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European Journal of Pharmacology 495 (2004) 111–118

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Protection by cyclosporin A of mitochondrial and cellular functions during a cold preservation–warm reperfusion of rat liver

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Received 8 April 2004; received in revised form 19 May 2004; accepted 25 May 2004

Abstract

Liver transplantation is an effective therapeutic option for end-stage liver disease, but initial poor graft function still occurs, often related to cold preservation–warm reperfusion (CPWR) conditions. Damages to mitochondria could be implicated in hepatocyte cell death since opening of the permeability transition pore (PTP) can lead to necrosis and apoptosis. The purpose of this study was to test the hypothesis that inhibition of mitochondrial permeability transition by cyclosporin A could improve rat liver mitochondrial and hepatocellular parameters after 24-h cold preservation followed by a warm reperfusion in Krebs-Henseleit Buffer. Mitochondrial functions were assessed by measuring respiratory parameters, swelling, cytochrome *c* release and caspases activation. Hepatocyte injury was assessed by evaluation of ATP energetic charge, lactate dehydrogenase (LDH) leakage, apoptosis and necrosis. Results show that CPWR induces liver mitochondrial and cellular damages. CPWR induced damages on the mitochondrial respiratory chain, leading to mitochondrial swelling. The consequences are the loss of ATP energetic charge, the initiation of apoptosis through cytochrome *c* release and the activation of caspases. Cyclosporin A partially protects respiratory chain integrity and totally prevents mitochondrial swelling, allowing better recovery of energetic charge. It also partially limits the activation of the apoptotic machinery and subsequent cell death by apoptosis in both the organ and isolated hepatocytes. Inhibition of permeability transition thus provides only partial protection against CPWR. However, this target can be considered as a promising adjunct therapeutic approach to improve the primary function of the grafted liver after transplantation.

Keywords: Necrosis; Apoptosis; Cyclosporin A; Permeability transition pore; Preservation solution; Rat, liver

1. Introduction

Liver transplantation is now the last resort treatment for end-stage acute and chronic liver diseases (Krasko et al., 2003). Organ preservation, transport and transplantation involve a period of global hypothermic tissue ischemia leading to tissue injury, which is exacerbated by the reperfusion that accompanies the transplantation. This can result in primary graft dysfunction, which is an important cause of death after liver transplantation (Strasberg et al., 1994). The pathophysiological mechanism underlying such tissue injury and cell death remains unclear.

It has been shown that mitochondria are involved in cell death via the mitochondrial permeability transition (Lemasters et al., 1999). The latter is characterized by an abrupt increase in permeability of the mitochondrial inner membrane to solutes of molecular mass less than about 1500 Da (Zoratti and Szabo, 1995). Mitochondrial permeability transition appears to be due to the opening of a pore, called permeability transition pore (PTP) that leads to membrane depolarization, uncoupling of oxidative phosphorylation, ATP depletion and consequently to cell death by necrosis (Crompton, 1999). PTP opening also causes mitochondrial swelling by massive entrance of solutes into the matrix. The consequence is the destruction of the external membrane of mitochondria and the release of apoptotic factors like cytochrome *c* from inter-membrane space. Once in the cytosol, this molecule leads to activation of cell apoptosis via the activation of mitochondrial caspase 9 and subsequent

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doi:10.1016/j.ejphar.2004.05.042

cellular caspase 3 (Parone et al., 2002; Zou et al., 1999). The progression to apoptosis rather than necrosis appears to depend on the cell's energy charge and evidence suggests that both death phenomena may be part of a continuum (Kim et al., 2003).

An interesting hypothesis is that PTP opening could be involved in cell death occurring during cold preservation–warm reperfusion (CPWR). Thus, the purpose of this study was to determine whether the inhibition of PTP opening constitutes a relevant pharmacological approach to protect hepatocytes during liver CPWR. We used cyclosporin A, which is a highly specific inhibitor of PTP (Broekemeier et al., 1989), acting by a mechanism distinct from its immunosuppressive effect. The molecule was tested on two models: a model of isolated-perfused rat liver (ex-vivo experiments) and a primary culture of hepatocytes (in vitro experiments). Isolated cells or whole livers were subjected to 24-h cold preservation followed by short-term warm reperfusion. The effects on mitochondrial functions and on hepatocytes viability and integrity were assessed after CPWR. Cyclosporin A was included in two simple preservation media to test its potential beneficial effect: the extracellular Krebs-Henseleit Buffer (eKHB), in which Na^+/K^+ concentrations are similar to blood concentrations, and the intracellular Krebs-Henseleit Buffer (iKHB), in which Na^+/K^+ concentrations are similar to cytoplasmic concentrations.

2. Materials and methods

2.1. Animals

All animal procedures used in this study were in strict accordance with the European Community council Directive of 24 November 1986 (86-609/EEC) and Decree of 20 October 1987 (87-848/EEC). Male Wistar rats (220–280 g) were obtained from Janvier (Le Genest-St-Isle, France).

2.2. Isolation of liver and hepatocytes and CPWR procedure

Rats were anaesthetized with sodium pentobarbital (65 mg/kg), the abdomen was opened and the portal vein was cannulated with a 16 GA catheter.

For ex vivo experiments, the treated liver was rinsed during 13 min at a flow rate of 30 ml/min with warm extracellular or intracellular Krebs-Henseleit Buffer (eKHB (120 mM NaCl, 5 mM KCl) or iKHB (120 mM KCl, 5 mM NaCl) plus 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4–7H_2O$, 11.1 mM D-Glucose, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$). The pH was maintained at 7.2–7.4 by gassing the solution with O_2/CO_2 (95:5%). The liver was cold-preserved 24 h in the same solution (under hypoxia because oxygenated solution was not circulating through the organ) and rewarmed gradually from 4 to 37 °C by reperfusion over 10 min followed by a 30-min perfusion at 37 °C at the rate of 30 ml/min with gassed eKHB in a recirculating mode (reoxygenation). The non-treated control liver (0-h preservation) was taken from a decapitated rat and mitochondria immediately isolated as described below.

