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Hyperoxia fully protects mitochondria of explanted livers

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Abstract Liver ischemia-reperfusion injury is still an open problem in many clinical circumstances, including surgery and transplantation. This study investigates how mitochondrial structure, mass and oxidative phosphorylation change and may be preserved during a brief period of ischemia followed by a long period of reperfusion, an experimental model that mimics the condition to which a liver is exposed during transplantation. Livers were explanted from rats and exposed for 24 h to three different oxygen availability conditions at 4 °C. Mitochondrial mass, respiration, oxidative phosphorylation (OXPHOS), and levels of OXPHOS complexes were all significantly altered in livers stored under the currently used preservation condition of normoxia. Remarkably, liver perfusion with hyperoxic solutions fully preserved mitochondrial morphology and function, suggesting that perfusion of the graft with hyperoxic solution should be considered in human transplantation.

Keywords Mitochondria · Ischemic injury · Hypoxia · Hyperbaric oxygen · Autophagy · Oxidative phosphorylation

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Abbreviations

ATA	Atmospheres Absolute
Complex I-IV	Respiratory Chain Complexes
Complex V	Mitochondrial ATP synthase or F_1F_0-ATPase
DTNB	5,5'-dithio-bis(2-nitrobenzoic) acid
HHMP	Hyperbaric Hypothermic Machine Perfusion
I/R	Ischemia/Reperfusion
MMP	Mitochondrial Membrane Potential (inner membrane)
NO	Nitric Oxide
OXPHOS	Oxidative Phosphorylation
RCR	Respiratory Control Ratio
ROS	Reactive Oxygen Species
RH-123	Rhodamine-123

Introduction

The pathophysiology of liver injury following ischemia/reperfusion (I/R) is not completely understood, particularly upon application of long term perfusion. On the other hand,

I/R injury remains a significant clinical problem in a wide range of medical specialties representing a challenge in many circumstances. The liver has been extensively studied due to the many clinical conditions where it is susceptible to I/R damage including surgery, trauma, and liver transplantation. In this respect, I/R injury may dramatically influence short-term and long-term outcomes after transplantation. The critical step initiating I/R injury is known to involve significant reprogramming of the cellular metabolism during the ischemic period (Schneider et al. 2010; Anaya-Prado and Delgado-Vázquez 2008). Variation in the energy metabolism is an important feature of preservation injury (Vajdovà et al. 2002). Hypothermia at 0–4 °C is a key factor for organ preservation by reducing the cellular metabolic activity by about 90–95% in rats (Van der Plaats et al. 2004). However, even at low temperature, metabolism still requires 0.27 mol oxygen/min/g of liver (Van der Plaats et al. 2004), which is not provided by the current static storage. Oxygen is mainly taken up by mitochondria for synthesis of adenosine triphosphate (ATP); therefore, the lack of oxygen results in failure of the respiratory chain and consequently ATP can only be generated by anaerobic glycolysis. Thus, once cellular glycogen stores are consumed, ATP depletion rapidly ensues leading to a series of events that eventually cause irreversible cell injury and death (Rosser and Gores 1995).

Reduced oxygen level (hypoxia) enhances anaerobic glycolysis, decreases the mitochondrial respiration rate and impairs oxidative phosphorylation, resulting in cellular energy deficiency and increased production of reactive oxygen species (ROS) (reviewed in Solaini et al. 2010). However, the reperfusion step is even more dangerous for the cells due to the dramatic ROS production caused by the leak of electrons from the redox centers of the respiratory chain as a consequence of the respiratory chain complexes impairment due to the previous hypoxic insult (Solaini and Harris 2005). Therefore, reduced energy availability to the cells and enhanced ROS production contribute to cellular dysfunction and death (Glantzounis et al. 2005).

If an explanted organ has to be re-implanted, in order to limit its damage over a long preservation time (at least 12 h), a supply of oxygen (and nutrients) is required. This may be accomplished by reperfusing the organ with different kinds of solutions obtaining some improvement, but oxygen might still be deficient within cells (Anaya-Prado and Delgado-Vázquez 2008). Indeed some years ago it was shown that nitric oxide, an efficient vasodilator, protects rat hepatocytes against reperfusion injury presumably by allowing an increased oxygen supply (Kim et al. 2004), and recent findings indicate protective effects in human liver transplantation by inhalation of a non toxic dose of nitric oxide in recipients (de Rougemont et al. 2010). Concomitantly, a study in our laboratory has evidenced that explanted rat livers could be better preserved for a 24 h period if continuously perfused with a hypothermic and hyperbaric Celsior solution (Giannone et al. unpublished results). In these previous studies, we observed a general amelioration of the hepatocytes structure, reduced glycogen depletion, and reduced oxidative stress. On the basis of these results, in particular the "salvage" of glycogen and the reduced oxidative stress, we postulate that in the explanted livers dynamically preserved with hyperoxic solutions, mitochondria might be better preserved leading to improved energy production. To verify this, we examined the structure and the main oxidative phosphorylation parameters of mitochondria isolated from explanted rat livers that were preserved under normoxic and hyperoxic experimental conditions. This study provides further insights into the characterization of the effects of I/R on hepatocytes at molecular levels, and will possibly be valuable for optimization of graft preservation and survival.

