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Role of the permeability transition pore in cytochrome *C* release from mitochondria during ischemia-reperfusion in rat liver

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Abstract

Ischemia and reperfusion cause mitochondrial dysfunctions that initiate the mitochondrial apoptosis pathway. They involve the release of cytochrome *C* and the activation of the caspase cascade but the mechanism(s) leading to cytochrome *C* release is(are) poorly understood. The aim of this study was to analyse the relation between cytochrome *C* release and the opening of the permeability transition pore (PTP) during in situ liver ischemia and reperfusion. Liver ischemia was induced for 30, 60 and 120 min and blood re-flow was subsequently restored for 30 and 180 min. Ischemia hugely altered mitochondrial functions, i.e., oxidative phosphorylation and membrane potential, and was accompanied by a time-dependent mitochondrial release of cytochrome *C* into the cytosol and by activations of capases-3 and -9. PTP opening was not observed during ischemia, as demonstrated by the absence of effect of an in vivo pre-treatment of rats with cyclosporin A (CsA), a potent PTP inhibitor. Cytochrome *C* release was due neither to a direct effect of caspases onto mitochondria nor to an interaction of Bax or Bid with the mitochondrial membrane but could be related to a direct effect of oxygen deprivation. In contrast, during reperfusion, CsA pre-treatment inhibits cytochrome *C* release, PTP opening and caspase activation. At this step, cytochrome *C* release is likely to occur as a consequence of PTP opening.

In conclusion, our study reveals that cytochrome *C* release, and thus the induction of the mitochondrial cell death pathway, occur successively independently and dependent on PTP opening during liver ischemia and reperfusion, respectively.

Keywords: Anoxia; Caspases; Cytochrome *C*; Cyclosporin A; Ischemia; Liver; Mitochondrial membrane permeability

1. Introduction

Ischemia-reperfusion injury is a major clinical problem after liver transplantation, major resections of the liver and hemorrhagic shock [1–4]. Until recently, sustained ischemia has been assumed to cause necrotic cell death. However, recent studies indicate that the reduction of oxygen supply may also lead to apoptosis in various organs including liver [5–8] and apoptosis has been suggested to be the first commitment to death following acute cerebral ischemia [8]. Thus, during ischemia followed by reperfusion, apoptosis or necrosis may simultaneously occur, probably depending on the severity of the insult and on the ability of the cell to maintain ATP synthesis. Indeed, ischemia decreases oxygen availability for mitochondria and leads to inhibition of mitochondrial respiration, resulting in a rapid depletion of ATP synthesis.

Mitochondria play a key role in the apoptotic process [9,10] and the mitochondrial pathway appears to be involved in liver ischemia-reperfusion injury [11]. Mitochondria release several apoptogenic factors such as cytochrome *C* [12], apoptosis inducing factor [13], smac [14] and caspases [15], which all activate the apoptotic cascade. Among these factors, cytochrome *C* plays a key role, since it activates caspase-9 in concert with ATP and the cytosolic apoptotic protease activating factor-1 [12,16,17].

The molecular mechanisms controlling the mitochondrial release of cytochrome *C* remain elusive. One hypothesis involves the opening of a large channel, the permeability transition pore (PTP), which is a multi-protein structure located at the level of the contact sites between the inner and the outer membranes of the

Abbreviations: CsA, cyclosporin A; RCR, respiratory control ratio; $\Delta\Psi$, mitochondrial membrane potential; PTP, permeability transition pore; ZVAD.fmk, Z-Val-Ala-Asp(Ome)-fluoromethylketone

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mitochondria [18,19]. PTP opening leads to mitochondrial swelling and to mitochondrial membrane potential disruption. It has been shown to occur during oxidative stress and is now considered a crucial event leading to cell injuries during ischemia-reperfusion [19]. However, there is evidence that cytochrome *C* release could occur independently of PTP opening with or without membrane potential disruption [20,21]. While this process has been widely studied in free cells or cultured cells models, little is known about mitochondrial cytochrome *C* release in situ, i.e., in animal submitted to ischemia-reperfusion. So, this study was designed to study the role of PTP opening in cytochrome *C* release during warm ischemia followed by a reperfusion period in rat liver.

2. Materials and methods

2.1. Animals and surgical procedure

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).

Wistar rats weighing 250–280 g were purchased from Janvier. They were housed in a room maintained under constant environmental conditions (temperature 22–25 °C and a constant cycle of 12 h light:12 h dark) and were acclimatised to the animal room before being used. They received standard pelted rat diet and water ad libitum.

