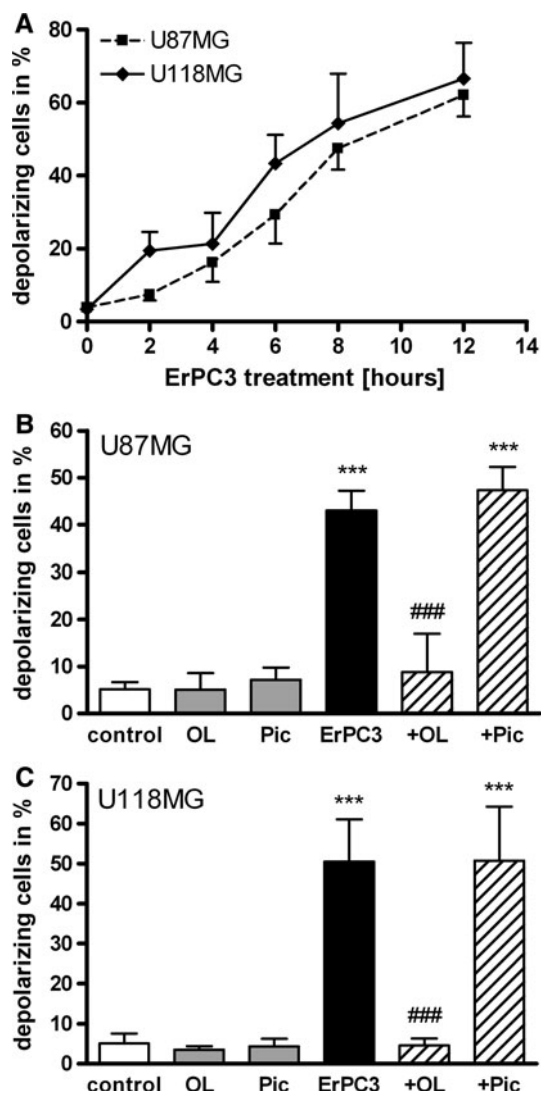


Fig. 9 Effects of ErPC3, oligomycin and piceatannol on the inner mitochondrial membrane potential ($\Delta\Psi_m$) in the glioblastoma cell lines U87MG and U118MG using the dye JC-1. In **a**, the influence on the $\Delta\Psi_m$ of ErPC3 treatment of U87MG and U118MG cells after various time periods is shown. For clarity, *error bars* are shown either above or below the lines. In **b**, **c** the effects of ErPC3, oligomycin and piceatannol on $\Delta\Psi_m$ are shown. U87MG (**b**) and U118MG (**c**) cells were treated with ErPC3 (45, 15 μ M, respectively) and/or oligomycin or piceatannol for 6 h (U87MG) and 8 h (U118MG). Each column represents the mean values of cell percentages derived from each time 9 independent experiments in the LR quadrant of the $\Delta\Psi_m$ analysis plot (depolarized mitochondria). In both cell lines co-treatment with oligomycin reduced the incidence of $\Delta\Psi_m$ collapse otherwise induced by ErPC3 itself, while piceatannol had no such effect. *** $P < 0.001$ versus control. ### $P < 0.001$ versus ErPC3

Effect of oligomycin and piceatannol on ErPC3-induced decrease of intracellular ATP levels

As oligomycin inhibits ATP production by oxidative phosphorylation, which may play a role in the apoptotic process, we investigated changes of intracellular ATP levels during ErPC3 treatment in the presence of

oligomycin or piceatannol. The luciferin/luciferase-based detection procedure used revealed on the one hand an increase of intracellular ATP levels by 28% in U87MG cells and, on the other hand, a reduction by 31% in U118MG cells upon treatment with oligomycin alone (Fig. 11a). Treatment with piceatannol alone caused decreases of intracellular ATP levels in U87MG cells as well as in U118MG cells, by 37% and 16%, respectively, compared to untreated control cells (Fig. 11b). In parallel to its pro-apoptotic effects, ErPC3 caused robust and significant reductions in ATP levels in both cell lines (Fig. 11). Co-incubation with oligomycin rescued from the ErPC3-induced decrease in intracellular ATP levels by a factor of 3 for U87MG cells and a factor of 6 for U118MG cells (Fig. 11a). Indeed, in U87MG cells, co-incubation of ErPC3 with oligomycin even restored ATP levels to control levels (Fig. 11a). In U87MG cells, co-incubation with piceatannol provided modest protection against ErPC3 induced ATP depletion (from a 6 fold reduction to a 4.5 fold reduction; Fig. 11b). In U118MG cells piceatannol was ineffective in this respect (Fig. 11b).

