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# Resveratrol protects against cold ischemia–warm reoxygenation-induced damages to mitochondria and cells in rat liver

Catherine Plin [a,b], Jean-Paul Tillement [a,b], Alain Berdeaux [a,b], Didier Morin [a,b,*]

[a] *INSERM, U660, Créteil, F-94010 France*
[b] *Université Paris XII, Créteil, F-94010 France*

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**Abstract**

Ischemia–reperfusion is a critical event in the development of primary graft dysfunctions after liver transplantations. Ischemia–reperfusion causes cell injuries which are related to the successive cold preservation–warm reperfusion (CPWR) periods required by the graft. Recent evidences suggest that oxidative stress plays an important role in the development of these injuries and that mitochondrial dysfunctions are involved. The purpose of this study was to investigate the effect of the natural phytoalexin resveratrol on the prevention of liver injuries induced by 40-h cold preservation followed by a warm reperfusion. CPWR induced liver mitochondrial and cellular damages as attested by the increase in lipid peroxidation of liver membranes, the alteration of oxidative phosphorylation parameters, mitochondrial swelling and the activation of the cellular markers of necrosis and apoptosis, i.e., lactate dehydrogenase (LDH) leakage, mitochondrial cytochrome *c* release and caspase activation.

Resveratrol inhibits lipid peroxidation and protects mitochondrial functions. It improves respiratory chain activity and prevents opening of the permeability transition pore, allowing better recovery of ATP energetic charge. Resveratrol also limits the activation of the cellular markers of necrosis and apoptosis. These protective effects could be related to the antioxidant properties of the drug but also to its membrane-stabilizing activity. Indeed, further experiments demonstrate that resveratrol is able to prevent the release of cytochrome *c* caused by oxygen deprivation in isolated liver mitochondria. These data demonstrate that resveratrol ameliorates the liver injury induced by CPWR and appears as a promising drug to improve the primary function of the grafted liver after transplantation.


*Keywords:* Resvaratrol; Rat liver; Mitochondria; Antioxidant; Permeability transition pore; Reactive oxygen species; Ischemia–reperfusion

## 1. Introduction

Orthotopic liver transplantation has become an established therapeutic regimen for end-stage liver diseases, but despite the success of this new therapy, several problems still remain. A major problem is the functional and structural damages caused to endothelial and hepatic liver cells by the successive cold preservation–warm reperfusion (CPWR) periods required by the graft. These ischemia–reperfusion injuries (Clavien et al., 1992) can result in primary graft dysfunction (Deschenes et al., 1998) or non-function (Strasberg et al., 1994), which are one of the most important causes of transplantation failure.

There are increasing amount of recent evidences that mitochondria are involved in the molecular events leading to the tissue damage occurring during CPWR. First, it has been shown that mitochondria are involved in cell death by necrosis and by apoptosis, via the opening of the permeability transition pore (PTP) (Lemasters et al., 2002; Rauen et al., 2003; Plin et al., 2004). Second, mitochondria are an important source of reactive oxygen species, particularly after reperfusion, when its anti-oxidant system is exhausted (Gonzáles-Flecha et al., 1993; Jassem et al., 2002). These reactive oxygen species are able to induce PTP opening (Kowaltowski et al., 2001), to alter mitochondrial membranes, proteins or DNA (Richter et al., 1995) and have been shown to mediate apoptosis during cold preservation (Rauen et al., 1999). Thus, it is essential to protect mitochondria from oxidative stress generated during CPWR.

*Trans*-resveratrol (*trans*-3,5,4′-trihydroxystilbene) (Fig. 1) is a natural phytoalexin found in a wide variety of plant species

* Corresponding author. INSERM U660, Faculté de Médecine de Paris XII, 8 rue du Général Sarrail, F-94010, Créteil, France. Tel./fax: +33 1 49 81 36 61.
*E-mail address:* didier.morin@im3.inserm.fr (D. Morin).