Normothermic liver ischemia was performed as described by Nauta et al. [22]. Briefly, the abdomen was opened through a midline incision under general anesthesia. After sectioning the ligaments of the liver, hepatic normothermic ischemia was induced for increasing times by hilum clamping of the hepatic pedicles of segments I–V. In order to preclude the vascular congestion of the alimentary tract, the blood supply by the portal pedicles of segments VI and VII was not interrupted. During the period of ischemia, 0.5 ml of saline was given through the dorsal vein of the penis every 30 min to maintain hemodynamic stability and to replace losses due to portal stasis. Reperfusion was established by the removal of the clamp. The abdomen was closed in two layers with silk and the animals were returned to their cages. Rats were killed 0 (sham-operated), 30, 60 and 120 min after the start of the ischemia and at 30 and 180 min after reperfusion. Sham-operated animals underwent mobilization of the liver but had no clamp application. Samples of the liver lobes suffering ischemic injury were immediately removed and were used for the experiments described below.

2.2. Isolation of mitochondrial and cytosolic fractions

Rat liver mitochondria were isolated as described by Johnson and Lardy [23]. Liver samples were placed in medium containing 250 mM sucrose, 10 mM Tris and 1 mM of the chelator EGTA, pH 7.2 at 4 °C. The tissue was scissor minced and homogenized on ice using a Teflon Potter homogenizer. The homogenate was centrifuged at 600 × *g* for 10 min (Sorvall RC 28 S) to remove unbroken tissue and nuclei. The supernatant was centrifuged for 5 min at 15,000 × *g* to obtain the mitochondrial pellet. The latter was washed with the same medium and centrifuged at 15,000 × *g* for 5 min. The resulting mitochondrial pellet was washed with medium, from which the EGTA was omitted, and centrifuged for 5 min at 15,000 × *g* resulting in a final pellet containing approximately 70 mg protein/ml. The 15,000 × *g* supernatant was centrifuged at 100,000 × *g* for 30 min. The resulting supernatant was used as the particulate-free cytosolic fraction. The protein content was determined by the method of Lowry et al. [24]. The mitochondrial suspension was stored on ice until assaying for mitochondrial swelling, membrane potential, mitochondrial respiration and superoxide anion ($O_2^{\bullet -}$) production.

2.3. Measurement of mitochondrial respiration and swelling

O_2 consumption was measured by a Clark-type oxygen microelectrode (Eurosep Instruments) in a thermostat controlled chamber. Mitochondria (1 mg) were added to 1 ml of phosphate buffer (250 mM sucrose, 5 mM KH_2PO_4, pH 7.2 at 25 °C) including 2 μM rotenone. Mitochondrial respiration was initiated by addition of succinate (6 mM final concentration), and oxidative phosphorylation was initiated by addition of ADP to a final concentration of 0.2 mM. O_2 consumption recordings allowed the calculation of V_3, the rate of state 3 (ADP-stimulated) respiration, of V_4, the rate of state 4 (non-ADP-stimulated) respiration, of the respiratory control ratio (RCR = V_3/V_4), and the P/O ratio, (the ADP consumed divided by O_2 used in state 3 respiration).

Mitochondrial swelling was assessed by measuring the change in absorbance of the suspension at 540 nm by using a Hitachi model U-3000 spectrophotometer. Mitochondria (4 mg) were added to 3.6 ml of the phosphate buffer including 2 μM rotenone. A quantity of 1.8 ml of this suspension was added to both sample and reference cuvettes, then 6 mM of succinate were added to the sample cuvette only and the A_{540} scanning was started.

2.4. Optical monitoring of mitochondrial membrane potential and determination of $O_2^{\bullet -}$ production

Mitochondrial membrane potential ($\Delta\Psi$) was evaluated by the uptake of rhodamine 123, which accumulates electrophoretically into energized mitochondria in response to their negative-inside membrane potential [25]. 1.8 ml of the phosphate buffer, 2 μM rotenone,

3 mM succinate and 0.3 μM rhodamine 123 were added to the cuvette, and the fluorescence scanning of the rhodamine 123 was monitored using a Perkin-Elmer SA LS 50B fluorescence spectrometer. After 30 s, mitochondria (0.5 mg/ml) were added. The $\Delta\Psi$ was calculated according to the Nernst equation.

The generation of $O_2^{\bullet -}$ was achieved as previously reported [26] by measuring the reduction of nitro blue tetrazolium in monoformazan that absorbed at 560 nm. Mitochondria (1 mg/ml) were incubated for 1 min in 1.2 ml the phosphate buffer supplemented with 2 μM rotenone, 1 μM cyclosporin A (CsA) and 100 μM nitro blue tetrazolium. For this particular experiment, CsA was added to inhibit mitochondrial swelling, which slightly interfered with the spectroscopic detection of the reduction reaction. The production of $O_2^{\bullet -}$ was then initiated by the addition of 6 mM succinate.

