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Mitochondrial permeability transition induced by the anticancer drug etoposide

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Abstract

Etoposide (VP-16) is widely used for the treatment of several forms of cancer. The cytotoxicity of VP-16 has been assigned to the induction of apoptotic cell death but the signaling pathway for VP-16-induced apoptosis is essentially unknown. There is some evidence that this process depends on events associated with the loss of mitochondrial membrane potential ($\Delta\Psi$) and/or release of apoptogenic factors, putatively as a consequence of mitochondrial permeability transition (MPT) induction. This work evaluates the interference of VP-16 with MPT in vitro, which is characterized by the Ca^{2+}-dependent depolarization of $\Delta\Psi$, the release of matrix Ca^{2+} and by extensive swelling of mitochondria. $\Delta\Psi$ depolarization and Ca^{2+} release were measured with ion-selective electrodes, and mitochondrial swelling was monitored spectrophotometrically. Incubation of rat liver mitochondria with VP-16 results in a concentration-dependent induction of MPT, evidenced by an increased sensitivity to Ca^{2+}-induced swelling, depolarization of $\Delta\Psi$, Ca^{2+} release by mitochondria and stimulation of state 4 oxygen consumption. All of these effects are prevented by preincubating the mitochondria with cyclosporine A, a potent and specific inhibitor of the MPT. Therefore, VP-16 increases the sensitivity of isolated mitochondria to the Ca^{2+}-dependent induction of the MPT. Together, these data provide a possible mechanistic explanation for the previously reported effects of VP-16 on apoptosis induction.

Keywords: Etoposide; Anticancer; Apoptosis; Liver mitochondria; Mitochondrial permeability transition

1. Introduction

Etoposide (VP-16) is an antitumor drug used as a single agent or in association with other chemotherapeutic drugs against a variety of hematopoietic and solid tumors (O'Dwyer et al., 1985). The inhibition of topoisomerase II, induction of protein-cross-linked DNA strand breaks, sister chromatid exchanges (Chatterjee et al., 1990) and formation of free radicals (Kalyanaraman et al., 1989; Kagan et al., 1994) have been implicated in its cytotoxicity, but the exact mechanism of VP-16-induced cell death has not yet been clearly defined.

The cytotoxicity of VP-16 has been suggested to be mediated by apoptosis (Lindsay and Wallace, 1999; Ferrano et al., 2000). Several studies with different cell types attempting to identify apoptotic mechanisms of VP-16 indicate early events occurring in mitochondria, for example, release of mitochondrial cytochrome c (Chen et al., 2000), formation of ultracondensed mitochondria, development of outer membrane discontinuities, reduction of mitochondrial membrane potential ($\Delta\Psi$) (Zhuang et al., 1998) and release of intermembrane apoptogenic proteins, triggering a caspase-dependent cascade (Meng et al., 2000). The role of mitochondria is putatively related with the release of cytochrome c and/or other apoptogenic factors that initiate or amplify the apoptotic cascade (Chen et al., 2000; Perkins et al., 2000). However, the mechanism responsible for these VP-16-induced mitochondrial events is essentially unknown.

The mitochondrial permeability transition (MPT), characterized by mitochondrial swelling, depolarization of $\Delta\Psi$, Ca^{2+} release and stimulation of state 4 oxygen consumption, has been implicated in the toxicity

Abbreviations: $\Delta\Psi$, mitochondrial membrane potential; BSA, bovine serum albumin; CyA, cyclosporine A; EGTA, ethylene glycol-bis(β-aminoethyl ether)*N,N,N′,N′*-tetraacetic acid; MPT, mitochondrial permeability transition; TPP^+, tetraphenylphosphonium; VP-16, etoposide.