For in vitro experiments, the liver was cleared of blood by washing at the rate of 20–30 ml/min with 200 ml of warm Hank's solution pH 7.4 gassed with O_2/CO_2 (95:5%) and containing 0.5 mM EGTA and 10 U/ml heparin. The perfusate was drained away by opening the right atrium. The liver was then continuously perfused in a recirculating mode for 10–12 min with 100 ml of warm Hank's solution containing 50 mg of collagenase to detach hepatocytes. The cells were filtered serially on 250- and 80-μm filters and washed three times with William's E medium pH 7.4 containing 5 U/ml pennicilin G-steptomycin and 10 mM Hepes. The hepatocytes were cold-preserved for 24 h in eKHB or iKHB pH 7.4. The preservation solution was not gassed in order to submit cells to hypoxia to mimic the cold preservation of the liver. The pH was checked regularly and was stable probably because the alkalinisation due to lack of gassing was counter-balanced by the acidification of the medium by cells. The reperfusion was initiated by incubation of cells in warm William's E medium, pH 7.4, complemented with 1% bovine serum albumin, for 1 hour under an atmosphere of O_2/CO_2 (95:5%), the time required for them to adhere to the collagen-coated plate.

2.3. Isolation of mitochondria and cytosol

Mitochondria were isolated from whole livers as described by Johnson and Lardy (1967). Briefly, tissues or cells were homogenized on ice using a Teflon Potter homogenizer in Tris–Sucrose–EGTA medium (10 mM Tris, 250 mM Sucrose, 1 mM EGTA, pH 7.2 at 4 °C). Cellular fragments were precipitated at $600 \times g$ for 10 min. The supernatant was centrifuged at $15\,000 \times g$ for 5 min. The mitochondrial pellet was washed once with the same medium and another time with medium from which EGTA was omitted, the same centrifugation procedure being used. The supernatant obtained after the first centrifugation at $15\,000 \times g$ was centrifuged at $100\,000 \times g$ for 20 min to precipitate microsomes and to obtain cytosolic fraction of cells which was frozen at −80 °C for subsequent measures. Protein content was determined according to the method of Lowry et al. (1951).

2.4. Determination of mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi$) was evaluated according to Emaus et al. (1986) from uptake of the fluorescence dye rhodamine 123, which accumulates into energized mitochondria in response to their negative-inside membrane potential. Measures were performed in 1.8 ml of phosphate buffer (250 mM sucrose, 5 mM KH_2PO_4, 1 μM rotenone, pH 7.2 at 25 °C) including 6 mM succinate, and 0.3 μM rhodamine 123. The fluorescence of rhodamine 123

was monitored using a Perkin Elmer LS 50B fluorescence spectrometer. Excitation and emission wavelengths were 503 and 527 nm, respectively. After 30 s, mitochondria (0.5 mg/ml) were added. $\Delta\Psi$ was calculated according to the following relationship: $\Delta\Psi = -59 \log (\text{rhodamine } 123)_{in}/(\text{rhodamine } 123)_{out}$, assuming that the distribution of rhodamine 123 between mitochondria and medium follows the Nernst equation. A matrix volume of 1 μl/mg protein (Hackenbrock, 1968) was used to estimate $(\text{rhodamine } 123)_{in}$.

2.5. Determination of mitochondrial respiration

O_2 consumption was measured at 25 °C by a Clark-type oxygen microelectrode in a thermostat controlled chamber. Mitochondria (1 mg/ml) were added to 1 ml of phosphate buffer containing 5 mM Mg^{2+} and 1 mM EGTA. Mitochondrial respiration was initiated by addition of 6 mM succinate and oxidative phosphorylation was initiated by addition of 0.2 mM ADP. O_2 consumption recordings allowed the calculation of the respiratory control ratio (RCR) corresponding to the ratio between the state 3 (ADP-stimulated) respiration rate (V3) and the state 4 (resting) respiration rate (V4). The P/O ratio was also assessed from the ADP consumed divided by the O_2 used in state 3 respiration.

2.6. Determination of mitochondrial swelling

Swelling was assessed by measuring the change in absorbance of the mitochondrial suspension at 540 nm by using a Jasco V-350 spectrophotometer, according to Elimadi et al. (1997). Briefly, the measures were performed in a final volume of 1.8 ml of phosphate buffer. Mitochondria (1 mg/ml) were added in sample and reference cuvettes and 6 mM succinate was added to the sample cuvette only in order to measure spontaneous swelling. When swelling was induced by Ca^{2+}, succinate was added in both cuvettes and Ca^{2+} (25 or 50 μM) was added in the sample cuvette only to induce swelling.

2.7. Determination of intracellular ATP concentrations

ATP concentrations were measured enzymatically by means of an ATP-detection kit (Sigma Diagnostics) on thawed piece of liver: 500 mg of liver was homogenised with a Teflon Potter homogenizer in 3 ml of NaCl 0.9%. The mixture was then filtered, the proteins precipitated by addition of trichloracetic acid and the transformation of NADH (which is proportional to ATP content) quantified by spectrophometry at 340 nm.