2.5. Western blot analysis

Samples containing equal amounts (25 μg) of protein from the cytosolic and the mitochondrial fraction were boiled at 100 °C in a buffer containing sucrose (20%), SDS (2.4%), β-mercaptoethanol (5%) and bromophenol blue (5%). They were subjected to electrophoresis on 4–15% gradient SDS–PAGE gel and then transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 5% non-fat dry milk in TBS buffer (Tris 0.02 M, NaCl 0.2 M, pH 8) containing 0.05% Tween-20 and incubated overnight at 4 °C with monoclonal anti-mouse cytochrome C antibody (MAB897, R&D systems), monoclonal anti-Bax antibody (2281-MC, R&D systems), polyclonal rabbit anti-Bid antibody (559681, Pharmingen), or monoclonal anti-cytochrome oxidase subunit IV antibody (A-21348, Molecular Probes). Anti-Bid antibody was raised against a synthetic peptide corresponding to the amino acids 129–146 of Bid and thus recognizes both the full-length and the cleaved forms of Bid. After incubation with mouse or rabbit HRP (1/10,000; Amersham Pharmacia Biotech) 1 h at room temperature, the blots were revealed by enhanced chemiluminescence reaction (Amersham ECL+) and exposed to X-rays films (Sigma, Biomax MS-1 film).

2.6. Caspase activity assay

A sample of liver tissue (1 g) was homogenized on ice using a Teflon Potter homogenizer in 6 ml of a buffer containing 25 mM Tris, 5 mM $MgCl_2$, 1 mM EGTA and 50 μl of a protease inhibitor cocktail (Sigma, product number P8340). The homogenate was centrifuged at 600 × *g* for 10 min (Sorvall RC 28 S). The supernatant was centrifuged for 15 min at 40,000 × *g* and the resulting supernatant (cytosolic fraction) was collected to determine caspase activity. Dithiothreitol (10 mM) was immediately added to the samples before freezing.

Caspase activity was assayed in a total volume of 100 μl. Briefly, 30 μg of cytosolic protein were incubated in a buffer containing 30 mM HEPES, 0.3 mM EDTA, 100 mM NaCl, 0.15 % Triton X-100 and 10 mM dithiothreitol. The samples were incubated at room temperature for 15 min. The reaction was started by adding 200 μM of either *N*-acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarine (LEHD-AFC; Tebu) for caspase-9 or *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarine (DEVD-AFC; Tebu) for caspase-3.

The samples were incubated at 37 °C for 120 min and the reaction was stopped by adding 1.2 ml of distilled water. Hydrolysis reactivities were determined by the measure of the fluorescence of 7-amino-4-trifluoromethylcoumarine (AFC) released. The excitation and emission wavelengths were 400 and 505 nm, respectively.

2.7. Inhibition of PTP opening and caspase activity

To inhibit PTP opening, CsA, a potent PTP inhibitor, was dissolved in a mixture of water–polyethylene glycol (50/50 v/v) and was injected intravenously (10 or 20 mg/kg) via the dorsal vein of the penis, 10 min before the induction of ischemia. In the same way, the inhibition of caspase activity was obtained by injection (0.5 mg/kg) of the general caspase inhibitor Z-Val-Ala-Asp(Ome)-fluoromethylketone (ZVAD.fmk; FMK001, R&D systems) 2 min before induction of ischemia.

3. Results and discussion

3.1. Ischemia-reperfusion alters mitochondrial functions

Mitochondrial functions were hugely altered by ischemia. We observed a deficiency in the respiratory chain as suggested by the decrease in the RCR and in the ability of energized mitochondria isolated from ischemic liver to generate $O_2^{\bullet -}$ (Fig. 1). This led to a time-dependent decrease in the capacity of mitochondria to maintain membrane potential, and to produce ATP as attested by the decrease in P/O values (Fig. 1). This resulted in an increase in the mitochondrial membrane permeability. Indeed, when mitochondria obtained from ischemic livers were introduced in a phosphate buffer, they swelled and the swelling was proportional to the extent of the ischemic period (Fig. 2(A)). This effect was not observed with mitochondria prepared from sham-operated animals. This swelling was due to the opening of the PTP since it was completely prevented (Fig. 2(A), line f) by the addition to the incubation medium of 1 μM CsA, the most potent inhibitor of PTP [27,28]. This was confirmed by the fact that mitochondria isolated from ischemic livers did not swell in a buffer, which did not include a PTP inductor as phosphate (Fig. 2(A), line h). These data demonstrate that conditions prevailing during ischemia, i.e., adenine nucleo-

