Biochemical Pharmacology, Vol. 44, No. 4, pp. 833–835, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

Protection by cyclosporin A of cultured hepatocytes from the toxic consequences of the loss of mitochondrial energization produced by 1-methyl-4-phenylpyridinium

(*Received* 21 *February* 1992; *accepted* 8 *May* 1992)

Abstract—Cyclosporin A prevented the killing of cultured rat hepatocytes by 1-methyl-4-phenylpyridinium (MPP^+). However, in the presence of both cyclosporin and atractyloside, there was no protection. Cyclosporin had no effect on the depletion of ATP or the loss of mitochondrial energization by MPP^+. Cyclosporin, however, did prevent the increase in the molecular order of hepatocyte membranes produced by MPP^+. These data suggest that mitochondrial de-energization produced by MPP^+ is accompanied by a "permeability transition" analogous to that which occurs *in vitro* in the presence of calcium. By preventing this transition, cyclosporin protects the cells. By antagonizing this action of cyclosporin, atractyloside restores the cell killing. The mitochondrial transition is causally linked to cell killing by a mechanism that increases the molecular order of the hepatocyte plasma membrane.

In the presence of Ca^{2+}, de-energized mitochondria undergo a reversible "permeability transition" associated with the formation of a nonspecific, inner-membrane pore [1–4]. Opening of this pore seems to be related to the binding of peptidyl-prolyl *cis-trans* isomerase (cyclophilin) [5, 6] to the adenine nucleotide translocase [7]. The immunosuppressive peptide cyclosporin inhibits the permeability transition and subsequent mitochondrial swelling by binding to cyclophilin, thereby preventing its interaction with the translocase [7]. Atractyloside, an inhibitor of the adenine nucleotide translocase [8], prevents this effect of cyclosporin A [7].

Whether this permeability transition occurs in intact cells under conditions of toxic or ischemic cell injury, and the role it may play in the loss of viability, are unknown. We have emphasized previously [9] that the killing of cultured hepatocytes by agents that interfere with energy metabolism is not the consequence of the depletion of ATP alone. Rather, cell killing is better correlated with collapse of the mitochondrial membrane potential. In the present report, we present evidence that under conditions of the loss of mitochondrial energization occurring in the course of the killing of cultured hepatocytes by 1-methyl-4-phenylpyridinium (MPP^+),* an agent known to inhibit NADH dehydrogenase [10, 11], it is likely that a mitochondrial "permeability transition" occurs and that it is related to the loss of viability.

Materials and Methods

Male Sprague–Dawley rats (150–200 g, Charles River Breeding Laboratory) were fed *ad lib.* and then fasted overnight prior to use. Isolated hepatocytes were prepared by the collagenase (Sigma) perfusion according to Seglen [12]. Yields of $2–4 \times 10^8$ cells/liver with 85–90% viability by trypan blue exclusion were routinely obtained. Hepatocytes were plated in 25 cm^2 flasks (Corning) at a density of 1.33×10^6 cells/flask in Williams E medium (GIBCO) containing 10 IU/mL penicillin, 10 μg/mL streptomycin, 0.05 mg/mL gentamycin, 0.02 U/mL insulin and 10% heat-inactivated (55° for 15 min) fetal bovine serum (Hazelton Research products) (complete Williams E). After incubation for 2 hr at 37° in an atmosphere of 5% CO_2–95% air, cultures were washed twice with prewarmed HEPES [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid] (Sigma) buffer (0.14 M NaCl, 6.7 mM KCl, 1.2 mM $CaCl_2$ and 2.4 mM HEPES, pH 7.4) to remove unattached dead cells. Complete Williams E was replaced and flasks were further incubated for 24 hr. Cultures were then washed and incubated in serum-free Williams E with additions indicated in the text. All experiments were performed on at least three separate cultures.

The viability of the cultured cells was determined by the release of lactate dehydrogenase as described previously [13, 14]. ATP was determined by the luciferin–luciferase method [15] using an Aminco Chem-Glow spectrometer. Protein content was determined by the method of Lowry *et al.* [16] using bovine serum albumin as the standard.

Changes in mitochondrial membrane potential were assessed by measuring the release of incorporated [^{3}H]-TPP^+ (tetraphenylphosphonium cation) into the culture medium according to the method of Hoek *et al.* [17]. Monolayers were loaded (60 min) with [^{3}H]TPP^+ (0.0125 μCi/mL, Amersham International) in the presence of serum-free Williams E medium. Release of [^{3}H]TPP^+ was measured by counting a 50-μL aliquot of the culture medium.