Materials and methods

Experimental design

Fed Sprague–Dawley rats (Charles-River Laboratories, Calco, Italy), weighing 250–300 g, were anesthetized (Zoletil-100, Virbac, France) between 9:00 and 10:00 a.m. and the abdomen opened with a mid-line incision. Afterwards, heparin 500 IU/liter was injected through the infrahepatic vena cava and the portal vein cannulated with a 16 G angiocath. The liver was then flushed out with 20 ml of cold Celsior solution, immediately explanted and flushed out again with 30 ml of cold Celsior solution (IMTIX SangStat Company, Lyon, France). The livers were finally assigned to the following experimental groups:

1. *Baseline controls*: liver tissue samples were collected immediately after excision for mitochondrial isolation and for the ultrastructural, histological, and biochemical analysis.
2. *Normobaric static preservation*: livers were kept at 4 °C in the Hyperbaric Hypothermic Machine Perfusion (HHMP) under normobaric conditions (1 atmospheres absolute or 1 ATA) immersed in the Celsior solution for 24 h. This group mimics the current static storage used in clinical transplantation.
3. *Hyperbaric static preservation*: livers were kept at 4 °C in the HHMP under hyperbaric conditions (2 ATA) immersed in the Celsior solution for 24 h. Compression and decompression were carried out progressively at a rate of 0.2 ATA/min.
4. *Hyperbaric dynamic preservation*: livers were kept at 4 °C in the HHMP under hyperbaric conditions (2

ATA) and continuously perfused with Celsior solution at 1 ml/min/g liver for 24 h. Compression and decompression were carried out progressively at a rate of 0.2 ATA/min.

After 24 h of cold preservation, tissue samples were immediately collected for mitochondrial isolation and for the ultrastructural, histological, and biochemical analysis. This preservation time was chosen based on preliminary experiments showing that liver damage increased progressively with the preservation period (6, 12, and 24 h), under normobaric conditions All procedures involving rats were conducted in accordance with internationally accepted principles for care of laboratory animals (EEC Council Directive 86/609, OJ L358, 1, December 12, 1987) and the guidelines approved by the ethical committee of our University.

Hyperbaric hypothermic machine perfusion (HHMP)

The HHMP, designed and patented by "Centro Iperbarico s.r.l.", Ravenna, Italy, consists of a hyperbaric container where the organ is stored, totally immersed in the preservation solution, with a residual free volume in the upper part containing a gas mixture of 95% O_2 and 5% CO_2. The hyperbaric chamber is enclosed into a conditioning system, which allows the exact regulation and control of the temperature and pressure (ranging from 0 to 2.5 ATA). The perfusion of the organ with the preservation solution throughout the portal vein is guaranteed by a peristaltic pump (Gilson Minupulse, Villiers Le Bel, France) located outside the hyperbaric chamber. A second peristaltic pump is used to generate a continuous movement of the preservation solution aiming to enhance oxygen diffusion within the solution.

Liver ultrastructure

Liver samples were cut into small pieces, fixed in 4% formaldehyde solution and post-fixed in 1% OsO_4 in 0.1 M Sörensen buffer. All the samples were then dehydrated in alcohol and embedded in Epon. Thin sections were double stained with uranium and lead, and observed with a Philips 410 Electron Microscope.

Assessment of mitochondrial function and energy metabolism

Respiration and oxidative phosphorylation rate measurements

Mitochondria (RLM) were isolated from rat liver according to a previously reported procedure (Barogi et al. 1995). The respiratory rate of liver mitochondria (1 mg/ml) was assayed polarographycally using a Clark-type oxygen electrode with either 10 mM glutamate/malate or 20 mM succinate as substrate (Aleardi et al. 2005). State 3 respiration was obtained by addition of 0.4 mM ADP. The respiratory control ratio (RCR) is defined as the State 3/State 4 (in the absence of ADP) respiratory rates ratio. ATP synthesis rate was monitored by the luciferin-luciferase chemiluminescent method, as recently described (Spinazzi et al. 2008). Essentially liver mitochondria at 0.4 mg/ml protein concentration were incubated with10 mM glutamate/malate or 20 mM succinate as respiratory substrates and then 0.5 mM ADP was added to start the reaction. Incubation was carried out for 3 min at 30 °C, and the ATP content of the sample was assayed by the luciferin/luciferase method.

Citrate synthase and protein assay

Citrate synthase activity of rat liver was detected by incubating 10–50 μg mitochondrial protein in 1 ml of 0.125 M Tris–HCl, 0.2% triton, 0.1 mM acetyl-coenzyme A, 0.1 mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), and 0.5 mM oxaloacetate. The activity was assessed by monitoring at 412 nm the release of 2-nitro-5-thiobenzoate (ε=13.6 mM^{-1} cm^{-1}) (Baracca et al. 2007). Protein concentration was measured by the Biuret method using bovine serum albumin as standard.

Mitochondrial membrane potential measurements

The mitochondrial membrane potential was assayed using the potentiometric fluorescent probe Rhodamine 123 (RH-123). Rhodamine was dissolved in ethanol and the concentration was assayed spectrophotometrically at 507 nm (ε=101 mM^{-1} cm^{-1}). Fluorescence measurements were carried out at 25 °C with a Jasco FP-777 spectrofluorometer using a thermostatic apparatus, to avoid differential temperature-dependent unspecific binding of the fluorescent cation to mitochondria. RH-123 was excited at 503 nm and fluorescence emission was recorded at 527 nm. During the measurements, the reaction medium containing mitochondria was continuously stirred. Mitochondrial membrane potential changes were evaluated by measuring RH-123 fluorescence quenching under the following conditions: 0.03 mg rat liver mitochondrial protein were added to 0.5 ml buffer (250 mM sucrose, 10 mM HEPES, 100 μM K-EGTA, 2 mM $MgCl_2$, 4 mM KH_2PO_4, pH 7.4) containing an ADP regenerating system (10 mM glucose and 2.5 U hexokinase). Before rhodamine (50 nM) addition, samples were incubated with 33 nM cyclosporin A, and 0.2 mM ADP. Finally, mitochondria were energized by 10 mM glutamate/malate in the presence of oligomycin (2 μM) to detect membrane potential changes associated with State 4

respiratory conditions. The steady-state fluorescence quenching of RH-123 was then measured (Solaini et al. 2007).

SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting analysis

To evaluate the OXPHOS complexes content, mitochondria were treated with two cycles of sonic radiations (5 s each) and finally were resuspended in the SDS loading buffer. Samples were electrophoretically separated by SDS-PAGE and blotted onto nitrocellulose membrane.

The amount of oxidative phosphorylation (OXPHOS) complexes of rat liver mitochondria was determined by Western blotting analysis as previously described (Baracca et al. 2010). Briefly, mitochondrial proteins were exposed to a cocktail of monoclonal antibodies (MitoSciences Inc., Eugene, OR, USA) specific for single subunits of each OXPHOS complex, as follows: NDUFA9 (39 KDa) of Complex I, SDHA (70 KDa) of Complex II, Rieske protein (22 KDa, apparent molecular weight is 30 KDa) of Complex III, COX-I (57 KDa, apparent 45 KDa) of Complex IV, β-subunit (52 KDa) of Complex V. Detection of those primary antibodies was achieved using a secondary goat anti-mouse IgG_{H+L} antibody labeled with horseradish peroxidase (Molecular Probes, Eugene, OR, USA) and a chemiluminescent technique based on the ECL^{TM} Advance Western Blotting Detection Reagent Kit (Amersham Biosciences, Piscataway, NJ, USA).

Statistical analysis

Data were analyzed by means of the two-way analysis of variance (ANOVA) with Bonferroni's post-hoc test. Statistical analysis was performed by running the OriginPro 7.5 (OriginLab Corporation, MA, USA) statistical package on a personal computer. Data are reported as mean values ± SD. Two-tailed P values of less than 0.05 were regarded as statistically significant.

Results

Structure of mitochondria

Figure 1 shows the typical ultrastructural patterns of hepatocytes mitochondria obtained from livers exposed to the different conditions examined. Mitochondria of control hepatocytes exhibit a normal organization of external and internal membranes, and of the cristae, together with a matrix characterized by intrinsic light electron opacity (Fig. 1a). Figure 1d shows the mitochondrial morphology of rat hepatocytes after hyperbaric dynamic preservation of livers. No changes are visible in mitochondria if compared with panel la, thus indicating that the hyperbaric dynamic condition allows a very good preservation of the cytoplasmic components of hepatocytes. This was not the case when the livers were stored under other experimental conditions. In fact, in rat liver preserved under hyperbaric static conditions (Fig. 1c), lightly swollen mitochondria can be observed, together with the reduction of the electron-opacity of the matrix. In hepatocytes of rat stored 24 h under the currently used normobaric static preservation conditions (Fig. 1b), the mitochondrial changes appear more severe: mitochondria are deeply swollen, with a reduced matrix electron-opacity, and with the occurrence of clusters of electron-opaque granules, quite similar to calcium salts inclusions.

Mitochondrial content and function

To examine how hyperoxia influences mitochondrial function in explanted organs, after 24 h hypothermic storage, rat livers were homogenized to evaluate mitochondrial mass by assaying the citrate synthase activity, which is solely present in the mitochondrial matrix (Sgarbi et al. 2006). To analyze mitochondrial dysfunction induced by the different organ storage conditions, mitochondria were isolated from the homogenates. Activities from the excised livers of control rats (mitochondria isolated immediately after explantation) were regarded as the baseline for comparison.

The specific activity of citrate synthase assayed on isolated mitochondria showed slight changes under the different liver preservation conditions (Fig. 2a), reaching the statistical significance only when controls were compared with livers stored under the currently used conditions. However, the total citrate synthase activity dramatically decreased (−60%) in crude extract of livers preserved for 24 h under the currently used static storage as compared to baseline controls (Fig. 2b). This is consistent with recently reported data, showing reduced mitochondrial mass caused by autophagy in hypoxic cells (Solaini et al. 2010; Semenza 2011; Rautou et al. 2010). In addition, hyperbaric static preservation was not protective, but the combination of continuous perfusion and hyperbaric oxygen could efficiently preserve mitochondria (about 80%) in explanted livers stored as long as 24 h. Incidentally, dynamic perfusion with normoxic solutions could not significantly exert effective protection of the mitochondria (data not shown).

Respiration using either NAD-dependent substrates (glutamate plus malate) or succinate was assayed in isolated mitochondria (Figs. 3 and 4). The respiratory control ratio, RCR (i.e. the ratio of respiratory rate in State 3 (in the presence of ADP) to State 4 in the absence of ADP), is an index of the functional integrity of the inner mitochondrial