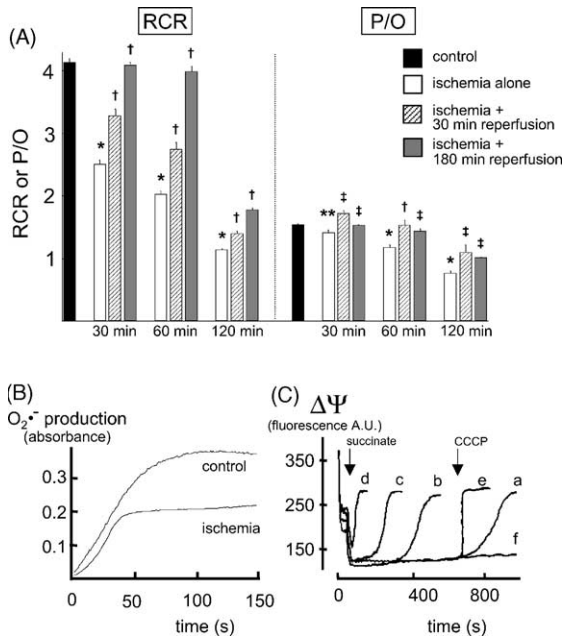

Fig. 1. Alteration of mitochondrial functions during liver ischemia-reperfusion. (A) Rat livers were subjected to increasing periods of ischemia (30, 60 or 120 min) followed or not by reperfusion. After isolation of mitochondria, oxygen consumption was measured polarographically and respiration parameters, RCR and P/O, were determined; (B) representative kinetics of mitochondria-induced $O_2^{\bullet -}$ production. Mitochondria were isolated from control liver or from liver subjected to 60 min ischemia and $O_2^{\bullet -}$ production was determined as described in the method section; (C) ischemia altered the capacity of mitochondria to maintain membrane potential ($\Delta\Psi$). Mitochondria were incubated with the fluorescent dye rhodamine 123 (0.3 μM), which exhibits membrane potential accumulation, and $\Delta\Psi$ was measured after addition of 6 mM succinate. (a) Control liver; (b–d) liver subjected to 30, 60 or 120 min ischemia, respectively; (e) addition of the mitochondrial uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 1 μM) induced a complete $\Delta\Psi$ depolarisation; (f) whereas 1 μM CsA maintained $\Delta\Psi$. $^{*}P < 0.001$ vs. control; $^{**}P < 0.02$ vs. control; $^{\dagger}P < 0.001$ vs. ischemia alone; $^{\ddagger}P < 0.02$ vs. ischemia alone; A.U., arbitrary units.

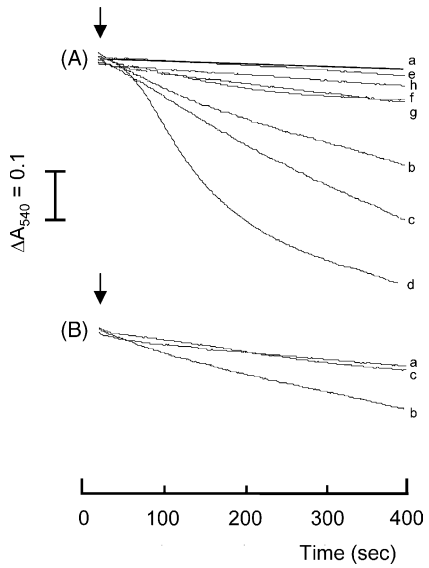

Fig. 2. Effect of ischemia-reperfusion on mitochondrial swelling. Liver mitochondria were preincubated for 2 min in a buffer containing 0.25 M sucrose, 5 mM KH_2PO_4, pH 7.2 at 25 °C and swelling was induced by the addition of 6 mM succinate (arrow). (A) Mitochondria were isolated from liver subjected to ischemia alone for 30 min (b), 60 min (c) or 120 min (d). Incubation of mitochondria in a free phosphate buffer (e); preincubation of mitochondria with 1 μM CsA (f); or pre-treatment of rats with either 10 mg/kg CsA (g); or 20 mg/kg CsA (h) counteracted the effect of 60 min ischemia; (a) control without ischemia. (B) Mitochondria were isolated from control liver (a) or from liver subjected to 60 min ischemia followed by a 30 min reperfusion period (b). Pre-treatment of rats with 20 mg/kg CsA (c) counteracted the effect of ischemia-reperfusion.

tide depletion, mitochondrial depolarisation [29] sensitise mitochondria to PTP opening.

Under these experimental conditions it was interesting to establish whether cytochrome *C* was released from mitochondria. Consistent with a previous report [11], cytochrome *C* could not be detected in sham-operated liver samples but appeared in the cytosol in the earlier phases of the ischemic period (Fig. 3). The amount of cytochrome *C* released was proportional to the duration of the ischemia. Immunoblotting for cytochrome oxidase subunit IV, a mitochondrial marker, failed to detect the enzyme in the cytosolic fraction (not shown), indicating that there was no contamination of mitochondria in the cytosolic fraction.

The increase in cytosolic cytochrome *C* was associated with a time-dependent concomitant decrease in the mitochondrial fraction whereas there was no difference in the amount of cytochrome oxidase subunit IV. These results indicate that cytochrome *C* dissociates from the electron transfer chain in the first time of the ischemic period, explaining why we observed a deficit of its function, i.e., the decrease in RCR, in ADP/O and the production of $O_2^{\bullet -}$.