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mechanisms of tissue injury and cell death caused by a number of compounds, the majority of which are oxidants (Bernardi and Petronilli, 1996; Bernardi et al., 1998). The induction of the MPT, causing the release of cytochrome c, depression of $\Delta\Psi$, extensive swelling with subsequent release and activation of intermembrane apoptogenic factors, is considered a critical and essential step for triggering efficient apoptosis in some cell types (Hirsch et al., 1998; Perkins et al., 2000). Additionally, the oxidation state of cysteine residues in the immediate vicinity of the voltage-sensing structures by reactive oxygen species and other oxidants has been proposed as a critical factor for MPT induction (Petronilli et al., 1994). As VP-16 oxidation leads to intermediate formation of hydroxyl and phenoxyl radicals (Kalyanaraman et al., 1989; Kagan et al., 1994), increases the peroxide formation and glutathione depletion in cells (Siitonen et al., 1999), it is tempting to propose that VP-16 may induce MPT and subsequent release of the mitochondrial apoptogenic factors involved in the signaling pathway of apoptosis. This study, reporting the effects of VP-16 on Ca^{2+}-dependent MPT in liver mitochondria, warrants significant support to this hypothesis.

2. Materials and methods

2.1. Materials

Etoposide (VP-16), HEPES, cyclosporine (CyA) and oligomycin were purchased from Sigma Chemical Co. (St Louis, MO, USA). Ethylene glycol-bis(β-aminoethyl ether)*N,N,N′,N′*-tetraacetic acid (EGTA), tetraphenylphosphonium (TPP^+) and sucrose were from Merck (Darmstadt, Germany). All the other chemicals were of research grade.

2.2. Rat liver mitochondria

Mitochondria were isolated from the liver of fasted male Wistar rats (200–300 g) by differential centrifugation as described previously (Custódio et al., 1998a). The liver tissue was quickly homogenized in 250 mM sucrose, 10 mM HEPES (pH 7.4), 1 mM EGTA and 0.1% bovine serum albumin. The homogenate was centrifuged for 10 min at 1200 ***g*** and 4°C. The mitochondria fraction was recovered from the supernatant centrifuged at 10 000 ***g*** for 10 min and the mitochondrial pellet was washed twice in the homogenization medium adjusted to pH 7.2 in the absence of EGTA and BSA.

2.3. Standard incubation procedure

Mitochondria were suspended in a standard reaction medium containing 200 mM sucrose, 10 mM Tris–Mops (pH 7.4), 1 mM KH_2PO_4 and 10 μM EGTA, supplemented with 2 μM rotenone and 0.5 μg oligomycin/ml. VP-16, prepared in dimethyl sulfoxide and diluted in standard reaction medium immediately prior to each experiment, was added to the reaction medium after protein addition and incubated for 5 min before starting the reactions. The results shown represent typical recordings from experiments with at least three different mitochondrial preparations.

2.4. Mitochondrial swelling

Changes in mitochondrial volume were monitored by the decrease in apparent absorbance (light scattering) at 540 nm (Custódio et al., 1998b). Mitochondria were suspended at 0.5 mg protein/ml in 2 ml of standard reaction medium supplemented with 140 nmol Ca^{2+}/mg protein before energization with 5 mM succinate.

2.5. Membrane potential and oxygen uptake

Mitochondria (1 mg/ml) was suspended in the reaction medium and the incubations were carried out at 30°C in an oxygen-electrode chamber with magnetic stirring. Mitochondrial $\Delta\Psi$ and oxygen consumption were monitored simultaneously using a TPP^+ sensitive electrode (Kamo et al., 1979; Custódio et al., 1998b) and a Clark-type electrode (Yellow Springs Instrument, Model YSI 5331) (Custódio et al., 1994) placed in the same closed reaction chamber.

2.6. Measurements of Ca^{2+} fluxes

Mitochondrial Ca^{2+} fluxes were evaluated by monitoring the changes in Ca^{2+} concentration in the reaction medium using a Ca^{2+}-sensitive electrode, according to procedures previously described (Madeira, 1975; Custódio et al., 1998b). The reactions were performed with 1 mg protein in 2 ml of the reaction medium in an open vessel with magnetic stirring and started with 5 mM succinate after 2 min of Ca^{2+} addition (200 nmol/mg protein) in the absence and presence of VP-16.