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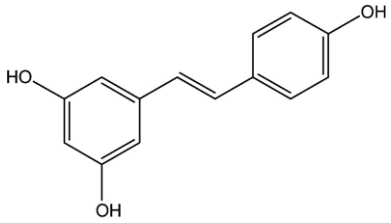


Fig. 1. Chemical structure of *trans*-resveratrol.

including grapes. It is synthesized in the seeds and skin of grapes as a response to environmental stress or fungal infection. It constitutes one of the components of red wine and would be responsible for the reduced risk of coronary heart disease associated with moderate wine consumption (Gaziano et al., 1993; Goldberg et al., 1995). Among its numerous properties (for review, see Granados-Soto, 2003), like many other phenolic compounds, resveratrol has anti-oxidant activities through a hydrogen-electron donation from its hydroxyl groups (Tadolini et al., 2000; López-Vélez et al., 2003). The consequence is a capacity to scavenge reactive oxygen species, a protective effect against DNA damage and lipid peroxidation in cell membrane (Leonard et al., 2003).

Resveratrol has shown beneficial effects on several experimental models of ischemia–reperfusion (Ray et al., 1999; Hung et al., 2000; Giovannini et al., 2001), and these effects appear to be, at least in part, related to its antioxidant properties (Hung et al., 2002). However, the protective properties of resveratrol have never been used to try to improve the damages induced by CPWR in liver. Accordingly, this study was designed to evaluate the possible role of resveratrol in protecting mitochondrial and cellular functions during liver CPWR.

## 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). Animals were housed in small groups on a 12-h:12-h light/dark cycle and given food and water ad libitum.

### 2.2. Liver isolation and CPWR procedure

Rats were anaesthetized with sodium pentobarbital (65 mg/kg), the abdomen was opened and the portal vein was cannulated with a catheter 16 GA. The liver was rinsed (30 ml/min) with 400 ml of warm Krebs–Henseleit Buffer pH 7.2–7.4 gassed with $O_2$: $CO_2$ (95%:5%). It was cold-preserved 40 h in the same solution and rewarmed gradually from 4 °C 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 Krebs–Henseleit buffer in a recirculating mode. *Trans*-resveratrol was dissolved in dimethylformamide and introduced at the concentration of 1 μM both in the preservation and reperfusion media. The final percentage of dimethylformamide in the medium was less than 0.1%. When resveratrol was omitted, the same quantity of dimethylformamide was added in the preservation and reperfusion media.

Control livers were taken from decapitated rats and mitochondria immediately isolated as described below.

### 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 a medium containing 10 mM Tris, 250 mM Sucrose, 1 mM EGTA, pH 7.2 at 4 °C. Cellular fragments were precipitated at 600×*g* for 10 min. The supernatant was centrifuged at 15,000×*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×*g* was centrifuged at 100,000×*g* for 20 min to precipitate microsomes and to obtain cytosolic fractions of cells which were 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 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 and oxidative phosphorylation were initiated by addition of 6 mM succinate and 0.2 mM ADP, respectively. $O_2$ consumption recordings allowed the calculation of the respiratory control ratio corresponding to the ratio between the state 3 (ADP-stimulated) and state 4 (resting) respiration rate. The ADP/oxygen ratio was also calculated as the quotient of ADP consumed over oxygen used in state 3 respiration.

### 2.5. Determination of PTP opening

PTP opening was assessed by the measure of mitochondrial swelling. Swelling was monitored 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. In order to measure spontaneous swelling, mitochondria (1 mg/ml) were added in sample and reference cuvettes and 6 mM succinate was added to the sample cuvette only. 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.

### 2.6. Determination of intracellular ATP concentrations

ATP concentrations were measured enzymatically by means of an ATP-detection kit (Sigma Diagnostics) on thawed pieces

of liver: 500 mg of liver was homogenized with a Teflon Potter homogenizer in 3 ml of NaCl 0.9%. The mixture was then filtered, the proteins were precipitated by the addition of trichloracetic acid and the suspension was centrifuged at 8000×*g* for 5 min. ATP was measured in the supernatant by means of an enzymatic system including glyceraldehyde 3-phosphate dehydrogenase and 3-phosphoglycerate kinase in the presence of 3-phosphoglycerate according to the following reactions:

$$\mathrm{ATP} + 3-\mathrm{phosphoglycerate} \rightarrow \mathrm{ADP} + \mathrm{glyceraldehyde}1,3-\mathrm{diphosphate} \quad (1)$$

$$\mathrm{glyceraldehyde}1,3-\mathrm{diphosphate} + \mathrm{NADH} \rightarrow \mathrm{glyceraldehyde}3-\mathrm{phosphate} + \mathrm{NAD}. \quad (2)$$

The transformation of NADH (which is proportional to ATP content) was quantified by spectrophometry at 340 nm.