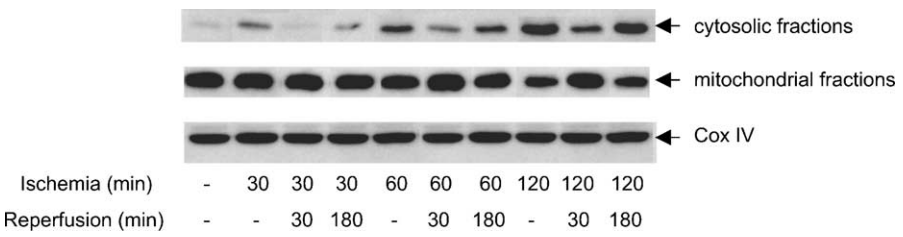

Fig. 3. Cytochrome *C* release during liver ischemia-reperfusion. Rats were subjected to different times of ischemia followed or not by a reperfusion period. After isolation of the liver, cytosolic and mitochondrial fractions were separated as described in Section 2 and were analysed by western blot using a monoclonal anti-mouse cytochrome *C* antibody. Labeling with the monoclonal anti-cytochrome oxidase (COX) subunit IV antibody demonstrate that the same amount of mitochondria was loaded in each lane.

In order to confirm the specific role of cytochrome *C* release during ischemia, we assessed whether the re-addition of exogenous cytochrome *C* would be able to improve oxidative phosphorylation and $\Delta\Psi$ in mitochondria damaged by ischemia. Mitochondria obtained after 1-h ischemia were incubated in respiratory experimental conditions and were monitored for respiratory parameters and $\Delta\Psi$ in the presence or in the absence of added cytochrome *C*. Fig. 4 shows that cytochrome *C* enhanced both states 3 and 4 respiration rates and increased the capacity of these mitochondria to maintain $\Delta\Psi$. This confirmed the permeabilization of the outer membrane and revealed that re-addition of exogenous cytochrome *C* was able to stimulate oxygen consumption. However, RCR and P/O values remained constant indicating that cytochrome *C* addition was not sufficient to restore entirely the responsiveness of the respiratory chain. This is probably due to the fact that other components of the mitochondrial membrane are also altered during ischemia. For instance, it was recently shown that ischemia induced a loss of cardiolipin, a phospholipid required for maximal activity of the respiratory chain [30,31].

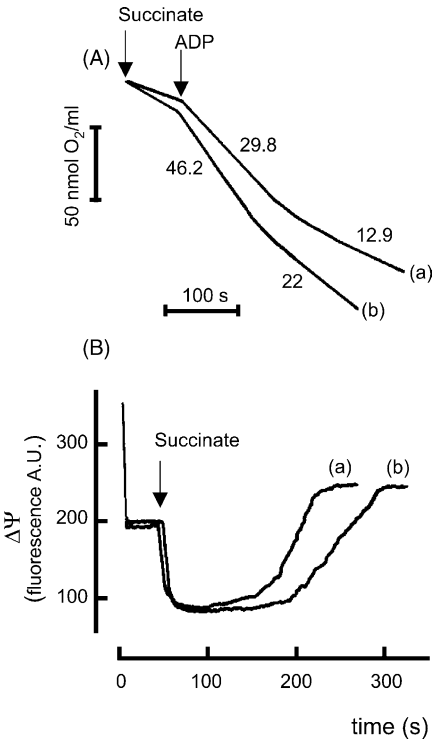

Fig. 4. Effect of exogenous cytochrome *C* on mitochondrial oxygen consumption and membrane potential. Mitochondria were isolated from liver subjected to 60 min ischemia and oxygen consumption and $\Delta\Psi$ were measured in the absence (curve (a)) or in the presence (curve (b)) of 10 μM cytochrome *C* (Sigma, product number C7752). (A) Oxygen consumption was initiated by addition of 6 mM succinate and state 3 respiration by the addition of 0.2 mM ADP. Rates of oxygen consumption, in nmol of oxygen/(min mg protein), are given alongside each trace. In these particular experiments, RCR and P/O values were 2.31 and 1.37 (curve (a)) and 2.1 and 1.4 (curve (b)), respectively; (B) experiments were performed as described in the legend to Fig. 1. The data shown are typical of three such experiments.

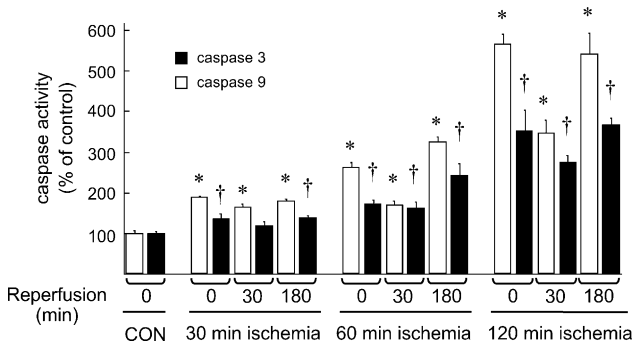

Fig. 5. Caspase activation during liver ischemia-reperfusion. Rats were subjected to different times of ischemia followed or not by a reperfusion period. After isolation of the liver, cytosols were prepared at 4 °C and assayed for enzymatic activity. Data are expressed as percentage of control (CON) value (no ischemia, 100%) and represent means ± S.E. of at least $n = 6$ animals per group. $^{*}P < 0.05$ vs. caspase-9 control value; and $^{\dagger}P < 0.05$ vs. caspase-3 control value. Control values were 266.6 ± 66.3 and 152.7 ± 44.7 nmol AFC/(h mg) protein for caspase-3 and caspase-9, respectively.

