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ORIGINAL ARTICLE

Prevention of I/R injury in fatty livers by ischemic preconditioning is associated with increased mitochondrial tolerance: the key role of ATPsynthase and mitochondrial permeability transition

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Summary

Ischemia/reperfusion (I/R) injury is a commonly encountered clinical problem and occurs probably as a consequence of irreversible mitochondrial injury. The increased susceptibility of fatty livers to ischemic injury is associated with depletion of adenosine triphosphate (ATP) content, which is preserved by preconditioning. Mitochondria being the main ATP production source for the cell, we aimed to evaluate whether ischemic preconditioning (IPC) of fatty livers prevents the impairment in mitochondrial function induced by I/R. Lean and steatotic animals were subjected to 90 min of hepatic warm ischemia and 12 h of reperfusion. IPC effect was tested in fatty livers. After reperfusion, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured. Mitochondrial membrane potential, mitochondrial respiration and susceptibility to mitochondrial permeability transition (MPT) were evaluated, as well as ATPase activity and adenine nucleotides. IPC of fatty livers decreased serum AST and ALT levels. Fatty animals subjected to I/R exhibited decreased mitochondrial membrane potential and a delay in the repolarization after a phosphorylation cycle, associated with increased state 4 respiration. Increased tolerance to MPT induction, preservation of F_1F_o-ATPsynthase activity and mitochondrial bioenergetics were observed in ischemic preconditioned fatty livers. Thus, IPC is an endogenous protecting mechanism that preserves mitochondrial function and bioenergetics in fatty livers.

Introduction

Hepatic ischemia/reperfusion injury (I/R) during hepatectomy and liver transplantation is a major cause of liver dysfunction. Hepatic steatosis (fatty livers) causes an increased susceptibility to I/R injury, thus bearing additional risks of primary nonfunction subsequent to liver surgery or transplantation [1–5].However, in spite of ongoing intensive research efforts, only a few pharmacologically protective strategies are currently available [6,7]. The development of effective strategies to protect fatty livers against ischemic injury is further justified by the increased prevalence of fatty livers mainly related to the overall increase of obesity and alcohol consumption in all industrialized countries [8].

Several hypotheses have been advanced for explaining the increased susceptibility of fatty organs to ischemic injury. These include impaired microcirculation, decreased intracellular energy level, Kupffer's cell dysfunction, and increased adhesion of leukocytes [2,3,5,9–13]. However, the role that each of these mechanisms play in I/R injury is not yet elucidated. Fatty liver is associated

with decreased ability to generate adenosine triphosphate (ATP) [14–19], thus being unable to restore ATP content after reperfusion.

Brief, intermittent episodes of ischemia and reperfusion, also known as ischemic preconditioning (IPC), have been shown to prepare the organ for exposure to a subsequently more prolonged lethal ischemic insult [20,21]. IPC offers a high degree of protection to fatty livers by preventing massive necrosis, associated with preservation of ATP content [22–24]. In the liver, the preconditioning effect has been demonstrated in animal models, as well as clinically during hemihepatectomies and in deceased donors [25–27].

Despite intensive investigation, the actual role of key mitochondrial proteins involved in bioenergetics, metabolism and cell death [20] in the protection afforded by IPC is still elusive. A potential target in this scheme may be the mitochondrial permeability transition (MPT), which represents a fundamental event in the pathway to reperfusion-induced cell death [20]. MPT induction causes an increase in the permeability of the inner mitochondrial membrane that collapses the mitochondrial membrane potential, uncouples oxidative phosphorylation and consequently results in ATP depletion. MPT induction also leads to cell death caused by the release of apoptosis-inducing factors. Interestingly, I/R results in oxidative stress, a high mitochondrial calcium and inorganic phosphate, conditions that favor MPT induction [28].

An ATP-dependent mechanism has been shown to be associated with the damage-preventive functioning of the IPC in the fatty livers subjected to ischemia/reperfusion [22]. As impaired mitochondrial function has been described in fatty livers, increased susceptibility to mitochondrial dysfunction and consequent ATP depletion, maybe a condition that potentiates I/R damage in fatty livers. However, a systematic investigation of mitochondrial function in fatty liver subjected to IPC is still lacking, considering that mitochondria is a probable target for IPC protection in the setting of I/R.

The aim of this study was to test whether the protection by IPC against I/R injury in fatty livers is associated with increased efficiency of mitochondrial function. Key parameters of mitochondrial bioenergetics, as well as susceptibility to MPT induction were evaluated.

Materials and methods

Materials

All compounds were purchased from Sigma Chemical Co. St Louis, MO, USA. All other reagents and chemicals used were of the highest grade of purity commercially available.

Animals and induction of fatty liver

Male Wistar rats 5 weeks old (Charles River, France) weighing 150 g, were allowed to acclimate to the animal quarters and given free access to a standard chow diet for 1 week. Animals were then randomly divided into two groups and fed a choline-deficient diet (CDD) or regular diet (lean animals) for an additional 16 weeks (Dyets Inc., Bethlehem, PA, USA). The animals were weighed immediately before induction of hepatic ischemia/reperfusion.