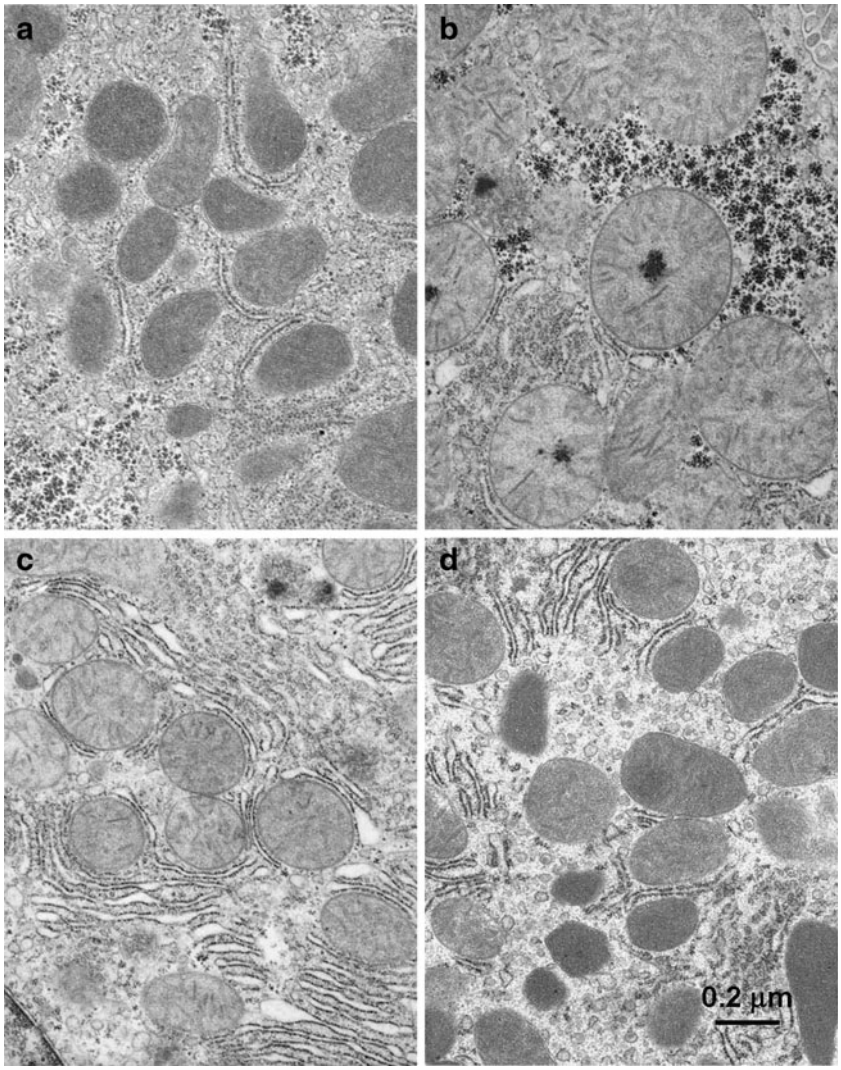

Fig. 1 Ultrastructural Pattern of Mitochondria in the Different Conditions Examined. **a** *normal rat liver.* Numerous mitochondria are visible, exhibiting a normal pattern of internal and external membranes. Mitochondrial matrix is lightly electron-opaque. **b** *rat liver after normobaric static preservation.* A marked enlargement of mitochondria is visible, together with the reduction of electron-opacity of the matrix. Electron-opaque deposits, very likely inorganic in nature, are visible in the mitochondrial matrix. **c** *rat liver after hyperbaric static preservation.* Mitochondria appear to be lightly swollen with a reduction of the electron opacity of the matrix. **d** *rat liver after hyperbaric dynamic preservation.* The ultrastructural pattern of mitochondria appears to be well preserved. Both the mitochondrial membranes and the matrix appear unchanged relative to controls. Bar, 0.2 μm

membrane and indirectly of the OXPHOS efficiency. After the current static liver storage, RCR was significantly decreased using either glutamate/malate (−30%) or succinate (−35%) as substrates. While the addition of hyperbaric oxygen alone produced a limited effect (RCR was nearly −20% of the control mean), hyperbaric dynamic preservation of livers fully restored the baseline RCR (Figs. 3 and 4). It is noteworthy that the respiratory rate was significantly higher in mitochondria isolated from liver preserved under hyperbaric dynamic conditions even compared to the untreated controls; however, since the rate was similarly affected in both State 3 and State 4, the RCR was comparable to the controls. At variance, as it was suggested from the increase of the solely state 4 respiratory rate, the significant decrease of the RCRs in mitochondria of liver stored under both normobaric and hyperbaric static conditions was mainly due to uncoupling of ATP synthesis and respiration.

Proper mitochondrial membrane potential (MMP) is critical for appropriate cellular bioenergetic homeostasis, and its anomalous change is an important event associated with progression of mitochondrial dysfunction. Therefore, by the Rhodamine fluorescence quenching approach (Baracca et al. 2007), we evaluated the mitochondrial membrane potential in State 4 respiratory conditions using glutamate plus malate as substrates (Fig. 5). After the static liver storage MMP was lower with respect to controls, supporting the uncoupling hypothesis based on the respiration measurements described above. Significantly, only hyperbaric dynamic preservation was able to restore the control conditions.

As compared to baseline controls, the current static storage induced a significant decrease (−20 or −30% depending on the respiratory substrate) of the mitochondrial ATP synthesis rate; indeed, dynamic preservation and hyperbaric conditions induced an enhancement of ATP synthesis rate (+70%) even above the control (Fig. 6), in accord with the respiration rate measured in State 3. Also, in accordance with the respiration rate described above, the