These results regarding restoration of ATP levels otherwise depleted by ErPC3 parallel the specific capacity of oligomycin to block ErPC3-induced apoptosis. Consistently, as piceatannol did not protect against apoptosis induced by ErPC3 in both cell lines, it did not effectively counteract ATP depletions due to ErPC3 treatment either.

Discussion

It is well known that loss of the $\Delta\Psi_m$ can lead to the release of apoptogenic factors, e.g. cytochrome *c*, apoptosis-inducing factor (AIF), and Smac/Diablo from the mitochondrial intermembrane space [56]. When the MPTP opens, the $\Delta\Psi_m$ collapses as a consequence of the dissipation of the proton gradient generated in the mitochondrial intermembrane space. In addition, it has been suggested that opening of the MPTP uncouples the respiratory chain and can lead to overproduction of superoxide anions [56]. In previous studies we have shown that ErPC3, as well as various other agents, requires TSPO to induce apoptosis. TSPO is a protein in the outer mitochondrial membrane that is associated closely with the MPTP, and appears to be involved in apoptosis [15, 16, 18, 57]. In particular, knockdown of the TSPO by genetic manipulation, as well as application of TSPO inhibitors, was found to prevent opening of the MPTP, generation of ROS at mitochondrial levels, including cardiolipin oxidation, and subsequent cell death, including activation of the mitochondrial apoptosis pathway [7, 8, 17, 18, 38, 45]. These studies also showed that ErPC3 can induce ROS generation via the TSPO. Our present study is consistent with this

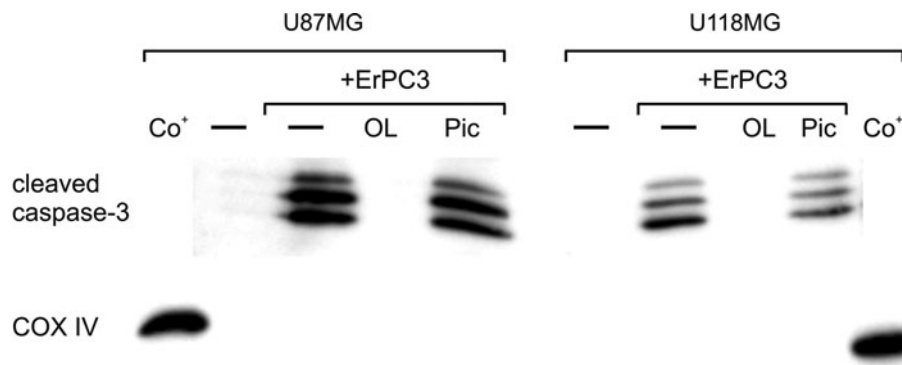


Fig. 10 Western blot protein analysis of effects of oligomycin (OL) and piceatannol (Pic) on ErPC3-induced caspase-3 processing. U87MG and U118MG cells were incubated for 12 h with ErPC3 alone, or in combination with either oligomycin or piceatannol, or treated with vehicle only (control). Co-treatment with oligomycin

blocked the effects of ErPC3 on caspase-3 processing, whereas piceatannol did not. COX IV was used as quality control for the purity of the cytoplasmic fractions. Co⁺ denotes glioma whole cell lysate used as a positive control for COX IV

