SYNERGISTIC INHIBITION OF MITOCHONDRIAL RESPIRATION BY AN ANTICANCER AGENT ERUCYLPHOSPHOHOMOCHOLINE AND CYCLOSPORIN A
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Alkylphosphocholines are a new class of anticancer agents. The mechanisms by which these drugs display their antitumor activities are not known. In this work, we show that erucylphosphohomocholine, a new antineoplastic compound, significantly decreased ATP synthesis in isolated rat liver mitochondria at a concentration of 50 µM or higher via permeabilization of the inner membrane. At concentration of 25 µM, it induced a moderate swelling of mitochondria, a slight decrease of the inner membrane potential and an increase in state 4 respiration without an essential influence on state 3 respiration or the outer membrane permeability to cytochrome c. We found that cyclosporin A did not prevent mitochondrial swelling induced by 25-100 µM erucylphosphohomocholine. Moreover, cyclosporin A induced a fast drop of the inner membrane potential in the presence of 25-50 µM erucylphosphohomocholine that seems to be due to a strong synergistic inhibition of the respiratory activity. The ratio of uncoupled to state 3 respiration rates increased from 1.3±0.1 with 25 µM erucylphosphohomocholine and from 1.5±0.1 with 1 µM cyclosporin A to 4.5±0.3 in the presence of both drugs. On the other hand, oligomycin or cyclosporin A protected certain cancer cell lines against erucylphosphohomocholine-induced apoptosis. This protection might be related to a prevention of cellular ATP hydrolysis by permeabilized mitochondria and to the inhibition of the classical permeability transition pore, respectively. Our findings provide new insight into the mechanisms by which these unusual alterations of mitochondria might be involved in anticancer activity of alkylphosphocholines.

EXPERIMENTAL PROCEDURES

Drugs and assay material. Erucylphosphohomocholine (ErPC3, etc.)
erufosine™) was generously provided by Genzyme Pharmaceuticals (Liestal, Switzerland). Cyclosporin A, bongkrekate, atractyloside, oligomycin, antimycin A, myxothiazol, rotenone and other chemicals were obtained from Sigma-Aldrich. The Cell Death Detection ELISAPLUS (Cell Death Kit) was from Roche Applied Science.

Isolation of mitochondria. Liver mitochondria from male Sprague Dawley rats (4-6 month, starved overnight) were isolated according to the method of Schneider and Hogeboom [13] with modifications. Cooled liver was homogenized in the medium containing 210 mM mannitol, 70 mM sucrose, 2.5 mM MgCl₂, 1 mM EGTA-KOH, 0.3 mg/ml bovine serum albumin BSA (free fatty acids), 10 mM Hepes-KOH, pH 7.2, at 2-4 °C. MgCl₂ was included in the homogenization medium to prevent cytochrome c adsorption on mitochondria [14], thus decreasing the respiration of the fraction of damaged mitochondria. After two washes with the medium containing 210 mM mannitol, 70 mM sucrose, 50 µM EGTA-KOH, 0.3 mg/ml BSA, 10 mM Hepes-KOH, pH 7.2, mitochondria were finally resuspended in 1 ml of the same medium lacking BSA. Protein content was evaluated by a fast volumetric test described earlier [15] taking the average concentration of protein in the final mitochondrial pellet for this protocol equal to 200 mg/ml, determined by the biuret method with BSA as standard.

Measurement of respiration. The oxygen consumption was measured using a Clark-type electrode as described earlier [14]. Incubation medium was composed of 100 mM sucrose, 75 mM KCl, 10 mM potassium phosphate, 10 mM Hepes-KOH, pH 7.2 (SKPH medium). Mitochondria, 0.5 mg protein/ml, 2.5 µM rotenone, 6.5 mM succinate-KOH, 500 µM ADP, 1 µM FCCP, 0.5 µM antimycin A plus 0.5 µM myxothiazol, 7 mM potassium ascorbate, 20 µM cytochrome c, and 0.5 mM N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) were added sequentially to obtain data presented in Table 1. For other additions see also figure legends. ErPC3, dissolved in 10% ethanol, or/and CsA, dissolved in absolute ethanol, were added to the medium to final concentrations of 25-50 µM and 1 µM, respectively, wherever indicated, with a final concentration of ethanol not higher than 0.1%.