3. Results

Adding Ca^{2+} (140 nmol/mg protein) to rat liver mitochondria energized with succinate (Fig. 1) causes a minimal mitochondrial swelling (trace 2). The incubation of mitochondria with VP-16 (1 μmol/mg protein) followed by Ca^{2+} addition induces a large decrease in the absorbance (trace 4), characteristic of the MPT activation. The induction of MPT is evidenced by incubating mitochondria with CyA, a potent and specific inhibitor of the MPT, since CyA added prior to VP-16 (trace 1) or after energization (trace 3) completely prevents or stops the mitochondrial swelling. Therefore,

VP-16 promotes the Ca^{2+}-dependent mitochondrial swelling as a consequence of MPT induction.

The MPT triggering by VP-16 is further evidenced by the effects on $\Delta\Psi$ (Fig. 2). Adding 200 nmol Ca^{2+} to mitochondria energized with succinate causes a transient depolarization of $\Delta\Psi$, which recovers within 2 min (Fig. 2-1). However, mitochondria preincubated with VP-16 fail to repolarize after Ca^{2+} loading and undergo an irreversible depolarization over the ensuing 12 min (Fig. 2-2). This extensive depolarization is completely protected by CyA added prior to VP-16 (Fig. 2-3). Moreover, when added to depolarized mitochondria, CyA induces repolarization of mitochondrial $\Delta\Psi$ depressed by VP-16 (Fig. 2-4). These events clearly indicate MPT induction by VP-16, according to data of mitochondrial swelling (Fig. 1).

Fig. 3 reports the effect of VP-16 on mitochondrial respiration recorded simultaneously to $\Delta\Psi$ with both oxygen- and TPP^{+}-selective electrodes placed in the same closed reaction chamber. The addition of Ca^{2+} (200 nmol/mg protein) to mitochondria energized with succinate induces a transient increase in the rate of oxygen consumption, which returns to respiratory state 4 within 2.5 min (trace 1), that is, the time required for accumulation of added Ca^{2+}. However, the previous addition of VP-16 to mitochondria causes full uncoupling of respiration (trace 2) which parallels the irreversible depolarization of membrane potential (Fig. 2). CyA added prior to VP-16 (trace 3) or during stimulated respiration induced by Ca^{2+} (trace 4) avoids the Ca^{2+}-induced uncoupling of mitochondrial respiration promoted by VP-16.

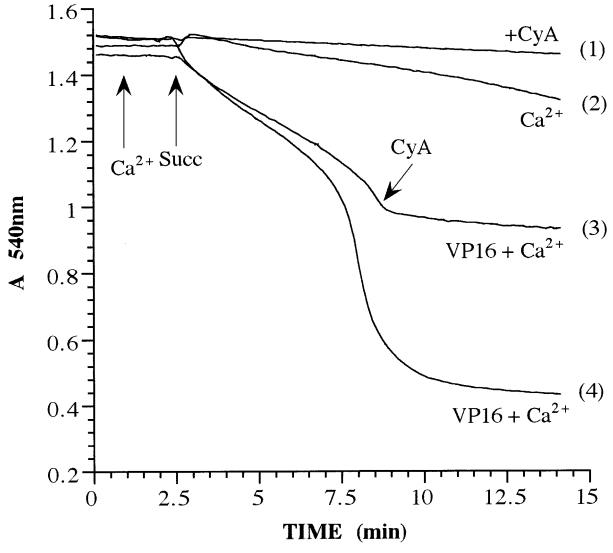

Fig. 1. Ca^{2+}-dependent induction of the mitochondrial swelling by etoposide (VP-16). Rat liver mitochondria (0.5 mg protein/ml) were incubated at 30°C in 2 ml of reaction medium containing 200 mM sucrose, 10 mM Tris–Mops (pH 7.4), 1 mM KH_2PO_4 and 10 μM EGTA, supplemented with 2 μM rotenone and 0.5 μg oligomycin/ml. Mitochondria were energized with 5 mM potassium succinate after 2 min of Ca^{2+} addition (140 nmol/mg protein) in the absence (2) and presence of VP-16 (1 μmol/mg protein) (4) preincubated for 5 min with mitochondria. CyA (1 μM) was added before VP-16 (1) or after mitochondria energization as indicated by the arrow (3). The traces were obtained by following continuously the light scattering at 540 nm and are typical direct recordings from several separate experiments.