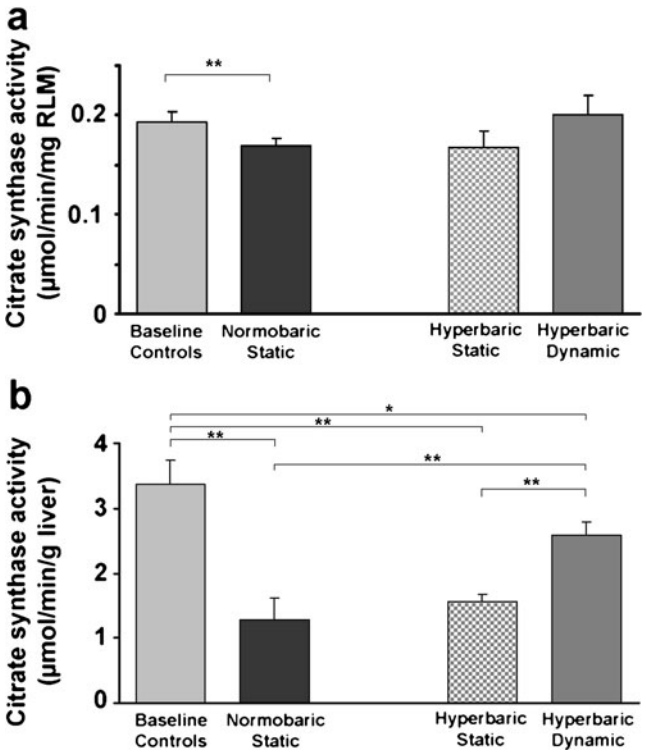

Fig. 2 Assay of the Citrate Synthase Activity. The citrate synthase activity was assayed essentially as described in (Baracca et al. 2007) by incubating either 10 µg protein of isolated mitochondria (**a**) or 50 µg of crude wet liver homogenate (**b**) with the reaction mixture. Enzymatic activity was expressed either as µmol/min/mg mitochondrial protein or µmol/min/g dry liver. The assays were performed in either duplicate or triplicate for each liver sample. Data are expressed as means ± SD. Data were considered significantly different when $P \leq 0.05$ (*) or $P \leq 0.01$ (**)

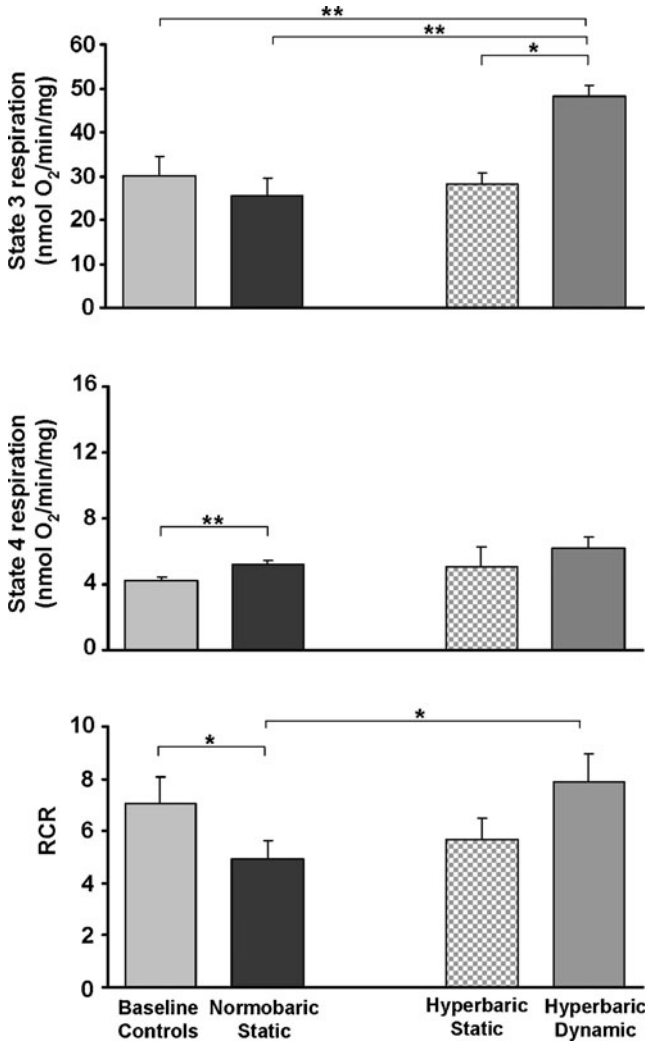

Fig. 3 Respiration Rate of Mitochondria Energized with Complex I-dependent Substrates. Oxygen consumption rates were measured in isolated mitochondria from livers stored under the different experimental conditions tested. Mitochondria were energized with glutamate/malate as substrates under State 3 and State 4 respiratory conditions. The respiratory control ratio (RCR) is reported in the bottom panel. The data are averages +/−SD of four independent experiments. Data were considered significantly different when $P \leq 0.05$ (*) or $P \leq 0.01$ (**)

OXPHOS rate was not significantly preserved when livers were exposed to static hyperbaric oxygen.

To state whether the different rates of respiration and ATP synthesis were due to changes of the mitochondrial content of OXPHOS complexes, liver mitochondria were run on a SDS-polyacrylamide gel electrophoresis and the five complexes were quantified by Western blotting (Fig. 7). The current static storage was associated with an approximately 25–30% reduction of Complexes I, II, IV, and V, which was fully reversed by dynamic preservation under hyperbaric conditions. Again, the addition of hyperbaric oxygen alone was not sufficient to preserve the OXPHOS complexes.

Discussion

The novel findings from this study indicate that preservation of an explanted liver over a prolonged period as 24 h can be achieved when functional mitochondria are preserved in hepatocytes. Indeed, only in conditions of dynamic hyperoxia mitochondrial mass was preserved during liver storage, suggesting that under all the other experimental conditions, mitochondria were damaged and possibly removed by autophagy in response to stress signals such as reduced oxygen levels (Youle and Narendra 2011; Ferraro and Cecconi 2007).