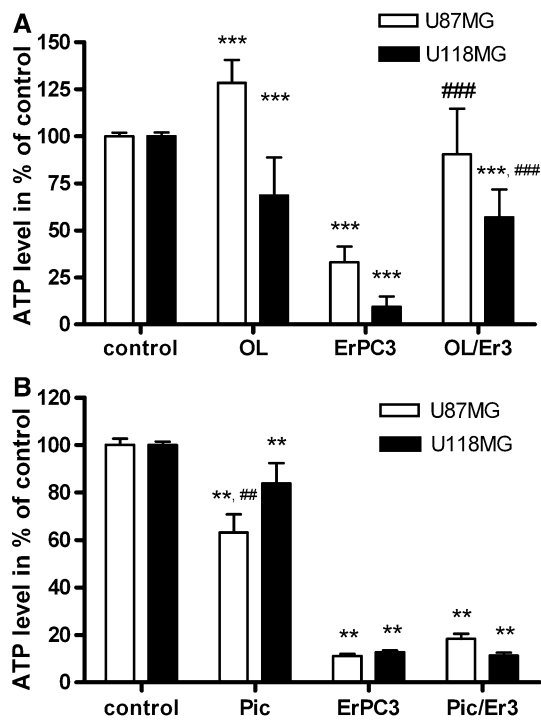


Fig. 11 Effects of F_0F_1 -ATP(synth)ase inhibitors on ErPC3-induced decrease in cellular ATP levels. U87MG and U118MG cells were treated for 12 h with oligomycin (a, $n = 9$), or piceatannol (b, $n = 6$), or ErPC3, or ErPC3 in combination with either oligomycin or piceatannol (OL/Er3, Pic/Er3), or left untreated (control). Cellular ATP levels were determined by bioluminescence measurement and the data were normalized as a percentage of the value of the corresponding untreated cells. *** $P < 0.001$ versus control; ** $P < 0.01$ versus control; ### $P < 0.001$ versus ErPC3; ## $P < 0.01$ versus ErPC3

observation, as application of the ROS scavenger BHA prevents ROS generation as well as $\Delta\Psi_m$ collapse and apoptosis otherwise induced by ErPC3. Thus, our findings strongly support the notion that ROS generation constitutes

a signal required for the execution of apoptosis in U87MG and U118MG glioblastoma cells upon activation of the TSPO by ErPC3.

We also assayed ROS generation other than indicated by cardiolipin oxidation after ErPC3 application [34]. The results were at best equivocal. In particular, iron chelators (deferoxamine, phenanthroline) could occasionally block apoptosis induced by ErPC3 in U373MG glioblastoma cells, but never in A172 glioblastoma cells. Sulfhydryl reagents (dithiothreitol, glutathione), superoxide dismutase, *N*-acetylcysteine, vitamin E (α -tocopherol) and its water-soluble derivative, Trolox, did not have any effect on ErPC3-induced cell death. Catalase occasionally inhibited ErPC3-mediated apoptosis in A172 cells, but never in U373MG cells [34 and unpublished data]. The present data combined with previous studies [8, 18, 44] suggest that ROS generation by ErPC3 may be a rather specific process taking place at the mitochondrial level, including cardiolipin oxidation.

One question emerging from these studies was by which mechanism TSPO activation by ErPC3 can lead to mitochondrial ROS generation. It has been implicated that TSPO by itself, via interactions with its ligands, may be able to generate electrons that subsequently may participate in the generation of ROS [8, 44]. Furthermore, TSPO is known to interact with O_2 [58], which may present a further contribution to ROS generation. Another suggestion was that the reported activation of the mitochondrial respiration chain by TSPO could also participate in ROS generation and subsequent induction of apoptosis [44–46, 59–61]. To study whether the mitochondrial respiratory chain could affect MPTP opening and the induction of the mitochondrial apoptosis cascade by ErPC3 in human glioblastoma cells, we applied the following mitochondrial respiration chain inhibitors and uncouplers: antimycin A (complex III inhibitor), myxothiazol (complex III and I

inhibitor), rotenone (complex I inhibitor), and CCCP (uncoupler of oxidative phosphorylation) [48]. These compounds did not affect ErPC3's typical induction of $\Delta\psi_m$ collapse i.e. opening of the MPTP, and subsequent activation of the mitochondrial apoptosis pathway. As the mitochondrial respiration chain inhibitors and uncouplers do not mimic the pro-apoptotic effect of ErPC3 in U87MG and U118MG cells in our study, and neither do inhibit the pro-apoptotic effects of ErPC3, it appears that the mitochondrial respiration chain does not play a major role in apoptosis induction by ErPC3. This further suggests that these components of the mitochondrial respiration chain do not participate in ROS generation by ErPC3, i.e. TSPO activation, in human glioblastoma cells. This is in accord with other studies that have shown that respiration is not required during apoptosis in yeast and in some mammalian cells [30, 62]. Interestingly, studies have suggested that some components of the mitochondrial respiration chain may malfunction in brain tumors [63], giving further credence to our postulation that ErPC3 does not require complex I–IV of the mitochondrial respiration chain to induce apoptosis.