Further evidence that VP-16 induces MPT is provided by the release of Ca^{2+} accumulated by mitochondria (Fig. 4). Mitochondria energized with succinate retain Ca^{2+} taken up within about 20 min (trace 1). After exposure to VP-16 mitochondria release the accumulated Ca^{2+} (trace 2), but co-incubation with CyA prevents this effect, affording complete capacity to accumulate and retain the added Ca^{2+} (trace 3). Furthermore, adding CyA during the course of the MPT reverses the Ca^{2+} release (trace 4), in agreement with the repolarization effect (Fig. 3), occurring accumulation of Ca^{2+} as in mitochondria energized in the absence of VP-16 (trace 1).

4. Discussion

Mitochondria have a large capacity for Ca^{2+} uptake and function as an intracellular buffer by actively sequestering Ca^{2+} from the cytoplasm before undergoing the Ca^{2+}-dependent MPT (Bernardi et al., 1998). Exposure to oxidants or a number of different chemicals is known to increase the sensitivity of isolated mitochondria to the Ca^{2+}-dependent induction of MPT (Petronilli et al., 1994; Bernardi et al., 1998).

VP-16 increases the sensitivity of mitochondria to Ca^{2+}-induced swelling (Fig. 1), depolarization of $\Delta\Psi$ (Fig. 2), Ca^{2+} release (Fig. 4) and renders the mitochondria highly susceptible to the uncoupling effect of Ca^{2+} (Fig. 3). All these effects of VP-16 are inhibited by CyA that also affords complete capacity of mitochondria to sequester the Ca^{2+} released, to recover membrane potential and to prevent the Ca^{2+}-induced uncoupling of mitochondrial respiration when added during the time course of the reactions. As VP-16 does not alter state 4 respiration (Fig. 3) and $\Delta\Psi$ (Fig. 2), these effects clearly demonstrate that VP-16 induces the CyA-sensitive MPT as the result of a direct effect on the MPT megachannel complex.

VP-16-induced MPT is also prevented by ascorbate (data not shown), the primary reductant of the phenoxyl radical of etoposide (Kagan et al., 1994), suggesting the generation of free radicals as a potential mechanism underlying MPT and apoptosis induction, in agreement with other studies (Siitonen et al., 1999; Perkins et al., 2000). Moreover, recently it has been shown that the pro-apoptotic factors Bax and Bak induce mitochondrial changes such as cytochrome c release and the loss of $\Delta\Psi$ by direct interaction with MPT, and overexpression of Bax promotes apoptosis