Hepatic ischemia/reperfusion

Lean and fatty animals were anesthetized with ketamine (50 mg/kg) and chlorpromazine (50 mg/kg). A model of partial ischemia (70%) was used in order to prevent mesenteric venous congestion by permitting portal decompression through the right and caudate lobes. After a midline laparotomy, the hepatic artery and portal vein to the left and median liver lobes were occluded for 90 min. Reperfusion (12 h) was initiated by removal of the clamp. Preconditioning of livers in animals was applied with 5 min of ischemia and 10 min of reperfusion prior to the 90-min ischemic insult, as previously established [23]. Six animals were included in each experimental group. The following experimental groups were studied: lean (Lean) or fatty (CDD) animals were subjected to anesthesia and laparotomy, lean (Lean I/R) or fatty (CDD I/R) animals were subjected to 60 min of ischemia followed by 12-h reperfusion and lean (Lean IPC) or fatty (CDD IPC) subjected to I/R but with a previous preconditioning. All procedures were conducted according to the guidelines for the care and use of laboratory animals approved by our Institution.

Plasma biochemical determinations and hepatic triglyceride content

Following 12 h of reperfusion, animals were killed by decapitation. Plasma samples were collected and enzymatic determinations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) performed using commercial kits. Hepatic triglyceride content was determined by using a commercially available triglyceride detection kit (Linear Chemicals, Spain), according to manufacturer's suggestions.

Liver histology

Livers from the animals were removed quickly and immediately after the animal sacrifice, fixed in 10% buffered formalin and stained with hematoxylin and eosin (H & E) [25].

Isolation of liver mitochondria

Mitochondria were isolated from animals of the six experimental groups by differential centrifugation, as previously described [29,30]. Homogenization medium contained 250 mM sucrose, 10 mM HEPES (pH 7.4), 0.5 mM EGTA, and 0.1% fat-free bovine serum albumin. EGTA and bovine serum albumin were omitted from the final washing medium, adjusted at pH 7.4. Briefly, after homogenization of the minced blood-free hepatic tissue, the homogenate was centrifuged at 500 *g* for 10 min at 4 °C. The resulting supernatant was spun at 10 000 *g* for 10 min at 4 °C to pellet mitochondria, which were resuspended in a final washing medium. Protein content was determined by the biuret method [31], calibrated with bovine serum albumin.

Mitochondrial respiration

Oxygen consumption was polarographically determined with a Clark type polarographic oxygen sensor [32] with a PC-operated electrode control unit (Oxygraph, Hansatech Instruments Ltd, UK). Mitochondria (1 mg) were suspended under constant stirring, at 25 °C, in 1 ml of standard respiratory medium (130 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 5 mM KH_2PO_4, 50 μM EDTA, and 5 mM HEPES [pH 7.4]) and energized by adding glutamate/malate or succinate to a final concentration of 5 mM. For succinate assays, 2 μM rotenone, an inhibitor of complex I, were previously added. State 3 respiration was induced by adding 200 nmol ADP. The oxygen consumption was also measured in the presence of oligomycin (0.5 μg/mg protein) and 1 μM carbonylcyanide-*p*-trifluoromethoxyphenylhydrazon (FCCP). States 3 and 4 and respiratory control ratio (RCR) were calculated according to Chance and Williams [33].

Mitochondrial transmembrane potential ($\Delta\Psi$) measurements

Mitochondrial transmembrane potential ($\Delta\Psi$) was estimated using an ion-selective electrode to measure the distribution of tetraphenylphosphorium (TPP^+) according to previously established methods [34,35]. The voltage response of the TPP^+ electrode to log (TPP^+) was linear with a slope of 59 ± 1, in conformity with the Nernst equation. ° Reactions were carried out at 25 °C, in a temperature-controlled water-jacketed chamber with magnetic stirring. Mitochondria (1 mg) were suspended in 1 ml of standard respiratory medium (as in mitochondrial respiration) supplemented with 3 μM TPP^+. A matrix volume of 1.1 μl/mg protein was assumed.

ATPase activity

ATPase activity was evaluated spectrophotometrically at 660 nm, in association with ATP hydrolysis. The reaction was carried out at 37 °C, in 2 ml reaction medium (125 mM sucrose, 65 mM KCl, 2.5 mM $MgCl_2$ and 50 mM HEPES, pH 7.4), supplemented with 0.25 mg of mitochondrial protein. The reaction was initiated by adding 2 mM Mg^{2+}-ATP. The released phosphate was quantified by reaction with ammonium molybdate. ATPase activity was calculated as the difference in total absorbance and absorbance in the presence of oligomycin (1 μg/mg protein).

Mitochondrial permeability transition induction

Mitochondrial swelling associated to MPT induction was estimated by changes in light scattering as monitored spectrophotometrically at 540 nm [36]. Reactions were carried out at 25 °C. Experiments were started by the addition of mitochondria (1 mg) to 2 ml of reaction medium (200 mM sucrose, 10 mM Tris-MOPS, 1 mM KH2PO4, 10 μM EGTA, pH 7.4) supplemented with 3 μM rotenone, 0.5 μg oligomycin and 5 mM succinate.