### 2.7. 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 (pH 7.5) containing 25 mM Tris, 5 mM $MgCl_2$, 1 mM EGTA and 50 μl of protease inhibitor cocktail (Sigma, product number P8340). Cellular fragments were precipitated at 600×*g* for 10 min. The supernatant was centrifuged at 100,000×*g* for 20 min. After addition of 2 mM dithiothreitol, the supernatant was stored at − 80 °C. 30 μg protein were 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 *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarine (Ac-DEVD-AFC, Tebu, Le Perray-en-Yvelines) or *N*-acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarine (Ac-LEHD-AFC, Tebu, Le Perray-en-Yvelines), the substrates of caspases 3 and 9, respectively. 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 nanomoles of AFC released per hour per milligram of protein.

### 2.8. Anoxia-induced cytochrome c release from mitochondria

Isolated liver mitochondria (4 mg/ml) were incubated 30 min in a medium containing 250 mM sucrose, 10 mM Tris, 1 mM EGTA, 1 μM rotenone, 6 mM succinate and submitted or not to oxygen deprivation. Oxygen deprivation was induced by nitrogen purging. At the end of the incubation, the mitochondrial suspension was centrifuged and the supernatant was analysed by Western blot using a monoclonal anti-mouse cytochrome *c* antibody.

### 2.9. Determination of cytochrome c content

Cytochrome *c* content was measured on thawed cytosolic fraction or in the supernatant of mitochondrial suspensions (in vitro experiments) by Western blot analysis: 25 μg proteins or 20 μl of reaction buffer were loaded onto a 15% sodium dodecyl sulfate–polyacrylamide gel. After fractionation, the proteins were electroblotted onto a polyvinylidene 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 a horseradish peroxidase-linked whole secondary antibody (NA931-1ML, Amersham Biosciences, Orsay, France). For detection, the membrane was incubated with peroxidase-linked antibody (ECL Plus Western Blotting Detection Reagents, Amersham Biosciences, Orsay, France) for 5 min and revealed on photographic film.

### 2.10. Assays of lipid peroxidation

Lipid peroxidation was assessed as the generation of thiobarbituric acid-reactive substances, i.e., lipid peroxides, according to Ligeret et al. (2004). Briefly, mitochondria (0.2 mg/ml) were suspended in NaCl (0.9%) supplemented with different concentrations of resveratrol in a total volume of 1 ml and were incubated for 10 min at 37 °C. Following the addition of 100 μl of a mixture containing $FeCl_2$ (500 μM)/$FeCl_3$ (1500 μM), mitochondrial suspensions were incubated for 30 min at 37 °C. After the addition of 1 ml trichloracetic acid (3%), mitochondrial suspensions were centrifuged at 20 °C for 15 min at 3000 rpm and 1 ml of each supernatant was added to 1 ml of thiobarbituric acid (1%) and incubated for 30 min at 95 °C. After recooling on ice, the generation of thiobarbituric acid-reactive substances was determined by measuring the absorbance at 530 nm.

In CPWR experiments, thiobarbituric acid-reactive substance levels were determined in the reperfusion medium at the end of the warm reperfusion. Thiobarbituric acid-reactive substance levels were expressed as nanomoles of malondialdehyde equivalents.

### 2.11. Determination of lactate dehydrogenase release

LDH release was measured in the perfusate during warm reperfusion. It was quantified enzymatically at 340 nm by measuring the decrease in NADH which is transformed in $NAD^+$ in presence of LDH and pyruvic acid. Measures were performed in 1.5 ml of Tris 25 mM buffer including 22.7 mM pyruvic acid and 0.167 mM NADH. The reaction was started by addition of a sample (100 μl) of reperfusion medium.

### 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. Resveratrol inhibits lipid peroxidation

In a first step, we checked the antioxidant properties of resveratrol on isolated liver mitochondria from control animals. Lipid peroxidation was induced by the addition of a mixture of $Fe^{2+}/Fe^{3+}$ in the absence or in the presence of increasing concentrations of resveratrol. Resveratrol inhibited lipid peroxidation in a concentration-dependent manner with an $IC_{50}$ value of 2.6 μM (Fig. 2A). Interestingly, these antioxidant properties were observed ex vivo in the rat liver model after 40 h cold preservation followed by 30 min warm reperfusion. Resveratrol (1 μM) inhibited lipid peroxidation during warm reperfusion as attested by the decrease in thiobarbituric acid-reactive substances released in the reperfusion medium (Fig. 2B).