ATP synthesis monitoring. Real-time ATP synthesis by mitochondria was monitored through accumulating NADPH by registering its fluorescence (365/450 nm) from one corner of the cuvette as described earlier [16]. The incubation medium was composed of 100 mM sucrose, 50 mM KCl, 10 mM potassium phosphate, 10 mM succinate, 40 mM D-glucose, 10 mM Hepes-KOH, pH 7.4 (SKPS medium), without or with ErPC3 at 50 or 100 µM. The components of an ATP-registering system (AS) (5 U hexokinase, 2.5 U glucose-6-phosphate dehydrogenase, 0.5 mM NADP⁺, 0.35 mM AMP, 5 µM ADP and 2 mM MgCl₂) were added 1 min after mitochondria, 0.5 mg protein/ml, and 2.5 µM rotenone addition. As a control, 0.5 mM ATP was added to be sure that the AS activity was not a limiting factor.

Measurement of the inner membrane potential. The inner membrane potential of mitochondria was monitored fluorimetrically using safranin O as previously described [17]. Mitochondria, 0.5 mg protein/ml, were added to SKPH medium supplemented with 5 mM succinate-KOH and 10 µM safranin O.

Mitochondrial swelling. Mitochondrial swelling was monitored simultaneously with the inner membrane potential using a modified cuvette holder for the Amino-Bowman-2 luminescence spectrometer to allow the measurement of 90°-dispersion of light emitted by the red light emitting diode attached as described earlier [18].

All mitochondrial samples were constantly stirred with a magnetic stirrer and maintained at constant temperature of 30 °C.

Cell culture. The human cell lines U87MG and U118MG were generously provided by Dr. G. Bernhardt, University of Regensburg, Germany. U87MG cells were maintained in EMEM, U118MG cells in DMEM, and Jurkat cells in RPMI 1640. The following components were also added to the culture media: heat-inactivated fetal bovine serum (FBS) at 10% (U87MG, Jurkat) or 5% (U118MG), 100 U/ml penicillin, and 100 µg/ml streptomycin. DMEM and EMEM media were supplemented with 1% of sodium pyruvate and 1% of non-essential amino acids. All cells were incubated at 37 °C in an atmosphere of 5% CO₂ and 90% relative humidity.

Quantitative analysis of apoptosis. Cytoplasmic histone-associated DNA fragments indicative of ongoing apoptosis were measured using the Cell
Death Kit following the manufacturer’s instructions as described previously [8]. Briefly, for apoptosis induction, 1 x 10^6 human glioma or Jurkat cells were seeded in 96-well microtiter plates and treated after 24 hrs with various drugs or with vehicle as control for 12 hours. To analyze the effects of the inhibitors on ErPC3-induced apoptosis the cells were pre-incubated for 1 h with CsA or oligomycin before treatment with ErPC3 at 45 µM (U87MG), 15 µM (U118MG), or 25 µM (Jurkat). Under these conditions the cell lines showed clear signs of apoptosis using the Cell Death Kit and caspase-3 processing as a read-out (data not shown). Absorption indicative of apoptosis was measured using a SUNRISE microplate reader (TECAN).

RESULTS AND DISCUSSION

Alkylphosphocholines induce apoptosis in a variety of glioblastoma cell lines [8, 19, 20], which are known to be resistant to various treatments. We have examined the effects of ErPC3, one of the most effective anticancer alkylphosphocholines, on the cells and found that ErPC3 induced tumor cell death at concentrations of 15 µM (U118MG), 45 µM (U87MG) and 25 µM (Jurkat cells) (Fig. 1). Oligomycin, a specific inhibitor of mitochondrial H^+-ATPase, essentially increased the viability of ErPC3-treated cells. This might be explained by a prevention of mitochondrial hydrolysis of glycolytically produced ATP, assuming that ErPC3 caused an uncoupling of the oxidative phosphorylation system.