Mitochondrial adenine nucleotides content

Mitochondrial endogenous ATP, ADP, and AMP were extracted using an alkaline extraction procedure and were separated by reverse-phase high-performance liquid chromatography as described by Stocchi [37]. The chromatographic apparatus was a Beckman-System Gold, consisting of a 126 Binary Pump Model and a 166 Variable UV detector, controlled by a computer. The detection wavelength was 254 nm, and the column was a 5-μm Lichrospher 100RP-18 from Merck (Darmstadt, Germany). An isocratic elution with 100 mM phosphate buffer (KH_2PO_4, pH 6.5) and 1.0% methanol was performed with a flow rate of 1 ml/min. The time required for each analysis was 5 min.

Statistical analysis

Data are presented as mean ± SEM of experiments with six different animals in each group. The difference between the six experimental groups was calculated by using a two-way repeated-measure analysis of variance (ANOVA) followed by Bonferroni's post-test. A $P < 0.05$ was considered statistically significant.

Results

Animal model

The use of a choline-deficient diet is a well-known experimental model to induce fatty liver [38]. Previously, we

Table 1. Characterization of the animal model.

	Lean	CDD
Liver/body weight	15.5 ± 0.5	21 ± 1*
Intrahepatic triglyceride content (mg/g tissue)	9.83 ± 0.35	199.93 ± 1.37*
Macrovesicular steatosis (%)	–	20
Microvesicular steatosis (%)	–	70

Data are means ± SEM of six animals in each group.
*Statistically significant difference.

have shown that the mitochondrion plays a central role in the development of nonalcoholic steatosis [19]. After 16 weeks of CDD feeding, there is a decrease in the efficiency of oxidative phosphorylation and increased susceptibility to MPT induction. As the study of the progression of this pathology unveiled an extremely interesting response designed to prevent and revert the mitochondrial damage at 12 weeks of CDD feeding, we decided to study IPC effects on CDD animals at 16 weeks, when mitochondrial dysfunction is evident.

Livers from animals fed a choline-deficient diet (CDD) were heavier than animals fed a regular diet (lean), as shown by the increased liver/body weight ratio, probably because of trigliceryde accumulation (Table 1). CDD animals developed fatty liver infiltration, characterized by both macro- and microvesicular steatosis, without evidence for inflammation and/or fibrosis.

Serum markers of liver injury

Ischemia/reperfusion caused an increase in serum levels of AST and ALT in both fatty and lean animals subjected to I/R (CDD I/R and Lean I/R, respectively). The serum levels of AST in ALT were higher in CDD I/R animals than in Lean I/R, indicating more severe liver injury caused by I/R in fatty animals. However, IPC of both fatty and lean livers (Fatty IPC and Lean IPC, respectively) was effective in preventing I/R damage, as reflected by decreased serum AST and ALT levels (Fig. 1).

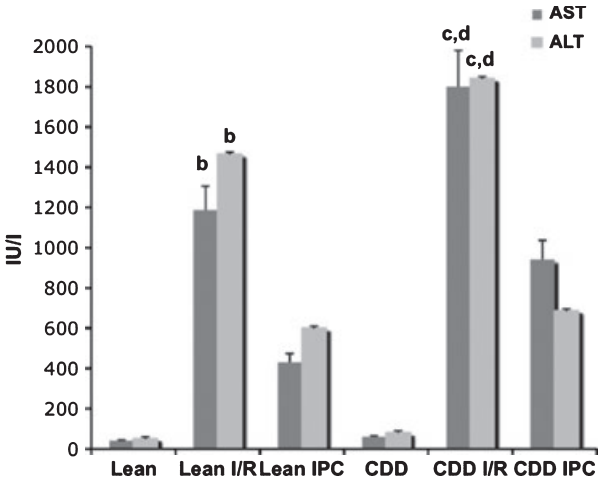


Figure 1 Aspartate aminotransferase (AST) and ALT levels after ischemia/reperfusion. Ischemic preconditioning (IPC) of fatty livers (CDD IPC) decreased AST and ALT release after I/R, which were higher in non-preconditioned CDD livers (CDD I/R)than in non-preconditioned lean livers subjected to I/R (Lean I/R). Data are means ± SEM of six animals in each group. [b]Statistically significant difference ($P < 0.05$) of Lean I/R versus Lean and Lean IPC, [c]Statistically significant difference ($P < 0.05$) of CDD I/R versus CDD and CDD IPC, [d]Statistically significant difference ($P < 0.05$) of CDD I/R versus Lean I/R.