### 3.2. Resveratrol improves mitochondrial oxidative phosphorylation parameters

We studied the evolution of mitochondrial parameters after 40 h cold preservation followed by 30 min warm reperfusion in the absence or in the presence of 1 μM resveratrol. CPWR hugely altered oxidative phosphorylation parameters as demonstrated by the extensive decrease in state 3 respiration rate, respiratory control ratio and ADP/oxygen values (Table 1). The presence of resveratrol in the preservation and the reperfusion media limited the drop in respiratory control ratio by both increasing the state 3 respiration rate and slightly lowering the state 4 respiration rate, although the latter was not statistically significant.

Similarly, resveratrol improved the capacity of mitochondria to produce ATP as evidenced by the increase in ADP/oxygen value and allowed to preserve, at least partially, the hepatic energy charge. Indeed, ATP liver content was decreased by more than half after CPWR and resveratrol limited ATP depletion to 32% (Table 1).

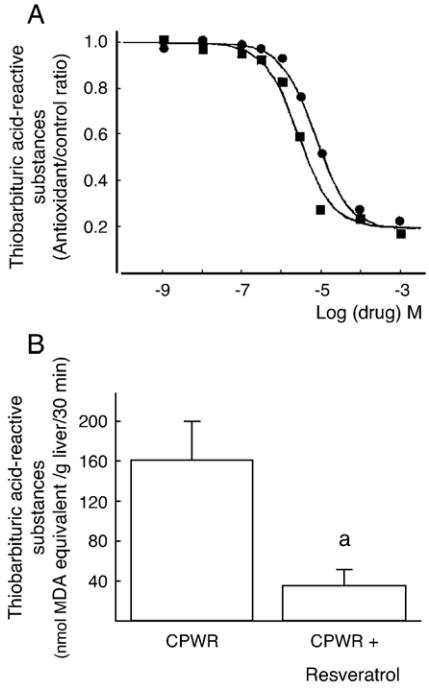


Fig. 2. Resveratrol inhibits lipid peroxidation. (A) Lipid peroxidation was induced by a mixture of $Fe^{2+}/Fe^{3+}$ on mitochondria isolated from control liver. The effect of resveratrol (■, $IC_{50}$=2.60 μM) was compared to that of butylhydroxytoluene (●, $IC_{50}$=7.60 μM). Control value (in the absence of drug) corresponds to 80 nmol malondialdehyde equivalent/g liver/30 min. (B) Thiobarbituric acid-reactive substances generation was measured in the perfusion medium at the end of the CPWR experiment. Data represent means of 4 experiments±S.E.M. [a]$P<0.01$ vs. CPWR.

Table 1
Effect of 1 μM resveratrol on oxidative phosphorylation parameters of mitochondria and cellular energetic charge of livers subjected to 40 h cold preservation followed by 30 min warm reperfusion

| | Control | CPWR | CPWR+1 μM resveratrol |
|---|---|---|---|
| Mitochondrial parameters | | | |
| $V_3$ (nmol/mg/min) | 64.5±2.9 | 38.5±2.9 * | 47.5±3.3 ** |
| $V_4$ (nmol/mg/min) | 15.4±1.0 | 20.5±1.5 * | 19.0±1.0 |
| Respiratory control ratio | 4.13±0.18 | 2.01±0.17 * | 2.53±0.12 ** |
| ADP/oxygen | 1.55±0.08 | 1.11±0.06 * | 1.27±0.04 ** |
| Percentage of ATP content | 100±6.2% | 46.6±3.3% * | 68.3±6.7% ** |

Control: mitochondria isolated from liver not subjected to CPWR. Control ATP value (100%) corresponds to 2.4 μmol/g liver. Each value represents the mean ±S.E.M. of 6–7 experiments performed in triplicate.
* $P<0.05$ vs. control.
** $P<0.05$ vs. CPWR.

### 3.3. Resveratrol limits the activation of the cellular markers of necrosis and apoptosis

It is now well established that CPWR can induce cell death by both necrosis and apoptosis (Huet et al., 2004). Thus, we investigated the effect of resveratrol treatment on biochemical markers of necrosis–namely, LDH leakage from cellular membrane–and apoptosis–namely, release of cytochrome *c* from mitochondrial inner membrane and subsequent activation of caspases 9 and 3. CPWR induced a large release of LDH in the reperfusion medium (Fig. 3) which was inhibited by 46.7% in the presence of 1 μM resveratrol.