2.8. Determination of caspases 3 and 9 activity

Cytosolic caspase activity was determined by fluorometry using a Perkin Elmer LS 50B fluorescence spectrometer. Samples were obtained by homogenization of livers on ice using a Teflon Potter homogenizer in a buffer containing 25 mM Tris, 5 mM $MgCl_2$, 1 mM EGTA and 50 μl of a protease inhibitor cocktail, pH 7.5. Cellular fragments were precipitated at $600 \times g$ for 10 min. The supernatant was centrifuged at $100\,000 \times g$ for 20 min. After addition of 0.5% of 0.4 M dithiothreitol, the supernatant was stored at −80 °C. Thirty micrograms of protein was assayed in a buffer containing 30 mM Hepes, 0.3 mM EDTA, 100 mM NaCl, 10 mM dithiothreitol, and 0.15% Triton X-100. The reaction was started with the addition of 200 μM of the substrate of caspase 3, *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarine (Ac-DEVD-AFC, Tebu, Le Perray-en-Yvelines), or of caspase 9, *N*-acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarine (Ac-LEHD-AFC, Tebu, Le Perray-en-Yvelines). Fluorescence of 7-amino-4-trifluoromethylcoumarine (AFC) released was measured after 2-h incubation at 37 °C using an excitation wavelength of 400 nm and an emission wavelength of 505 nm. Fluorescence intensity was calibrated with standard concentrations of AFC. Protease activity was calculated from the slope of the recorder trace and expressed as nmol of AFC released/h/mg protein.

2.9. Determination of cytosolic and mitochondrial cytochrome c content

Cytochrome *c* content was measured on thawed cytosolic fraction by Western blot analysis: 25 μg proteins were loaded onto a 15% Sodium Dodecyl Sulfate-polyacrylamide gel. After migration, the proteins were electroblotted onto a Polyvinylidine Difluoride transfer membrane. Immunostaining of cytochrome *c* was carried out with an anti-cytochrome *c* monoclonal primary antibody (MAB897, Amersham Biosciences, Orsay, France) and with an HRP-linked whole secondary antibody (NA931-1ML, Amersham Biosciences). For detection, the membrane was incubated with peroxidase-linked antibody (ECL Plus Western Blotting Detection Reagents, Amersham Biosciences) for 5 min and exposed to X-rays films.

2.10. Determination of cell death

Lactate dehydrogenase (LDH) activity was measured spectrophotometrically in the preservation and culture media with the Cytotoxicity Detection Kit (Roche, Meylan, France) as an index of plasma membrane damage and loss of membrane integrity. Hepatocytes were seeded into 35-mm culture dishes (Fisher Bioblock, Illkirch, France) at a density of 4×10^5 cells in 1.5 ml William's E medium and allowed an attachment period of 1 h at 37 °C under an atmosphere of O_2/CO_2 (95:5%). Enzyme activity was expressed as the percentage of extracellular LDH activity relative to the total LDH activity on the plates.

Cell death was estimated after seeding 4×10^5 hepatocytes in warm Williams' E medium and allowing an attachment period of 1 h at 37 °C under an atmosphere of

O_2/CO_2 (95:5%). Necrosis and apoptosis were determined by fluorescence microscopy using propidium iodide (2 nM) and Hoechst 33342 (5 nM) staining, respectively.

2.11. Materials

When not specified, chemicals, reagents and buffers were of analytical grade and were obtained from Sigma-Aldrich (Saint-Quentin, France). Cyclosporin A was a generous gift of Novartis Laboratories (Basle, Switzerland) and was solubilized in dimethylformamide. When cyclosporin A was used, a similar volume of dimethylformamid was included in the control experiments.

2.12. Statistical analysis

All values are given ± S.E.M. Statistical comparisons were made using a test of analysis of variance (SigmaStat software package, Jandel Scientific). A P value <0.05 was considered statistically significant.

3. Results

3.1. Effect of preservation solution and cyclosporin A addition on mitochondrial parameters during CPWR on the model of isolated-perfused rat liver

We utilized a model of isolated-perfused rat liver to evaluate the potential role of PTP opening during cold preservation followed (or not) by warm reperfusion.

Table 1 summarizes the mitochondrial parameters measured after 24-h cold-preservation in eKHB followed or not by 30-min warm reperfusion of rat liver compared to control mitochondria isolated from a control liver. After 24-h cold preservation only, the mitochondrial parameters were close to the control data. After 24-h CPWR, state 3 respiration rate, RCR and P/O values fell from 87.2 ± 4.7 to 64.2 ± 8.2 nmol/mg/min, from 4.31 ± 0.10 to 2.92 ± 0.25, and from 1.61 ± 0.01 to 1.36 ± 0.07, respectively ($n=4$ to 8, $P<0.05$ vs. control). Consequently, $\Delta\Psi$ was slightly lowered after this treatment and the ability of mitochondria to maintain $\Delta\Psi$ was significantly altered as attested by the decrease in $t_{\Delta\Psi}$ from 637 ± 56 to 409 ± 43 s after 24 h ($n=4$ to 8, $P<0.05$ vs. control). Inversion of Na^+/K^+ concentrations did not show any improvement of functional parameters. For instance, after 24-h CPWR in iKHB medium, RCR and P/O values were 2.43 ± 0.19 and 1.21 ± 0.08, respectively ($n=4$).