Interestingly, long term reperfusion of livers in presence of energy substrates and hyper-oxygenation was shown to fully preserve the morphology of hepatocytes mitochondria, the membrane potential dynamics, the OXPHOS complexes composition, and the oxidative phosphorylation efficiency together with a significant enhancement of the ATP synthesis rate.

The present study shows that static cold preservation beyond reducing the mitochondrial mass of the hepatocytes, induces a decrease of both State 3 respiration and ATP

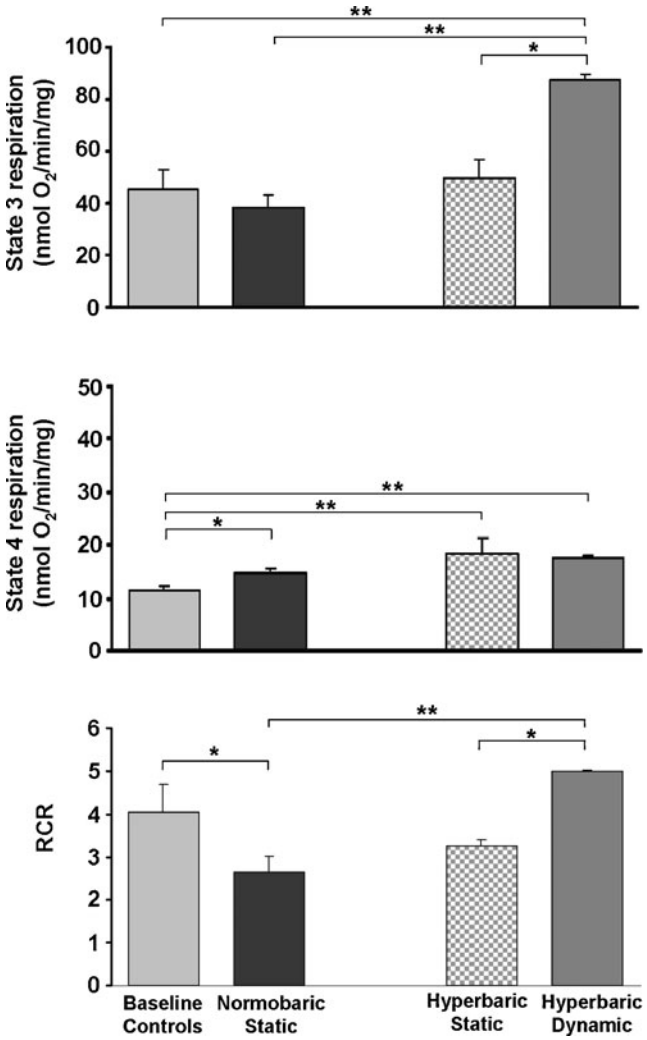

Fig. 4 Respiration Rate of Mitochondria Energized with Succinate. Oxygen consumption rates were measured in isolated mitochondria from livers stored under the different experimental conditions tested. Mitochondria were energized with the Complex II dependent substrate, succinate, in the presence (State 3) or absence of ADP (State 4), respectively. The respiratory control ratio (RCR) is reported in the bottom panel. The data are averages +/−SD of four independent experiments. Data were considered significantly different when $P \leq 0.05$ (*) or $P \leq 0.01$ (**)

synthesis rates compared to control. The decrease in the latter was largely due to an increase of State 4 respiration, indicating partial uncoupling, that was confirmed by the observed decrease of membrane potential in State 4 conditions. The impairment of the functional parameters was accompanied by a decrease in content of the respiratory complexes and ATP synthase. Neither normobaric perfusion (data not shown) nor hyperbaric oxygen alone could restore the control conditions. However, combining continuous perfusion and hyper-oxygenation of the cold preservation solution could induce a strong improvement of critical bioenergetic parameters beyond the positive effects on the mitochondrial morphology.

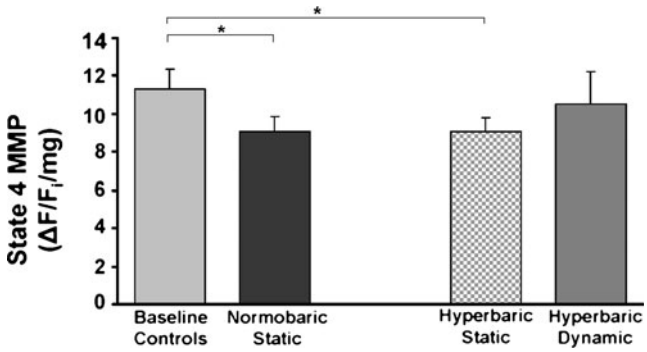

Fig. 5 Membrane Potential of Mitochondria Energized with Complex I-dependent Substrates. The membrane potential (MMP) was assayed in isolated mitochondria energized with glutamate/malate as substrates in presence of oligomycin to induce State 4 respiration condition. The mitochondria from the livers stored under the different conditions examined were loaded with RH-123, and the MMP was expressed by RH-123 fluorescence quenching evaluated upon energization. The data are averages +/−SD of four independent experiments. Data were considered significantly different when $P \leq 0.05$ (*).