The protective effect of CsA, which is known to block the opening of the classical permeability transition pore [21], was significantly less than that of oligomycin (Fig. 1). Theoretically, it is possible that ErPC3 causes uncoupling by two ways: by a direct increase of the inner membrane permeability to ions and by a further activation of the permeability transition pore opening. Only the latter should be inhibited by CsA, while oligomycin is able to inhibit ATP hydrolysis in mitochondria activated by both mechanisms. The relatively high concentrations of oligomycin and CsA needed to achieve maximum protection might be associated with the presence of serum albumin in the incubation media. We suggest that the difference in the protective effects of oligomycin and CsA against ErPC3-induced cell death for different tumor cell lines (Fig. 1) may relate to a specificity of the energy metabolism in each of these cell lines.

The mechanisms of antineoplastic activity of alkylphosphocholines are not known, although indirect evidence suggest that mitochondria might be involved in this process [1, 7, 9]. However, the direct effects of this class of anticancer drugs on isolated mitochondria have been not studied so far. Therefore, we have analyzed the influence of ErPC3 on respiration, inner membrane potential and swelling of isolated rat liver mitochondria. According to the data presented in Fig. 2 (panel A), ErPC3 at a concentration of 50 µM significantly decreased ATP synthesis in mitochondria. The inhibition at 100 µM ErPC3 increased progressively with time, leading to a complete collapse of this process presumably due to a direct permeabilization of mitochondrial membranes. Under these conditions, the fast response to exogenous ATP (Fig. 2, panel A) shows that ErPC3 indeed affected ATP synthesis, but not the activity of ATP-registering enzyme system.

ErPC3 at a concentration of 25 µM slightly increased state 4 respiration and decreased respiratory control ratio (RCR), without essential inhibition of state 3 respiration (Table 1, Fig. 1 and Fig. 2B, e). A higher increase in state 4 respiration, accelerating in time, and a marked inhibition of state 3 respiration were observed at 50 µM ErPC3 (Fig. 2B, f) resulting in a more significant decrease of RCR.

The inhibition of state 4 and especially state 3 respiration, resulting in a decrease of RCR, was observed in the presence of both 1 µM CsA and 25 µM ErPC3 (Table 1). A stronger synergistic inhibition of the ADP-dependent respiration was detected at 1 µM CsA and 50 µM ErPC3 (Fig. 2, f). We found that FCCP-uncoupled respiration was significantly less sensitive to this combined action of CsA and ErPC3 (State 3u in Table 1, Fig. 2B, e and f). The partially inhibited FCCP-uncoupled respiration at 50 µM ErPC3 could be slightly re-activated by exogenous 20 µM cytochrome c (Fig. 2B, f), indicating that at this and higher concentrations, ErPC3 might cause rupture or permeabilization of the outer membrane inducing cytochrome c release. The synergistic inhibition of state 3 respiration by CsA and ErPC3 was also accompanied by a significant increase in the ratio of FCCP-uncoupled to state 3 respiration rates (Table 1, 3u/3 ratio).
We have observed that ErPC3 at 25 µM did not permeabilize the outer mitochondrial membrane: the respiration rate in the presence of exogenous cytochrome c and ascorbate as substrate of oxidation was not altered by ErPC3 in the absence or presence of CsA (Table 1, Asc+CytC). In addition, maximum cytochrome oxidase activity of mitochondria was not changed under these conditions (Table 1, Asc+CytC+TMPD).