It is now well established that once released, cytochrome *C* in the presence of dATP stimulates the formation of APAF1, which can activate caspase-9, and the downstream caspase cascade [32]. Consistent with the observation that cytochrome *C* is released during ischemia, we were able to detect caspase-9 and caspase-3 activations as revealed by LEHD–AFC and DEVD–AFC cleavages, respectively (Fig. 5), confirming that the so-called mitochondrial apoptotic pathway was activated.

The same studies were performed during the reperfusion period. Whatever the duration of ischemia, reperfusion improved mitochondrial functions as attested by the increase in the RCR values after removal of the clamp (Fig. 1). In addition, the swelling of mitochondria isolated from ischemia-reperfused livers was less important than that of ischemic mitochondria (Fig. 2(B)) and we observed a reduction in cytochrome *C* release (Fig. 3) and a decrease in caspase-3 and caspase-9 activities (Fig. 5) in the first time of the reperfusion period. This indicates that immediately after the beginning of reperfusion, mitochondria preferentially used cytochrome *C* to transfer electrons from complex III to IV instead of releasing it. However, longer reperfusion time caused a further increase in cytochrome *C* release and caspase activities (Figs. 3 and 5).

3.2. CsA prevented cytochrome C release during reperfusion but not during ischemia

In order to evaluate the role of PTP opening in mitochondrial dysfunction and cytochrome *C* release, rats were injected with either 10 or 20 mg/kg CsA 15 min before clamping, and both parameters were analysed after 1-h ischemia followed (or not) by a reperfusion period. This time of ischemia was chosen because, in our model, it induced major but not complete inhibition of mitochondrial functions. CsA pretreatment protected mitochondria from the deleterious effect of ischemia-reperfusion. It

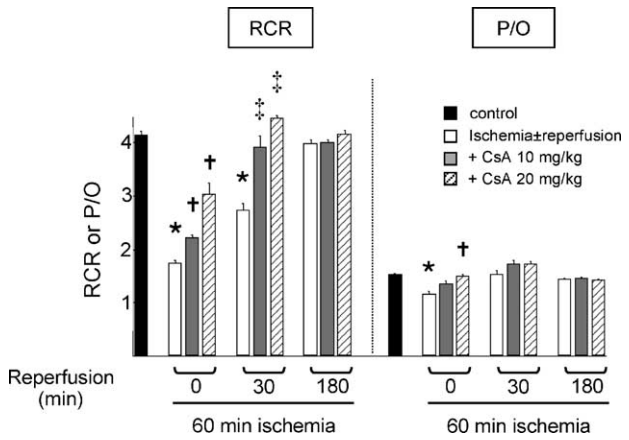

Fig. 6. Effect of cyclosporin A (CsA) pre-treatment on RCR and P/O values, in liver mitochondria of rats subjected to ischemia-reperfusion. Rat were pre-treated with CsA (10 or 20 mg/kg) before the induction of 60 min ischemia followed or not by increasing times of reperfusion. After isolation of mitochondria, oxygen consumption was measured polarographically and respiration parameters, RCR and P/O, were determined. $^{*}P < 0.05$ vs. control; $^{\dagger}P < 0.05$ vs. ischemia; and $^{\ddagger}P < 0.05$ vs. ischemia-reperfusion.

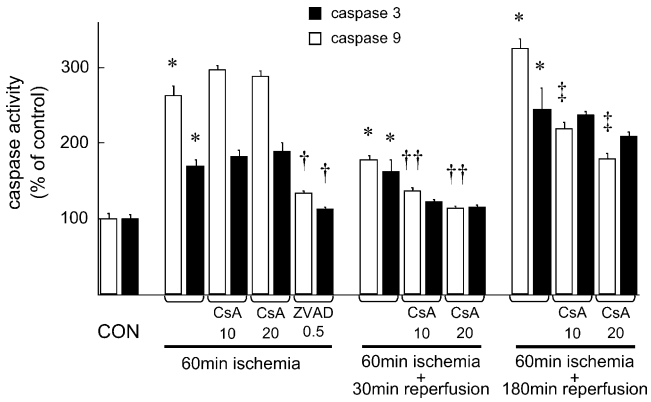

Fig. 8. Effect of cyclosporin A pre-treatment on caspase activity. CsA (20 mg/kg) or ZVAD.fmk (0.5 mg/kg) were injected intravenously before the induction of 60 min ischemia followed by 0, 30 or 180 min reperfusion. After isolation of the liver, cytosols were prepared at 4 °C and immediately assayed for enzymatic activity. Data are expressed as percentage of control (CON) value (no ischemia, 100%) and represent means ± S.E. of at least $n = 6$ animals per group. Control values were 266.6 ± 66.3 and 152.7 ± 44.7 nmol AFC/(h mg) protein for caspase-3 and caspase-9, respectively. $^{*}P < 0.05$ vs. the respective control values; $^{\dagger}P < 0.05$ vs. 60 min ischemia; $^{\dagger\dagger}P < 0.05$ vs. 60 min ischemia-30 min reperfusion; and $^{\ddagger}P < 0.05$ vs. 60 min ischemia-180 min reperfusion.