Under hypoxic conditions, OXPHOS can result in the generation of reactive oxygen species as byproducts. Furthermore, under normal conditions, 1–2% of the totally

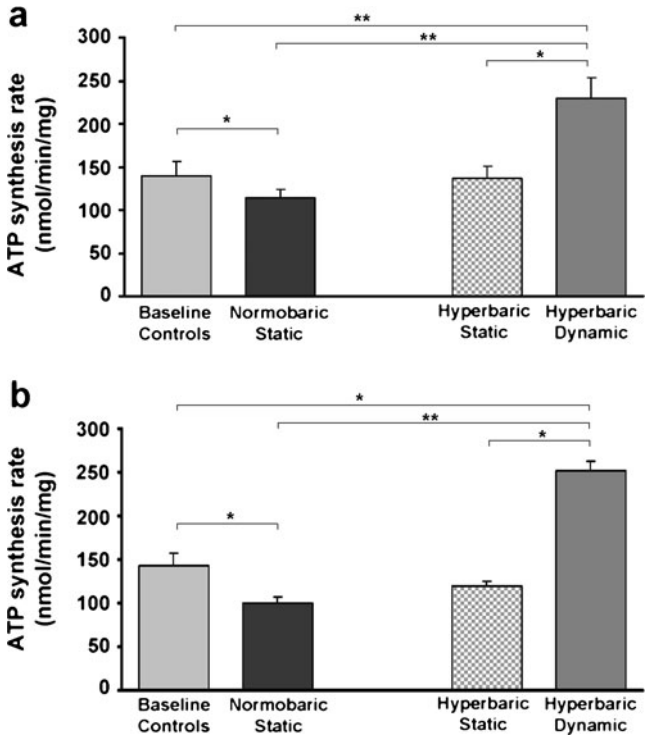

Fig. 6 Rate of Mitochondrial ATP Synthesis. The ATP synthesis rate was assayed as previously reported (Spinazzi et al. 2008) in mitochondria isolated from liver preserved under different experimental conditions. The oxidative phosphorylation rate was estimated by using as respiratory substrate both glutamate/malate (**a**) and succinate (**b**). The data are averages +/−SD of four independent experiments. Data were considered significantly different when $P \leq 0.05$ (*) or $P \leq 0.01$ (**)