These observations allow us to suppose that PTP opening could be involved in the alterations caused by CPWR. In order to confirm this hypothesis, spontaneous or induced swelling of mitochondria was measured. Fig. 1 shows that mitochondria subjected to 24-h CPWR in eKHB swell spontaneously when they are energized with succinate (line b, panel A). This effect was moderate and was not observed with either control mitochondria (line a, panel A) or mitochondria isolated from livers subjected to simple cold

Table 1
Functional parameters of liver mitochondria subjected to 24-h CPWR in the absence or in the presence of 1 μM cyclosporin A (CsA)

Time of preservation	0 h (control)	24 h		
Preservation solution	–	eKHB	eKHB	eKHB + CsA (1 μM)
Treatment		CP	CPWR	CPWR
V3 (nmol/mg/min)	87.2 ± 4.7	72.6 ± 3.2	64.2 ± 8.2[a]	69.0 ± 3.6[a]
RCR	4.31 ± 0.10	3.58 ± 0.23[a]	2.92 ± 0.25[a]	3.46 ± 0.15[a,b]
P/O	1.61 ± 0.01	1.44 ± 0.08[a]	1.36 ± 0.07[a]	1.56 ± 0.10[b]
$\Delta\Psi$ (mV)	− 189 ± 1	− 188 ± 1	− 184 ± 1[a]	− 190 ± 2[b]
$t_{\Delta\Psi}$ (s)	637 ± 56	590 ± 55	409 ± 43[a]	>1500[a,b]

The cold preservation was performed in eKHB medium. Control: mitochondria isolated from liver not subjected to CPWR. Each value represents the mean ± S.E.M. of four to eight experiments performed in triplicate.

[a] $P<0.05$ vs. control.

[b] $P<0.05$ vs. 24-h CPWR in eKHB.

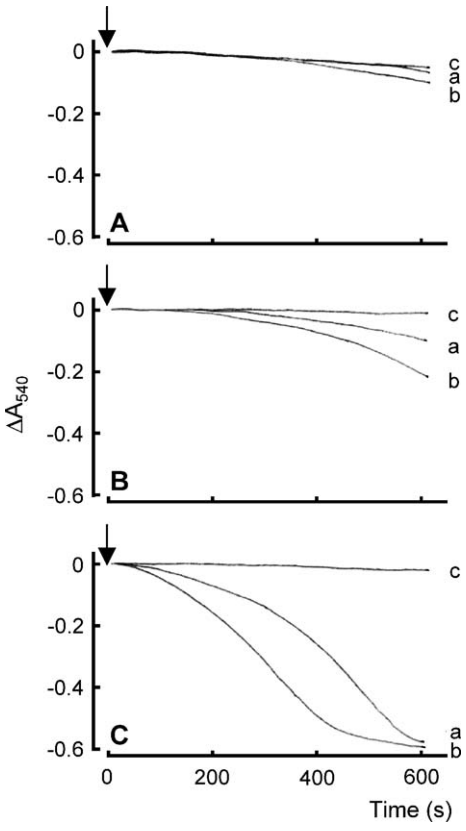

Fig. 1. Effect of 24-h CPWR on mitochondrial swelling. Swelling was induced by the addition (arrow) of 6 mM succinate (panel A), 6 mM succinate + 25 μM Ca^{2+} (panel B) or 6 mM succinate + 50 μM Ca^{2+} (panel C). ΔA_{540}: Change in absorbance of the mitochondrial suspension at 540 nm. Line a: control mitochondria. Line b: mitochondria isolated from 24-h eKHB CPWR liver. Line c: mitochondria isolated from 24 h eKHB + 1 μM cyclosporin A CPWR liver.

storage (not shown). The addition of increasing concentrations of Ca^{2+} amplified the phenomenon (compare lines a and b in panels B and C). In contrast, the presence of 1 μM of cyclosporin A (the specific inhibitor of PTP) in the preservation and reperfusion media completely inhibited mitochondrial swelling irrespective of the protocol used (line c in all panels), indicating that PTP opens during warm reperfusion and that CPWR sensitized mitochondria to Ca^{2+}-induced permeability transition. Cyclosporin A also restored $\Delta\Psi$ to the control value and the ability of mitochondria to maintain $\Delta\Psi$ over time (Table 1). In addition, cyclosporin A significantly limited the decrease in RCR and P/O values (3.46 ± 0.15 and 1.56 ± 0.10 after 24-h CPWR, $P < 0.05$) and tended to increase state 3 respiration rate but this was not statistically significant.

Interestingly, the improvement of mitochondrial functions by cyclosporin A also limited the decrease in liver ATP content measured after 24-h CPWR in eKHB as shown in Table 2. Indeed, the latter treatment induced a 40 ± 2.1% decrease ($n = 4$ to 6, $P < 0.05$) in ATP content (ATP content of a control liver was about 2.4 μmol/g liver). The presence of 1 μM cyclosporin A in the preservation and reperfusion media partially limited ATP depletion to 30 ± 3.3% ($n = 4$ to 6, $P < 0.05$).

3.2. Effect of preservation solution and cyclosporin A addition on biochemical markers of apoptosis during CPWR on the model of isolated-perfused rat liver

It is now well established that PTP opening plays a pivotal role in the early phase of apoptosis in mammalian cells (Bernardi et al., 1999). Thus, we investigated the consequences of CPWR on biochemical markers of apoptosis—namely, release of cytochrome *c* from mitochondrial inner membrane and subsequent activation of caspases 9 and 3—in the absence and in the presence of cyclosporin A.

The release of cytochrome *c* from mitochondria was studied by Western blot analysis (Fig. 2). Cold preservation of the liver did not modify the release of cytochrome *c* from mitochondria (lane 4). However, such a release was observed during the warm reperfusion period (lane 2). Moreover, addition of 1 μM cyclosporin A in 24-h preservation and reperfusion media (lane 3) significantly reduced this release. Cytochrome *c* release was followed by a significant increase in the activity of caspases 3 and 9 after 24-h CPWR in eKHB medium (Fig. 3). The presence of 1 μM cyclosporin A in the CPWR media limited significantly the activation of the two caspases after 24-h preservation.