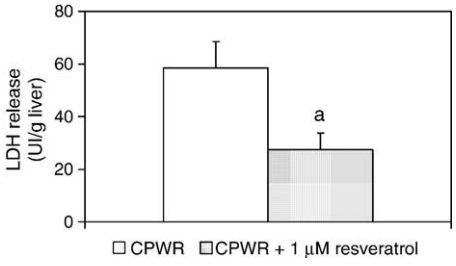


Fig. 3. Activity of lactate dehydrogenase (LDH) released in reperfusion media after CPWR in the absence (open bar) or in the presence of 1 μM resveratrol (dotted bar). Activity of LDH was estimated by enzymatic assay performed on a sample of 100 μl of reperfusion media at the end of CPWR ($n$=4). [a]$P<0.05$ vs. CPWR.

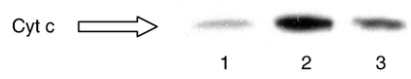


Fig. 4. Effect of resveratrol on cytochrome *c* (Cyt c) release into cytosol during CPWR in rat liver. Cytosolic fractions were obtained as described in Materials and methods and cytochrome *c* release was measured by Western blot analysis in cytosolic fractions. Western blots are representative of four independent experiments with similar results. Lane 1: control; lanes 2 and 3: cytochrome *c* released after 40 h CPWR in the absence and in the presence of 1 μM resveratrol, respectively.

In the same way, CPWR activated the biochemical markers which are characteristic of cell death by apoptosis. Indeed, CPWR induced the release of cytochrome *c* and the concomitant activation of caspase 9 and 3 (Figs. 4 and 5). Resveratrol limited cytochrome *c* release and this was related to a significant decrease in caspase 9 activation. Caspase-3 activity also tended to decrease but this effect did not reach significance.

Several mechanisms have been described to explain cytochrome *c* release from mitochondria (Desagher and Martinou, 2000). Among these mechanisms PTP opening is now considered to play a predominant role during ischemia–reperfusion (Crompton, 1999) and PTP opening has been involved in the cellular alterations caused by CPWR (Rauen et al., 2003; Plin et al., 2004). Thus, we investigated the possible effect of resveratrol on PTP opening which was evaluated by means of swelling experiments. When mitochondria isolated from livers after CPWR were energized with succinate, they swelled slightly (Fig. 6A, line b) and the swelling was due to the opening of the PTP since it was completely prevented by the addition to the incubation medium of 1 μM cyclosporin A (Fig. 6A, line c), the most potent inhibitor of PTP (Broekemeier et al., 1989).

This effect was not observed with mitochondria prepared from control animals (Fig. 6A, line a) and was completely inhibited when 1 μM resveratrol was present in the preservation and the perfusion media (Fig. 6A, line d) which contributed to the protective effect of resveratrol on cytochrome *c* release and caspase activation.

Moreover, when the phenomenon was amplified by the addition of 25 μM $Ca^{2+}$, the inhibiting effect of cyclosporin A persisted whereas resveratrol was inactive (Fig. 6B).

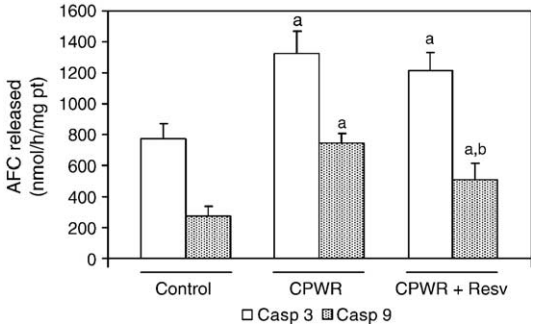


Fig. 5. Effect of resveratrol on activation of caspases 3 and 9 after CPWR. Caspase activities were studied after 40 h CPWR in the absence (CPWR) or in the presence of 1 μM of resveratrol (CPWR+Resv) in the incubation and the reperfusion medium. Caspase activities are expressed as nmol AFC released/h/mg of protein. $^{a}P<0.05$ vs. control; $^{b}P<0.05$ vs. CPWR.