CsA at 1 µM concentration caused a complete protection of mitochondria against permeabilization by 75 µM Ca²⁺ (Fig. 3A, a (potential) and b (light dispersion)). On the contrary, in the absence of CsA we have observed a fast decrease of the inner membrane potential (Fig. 3B, a) and subsequent opening of the classical Ca²⁺-potential-dependent permeability transition pore, accompanied with a high amplitude swelling of mitochondria (Fig. 3B, b). In the presence of CsA, the addition of 25 µM ErPC3 after completion of calcium accumulation or to the mitochondria without calcium addition caused a decrease of the inner membrane potential that developed with a lag-period (Fig. 3A, a and Fig. 3C, a, respectively). Before that, a decline in light dispersion, starting just after ErPC3 addition, was observed (Fig. 3A, a and Fig. 3C, a, respectively, for the presence and absence of calcium).

In the absence of CsA, 25 µM ErPC3 caused only a slight reduction of the inner membrane potential (Fig. 3D, a), although a decline in light dispersion (Fig. 3D, b) was similar to that caused by ErPC3 in the presence of CsA (Fig 3C, b). When CsA was added simultaneously with 25 µM ErPC3 the curves were almost identical to those presented in Fig. 3C (data not shown). On the other hand, the addition of CsA after a 2 min pre-incubation of mitochondria with 25 µM or 50 µM ErPC3 caused a very fast drop of the inner membrane potential (Fig. 3D, a and c, respectively) without an additional marked influence on light dispersion (Fig. 3D, b and d, respectively).

The moderate increase in state 4 respiration observed at 25 µM ErPC3 (Table 1, Fig. 2B, e) and 50 µM ErPC3 (Fig. 2B, f) was in agreement with the slight decrease of the inner membrane potential (Fig. 3D, a and c, respectively). We suggest that the inner membrane potential drop, caused by combined action of ErPC3 and CsA was the result of both a slight permeabilization of the inner membrane to ions and a relatively strong synergistic inhibition of mitochondrial respiration. According to the data of Azzone et al. [22], at a certain concentration of malonate, state 3 respiration of rat liver mitochondria was inhibited by approximately 90%, whereas the FCCP-dependent respiration (20 pmol FCCP/mg protein) was decreased by only 25%. In our experiments we have observed a similar effect: the addition of CsA in the presence of 25 µM ErPC3 caused inhibition of the respiration rate by 81% in state 3, but only by 14% in state 3u with FCCP (Fig. 2B, e). The inhibition was even stronger, by 93% and 41%, respectively, in the presence of 50 µM ErPC3 (Fig. 2B, e).

ErPC3 at a concentration of 100 µM caused the inner membrane potential drop even in the absence of CsA. The potential drop started with a lag-period of approximately 2 min (Fig. 3E, a) during which a relatively fast mitochondrial swelling of high amplitude was observed (Fig. 3E, b). A very striking additional phase of light dispersion decrease developed after an almost complete drop of the inner membrane potential. This suggests that ErPC3 acts on the mitochondrial membrane by more than one mechanism. The presence of CsA significantly shortened the lag-period of the inner membrane potential drop (Fig. 3E, c), but did not affect the initial phase of mitochondrial swelling. We have observed only slight retardation of the second phase of light dispersion decrease (Fig. 3E, d) observed in the presence of 100 µM ErPC3.