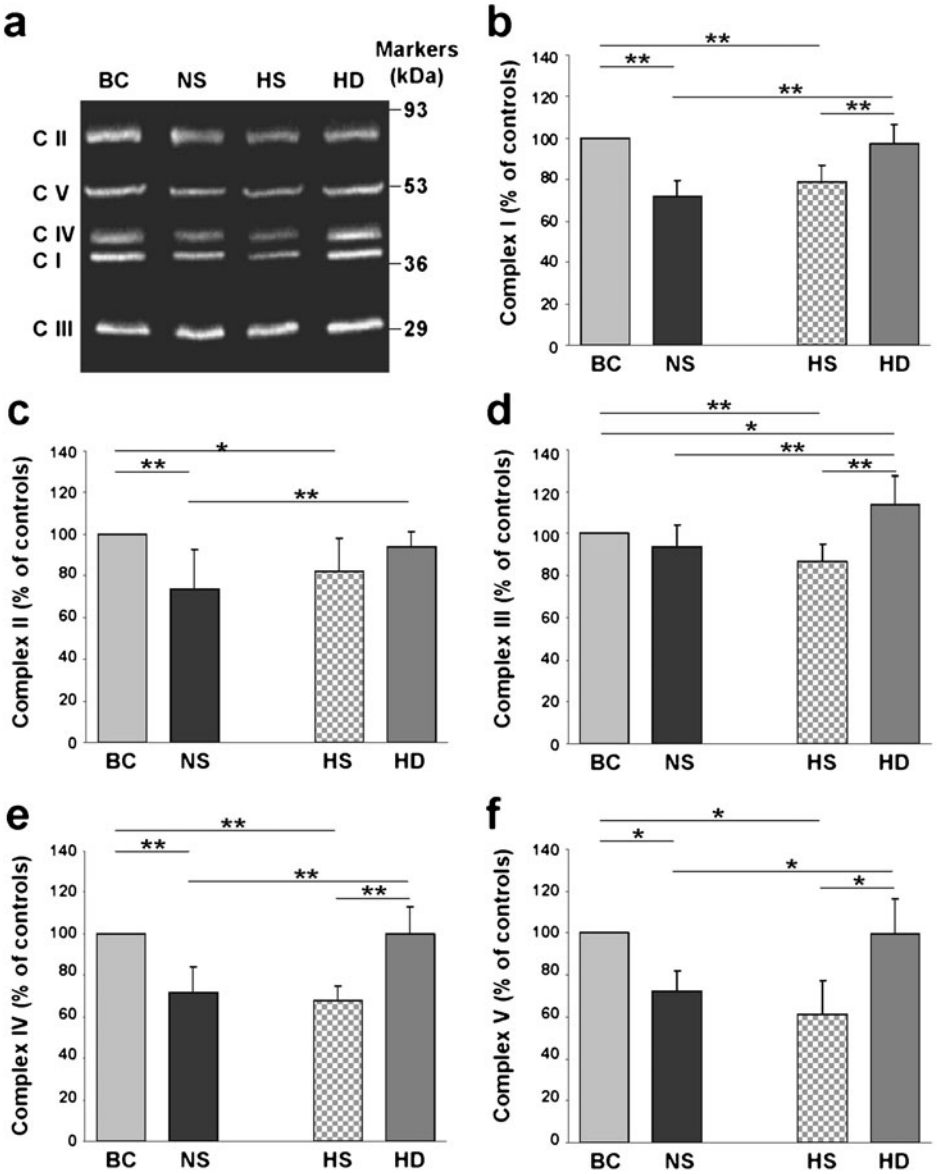

Fig. 7 SDS-PAGE Separation of OXPHOS Complexes. Mitochondrial proteins from rat livers were resolved by SDS-PAGE. Protein bands were then transferred onto nitrocellulose membrane and submitted to immunodetection with monoclonal antibodies specific for single subunits of the OXPHOS complexes: NDUFA9 (39 kDa) of Complex I, SDHA (70 kDa) of Complex II, Rieske protein (22 kDa, apparent molecular weight is 30 kDa) of Complex III, COX-I (57 kDa, apparent molecular weight is 45 kDa) of Complex IV, β-subunit (52 kDa) of Complex V (ATP synthase). Primary antibodies were then detected with a secondary antibody labelled with horseradish peroxidase by chemiluminescence. (**a**). The histograms show a semiquantitative estimation (relative to the baseline controls) of each OXPHOS complex in mitochondria of livers stored 24 h under the various experimental conditions (**b**–**f**). BC, Baseline Controls; NS, Normobaric Static preservation; HS, Hyperbaric Static preservation; HD, Hyperbaric Dynamic preservation

consumed oxygen yields superoxide (Solaini and Harris 2005) which amount may significantly increase in organs exposed to ischemia-reperfusion (Solaini et al. 2010; Huet et al. 2004). The enhanced production of reactive oxygen species (ROS) during cold ischemia has been already reported (Becker et al. 1999; Chandel and Budinger 2007) and only apparently it represents a paradox. Indeed, the decrease of O_2 tension to the hypoxic range of 5 to 0.5% would lower mitochondrial respiration and enhance the reduction state of the mitochondrial redox centres, favouring the electron leak to molecular oxygen and ROS production (Magalhaes et al. 2005), mainly by the respiratory complexes I and III and by cytochrome c (Lenaz et al. 2006). The rate of ROS generation seems to be inversely related to the rate of electron transfer, increasing when Complex I of the respiratory chain is at sub-optimal level as it occurs after prolonged cold ischemia (Kolamunne et al. 2011). It has been reported that under moderate hypoxic conditions the respiratory chain generates ROS that are normally detoxified by Mn- and CuZn-superoxide dismutases, catalase, glutathione peroxidase, and thioredoxins (Lenaz et al. 2006; Circu and Aw 2010), but following ischemia-reperfusion these molecules can accumulate generating oxidative stress that results in both mitochondrial structure damage and function impairment with consequent energy depletion and cell death (Glantzounis et al. 2005). Giannone et al. (unpublished results) observed a similar increased oxidative stress under the cold static preservation conditions of the livers.

The mechanisms linking hyperbaric oxygen and protection against oxidative injury and mitochondrial dysfunction during cold preservation are still elusive. However, we may hypothesize that dynamic hyperbaric oxygen supports the maintenance of an efficient electron transfer through the OXPHOS complexes. This view is supported by the improvement of RCR (i.e. similar to that of freshly prepared mitochondria), associated to an increase of the State 3 respiration, and reduced ROS formation (Huet et al. 2004), which eventually may both prevent the GSH oxidative consumption and allow the de novo synthesis of this vital compound.

It is interesting to note that perfusion under hyperbaric conditions enhances mitochondrial respiration in both State 3 and State 4 even above the untreated controls, with unchanged respiratory control ratio; this enhanced activity is not matched by any increased content above the controls of the respiratory complexes in the hyperbaric perfused livers. It is possible that the perfusion under hyperbaric condition allows a better preservation of the supramolecular organization of the respiratory chain in supercomplexes that improves the efficiency of electron transfer (Lenaz et al. 2010) and ATP synthesis (Chen et al. 2004), hypothesis currently under investigation. This view is supported by the notion that lipid peroxidation does not allow supercomplexes formation in reconstitution studies (Genova et al. 2008). On the other hand, supercomplexes stability is dependent on cardiolipin biosynthesis, which has been reported to decrease when MMP declines (Gohil et al. 2004), as observed in the present study (Fig. 5). In addition, liver dynamic hyper-oxygenation might enhance ATP availability to the cells, which in turn improves protein turnover and mitochondrial biogenesis. We cannot however exclude that perfusion of the stored liver with dynamic hyperbaric oxygen could result in the removal of NO inhibition of cytochrome oxidase (Brunori et al. 1999) that might persist in the mitochondria even after isolation from not well oxygenated tissues.

Finally, it is also possible that prolonged exposure of liver to high oxygen tension contributes to dissociation of the endogenous inhibitor protein (IF_1) from the catalytic domain, F_1, of Complex V (Solaini et al. 2010), enhancing the ATP synthesis rate and hence State 3 respiration also. In other words, even normal control livers that are isolated under partial hypoxic conditions, may have some IF_1 actively bound to F_1, so that State 3 respiration cannot be fully expressed.

Regarding the ATP synthase complex, it might be interesting to recall our previous observations in which severe effects on the enzyme activity were found by exposure of mitochondrial preparations to low temperatures (Solaini et al. 1984; Baracca et al. 1989). When measured at 30 or 8 °C, the structure and the catalytic properties of the ATP synthase complex were critically affected: the specific activity of the enzyme was found 7- or 9-fold decreased, in presence or absence of its membrane domain, F_0, respectively. All together, these observations suggest that warm perfusion with hyperbaric solutions might further improve the mitochondrial performance and consequently the storage conditions of explanted livers. Therefore, the present results support, at the molecular level, previous studies in which hyperbaric oxygen had been shown to be protective against warm ischemia-reperfusion injury in several organs, including the liver, but in which the cellular and molecular mechanisms were poorly addressed (Dutkowski et al. 2006; Monbaliu and Brassil 2010; Hara et al. 2010).

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