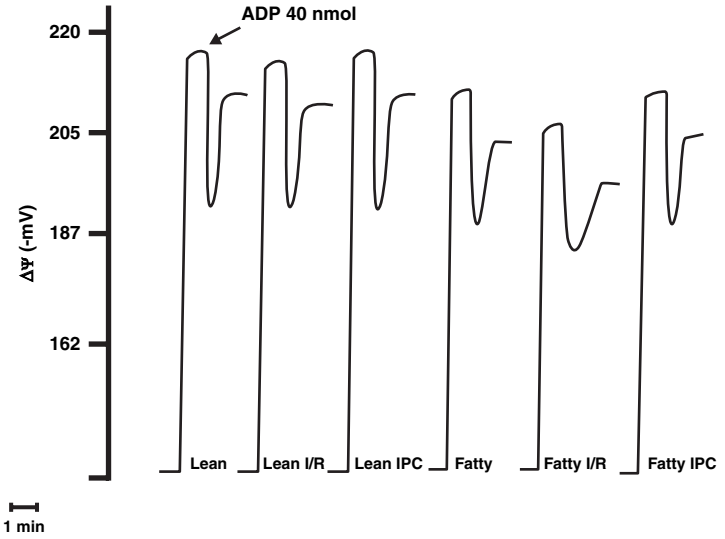
Mitochondrial transmembrane potential

Taking into account the fundamental role of mitochondrial transmembrane potential for the phenomenon of oxidative phosphorylation, $\Delta\Psi$ was evaluated in glutamate-/malate- or succinate-energized mitochondria (Fig. 2A, B respectively). CDD animals exhibited a basal decrease in $\Delta\Psi$, when compared with lean animals. In comparison to the mitochondria isolated from lean animals subjected to I/R (Lean I/R), $\Delta\Psi$ was decreased in non-preconditioned fatty livers, upon I/R (CDD I/R).Irrespective of the substrate used, mitochondria isolated from IPC animals developed higher $\Delta\Psi$ than non-preconditioned I/R fatty livers, the values being comparable to the lean group.

Figure 2 Mitochondrial transmembrane potential ($\Delta\Psi$) in glutamate/malate (A) or succinate-energized (B) mitochondria isolated from livers subjected to ischemia/reperfusion. $\Delta\Psi$ was measured with a TPP^+-selective electrode. Reactions were carried out in 1 ml of reaction medium, supplemented with 1 mg of freshly isolated mitochondria, as described in materials and methods. In assays with glutamate/malate, energization was achieved with 5 mm glutamate/malate and phosphorylation induced by 40 nmol ADP; in assays with succinate reaction media was supplemented with 2 μm rotenone and energization was achieved with 5 mm succinate, phosphorylation was induced by 200 nmol ADP. Animals fed a choline-deficient diet (CDD) and subjected to I/R (CDD I/R) had significantly decreased $\Delta\Psi$ when compared with the lean group. Ischemic preconditioning improved $\Delta\Psi$ in fatty animals (CDD IPC) to values comparable with lean animals. The traces represent typical direct recordings and data are means ± SEM of experiments performed with six different mitochondrial preparations. [a]Statistically significant difference ($P < 0.05$) of Lean versus CDD, [c]Statistically significant difference ($P < 0.05$) of CDD I/R versus CDD and CDD IPC, [d]Statistically significant difference ($P < 0.05$) of CDD I/R versus Lean I/R.

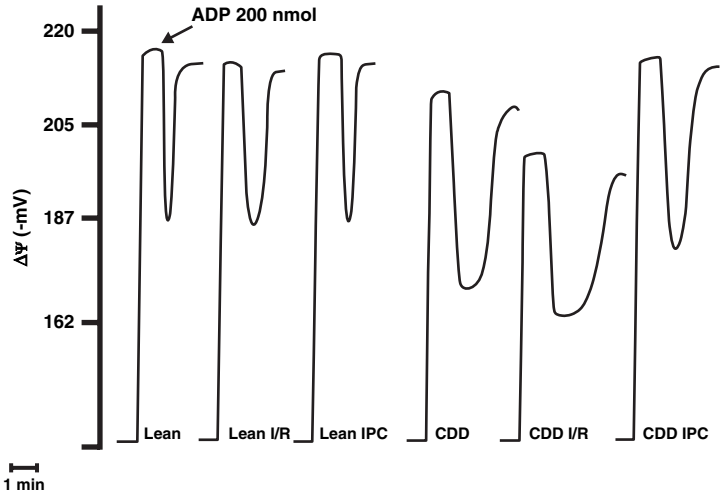
(A)

	Lean	Lean I/R	Lean IPC	CDD	CDD I/R	CDD IPC
ΔΨ upon energization (-mV)	215.67 ± 1.41	213.61 ± 1.51	215.88 ± 1.48	209.48 ± 2.21[a]	199.88 ± 1.29[c,d]	208.42 ± 1.13
Depolarization (-mV)	25.12 ± 0.91	24.39 ± 1.34	25.49 ± 1.26	18.6 ± 1.34[a]	17.25 ± 1.53	18.1 ± 2.3
Repolarization (-mV)	213.86 ± 1.42	211.36 ± 1.18	212.36 ± 0.99	198.38 ± 1.64[a]	193.26 ± 2.12[c,d]	199.42 ± 1.75