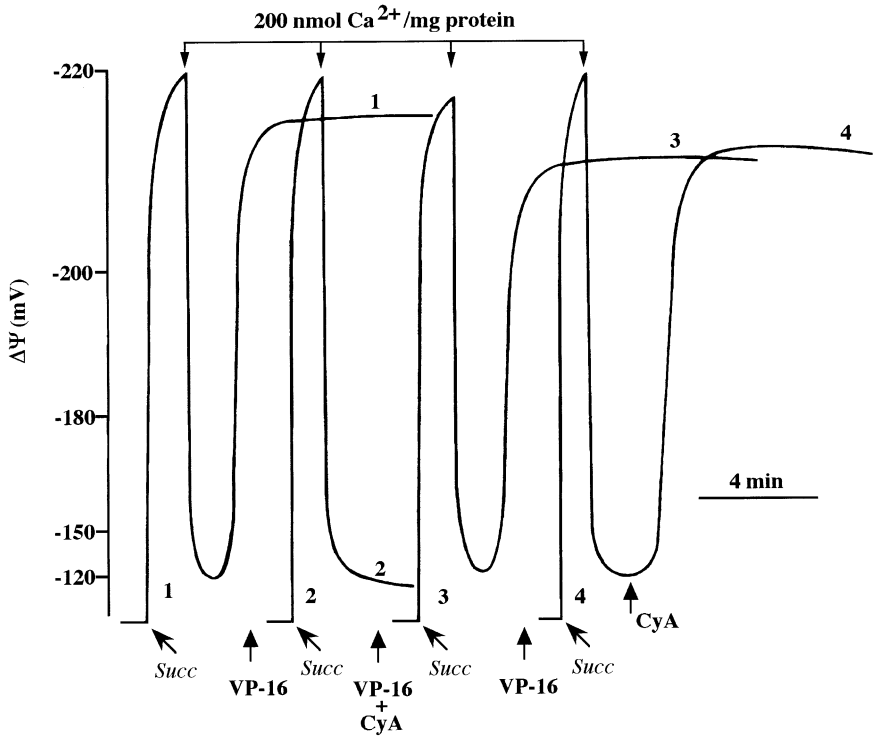

Fig. 2. Effect of etoposide (VP-16) on mitochondrial membrane potential ($\Delta\Psi$). Mitochondria were incubated at 30°C in a closed reaction chamber at 1 mg protein/ml in 200 mM sucrose, 10 mM Tris–Mops (pH 7.4), 1 mM KH_2PO_4 and 10 μM EGTA, supplemented with 2 μM rotenone, 0.5 μg oligomycin/ml and 4 μM TPP^+. $\Delta\Psi$ was recorded continuously using a TPP^+ selective electrode as described in Materials and Methods. The reactions were initiated by adding 5 mM succinate. Ca^{2+} (200 nmol/mg protein) was added after a steady-state of TPP^+ signal in the absence (1) or presence of 1 μmol VP-16/mg protein incubated with mitochondria for 5 min (2). CyA (1 μM) was preincubated with mitochondria (3) or added after Ca^{2+}-induced depolarization (4). All traces are representative of three independent experiments.

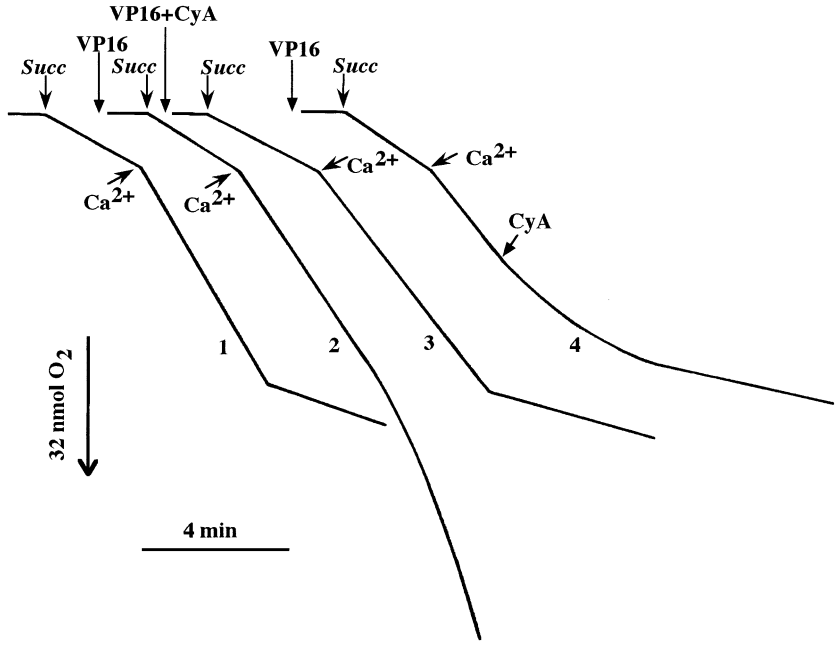

Fig. 3. Effect of etoposide (VP-16) on mitochondrial oxygen consumption. Oxygen uptake was monitored using a Clarke-type YSI electrode simultaneously with the recording of membrane potential. The reactions were carried out in a closed chamber under conditions identical to those described for Fig. 2. At the points indicated by the arrows, succinate, Ca^{2+}, VP-16 and CyA were added. The traces represent typical recordings from three separate experiments with different mitochondrial preparations.