inhibited completely mitochondrial swelling (Fig. 2) and maintained the mitochondrial membrane potential (Fig. 1). This demonstrated that CsA reached its target, i.e, the PTP after injection since it blocked its opening.

This protection is clearly seen on respiratory parameters. CsA improved both the mitochondrial coupling and ATP synthesis, measured after ischemia or after ischemia, plus reperfusion as demonstrated by the increase in RCR and P/O values, respectively (Fig. 6). The effect was dose-dependent and a maximal effect was obtained at 20 mg/kg. Increasing the dose to 30 mg/kg did not improve the effect (not shown).

During reperfusion, these protective effects could be related to the PTP blocking properties of the drug as CsA administration completely abolished mitochondrial swelling (Fig. 2(B)) and caused a pronounced reduction in cytochrome *C* release and caspase-9 activity (Figs. 7 and 8). This demonstrates that during reperfusion, PTP opens and plays a predominant role in mitochondrial alteration.

On the contrary, whatever the dose used, CsA pre-treatment did not prevent cytochrome *C* release during ischemia (Fig. 7), although the pre-treatment caused an inhibition of the swelling obtained after incubation in a phosphate buffer (Fig. 2(A)). In addition, the extent of the activation of caspases was identical in the presence or in the absence of CsA (Fig. 8), confirming that PTP was not involved in the mitochondrial apoptotic pathways during ischemia. Thus, liver ischemia sensitises mitochondria to PTP opening but PTP remains close. Similar results were observed in an in vitro model of cardiac ischemia [33].

Taken together, these data demonstrate that cytochrome *C* release is successively independent and dependent of PTP opening during ischemia and reperfusion, respectively. In this line, it is conceivable that mitochondria could utilize different processes to induce apoptotic protein

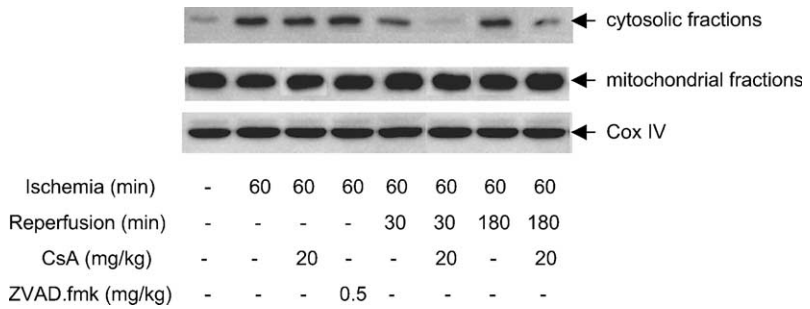

Fig. 7. Cyclosporin A (CsA) limited cytochrome *C* release during reperfusion but not during ischemia. CsA (20 mg/kg) or ZVAD.fmk (0.5 mg/kg) were injected intravenously before the induction of 60 min ischemia and 0, 30 or 180 min reperfusion. After isolation of the liver, cytosolic and mitochondrial fractions were separated as described in Section 2 and were analysed by western blot using a monoclonal anti-mouse cytochrome *C* antibody. Labeling with the monoclonal anti-cytochrome oxidase (COX) subunit IV antibody demonstrate that the same amount of mitochondria was loaded in each lane.

release as cytochrome *C* during ischemia and reperfusion, since the inducing signals are not the same.

3.3. Possible mechanisms by which ischemia promotes cytochrome C release

3.3.1. The effect of some proapoptotic Bcl-2 family members

To date, at least five models have been proposed to explain the permeabilization of the mitochondrial membranes and the release of soluble proteins in the cytosol [9,10] and several hypothesis have been raised to explain the efflux of cytochrome *C* from mitochondria without PTP opening [34]. One possibility includes the translocation of a member of the Bcl-2 family proteins from cytosol to mitochondria [10]. One of these proteins is Bax. Indeed, this protein is able to form a cytochrome *C*-conducting channel alone or in combination with the voltage-dependent-anion-channel [35,36] without PTP opening. Thus, we tested this hypothesis during ischemia. Western blot analysis revealed no significant modification of Bax level in the cytosol and no significant expression at the level of the mitochondrial membranes whatever the duration of ischemia (Fig. 9). These results differ from those of Ishigami et al. [37] who observed Bax translocation from cytosol to mitochondria after warm ischemia-reperfusion. However, their experimental conditions differed from ours since rat livers were subjected to more prolonged period of ischemia-reperfusion. In the same way, because cleaved Bid has been shown to target mitochondria [38] in different experimental conditions and to release cytochrome *C* during myocardial ischemia [39], we examined the possible involvement of this protein during liver ischemia. Our results show that the amount of Bid did not decrease in the cytosol and that cleaved Bid did not accumulate in the mitochondrial membrane (Fig. 9), indicating that the interaction of this protein at the level of the mitochondrial membrane did not seem to play a critical role in cytochrome *C* release in our model of liver ischemia. This is in accordance with previous results indicating that proteolysis of Bid only occurred during liver reperfusion [11].