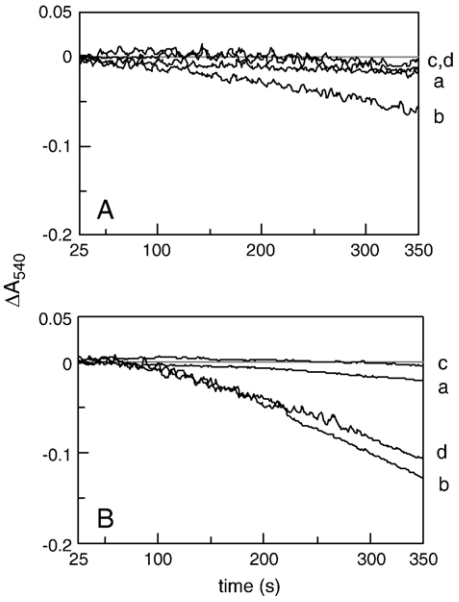


Fig. 6. Effect of resveratrol on mitochondrial swelling after 40 h CPWR. Mitochondria were isolated from livers subjected to CPWR and were incubated in phosphate buffer (250 mM sucrose, 5 mM $KH_2PO_4$, 1 μM rotenone, pH 7.2 at 25 °C). Mitochondrial swelling was monitored by measuring the change in absorbance of the suspension at 540 nm ($\Delta A_{540}$). Swelling was induced by the addition of 6 mM succinate (Panel A, spontaneous swelling), 6 mM succinate +25 μM $Ca^{2+}$ (Panel B). Line a: control mitochondria. Line b: mitochondria isolated from 40 h CPWR liver. Line c: mitochondria isolated from 40 h CPWR liver+1 μM cyclosporin A Line d: mitochondria isolated from 40 h CPWR liver +resveratrol.

These data indicate that resveratrol can limit PTP opening but this effect is not mediated by a direct interaction with PTP.

We recently demonstrated that at least two different mechanisms were responsible for cytochrome *c* release during ischemia–reperfusion (Morin et al., 2004). Indeed, the release of cytochrome *c* occurred successively independently and dependently of PTP opening during liver ischemia and reperfusion, respectively. During ischemia, cytochrome *c* release appeared to be triggered by oxygen deprivation by an unknown mechanism. Thus, we asked whether resveratrol would be able to interfere with this process.

To this end, we monitored the release of cytochrome *c* from isolated liver mitochondria submitted to 30 min anoxia. This time was shown to be sufficient to induce a relevant release of cytochrome *c*. Oxygen was exhausted from the

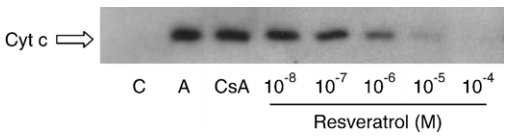


Fig. 7. Effect of resveratrol on anoxia-induced cytochrome *c* release from isolated mitochondria. Isolated liver mitochondria (4 mg/ml) were incubated 30 min under anoxia in the absence (A) or in the presence of either 1 μM cyclosporin A (CsA) or increasing concentrations of resveratrol. C: control experiment performed in normoxia. At the end of the incubation, the mitochondrial suspension was centrifuged and the supernatant was analysed by Western blot using a monoclonal antimouse cytochrome *c* antibody. The data shown are typical of four such experiments.

incubation medium through nitrogen purging. Oxygen deprivation promoted a release of cytochrome *c* which was not inhibited in the presence of cyclosporin A (Fig. 7) confirming our previous results (Morin et al., 2004). However, increasing concentrations of resveratrol clearly prevented cytochrome *c* release from mitochondria in a concentration-dependent manner. At $10^{-4}$ M, no cytochrome *c* could be detected in the incubation medium.

## 4. Discussion

Liver CPWR is an unavoidable process in liver transplantation and there is a general consensus that initial graft dysfunction is caused in great part by the so-called CPWR injury (Clavien et al., 1992). Thus, it is important to prevent the liver injury caused by CPWR. Reperfusion of ischemic tissues is known to generate reactive oxygen species which can lead to tissue injury (Zhao, 2004) through protein oxidation, lipid peroxidation and DNA damage. Mitochondria is a dominant site of reactive oxygen species production and mitochondrial damage and dysfunction is also a primary characteristic of reactive oxygen species-mediated cell death. Indeed, reactive oxygen species are also susceptible to attack mitochondrial membrane phospholipids, DNA and proteins (Richter et al., 1995) with the consequence of an uncoupled oxidative phoshorylation and an altered capacity of ATP synthesis (Du et al., 1998). Thus, a therapeutic option for limiting CPWR injury includes the inhibition of reactive oxygen species generation and the protection of mitochondrial functions during reperfusion.