In contrast to the non-effects of the inhibitors of the respiration chain discussed above, the application to the U118MG and U87MG cell lines of oligomycin, the classical inhibitor of mitochondrial F_0F_1 -ATP(synth)ase (complex V), prevented not only collapse of $\Delta\Psi_m$, but also the activation of caspase-3 and DNA fragmentation induced by ErPC3, as well as the morphological appearance of apoptotic cells. Thus, the mitochondrial F_0F_1 -ATP(synth)ase appears to be important for apoptosis induction by ErPC3 i.e. activated TSPO. Previously, Cleary et al. [64] demonstrated that the classical TSPO ligand PK11195 inhibits F_0F_1 -ATP(synth)ase activity in an oligomycin sensitivity conferring protein (OSCP)-dependent manner. To further investigate which one of the F_0 and F_1 subunits of ATP(synth)ase may be more important in this respect, we compared the effects of oligomycin, an F_0 inhibitor, with piceatannol, an F_1 inhibitor. While oligomycin consistently inhibited all apoptotic effects induced by ErPC3, piceatannol did not. Thus, our data show convincingly that piceatannol does not have an effect on ErPC3 function i.e. F_1 does not appear to affect ErPC3 function. This indicates that the F_0 component of the F_0F_1 -ATP(synth)ase is required for the pro-apoptotic effects of ErPC3 in these human glioblastoma cells, while the F_1 subunit is not. It is well known that piceatannol application does not only affect the F_1 of ATP(synth)ase, but also other systems at the concentration we used for the present study, and even more so at higher concentrations [65, 66]. Therefore we did not apply higher concentrations of piceatannol to avoid complications of the interpretations of the results. As piceatannol did not modulate pro-apoptotic

effects of ErPC3 at the concentration we used, our results also indicate that these other systems are also not involved in ErPC3-induced apoptosis. This latter observation is however not the subject of our study. Recently it was found that the catalytic F_1 subunit is downregulated in carcinomas from liver, kidney, colon, oesophagus, lung, breast and stomach [33, 67]. It would be interesting to determine whether the F_1 function actually is hampered in the two glioblastoma cell lines we used and, more generally, in glioblastoma multiforme. It has been suggested by others that in human glioblastoma both F_1 and F_0 expression may be affected [68, 69]. As our and other studies show that the F_0F_1 -ATP(synth)ase function may have tumorigenic implications, more studies on F_1 and F_0 expression and functions in human glioblastoma could be beneficial. Interestingly, it has been suggested in isolated mitochondria that the TSPO can affect the F_0F_1 -ATP(synth)ase function [70].

As our study indicates that the F_0 subunit of ATP(synth)ase rather than the F_1 subunit is needed for apoptosis induction by ErPC3, our data also suggest that the effect of ErPC3 on ROS generation is due to its effects on the F_0 proton pump. Two possibilities can be considered whereby ErPC3 treatment leads to generation of mitochondrial ROS via an effect on the F_0 proton pump. Mitochondrial ROS can be generated in the intermembrane space as well as the matrix [47]. At present it is not clear whether ErPC3 induces ROS generation in the matrix or intermembrane space. It, for example, has been suggested that a high pH in the matrix can cause ROS generation [71]. In such a scenario the ErPC3 may act by stimulating the F_0 to pump protons from the matrix to the intermembrane space, thereby alkalizing the matrix and allowing for generation of ROS. Alternatively, ErPC3 could cause protons to be pumped to the matrix, alkalizing the intermembrane space i.e. reverse the proton pump. In any case, oligomycin would inhibit the effects of ErPC3 by suppressing F_0 activity. Some of the protective effect of BHA may be due to its ability to make the inner mitochondrial membrane more permeable for protons [72], thus counteracting the effects ErPC3 on the F_0 proton pump. More studies are needed to exactly understand the mechanisms around F_0 in relation to ErPC3 regarding ROS generation at mitochondrial levels.