(B)

	Lean	Lean I/R	Lean IPC	CDD	CDD I/R	CDD IPC
ΔΨ upon energization (-mV)	217.76 ± 4.15	215.24 ± 4.36	218.87 ± 1.95	210.43 ± 12.53[a]	203.26 ± 1.86[c,d]	216.26 ± 1.92
Depolarization (-mV)	30.4 ± 1.87	29.41 ± 2.23	30.21 ± 2.25	31.6 ± 1.26	31.92 ± 2.75	29.34 ± 3.2
Repolarization (-mV)	214.25 ± 4.23	213.32 ± 3.65	214.2 ± 3.21	202.63 ± 6.45[a]	199.8 ± 3.84[c,d]	214.24 ± 2.53



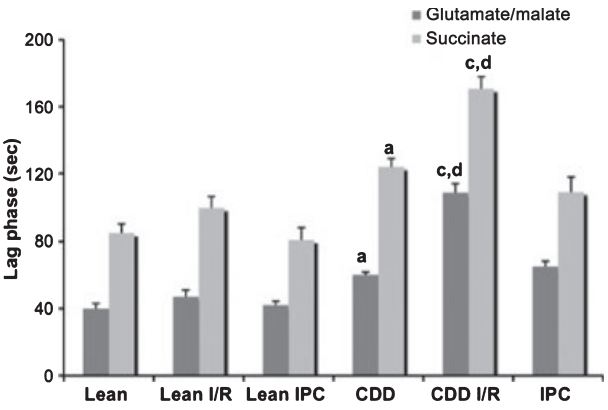


Figure 3 Lag phase in glutamate/malate and succinate-energized mitochondria isolated from livers subjected to ischemia/reperfusion. $\Delta\Psi$ was measured with a TPP^+-selective electrode as described in materials and methods. Phosphorylation was induced by 40 nmol (glutamate/malate) or 200 nmol ADP (succinate). Animals fed a choline-deficient diet (CDD) and subjected to I/R (CDD I/R) had significantly increased lag phase when compared with the lean group. Ischemic preconditioning improved the lag phase in fatty animals (CDD IPC) to values comparable with lean animals. Data are means ± SEM of six animals in each group. [a]Statistically significant difference ($P < 0.05$) of Lean versus CDD, [c]Statistically significant difference ($P < 0.05$) of CDD I/R versus CDD and CDD IPC, [d]Statistically significant difference ($P < 0.05$) of CDD I/R versus Lean I/R.

ADP-induced depolarization was similar among the three experimental groups. However, $\Delta\Psi$ after repolarization (mitochondrial capacity to establish $\Delta\Psi$ after ADP phosphorylation) was decreased in non-preconditioned I/R fatty livers. Both mitochondria from lean I/R and preconditioned fatty livers, displayed similar ability to recover $\Delta\Psi$ after ADP phosphorylation.

The lag phase (time necessary for ADP phosphorylation) was significantly affected by I/R in steatotic animals (Fig. 3), in relation to lean livers. IPC prevented the increase in the lag phase.

Mitochondrial respiration

Oxidative phosphorylation capacity was investigated by evaluating mitochondrial oxygen consumption in relation to glutamate-/malate- or succinate oxidation. Although the basal level decreased to state 3 in CDD animals when compared with lean, no differences were observed in mitochondrial state 3 respiration (ADP-induced oxygen consumption) after I/R in both animal models, irrespective of the substrate used. Similarly, oxygen consumption stimulated by FCCP, a well-known respiratory chain uncoupler, was also identical (Fig. 4A).

The consumption of oxygen after ADP phosphorylation (state 4 respiration) was increased in fatty livers, in relation to lean livers (Fig. 4B). IPC prevented the increase in state 4 respiration observed in fatty animals subjected to I/R (CDD I/R), decreasing this parameter to a value similar to the lean group. In both animals subjected to ischemia and preconditioning, no differences were observed in mitochondrial respiration in the presence of oligomycin, a known inhibitor of the mitochondrial F_1F_o-ATPsynthase (Fig. 4B).

The ratio between mitochondrial state 3 and state 4 respirations (RCR) was significantly decreased in fatty livers (CDD), as well as in lean and fatty livers after I/R (Lean I/R and CDD I/R, Fig. 4C). IPC of both fatty and lean livers preserved the RCR. After I/R, no statistically significant difference was observed in the ADP/O ratio, an indicator of oxidative phosphorylation efficiency, although the basal difference between lean and CDD animals (Fig. 4D).

Mitochondrial ATPase activity

The decreased performance of phosphorylation in non-preconditioned fatty livers, as reflected by an increased lag phase, suggested alterations in the F_1F_o-ATPsynthase, a key component of the phosphorylation system. ATPase activity was only decreased in mitochondria from non-preconditioned fatty livers after I/R, in relation to all experimental groups (Fig. 5). IPC preserved ATPase activity in a manner similar to that observed in lean livers.