The same studies were also performed after 48-h CPWR. Mitochondrial functions were extensively altered so that RCR and P/O values reached 1.40 ± 0.15 and 0.77 ± 0.07, respectively ($n = 4$). These effects were probably due to the membrane damages induced by the prolonged hypothermic storage and under these conditions, cyclosporin A was not able to induce any protective effect on mitochondrial parameters, loss of ATP, cytochrome *c* release or caspases activation (results not shown).

Table 2
Percentage of cellular ATP content after 24-h CPWR in eKHB in the absence or in the presence of 1 μM cyclosporin A (CsA)

Time of preservation	0 h (Control)	24 h	
Preservation solution	–	eKHB	eKHB + CsA (1 μM)
Percentage of ATP content	100 ± 6.2%	60.0 ± 2.1%[a]	70.0 ± 3.3%[a,b]

Percentage of cellular ATP content after 24-h CPWR in eKHB in the absence or in the presence of 1 μM cyclosporin A (CsA) was compared to control value (100%) which corresponds to 2.4 μmol/g liver. ($n = 4$ to 6).
[a] $P < 0.05$ vs. control.
[b] $P < 0.05$ vs. 24 h eKHB.

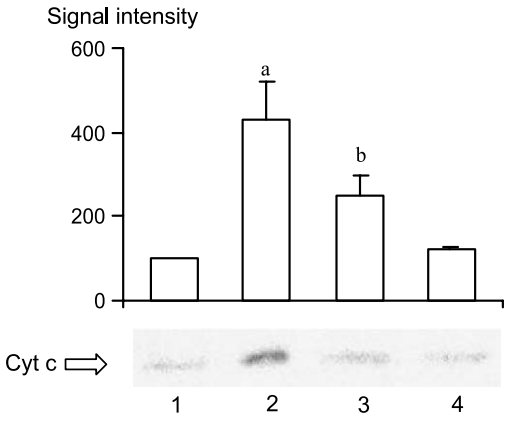

Fig. 2. Effect of cyclosporin A on cytochrome *c* (cyt *c*) release into cytosol during CPWR in rat liver. Cytosolic and mitochondrial fractions were separated as described in Material and methods and cytochrome *c* release was measured by Western Blot analysis in cytosolic fractions. Western Blots are representative of five independent experiments with similar results. Lane 1: Control; lanes 2 and 3: cytochrome *c* release after 24-h CPWR in the absence and presence of 1 μM cyclosporin A, respectively; lane 4: cytochrome *c* release after cold preservation only. The signal intensity was evaluated by densitometry ([a]$P < 0.05$ vs. control; [b]$P < 0.05$ vs. 24-h cyclosporin A ($n = 5$)).

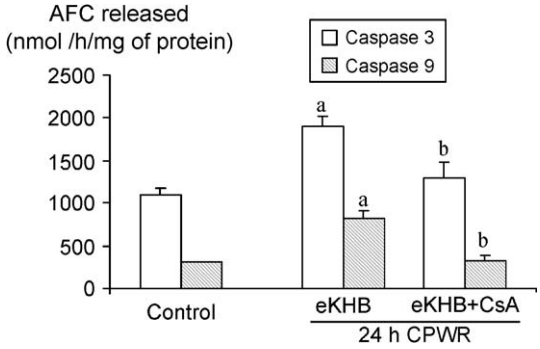

Fig. 3. Activities of caspases 3 and 9 after 24-h CPWR in eKHB. Caspase activities were studied in the absence or in the presence of 1 μM cyclosporin A (CsA) and are expressed as nmol of AFC released/h/mg of protein. [a]$P < 0.05$ vs. control; [b]$P < 0.05$ vs. 24 h eKHB ($n = 4$ to 6).

3.3. Effect of preservation solution and cyclosporin A addition on cell necrosis and apoptosis after CPWR on the model of the primary culture of rat hepatocytes

It has been previously demonstrated that cultured rat hepatocytes subjected to CPWR in vitro mimick the two stages of cell death observed in vivo; a slight decrease in viability as a function of simple cold storage time, which is greatly exacerbated by warm reperfusion (Serrar and Haddad, 1997; Serrar et al., 1999). However, the type of cell death, hence the extent of necrosis and apoptosis after CPWR, has not been examined. We therefore studied these parameters in primary cultures of hepatocytes by means of fluorescence microscopy.

Table 3 presents the data on the percentage of necrotic (propidium iodide staining) and advanced apoptotic cells (chromatin condensation/nuclear fragmentation stained with Hoechst 33342) after 24-h cold preservation followed by a 1-h warm culture period (reperfusion). In cells preserved hypothermically for 24 h followed by warm culture, the number of necrotic cells increased to 12.9% whatever the preservation solution used, in comparison to 7.6% for unstored controls. Very few advanced apoptotic hepatocytes could be observed in unstored control cells put in short-term culture. On the other hand, cell death by apoptosis progressed to 11.5% in cells cold-preserved in eKHB for 24 h. Hepatocytes stored hypothermically in iKHB exhibited a similar apoptotic rate of 9.9%.

In order to evaluate the impact of PTP opening on cell death, cyclosporin A was added to the preservation and to the culture media at five different concentrations between 0.1 and 5 μM. These concentrations are below the threshold of cyclosporin A toxicity for cells (Grub et al., 2000). Cyclosporin A had no effect on cell viability measured after simple cold storage for 24 h (not shown). Likewise, it did not affect necrosis measured after warm reperfusion. However, a dose-dependent inhibition of apoptosis by cyclosporin A was observed in cells subjected to 24-h CPWR, again irrespective of the preservation solution used (Fig. 4).