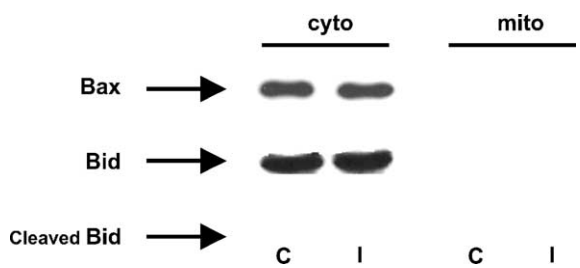

Fig. 9. One-hour ischemia does not cause translocation of either Bax or Bid from cytosol to mitochondria. Cytosolic and mitochondrial fractions were isolated from control (C) and ischemic (I) livers and were analysed by western blot analysis using anti-Bax and anti-Bid antibodies as described in Section 2. Similar results were found after 30-min and 120-min ischemia.

3.3.2. The caspase inhibitor ZVAD.fmk did not prevent cytochrome C release

Caspases were also recently shown to be able to release cytochrome *C* and other proapoptotic proteins from mitochondria [40,41]. To examine this possibility, rats were injected with general caspase inhibitor ZVAD.fmk 2 min prior to ischemia. This approach was already successfully used to inhibit caspase activation during liver ischemia-reperfusion [42]. Our results confirm that ZVAD.fmk pre-treatment suppress almost completely caspase activity (Fig. 8). However, it has no effect on cytochrome *C* release (Fig. 7) indicating that the release of cytochrome *C* was not triggered by a direct effect of caspases during liver ischemia.

3.3.3. Anoxia can directly release cytochrome C from mitochondria

Ischemia is first characterized by a reduction of oxygen availability for mitochondria. Therefore, in the last approach we postulated that the inhibition of the activity of the respiratory chain due to the absence of oxygen could be the cause of cytochrome *C* release. To this end, we monitored the release of cytochrome *C* from isolated liver mitochondria submitted to increasing times of anoxia. Oxygen was exhausted from the incubation medium through nitrogen purging. Anoxia promoted a time-dependent release of cytochrome *C*, and the presence of CsA did not modify the rate or the extent of the release (Fig. 10). These in vitro results demonstrate that the absence of oxygen is a sufficient signal to dissociate cytochrome *C* from the electron transfer chain of the cristae membranes and to release it from mitochondria suggesting that during liver ischemia cytochrome *C* release may be caused by a direct effect of oxygen deprivation on mitochondria. This

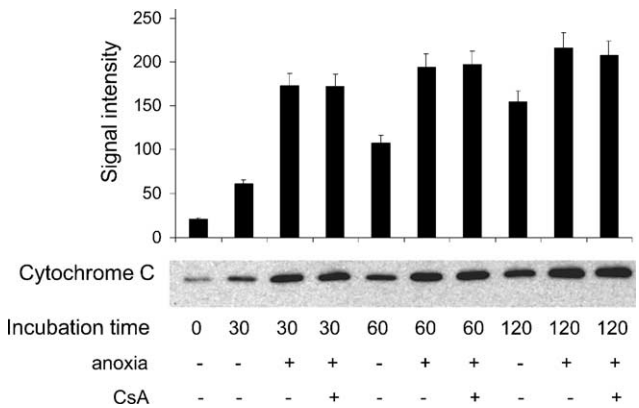

Fig. 10. Cyclosporin A do not prevent anoxia-induced cytochrome *C* release from isolated mitochondria. Isolated liver mitochondria (4 mg/ml) were incubated in a medium containing 250 mM sucrose, 10 mM Tris, 1 mM EGTA, 1 μM rotenone and 6 mM succinate and submitted or not to increasing times of anoxia in the presence or in the absence of 1 μM CsA. Anoxia 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. The signal intensity was evaluated by densitometry.

is in line with recent data in non-hepatic cells demonstrating that oxygen deprivation could be the initial event leading to cell death [43,44].

Taken together, these data suggest that different pharmacological strategies should be used to prevent cell death during ischemia and reperfusion. Indeed, whether an inhibitor of PTP opening should be useful during reperfusion, it will be inactive during ischemia. The challenge is probably to find molecules able to stabilize the association of cytochrome *C* with the respiratory chain.

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