In the present study, we showed that resveratrol protects mitochondria and cells against CPWR-induced injury. This protection is clearly seen on mitochondrial respiratory parameters. Resveratrol improved both mitochondrial coupling and ATP synthesis measured after CPWR as demonstrated by the increase in respiratory control ratio and ADP/oxygen values, respectively. These protective effects could be related to the antioxidant properties of the molecule which has been shown to be able to scavenge reactive oxygen species (Leonard et al., 2003) but also to inhibit their generation by limiting the activity of complex III of the respiratory chain during reperfusion (Zini et al., 2002). Our present data demonstrate that resveratrol protects mitochondrial membranes from oxidative stress in an in vitro experiment of peroxyl radical scavenging assay but also in an ex vivo model of CPWR.

The consequence of the improvement of the mitochondrial respiratory chain activity is an increase in the ATP energetic charge measured at the outcome of the reperfusion. This point is of particular interest since several studies have demonstrated that preservation of ATP energetic charge in liver during CPWR is well correlated with organ viability (Metzger and Lauterburg, 1988). Moreover, the lack of ATP leads to cell death by necrosis (Lemasters et al., 1999). Thus, the recovery of ATP obtained in the presence of resveratrol is a good sign of protection from cell death by necrosis. This is confirmed by the fact that resveratrol limits LDH leakage in the reperfusion media.

Another property of resveratrol can also contribute to the preservation of the energetic charge. Several studies have shown that micromolar concentrations of resveratrol can inhibit the $F_1F_0$-ATPase in isolated mitochondria (Zini et al., 1999; Kipp and Ramirez, 2001). The pharmacological inhibition of this enzyme could constitute a real advantage during ischemia since it is now well established that $F_1F_0$-ATPase is a major consumer rather than a producer of ATP during this period (Jennings et al., 1991; Grover et al., 2004). Indeed, mitochondria are believed to hydrolyse ATP when oxygen is lacking to maintain mitochondrial membrane potential.

The effect of resveratrol on cytochrome *c* release also favors oxidative phosphorylation. Indeed, resveratrol limits the leak of cytochrome *c* from the mitochondrial inner membrane and thus improves the electron flow between the complexes of the respiratory chain.

Different hypotheses could be advanced to explain this effect. The first one involves the inhibition of PTP opening which takes place during reperfusion of an organ (Morin et al., 2004; Halestrap et al., 2004). Our results support this hypothesis. Indeed, resveratrol, as well as the specific inhibitor of PTP, cyclosporin A, completely inhibited the spontaneous swelling of mitochondria subjected to CPWR. Resveratrol was ineffective when the swelling was amplified by the addition of a high concentration of $Ca^{2+}$ indicating that this effect did not result from a direct action on PTP, as observed with cyclosporin A. Resveratrol appears to decrease the sensitivity of mitochondria to PTP opening during CPWR and this might be related to the decrease in reactive oxygen species which are key inducers of PTP during reperfusion (Crompton, 1999).

Resveratrol might also prevent cytochrome *c* release during the cold preservation period and thus limit tissue injury during reperfusion. Indeed, resveratrol is able to prevent the release of cytochrome *c* caused by oxygen deprivation in a concentration-dependent manner. This effect is independent of PTP opening which does not occur during ischemia but could be due to the modulation of the mitochondrial membrane conformation. A similar effect was already observed in brain mitochondria (Zini et al., 2002) and was attributed to an effect of the drug on membrane fluidity. Indeed, resveratrol is a lipid-soluble molecule which can penetrate into mitochondrial membranes and could stabilize the association between cytochrome *c* and cardiolipine which is altered by lipid peroxidation (Shidoji et al., 1999).

Another hypothesis to explain the effect of resveratrol on cytochrome *c* release could be a direct interaction of the drug with the voltage-dependent anion channel which is located on the outer mitochondrial membrane and has been involved in the cytochrome *c* release (Shimizu et al., 1999). Indeed, resveratrol shows a distilbene chemical structure close to that of 4′4′-diisothiocyanatostilbene-2,2′-disulfonate a known inhibitor of the voltage-dependent anion channel (Shafir et al., 1998). This might provide a mechanism by which resveratrol could block the formation or opening of a cytochrome *c* release pathway. Additional experiments are in progress to verify this assumption.

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