Table 3
Percentage of cell death in primary culture of hepatocytes estimated after 0 h or 24-h CPWR in eKHB or iKHB

Time of preservation	0 h (control)	24 h	
Preservation solution	–	eKHB	iKHB
Necrosis	7.58 ± 2.81	12.93 ± 3.36[a]	12.92 ± 5.13[a]
Chromatin condensation/ nuclear fragmentation	0.75 ± 0.40	11.49 ± 1.08[a]	9.93 ± 1.10[a]

Necrosis and advanced stages of chromatin condensation/nuclear fragmentation (apoptosis) were estimated by fluorescence microscopy using the fluorochromes propidium iodide and Hoechst 33342, respectively. A two-way analysis of variance confirmed that cold preservation time had a significant effect on necrosis and apoptosis. On the other hand, preservation solution type had no significant effect, and the analysis revealed no interaction between the two parameters. ($n = 10$ to 13).

[a] $P < 0.05$ vs. 0-h preservation.

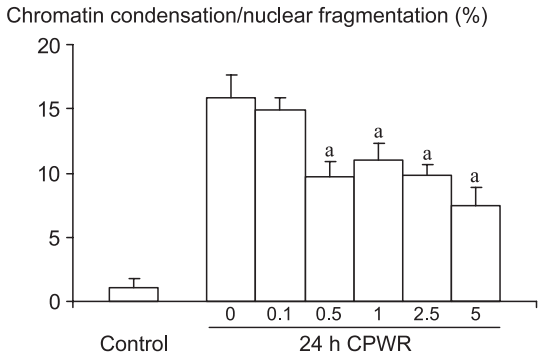

Fig. 4. Percentage of cell death by apoptosis (chromatin condensation/nuclear fragmentation) after 24-h CPWR. Chromatin condensation/nuclear fragmentation was estimated by fluorescence microscopy using Hoechst 33342 (5 nM) staining and was determined in the absence of cyclosporin A (0) or in the presence of five different concentrations of cyclosporin A: 0.1, 0.5, 1, 2.5, 5 μM. [a]$P < 0.05$ vs. control (0) ($n = 4$ to 6).

In order to ensure that this effect was restricted to apoptosis, we studied LDH release by hepatocytes, which is characteristic of cell death by necrosis (Fig. 5). During simple cold storage in eKHB and iKHB, 5.78 ± 1.34% and 6.38 ± 0.47% of total LDH were released, respectively. As expected, this effect was amplified after reperfusion (warm culture). Again, results confirm that the two preservation media were equivalent in terms of cell viability, estimated here by another parameter reflecting deterioration of plasma membrane integrity. The addition of 1 μM cyclosporin A to the cold preservation and warm culture media did not modify the LDH data, thereby confirming aforementioned results on necrosis obtained with propidium iodide staining (results not illustrated).

4. Discussion

Liver graft involves a period of cold ischemia followed by a warm reperfusion that is deleterious for hepatocytes and can lead to organ dysfunctions (Clavien et al., 1992). At the mitochondrial level, there is a good agreement to consider PTP as a crucial event leading to cell death by

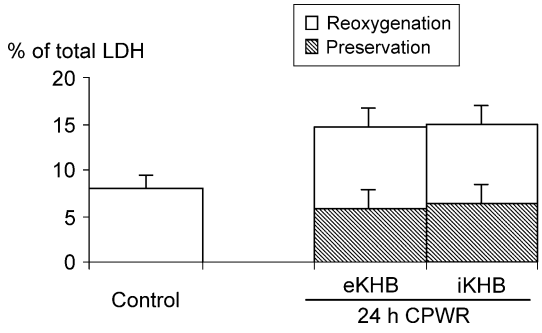

Fig. 5. Percentage of total hepatocellular lactate dehydrogenase (LDH) released after 24-h simple cold preservation in eKHB or iKHB (hatched bars) and after warm reperfusion (white bars) ($n = 6$ to 8).

necrosis or by apoptosis (Crompton, 1999; Lemasters et al., 1999). Thus, the aim of this study was to test the hypothesis that inhibition of PTP opening could improve rat liver mitochondrial and hepatocellular parameters after 24-h cold preservation followed by a warm reperfusion in Krebs-Henseleit Buffer.

Our study shows that the mitochondrial respiratory chain is highly affected after 24-h CPWR and that the capacity of ATP production is decreased, confirming the data of Sammut et al. (1998). Moreover, the lowered capacity of mitochondria to maintain the electrochemical gradient suggests that the mitochondrial membrane is damaged and thus allows the return of protons inside the mitochondria by another way than complex V. These alterations are at least in part due to the occurrence of PTP. Indeed, it is well established that PTP opening renders the inner membrane permeable to ions and solutes and thus induces swelling (Zoratti and Szabo, 1995). Our results show that mitochondria subjected to CPWR swell spontaneously and that this swelling is totally inhibited by addition of 1 μM cyclosporin A, a specific inhibitor of the PTP, in the preservation and in the reperfusion media. Moreover, the presence of cyclosporin A restored completely the mitochondrial membrane potential, limited the decrease in RCR and P/O as well as the loss of cellular ATP content showing that inhibition of the permeability transition protects mitochondrial functions and cellular energetic charge after 24-h CPWR.

These data are in line with those of Leducq et al. (1998) who demonstrated that cyclosporin A inhibited the rapid decrease in liver ATP content during the transition from hypothermia to normothermia. They also confirm the protective effect of cyclosporin A towards ATP decrease in other models of normothermic ischemia–reperfusion (Kurokawa et al., 1992; Sakr and Abdel-Aal, 1996).