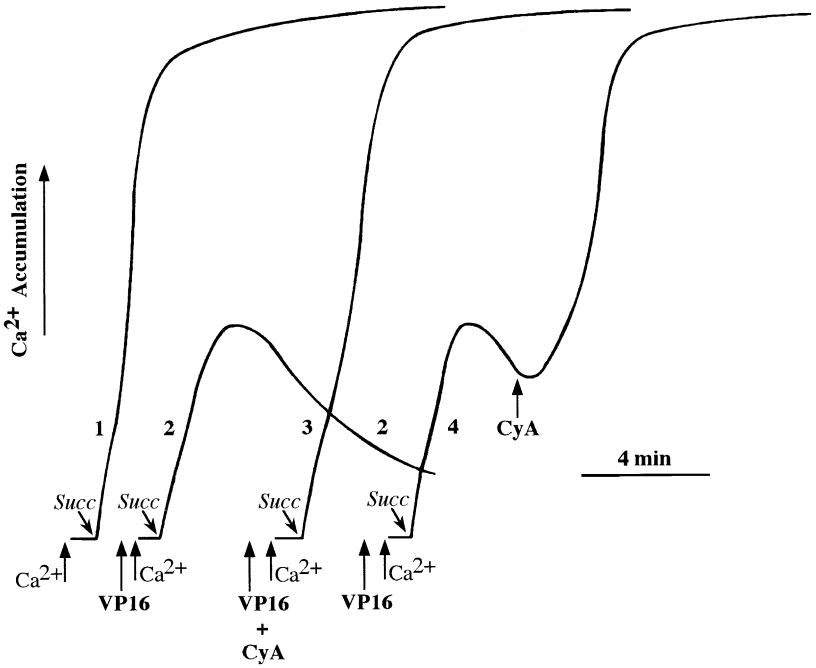

Fig. 4. Effect of etoposide (VP-16) on mitochondrial Ca^{2+} uptake. Mitochondria were preincubated for 5 min at 30°C in the standard reaction medium in the absence (1) and presence of 1 μmol VP-16/mg protein (2). 1 μM CyA was added prior (3) or after (4) addition of 200 nmol Ca^{2+}/mg protein. Mitochondria were energized with 5 mM potassium succinate and the uptake of Ca^{2+} and the release of sequestered Ca^{2+} by mitochondria were monitored continuously with a Ca^{2+}-selective electrode. All traces are representative of several independent experiments.

on induction of MPT (Pastorino et al., 1998). Some questions have been raised about the involvement of mitochondrial $\Delta\Psi$ collapse and cytochrome c release as markers for apoptosis, since they are cell type- and inducer-dependent phenomena (Tang et al., 1998; Salvioli et al., 2000). However, several studies point out that mitochondria play a key role in apoptotic cascade and that the induction of MPT leads to cell death by releasing several activators of apoptosis (Fadeel et al., 1999; Tafani et al., 2000). Therefore, the induction of the MPT by VP-16 can directly promote the release of mitochondrial apoptogenic factors involved in the signaling pathway for VP-16-induced apoptosis. Moreover, the irreversible (high-conductance) MPT induction causes prolonged mitochondrial $\Delta\Psi$ depolarization across the inner mitochondrial membrane (Petit et al., 1998), resulting either in the loss of MPT functions involved in Ca^{2+} signaling and in the regulation of Ca^{2+} (Bernardi and Petronilli, 1996) or uncoupling of mitochondria, stimulation of respiration, swelling and ATPase (Petit et al., 1998; Shinohara et al., 1998). The consequent disruption of mitochondria functions in cellular Ca^{2+} homeostasis, uncoupling of phosphorylation from respiration and activation of mitochondrial ATPase, depleting cellular ATP and accounting for accidental/toxic cell death (Ichas and Mazat, 1998), might also contribute to the antiproliferative activity of VP-16 observed in different cell types. In conclusion, the induction of apoptosis and/or cellular ATP depletion as consequence of VP-16-induced MPT might explain the cytostatic activity of this drug and side-effects in normal cells observed in vivo.

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