Mitochondrial adenine nucleotides content

As mitochondrial oxidative and phosphorylation pathways are controlled by the ATP/ADP ratio [39], endogenous adenine nucleotides were determined in both preconditioned and non-preconditioned fatty livers. As shown in Table 2, ATP content was increased and ADP content decreased in preconditioned livers. In fact, IPC significantly preserved mitochondrial energy levels (ATP/ADP ratio), which significantly decreased during I/R.

Susceptibility to MPT induction

Increased mitochondrial calcium uptake has been related with hepatocytic apoptosis caused by I/R [40]. In isolated mitochondria, the ability to tolerate a calcium challenge is an indicator of the susceptibility to MPT induction. Thus, as mitochondria possess a finite capacity for accumulating calcium before undergoing the MPT, calcium-induced mitochondrial swelling was evaluated. Mitochondria isolated from non-preconditioned fatty livers subjected to I/R were more susceptible to undergo mitochondrial swelling, when compared with lean animals also subjected to I/R (Fig. 6). Pretreatment with 1 μM

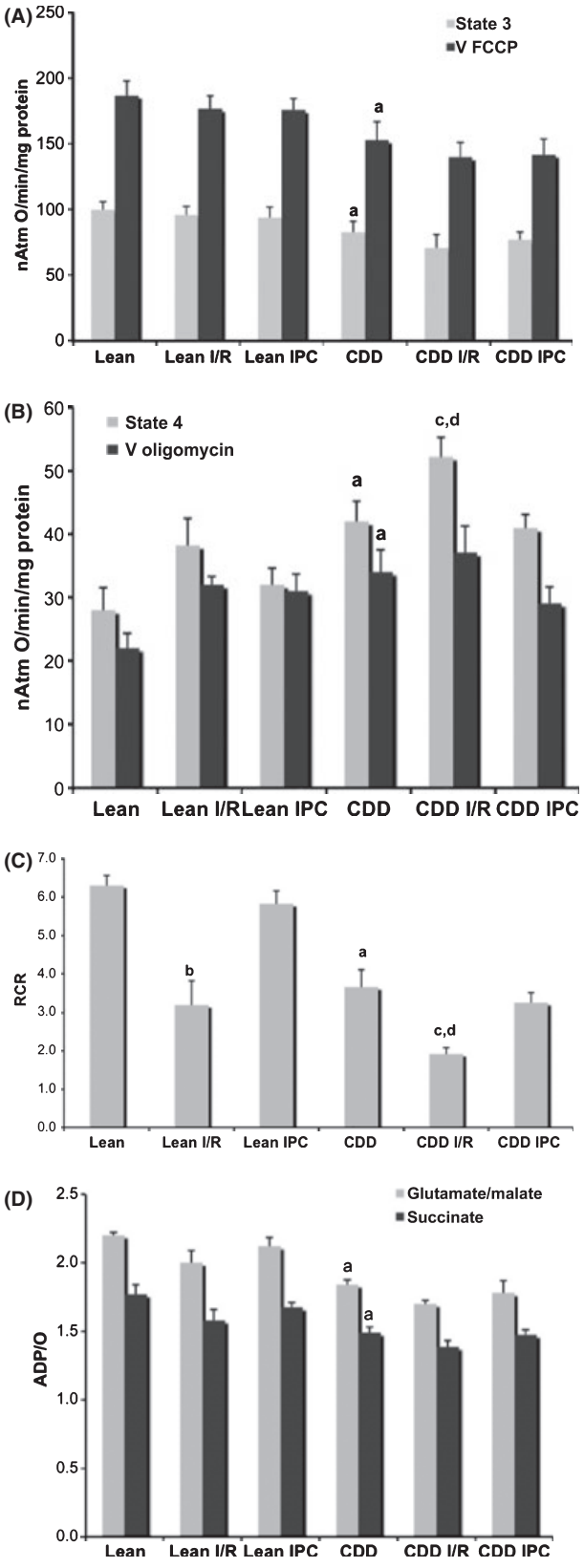


Figure 4 (A) State 3 respiration and FCCP-stimulated oxygen consumption (V FCCP), (B) State 4 respiration and oligomycin-inhibited oxygen consumption, (C) Respiratory control ratio (RCR) and (D) ADP/O in mitochondria isolated from livers subjected to ischemia/reperfusion. Reactions were carried out in 1 ml of reaction medium, supplemented with 2 μm rotenone and 1 mg of freshly isolated mitochondria, as described in materials and methods. Energization was achieved with 5 mm succinate (or glutamate/malate for ADP/O) and phosphorylation induced by 200 nmol ADP. No differences were observed on state 3 and FCCP-stimulated oxygen consumption in both lean and CDD animals subjected to I/R. IPC prevented the increase in state 4 respiration observed in CDD I/R group. RCR was significantly decreased in fatty livers (CDD), as well as in lean and fatty livers after I/R. IPC of both fatty and lean livers preserved the RCR. After I/R, no statistically significant difference was observed in the ADP/O ratio. Data are means ± SEM of six animals in each group. [a]Statistically significant difference ($P < 0.05$) of Lean versus CDD, [b]Statistically significant difference ($P < 0.05$) of Lean I/R versus Lean and Lean IPC, [c]Statistically significant difference ($P < 0.05$) of CDD I/R versus CDD and CDD IPC, [d]Statistically significant difference ($P < 0.05$) of CDD I/R versus Lean I/R.