Protection of cellular energetic charge is particularly interesting as Metzger and Lauterburg (1988) and He et al. (2003) have shown that the recovery of ATP concentration in liver after CPWR is well correlated with organ viability. Indeed, ATP is the energetic substrate of cells and the lack of this energetic source leads to cell death by necrosis (Lemasters et al., 1999). So, preservation of ATP content by inhibition of PTP could protect the organ from necrosis, allowing the execution stage of apoptosis to proceed.

It has been shown that PTP opening induces cytochrome *c* release and that it is followed by the activation of caspases that execute apoptosis (Borutaite et al., 2003; Thress et al., 1999). Our study reveals that the apoptotic process is initiated after CPWR and thus likely contributes to the ensuing liver damage. Indeed, the inhibition of PTP opening by cyclosporin A limited the activation of biochemical markers of liver apoptosis in our experimental conditions, namely cytochrome *c* release and concomitant caspase activation. Since parenchymal cells carry out the majority of hepatic functions (such as metabolism and bile secretion) and are sensitive to CPWR (Kukan and Haddad, 2001), we have examined in greater detail the mode of cell death in purified hepatocytes subjected to CPWR in short-term cultures (Elimadi and Haddad, 2001; Serrar and Haddad, 1997). The results show that cell viability is highly dependent on the duration of the cold preservation period preceding warm reperfusion. In control cells (0-h preservation), cell death was limited, but occurred mainly by necrosis. If cells were cold-preserved 24 h before this culture, cell death by necrosis increased and advanced stages of cell death by apoptosis (chromatin condensation/nuclear fragmentation) appeared.

Addition of increasing concentrations of cyclosporin A limits in a dose-dependent manner cell death by apoptosis thereby confirming the protective effect observed on cytochrome *c* release and caspase activation using the ex vivo approach. However, it did not limit cell death by necrosis evaluated by propidium iodide staining or by LDH release. However, we have shown in the first part of this study that cyclosporin A limited the loss of liver ATP content. Consequently, this should have protected from cell death by necrosis. We can suppose that this partial resumption of hepatocytes metabolism was not sufficient to afford protection against necrosis.

It should be noted that other proteins as calcineurin, cyclophilins and multidrug resistance protein are also targeted by cyclosporin A (Lemasters et al., 1998; Bernardi et al., 2001) and our data cannot exclude a combinatorial protective effect by cyclosporin A through the inhibition of PTP and the effect on these proteins.

The two preservation solutions tested were equivalent for the maintenance of cell viability. This observation is in accordance with the previous study of Serrar et al. (1999) where sodium–lactobionate–sucrose extracellular medium was compared to University of Wisconsin intracellular solution. The iKHB medium did not afford any improvement of mitochondrial parameters in the ex vivo model. The results even show a slight alteration of these parameters. Gibelin et al. (2002) have studied mitochondrial lesions after 24-h cold preservation followed by 90-min warm reperfusion of rat liver by means of histological examination. They demonstrated that the lesions were more important in an intracellular preservation medium rather than in an extracellular one. Several other publications mentioned the equal quality or the superiority of extracellular solutions on other models like the rat lung ischemia–reperfusion (Chiang, 2001) or the Langendorff-perfused heart (Drinkwater et al., 1995). Thus, preservation solutions of intracellular-like nature do not afford any protective advantage over extracellular ones.

In conclusion, mitochondrial PTP opening is clearly involved in CPWR lesions. These lesions are generated essentially after warm reperfusion, and are amplified as cold preservation time is lengthened. PTP inhibition protects the mitochondrial respiratory chain allowing better recovery of energetic charge and limits the activation of cell death by apoptosis in both the organ and the isolated liver cells. However, PTP is not the only element responsible of such damages. Therefore, inhibition of PTP provides only a

partial protection of the organ. Nevertheless mitochondrial protection should be considered as a promising adjunct therapeutic approach to improve the primary function of the grafted liver after transplantation.

References

Bernardi, P., Scorrano, L., Colonna, R., Petronilli, V., Di Lisa, F., 1999. Mitochondria and cell death. Mechanistic aspects and methodological issues. Eur. J. Biochem. 264, 687–701.

Bernardi, P., Petronilli, V., Di Lisa, F., Forte, M., 2001. A mitochondrial perspective on cell death. Trends Biochem. Sci. 26, 112–117.

Borutaite, V., Jekabsone, A., Morkuniene, R., Brown, G.C., 2003. Inhibition of mitochondrial permeability transition prevents mitochondrial dysfunction, cytochrome *c* release and apoptosis induced by heart ischemia. J. Mol. Cell. Cardiol. 35, 357–366.

Broekemeier, K.M., Dempsey, M.E., Pfeiffer, D.R., 1989. Cyclosporin A is a potent inhibitor of the inner membrane permeability transition in liver mitochondria. J. Biol. Chem. 264, 7826–7830.

Chiang, C.H., 2001. Comparison of effectiveness of intracellular and extracellular preservation solution on attenuation in ischemic–reperfusion lung injury in rats. J. Formos. Med. Assoc. 100, 233–239.

Clavien, P.A., Harvey, P.R.C., Strasberg, S.M., 1992. Preservation and reperfusion injuries in liver allografts. Transplantation 53, 957–978.

Crompton, M., 1999. The mitochondrial permeability transition pore and its role in cell death. Biochem. J. 341, 233–249.

Drinkwater Jr., D.C., Ziv, E.T., Laks, H., Lee, J.R., Bhuta, S., Rudis, E., Chang, P.,, 1995. Extracellular and standard University of Wisconsin solutions provide equivalent preservation of myocardial function. J. Thorac. Cardiovasc. Surg. 110, 738–745.