The effects of CsA on mitochondria are usually associated with the prevention of permeability transition pore opening, frequently attributed to a complex formed by ANT and the voltage-dependent anion channel. However, the exact nature of this pore is currently under strong revision [23]. According to an alternative concept of He and Lemasters [24], the permeability transition pore might be formed by aggregation of damaged membrane proteins including ANT as the most abundant protein of the inner mitochondrial membrane. In this work, we discovered a new effect of CsA, a fast drop of the inner membrane potential induced by the addition of CsA to mitochondria pre-incubated with the new anticancer compound ErPC3. To our knowledge this effect has been not described in the literature previously. The strong inhibition of the respiration rate in metabolic state 3 compared to state 3u by both CsA and ErPC3 might indicate a possible involvement of the ANT. This prompted us to evaluate the influence...
of BK and ATR on the reported synergistic effect of CsA and ErPC3. BK and ATR are specific inhibitors of the ANT known as an antagonist and agonist of the permeability transition pore, respectively. As shown in Fig. 3F, 10 µM BK and 10 µM ATR completely inhibited the oxidation of endogenous NAD(P)H in response to the addition of ADP. However, they did not prevent, but even accelerated the drop of the inner membrane potential induced by CsA and ErPC3 (Fig. 3G, a, and Fig. 3H, a, respectively for BK and ATR) without an essential influence on the light dispersion changes (Fig. 3G, b and Fig. 3H, b, respectively for BK and ATR). The oligomycin-sensitive H⁺-ATPase activity of mitochondria, measured in the presence of 50 µM ErPC3 and 0.1 mM 2,4-dinitrophenol, was also not affected by CsA (data not shown). Thus, these data show that the synergistic inhibition of mitochondrial respiration by CsA and ErPC3 did not result from the inhibition of the ANT or H⁺-ATPase. Nevertheless, according to our other findings, a knock-down of the ANT isoform 2 reduced the apoptotic effects of ErPC3 [25], indicating that the ANT might be involved in some ways in the mechanism of cell death induction by this anticancer compound. On the other hand, experiments with mice lacking the ANT isoforms 1 and 2 revealed that the ANT is not essential for permeability transition pore formation [26].

Our results demonstrate that the effect of ErPC3 on mitochondria depends on its concentration. At 25-50 µM, ErPC3 moderately increased the permeability of the inner membrane to ions, thereby causing a modest swelling of mitochondria and a mild uncoupling of the oxidative phosphorylation system. This led to a decrease of the inner membrane potential and to an increase of state 4 respiration. Most interesting is that the inhibitory action of CsA on mitochondrial respiration was strongly increased at these concentrations of ErPC3. At a concentration of 100 µM, ErPC3 itself inhibited almost completely ATP synthesis due to the induction of a progressive drop of the inner membrane potential. This might result from a possible lysophospholipid-like or fatty acid-like action [27,28] of ErPC3 leading to high amplitude swelling of mitochondria.

In summary, data in this paper present evidence for synergistic inhibition of mitochondrial state 3 respiration by CsA and ErPC3. This new finding is important for the understanding of the mechanism(s) of mitochondrial membrane permeabilization by alkylphosphocholines and of the known toxicity of CsA. This is also important in the light of evidence showing that the pro-apoptotic activity of ErPC3 might be blocked by ligands of the mitochondrial peripheral-type benzodiazepine receptor [11] or might be decreased by antioxidants [10]. The moderate permeabilization of the inner mitochondrial membrane for long time by relatively low concentrations of ErPC3 might lead to the opening of the classical potential-dependent permeability transition pore inducing cell death. Presumably, both CsA-sensitive and insensitive permeabilization are involved in cell killing by ErPC3. Further studies of the mechanism of mitochondrial membrane permeabilization by ErPC3 and other alkylphosphocholines are needed to better understand the pro-apoptotic action of this new class of anticancer agents.

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FOOTNOTES

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1. The abbreviations used are: ErPC3, erucylphosphohomocholine; CsA, Cyclosporin A; ANT, adenine nucleotide transporter; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine; FCCP, carbonyl-cyanide-p-trifluoromethoxy phenylhydrazone; RCR, respiratory control ratio.

FIGURE LEGENDS

**Fig. 1.** The induction of apoptosis in U118MG, U87MG and Jurkat cells by ErPC3 or by ErPC3 in combination with CsA (1 and 10 µM) or with oligomycin (oligo., 1 and 5 µM). Apoptosis was
quantitatively measured using the Cell Death Kit and expressed as 100 % for ErPC3 application at concentrations of 45 µM (U87MG), 15 µM (U118MG), and 25 µM (Jurkat). Results are expressed as mean values ± SEM; n=8 for the control, n=4 for treated cells.