The molecular order of hepatocyte membrane lipids was determined by electron spin resonance spectroscopy. The cells from five flasks were removed by trypsinization and pooled by centrifugation at 1250 rpm for 5 min. The pellet was resuspended in phosphate-buffered saline (PBS). The spin-label 12-doxyl stearic acid (Molecular Probes, Inc.) was dissolved in 5% bovine serum albumin in PBS and added to the final cell suspension. The membrane phospholipid to spin-label ratio was kept above 200:1. Following a 1-hr incubation with shaking at 37°, the sample was centrifuged three times, 1250 rpm for 5 min, and resuspended in PBS. ESR spectra were immediately obtained at 37° with an IBM spectrometer. Spectra were computer-averaged, and the order parameter S was calculated from the inner hyperfine splittings by using the equation of Gaffney [18].

MPP^+I^- was obtained from Research Biochemicals (Wayland, MA). Atractyloside, pyruvate, NADH, Na_2CO_3, NaH_2PO_4, Na_2HPO_4, H_3PO_4, ATP, ATP reagent (firefly extract), and bovine serum albumin were from Sigma. Cyclosporin A [dissolved in Cremephore (polyoxyethylated vegetable oil)] was obtained from Sandoz Pharmaceuticals (U.K.). Cremephore had no effect on the hepatocytes.

Results

Table 1 details the effect of cyclosporin and atractyloside on the killing of cultured hepatocytes by MPP^+. Neither

* Abbreviations: MPP^+, 1-methyl-4-phenylpyridinium; TPP^+, triphenylphosphonium cation; and PBS, phosphate-buffered saline.

Table 1. Effect of cyclosporin and atractyloside on the killing of cultured hepatocytes by MPP^+

Treatment	Percent dead cells
Control	6 ± 1
1 μM Cyclosporin	7 ± 1
5 μM Atractyloside	6 ± 1
1.5 mM MPP^+	62 ± 1
MPP^+ + atractyloside	66 ± 5
MPP^+ + cyclosporin	9 ± 1
MPP^+ + atractyloside + cyclosporin	60 ± 3
Atractyloside + cyclosporin	7 ± 2

Hepatocytes in culture for 24 hr were washed and placed in serum-free Williams E medium. The cells were treated with the additions indicated above. The viability of cells was determined 6 hr later. Results are the means ± SD of the determinations on three separate cultures.

1 μM cyclosporin nor 5 μM atractyloside had any effect on the viability of control hepatocytes. Over a 6-hr time course, MPP^+ killed over 60% of the cells. Whereas atractyloside was without effect on the toxicity of MPP^+, cyclosporin completely prevented the cell killing. A similar result was seen with concentrations as low as 1 μM cyclosporin A. However, in the presence of both cyclosporin and atractyloside, there was no protection against the cell killing by MPP^+. Cyclosporin and atractyloside had no effect on the viability of control cells.

Table 2 shows that cyclosporin was without effect on the depletion of ATP produced by MPP^+. Within 2 hr 1.5 mM MPP^+ reduced the content of ATP to less than 5% of the initial value. Cyclosporin did not prevent this loss of ATP after 2 hr, a time at which there was no loss of viability in MPP^+-treated cultures. ATP content was still depleted in cyclosporin-treated cultures after 6 hr (data not shown), a time at which cyclosporin prevented the killing of over 60% of the cells by MPP^+ (Table 1).

The intracellular distribution of the non-metabolizable lipophilic cation TPP^+ has been used to monitor the mitochondrial membrane potential in intact hepatocytes [17]. Table 3 shows that MPP^+ caused the release of most of the intracellular TPP^+ within 2 hr. Cyclosporin increased the TPP^+ content of the cells at 2 hr. However, cultures treated with both cyclosporin and MPP^+ released more than 85% of the intracellular TPP^+ at 2 hr.

The anisotropic motion of the spin-labeled fatty acid, 12-doxyl stearic acid, can be quantitated by determination by ESR spectroscopy of the order parameter S [18]. An increase in S may be interpreted as an increase in molecular order or a decrease in fluidity, even though S does not directly measure rates of molecular motion. The effect of MPP^+ and cyclosporin on the fluidity of hepatocyte membranes is documented in Table 4. MPP^+ increased the order parameter S. Although cyclosporin alone also decreased membrane fluidity, the order parameter S was significantly lower with MPP^+ and cyclosporin than with MPP^+ alone.