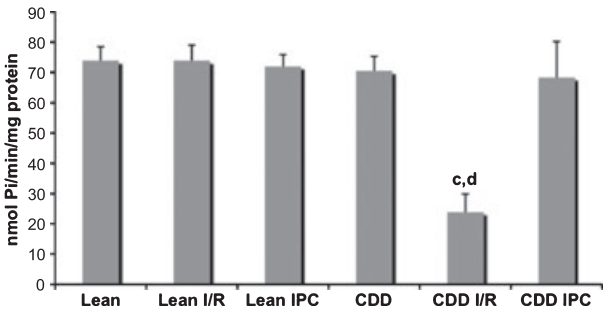


Figure 5 ATPase activity in mitochondria isolated from livers subjected to ischemia/reperfusion. ATPase activity was evaluated spectrophotometrically at 660 nm, in association with ATP hydrolysis. Reactions were carried out in 2 ml of reaction medium, supplemented with 0.25 mg of freeze–thawed mitochondria, as described in materials and methods. ATPase activity was significantly decreased in mitochondria from non-preconditioned fatty livers subjected to I/R (CDD I/R), when compared with lean livers. Ischemic preconditioning (CDD IPC) prevented the decrease in ATPase activity in fatty animals. Data are means ± SEM of six animals in each group. [c]Statistically significant difference ($P < 0.05$) of CDD I/R versus CDD and CDD IPC, [d]Statistically significant difference ($P < 0.05$) of CDD I/R versus Lean I/R.

Table 2. Endogenous adenine nucleotides content.

	CDD I/R	CDD IPC
ATP (nmol/mg protein)	2.09 ± 0.63*	3.28 ± 0.61
ADP (nmol/mg protein)	3.84 ± 0.84*	1.73 ± 0.39
AMP (nmol/mg protein)	3.42 ± 0.21*	2.21 ± 0.28
ATP/ADP	0.54 ± 0.13*	1.91 ± 0.19
(ATP + ADP/2)/(ATP + ADP + AMP)	0.42 ± 0.04*	0.57 ± 0.01

Data are means ± SEM of six animals in each group.
CDD, choline-deficient diet; IPC, ischemic preconditioning; I/R, ischemia/reperfusion.
*Statistically significant difference ($P < 0.05$).

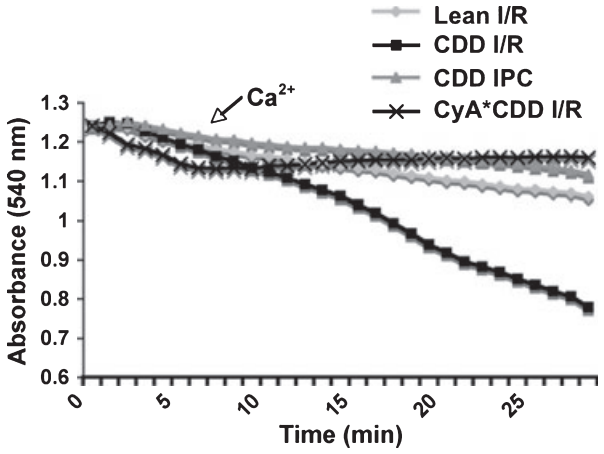


Figure 6 Susceptibility to induction of the mitochondrial permeability transition in mitochondria isolated from livers subjected to ischemia/reperfusion. Experiments were started by the addition of mitochondria (1 mg) to 2 ml of reaction medium supplemented with 3 μm rotenone, 0.5 μg oligomycin and 5 mm succinate. MPT was induced with 30 nmol $CaCl_2$ where indicated by the arrow. Cyclosporin A CyA (1 μm) was added to the reaction medium prior to calcium addition. The traces are representative of experiments performed with six different mitochondrial preparations. Mitochondria from non-preconditioned fatty livers (CDD I/R)were more susceptible to the MPT, when compared with lean animals subjected to I/R. Ischemic preconditioning (CDD IPC) prevented MPT induction in CDD I/R animals.

CyA (an *in vitro* inhibitor of MPT pore induction) completely prevented MPT induction. IPC restored the capacity of mitochondria from fatty livers to accumulate calcium, without inducing the MPT.

Discussion

This report demonstrates that the protection afforded by IPC to fatty livers that were subsequently subjected to I/R is associated with preservation of mitochondrial function efficiency. These results are in accordance with previous work by Selzner *et al.* that have demonstrated that in fatty livers, IPC preserves ATP content during ischemia and restores intrahepatic ATP after reperfusion [22]. In cold ischemia, IPC has also been shown to improve cellular energy metabolism, thus increasing survival of recipients of fatty organs [24,41]. However, the exact mechanism by which IPC ensures the preservation of ATP content in fatty livers is not yet elucidated. Here we demonstrate that the preservation of ATP content and the subsequent prevention of I/R damage in fatty livers are related with increased tolerance of mitochondrial function.