While our data suggest that the F_0 proton pump of ATP(synth)ase due to its potential effect on ROS generation is important for apoptosis induction by ErPC3, our data further indicate that ATP depletion due to ErPC3 application may also play a role. Since ErPC3 depletes ATP levels, and piceatannol actually does the same, this may explain in part the lack of a protective effects by piceatannol. In this context, the protective effects of oligomycin against ATP depletion otherwise caused by

ErPC3 are somewhat surprising. However, it is known that treatment with oligomycin can stimulate aerobic glycolysis due to blockade of the F_0 subunit of the F_0F_1 -ATP (synth)ase [33]. Thus, the resulting increase in ATP levels by this stimulation of aerobic glycolysis may compensate for ATP depletion otherwise caused by a reduced function of the F_0F_1 -ATP(synth)ase. Another question relates to the different effects of oligomycin by itself on ATP levels of U118MG cells and U87MG cells, respectively a decrease and an increase. We suggest that oligomycin can have different effects on ATP levels in different cell lines, depending on which metabolic pathway (glycolysis vs. oxidative phosphorylation) is mainly active. Furthermore, as inhibition of the mitochondrial respiration chain in the present study does not appear to affect apoptosis induction by ErPC3, the electron transport chain may also not be essential for ATP depletion by ErPC3. This is in agreement with studies reporting that ρ^0 cells lacking a functional electron transport chain still are able to generate ATP, as well as to undergo apoptosis [24–26].

Regarding processes other than ROS generation and ATP depletion leading to apoptosis, it was shown earlier that oligomycin can inhibit opening of the MPTP [73]. Furthermore, oligomycin can also block Bax dimerization [30, 74]. Indeed, intact F_0F_1 -ATP(synth)ase is necessary for optimal function of Bax [30, 74]. Bax is considered to be required for cytochrome *c* release, which contributes to the initiation of the mitochondrial apoptosis pathway [75, 76]. Furthermore, it was found previously that Bax is necessary for ErPC3-induced apoptosis [77]. Bax is also considered to be involved in TSPO-induced apoptosis [44]. In addition, using the Bax-negative prostate cancer cell line DU145, we were able to demonstrate drastically reduced apoptosis levels after ErPC3 treatment (unpublished results). Preliminary data by us further suggest that oligomycin may also reduce cytochrome *c* release otherwise induced in U118MG cells exposed to ErPC3. Thus, Bax and its related functions may also be important for apoptosis induction by ErPC3.

As a final point, our and other studies indicate that ROS generation at mitochondrial levels can lead to MPTP opening, as mentioned above [17, 60]. On the other hand, various studies have indicated that opening of the MPTP can lead to ROS generation as well [78]. This suggests that the effective apoptosis induction by ErPC3 via TSPO activation and, consequently ROS generation that opens the MPTP, may be due to a powerful reciprocal cycle of ROS generation leading to MPTP opening and vice versa. Such ROS-induced ROS release has also been described in other systems [79, 80].

In summary, this study provides evidence that ROS generation due to TSPO activation by ErPC3 may be involved in opening of the MPTP leading to activation of

the mitochondrial apoptosis pathway. One component of the mitochondrial respiratory chain, F_0F_1 -ATP(synth)ase may take part in this effect of ErPC3. In particular, the F_0 component of the F_0F_1 -ATP(synth)ase appears to be important in this respect, suggesting that proton exchange at mitochondrial levels takes part in the ROS generating and apoptotic effects of ErPC3 and TSPO. Furthermore, ATP depletion due to ErPC3 treatment may play a role in the induction of apoptosis. The present study, applying pharmacological means, may give directions to future research, for example knockdown studies to reduce expression of proteins involved in mitochondrial respiration in relation to ROS generation and apoptosis induction by ErPC3 and TSPO.

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