Table 2. Effect of cyclosporin on the ATP content of cultured hepatocytes intoxicated with MPP^+

Treatment	ATP content (% of coltrol)
1.5 mM MPP^+	3.1 ± 0.1
5 μM Cyclosporin	99.0 ± 2.5
MPP^+ + cyclosporin	6.5 ± 0.3

Cultured hepatocytes were treated with cyclosporin in the presence or absence of 1.5 mM MPP^+. The cellular content of ATP was measured 2 hr later. The ATP content of untreated controls was 11 nmol/mg protein. Results are the mean percentage of this control ± SD of the determination on at least three separate cultures.

Table 3. Effect of cyclosporin A on the mitochondrial membrane potential in hepatocytes intoxicated with MPP^+

Treatment	Intracellular [^{3}H]TPP^+ (% of initial content)
1.5 mM MPP^+	< 1.0
5 μM Cyclosporin A	168 ± 7
MPP^+ + cyclosporin	14 ± 6

Hepatocytes in culture for 24 hr were loaded with [^{3}H]-TPP^+ (0.0125 μCi/mL) for 60 min and then treated for 2 hr with 5 μM cyclosporin A in the presence or absence of 1.5 mM MPP^+. The loss of mitochondrial membrane potential was assessed by the release of [^{3}H]TPP^+ into the medium. Results are the mean percentage of the initial [^{3}H]TPP^+ ± SD of the determinations on three separate cultures.

Discussion

The killing of cultured hepatocytes by MPP^+ was prevented by cyclosporin A. In turn, the ability of cyclosporin to protect was blocked by atractyloside. These results suggest that mitochondria de-energized by MPP^+ undergo a permeability transition, an event that is linked in some manner to the killing of the hepatocytes.

Cyclosporin A binds to peptidyl-prolyl *cis-trans* isomerase (cyclophilin), a protein widely present in living systems [5, 6, 19]. This interaction is associated with inhibition of a permeability transition that is responsible for the large amplitude swelling of de-energized mitochondria [1–4, 7]. Atractyloside antagonizes the ability of cyclosporin to prevent mitochondrial swelling [7].

In the presence of adenine nucleotides, ion channels or "pores" that can be formed by mitochondrial adenine nucleotide translocase are normally closed [7]. In the absence of adenine nucleotides, the "c" (cytosolic)

Table 4. Effects of cyclosporin on the increase in molecular order of the membranes of cultured hepatocytes produced by MPP^+

Treatment	Order parameter S
Control	0.369 ± 0.003
1.5 mM MPP^+	0.394 ± 0.006*
10 μM Cyclosporin A	0.390 ± 0.009
MPP^+ + cyclosporin	0.381 ± 0.005†

Cultured hepatocytes were pretreated for 1 hr with cyclosporin prior to exposure to 1.5 mM MPP^+ for 2 hr. Trypsinized cells were spin-labelled with 12-doxyl stearic acid, and ESR spectra were obtained as described in Materials and Methods. Results are the means ± SD of the order parameter S from at least three separate experiments.

* $P < 0.005$ with respect to control.

† $P < 0.005$ with respect to MPP^+ alone.

conformation of the translocase binds Ca^{2+} and cyclophilin. The interaction alters translocase structure so as to open an ion channel that allows the permeability transition in de-energized mitochondria. The binding of cyclosporin prevents cyclophilin from interacting with the translocase, thereby inhibiting pore formation. In the presence of atractyloside, the translocase remains in the "c" (pore-forming) conformation, and cyclosporin can have no effect on the mitochondria. Thus, cyclosporin acts distal to ATP depletion (Table 2) and the loss of mitochondrial energization (Table 3).

The present study argues that two previously unidentified alterations, namely a mitochondrial permeability transition and a decrease in plasma membrane fluidity, are intermediary steps between the loss of mitochondrial energization and the increase in plasma membrane permeability that denotes the death of the cell. By preventing the permeability transition, cyclosporin also prevents the subsequent change in the fluidity of the plasma membrane and prevents the death of the cells.

Acknowledgement—This work was supported by Grant DK38305 and AA 07186 from the National Institutes of Health.

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