Elimadi, A., Haddad, P.S., 2001. Cold preservation–warm reoxygenation increases hepatocyte steady-state Ca^{2+} and response to Ca^{2+}-mobilizing agonist. Am. J. Physiol.: Gasterointest. Liver Physiol. 281, G809–G815.

Elimadi, A., Morin, D., Sapena, R., Chauvet-Monges, A.M., Crevat, A., Tillement, J.P., 1997. Comparison of the effects of cyclosporin A and trimetazidine on Ca^{2+}-dependent mitochondrial swelling. Fundam. Clin. Pharmacol. 11, 440–447.

Emaus, R.K., Grunwald, R., Lemasters, J.J., 1986. Rhodamine 123 as a probe of transmembrane potential in isolated rat-liver mitochondria: spectral and metabolic properties. Biochim. Biophys. Acta 850, 436–448.

Gibelin, H., Hauet, T., Eugene, M., Essique, D., Levillain, P., Carretier, M., 2002. Beneficial effects of addition of polyethylene glycol to extracellular type solutions to minimize ischemia/reperfusion injuries in an isolated-perfused rat liver model. Transplant. Proc. 34, 768.

Grub, S., Persohn, E., Trommer, W.E., Wolf, A., 2000. Mechanisms of cyclosporine A-induced apoptosis in rat hepatocyte primary cultures. Toxicol. Appl. Pharmacol. 163, 209–220.

Hackenbrock, C.R., 1968. Chemical and physical fixation of isolated mitochondria in low-energy and high-energy states. Proc. Natl. Acad. Sci. U. S. A. 61, 598–605.

He, X.S., Ma, Y., Chen, G.H., Zhang, J.X., Wu, J.L., Liang, Y.J., et al., 2003. The influence of warm ischemia injury on viability and posttransplantative outcome of liver graft from non-heart-beating donor in rats. Zhonghua Yixue Zazhi 83, 1236–1240.

Johnson, D., Lardy, H.A., 1967. Isolation of liver and kidney mitochondria. In: Estabook, R.W., Pullman, M. (Ed.), Methods in Enzymology, vol. 10. Academic Press, New York, pp. 94–96.

Kim, J.S., He, L., Qian, T., Lemasters, J.J., 2003. Role of the mitochondrial permeability transition in apoptotic and necrotic death after ischemia/reperfusion injury to hepatocytes. Curr. Mol. Med. 3, 527–535.

Krasko, A., Deshpande, K., Bonvino, S., 2003. Liver failure, transplantation, and critical care. Crit. Care Clin. 19, 155–183.

Kukan, M., Haddad, P.S., 2001. Role of hepatocytes and bile duct cells in preservation–reperfusion injury of liver grafts. Liver Transplant. 7, 381–400.

Kurokawa, T., Kobayashi, H., Nonami, T., Harada, A., Nakao, A., Sugiyama, S., et al., 1992. Beneficial effects of cyclosporine on post-ischemic liver injury in rats. Transplantation 53, 308–311.

Leducq, N., Delmas-Beauvieux, M.C., Bourdel-Marchasson, I., Dufour, S., Gallis, J.L., Canioni, P., Diolez, P., 1998. Mitochondrial permeability transition during hypothermic to normothermic reperfusion in rat liver demonstrated by the protective effect of cyclosporin A. Biochem. J. 336, 501–506.

Lemasters, J.J., Nieminen, A.L., Qian, T., Trost, L.C., Elmore, S.P., Nishimura, Y., et al., 1998. The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. Biochim. Biophys. Acta 1366, 177–196.

Lemasters, J.J., Qian, T., Bradham, C.A., Brenner, D.A., Cascio, W.E., Trost, L.C., et al., 1999. Mitochondrial dysfunction in the pathogenesis of necrotic and apoptotic cell death. J. Bioenerg. Biomembranes 31, 305–319.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275.

Metzger, J., Lauterburg, B.H., 1988. Postischemic ATP levels predict hepatic function 24 hours following ischemia in the rat. Experientia 44, 455–457.

Parone, P.A., James, D., Martinou, J.C., 2002. Mitochondria: regulating the inevitable. Biochimie 84, 105–111.

Sakr, M.F., Abdel-Aal, A.N., 1996. Protective effect of cyclosporine A (CyA) against the hepatic injury associated with ischemia and reperfusion. Int. Surg. 81, 180–183.

Sammut, I.A., Thorniley, M.S., Simpkin, S., Fuller, B.J., Bates, T.E., Green, C.J., 1998. Impairment of hepatic mitochondrial respiratory function following storage and orthotopic transplantation of rat livers. Cryobiology 36, 49–60.

Serrar, H., Haddad, P., 1997. Effects of cold preservation and rewarming on rat liver cell volume regulation and concentrative amino acid uptake. Gastroenterology 112, 1344–1353.

Serrar, H., Musallam, L., Haddad, P., 1999. Comparative effects of UW and SLS solutions on concentrative proline uptake in cold preserved rat hepatocytes. Therapie 54, 601–606.

Strasberg, S.M., Howard, T.K., Molmenti, E.P., Hertl, M., 1994. Selecting the donor liver: risk factors for poor function after orthotopic liver transplantation. Hepatology 20, 829–838.

Thress, K., Kornbluth, S., Smith, J.J., 1999. Mitochondria at the crossroad of apoptotic cell death. J. Bioenerg. Biomembranes 31, 321–326.

Zoratti, M., Szabo, I., 1995. The mitochondrial permeability transition. Biochim. Biophys. Acta 1241, 139–176.

Zou, H., Li, Y., Liu, X., Wang, X., 1999. An APAF-1.cytochrome *c* multimeric complex is a functional apoptosome that activates procaspase-9. J. Biol. Chem. 274, 11549–11556.