**Fig. 2.** The influence of ErPC3 on ATP synthesis and respiration of isolated rat liver mitochondria. **Panel A** (ATP synthesis): mitochondria (Mc), 0.5 mg protein/ml, and 2.5 µM rotenone were added to SKPS medium without ErPC3 (-E) (a), with 50 µM (b) or 100 µM (c) ErPC3 (+E). The ATP-registering system (AS) was added 1 min after mitochondria addition. NADPH level was monitored fluorimetrically (365/450 nm). **Panel B** (respiration): mitochondria (Mc), 0.5 mg protein/ml, and 2.5 µM rotenone were added to SKPH medium supplemented with 6.5 mM succinate-KOH without ErPC3 (d), with 25 µM ErPC3 (E25; e) or 50 µM ErPC3 (E50; f). ADP - 500 µM ADP, CsA - 1 µM CsA; FCCP - 1 µM FCCP, Cyt - 20 µM cytochrome c. RCR – respiratory control ratio. Numbers at curves – ATP synthesis rates (%) (**Panel A**) or respiration rates (nanoatoms O²·mg protein⁻¹·min⁻¹) (**Panel B**). Results are expressed as mean values ± SEM; n=5-6.

**Fig. 3.** The influence of ErPC3 on the inner membrane potential and swelling of mitochondria. Mitochondria (Mc), 0.5 mg protein/ml, and 2.5 µM rotenone were added to SKPH medium supplemented with 5 mM succinate-KOH and 10 µM safranin O. The inner membrane potential generation was accompanied with a decrease of safranin O fluorescence (520/580 nm, curves a and c) and mitochondrial swelling was reflected in a decrease of light dispersion (640 nm, curves b and d). **Panel A, B:** Permeabilization of mitochondria by 75 µM Ca²⁺ with or without CsA. **Panel C-E:** Permeabilization of mitochondria by different ErPC3 concentrations with or without CsA. **Panel F:** 10 µM bongkrekate (BK) or 10 µM atractyloside (ATR) completely inhibited the change in the redox state of endogenous pyridine nucleotides of mitochondria induced by the addition of 100 µM ADP and monitored by NAD(P)H fluorescence (365/450 nm) in SKPH medium; Suc - 5 mM succinate-KOH. **Panel G, H:** The influence of BK (G) and ATR (H) on the combined action of CsA and ErPC3. CsA - 1 µM cyclosporin A; E25 – 25 µM ErPC3; E50 – 50 µM ErPC3; E100 – 100 µM ErPC3; Ca - 75 µM CaCl₂. Results are expressed as mean values ± SEM; n=5-8.
Table 1. The influence of ErPC3 or and cyclosporin A (CsA) on the respiration rate of rat liver mitochondria in various metabolic states (nanoatoms O₂·mg protein⁻¹·min⁻¹. Mean±SEM, n=4-6)

| Metabolic state or combined parameter | Control | 1 µM CsA | 25 µM ErPC3 | 25 µM ErPC3 plus 1 µM CsA |
|---------------------------------------|---------|----------|-------------|-------------------------|
| State 4                               | 30±1    | 24±1     | 36±2        | 13±1                    |
| State 3                               | 188±7   | 159±4    | 179±6       | 40±4                    |
| State 3u                              | 234±10  | 237±12   | 227±11      | 175±10                  |
| RCR                                   | 6.2±0.2 | 6.6±0.2  | 5.1±0.2     | 3.1±0.3                 |
| States 3u/3 ratio                     | 1.2±0.1 | 1.5±0.1  | 1.3±0.1     | 4.5±0.3                 |
| Asc+CytC                              | 66±3    | 56±3     | 64±2        | 59±4                    |
| Asc+CytC+TMPD                         | 427±15  | 431±11   | 437±11      | 439±7                   |

RCR – respiratory control ratio; a - <0.01 with respect to the control; b - <0.01 with respect to ErPC3; d - <0.05 with respect to the control. Respiration rates for Asc+CytC or Asc+CytC+TMPD were determined after previous complete inhibition of succinate-dependent respiration by antimycin A and myxothiazol (see Materials and Methods section for details).
Lemeshko and Kugler, Figure 1.
Lemeshko and Kugler, Figure 2.
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