Fatty liver is associated with impaired ATP synthesis caused by intracellular accumulation of nonesterified fatty acids that increase mitochondrial uncoupling and inhibit gluconeogenesis [14–16]. Fat accumulation in the liver is also associated to decreased $\Delta\Psi$ and oxygen consumption, as well as decreased efficiency of the phosphorylation system, caused by depletion of the adenine nucleotide translocator [17,19]. Upon I/R, mitochondria isolated from fatty livers (CDD I/R) exhibited decreased $\Delta\Psi$ and a delay in the repolarization after a phosphorylation cycle, associated with increased state 4 respiration and increased susceptibility to MPT induction. In preconditioned fatty livers subjected to I/R (CDD IPC), mitochondrial function was improved to values comparable to lean animals subjected to I/R. $\Delta\Psi$ analysis revealed that the capability of creating and maintaining a potential was compromised on mitochondria from fatty livers, a pre-existing condition that was aggravated by I/R. This could be the result of increased permeability of the mitochondrial inner membrane to protons (proton leak]. In fact, the resting mitochondrial oxygen consumption, the state 4 respiratory rate, was also significantly increased in fatty livers upon I/R, in relation to lean and non-I/R fatty animals. IPC prevented the increase in state 4 respiration thus enabling the generation of higher $\Delta\Psi$.

Interestingly, regarding oxidative capacity as shown by ADP and FCCP-induced oxygen consumption, no differences were observed between fatty and lean livers subjected to I/R. However, the phosphorylative efficiency was affected in fatty livers upon I/R, as indicated by the increased lag phase. Such alteration caused a decrease in the ATP/ADP ratio, which was preserved by preconditioning. The decrease in ATPase activity in fatty livers is the probable cause for the loss of mitochondrial phosphorylative efficiency induced by I/R, as fatty animals that were not subjected to I/R did not exhibit impaired ATPase activity. Mitochondrial respiratory rate, in the presence of oligomycin, a specific inhibitor of the ATPsynthase, was not statistically different between lean and fatty animals subjected to I/R, indicating that the increase in the resting mitochondrial oxygen consumption and decreased $\Delta\Psi$, in non-preconditioned fatty livers subjected to I/R, is caused by impaired proton slip at the ATPsynthase. Caraceni *et al.* have shown that fatty infiltration exacerbates mitochondrial oxidative injury during I/R, by reducing F_0–F_1-ATPsynthase content [13]. Indeed, increased rates of fatty acid oxidation caused by fat accumulation enhance lipid and protein oxidation in fatty livers [19,42–44]. Depletion of antioxidant defenses in fatty liver further aggravates oxidative damage [44–46].The novelty in our study is that in spite of the increased susceptibility to oxidative damage, IPC is found to be capable of preserving ATPsynthase activity and thus the phosphorylative efficiency in fatty livers.

The protection elicited by IPC may be also mediated through the modulation of the MPT [20,47]. The MPT is a marker of impaired mitochondrial function that is

evident in hepatic I/R injury. MPT induction is promoted by high level of matrix calcium, ROS, fatty acids and a $\Delta\Psi$ reduction, which all occur during I/R, and result in mitochondrial swelling and uncoupling of oxidative phosphorylation. To evaluate whether mitochondria isolated from fatty animals subjected to I/R exhibited increased susceptibility to MPT induction, mitochondria were exposed to a pulse of calcium as an MPT inducer. Our data clearly shows that mitochondria isolated from non-preconditioned I/R fatty livers have decreased ability to withstand a calcium challenge, when compared with lean I/R animals. Thus, ATP depletion and necrotic cell death observed in fatty livers subjected to I/R [3,22] could also be related with sustained activation of the MPT, as mitochondria isolated from fatty animals subjected to I/R are more susceptible to MPT inducers. Non-preconditioned I/R fatty livers non-preconditioned I/R fatty livers non-preconditioned I/R fatty livers non-preconditioned I/R fatty livers.*In vivo*, by increasing the resistance to MPT induction in fatty livers subjected to I/R, IPC has been found to increase the number of cells that have suffered a sublethal injury during I/R that would recover**.**

In conclusion, although fatty livers have predisposing factors for enhanced risk of I/R injury, IPC is a protective strategy that preserves the efficiency of mitochondrial function. This study shows that in fatty livers, IPC is effective in preventing loss or alteration of mitochondrial function and energy metabolism caused by I/R. This prevention involves decreased susceptibility to MPT induction and preservation of ATPsynthase activity, thus increasing the tolerance of fatty livers to I/R injury.

Authorship

Anabela Pinto Rolo designed study, performed research, analysed data, wrote the paper; João Soeiro Teodoro collected data; Carmen Peralta designed study; Joan Rosello-Catafau designed research, discussed data; Carlos M. Palmeira designed research, discussed data.

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