



## Differential alterations in mitochondrial function induced by a choline-deficient diet: Understanding fatty liver disease progression <sup>☆</sup>

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### ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is an increasingly reported pathology, characterized by fat accumulation within the hepatocyte. Growing evidences suggest specific effects on mitochondrial metabolism, but it is still unclear the relationship between fatty liver progression and mitochondrial function. In the present work we have investigated the impact of fatty liver on mitochondrial bioenergetic functions and susceptibility to mitochondrial permeability transition (MPT) induction in animals fed a choline-deficient diet (CDD) for 4, 8, 12 or 16 weeks. Mitochondria isolated from CDD animals always exhibited higher state 4 respiration. Mitochondrial membrane potential was decreased in CDD animals at 4 and 16 weeks. At 12 weeks, oxidative phosphorylation was more efficient in CDD animals, suggesting a possible early response trying to revert the deleterious effect of increased triglyceride storage in the liver. However, mitochondrial dysfunction was evident in CDD animals at 16 weeks as indicated by decreased RCR and ADP/O, with a corresponding decrease in respiratory chain enzymes activities. Such loss of respiratory efficiency was associated with accumulation of protein oxidation products, in tissue and mitochondrial fraction. Additionally, although no differences in ATPase activity, the lag phase was increased in mitochondria from CDD animals at 16 weeks, associated with decreased content of the adenine nucleotide translocator. Increased susceptibility to calcium-induced MPT was evident in CDD animals at all time points. These results suggest a dynamic mechanism for the development of NALFD associated with altered mitochondrial function.

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### 1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is an increasing modern world disease, recognized as the basis for inflammatory hepatitis and induction of liver cirrhosis (Clark and Diehl, 2003). It is marked by the accumulation of fat, mainly in the form of free fatty acids and triglycerides, inside the hepatocytes (Pessayre et al., 2002; Diraison et al., 2003). This pathology may be caused by increased dietary fat consumption (an increasingly common phenomenon, especially in developed countries) or due to deficiencies in any of the steps of lipid metabolism. A deregulation in fatty acid's metabolism leads to an overproduction of triglycerides with subsequent fat accumulation inside the liver cells. The excessive abundance of free fatty acids causes a shift in metabo-

lism, turning these molecules into the main energy source of the cell (Haque and Sanyal, 2002; McClain et al., 2004). Such alteration increases lipid peroxidation, destabilizing the inner cell redox environment (Rao and Reddy, 2001).

The choline-deficient diet (CDD) model for fatty liver disease, relates with the fact that choline is essential for the correct assembly and export of the very low density lipoproteins (VLDLs), the main vehicle of fat exportation from the liver to the peripheral adipose tissue. Being unable to correctly produce and export VLDLs, the CDD-fed animals develop fat accumulation in the liver (Koteish and Diehl, 2001).

Being the main ATP production source for the cell (where almost 90% of the ATP is produced), mitochondria play a key role in the majority of metabolic pathways, including a central role in lipid metabolism (Schatz, 1995). It is one of the most susceptible organelles for damage with a profound involvement in the onset of diverse pathologies (Mahler et al., 1997; Jacobsen et al., 2001; Brealey et al., 2002; Sayeed, 2002; DiMauro and Schon, 2003; Duchon, 2004). Indeed, progressive decline of mitochondrial function correlates with disease progression. An example is microvesicular steatosis, which occurs in conditions characterized by severe impairment of the mitochondrial  $\beta$ -oxidation due to genetic and/or acquired causes (Fromenty and Pessayre, 1997).

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Previous work has demonstrated that fat accumulation is severely impairing to mitochondrial function (Leclercq et al., 2000; Caldwell et al., 2004; Douette et al., 2005; Begriche et al., 2006; Teodoro et al., 2006). However, the progression of the pathology has never been reported. As so, we hypothesize that excessive fat accumulation in hepatocytes will impair their mitochondrial bioenergetics, in a cause–effect relationship, in the CDD model. This could supply several pieces of essential information for the further understanding of the disease's onset and progression towards more aggressive pathological situations. By analyzing several parameters of proved relevance we demonstrate that there is an apparent response mechanism of the liver to fat accumulation. This response can revert almost all of the deleterious effects caused by the accumulation of fat. However, this is a transient and frail mechanism, since the continuous aggression caused by the fat destroyed the capacity of response of the organ, which ultimately was associated to, amongst other factors, a severe depletion on the mitochondrial content on adenine nucleotide translocator (ANT). This loss of ANT could be a cause for the observed loss of oxidative phosphorylative efficiency.

## 2. Materials and methods

### 2.1. Animals

Seven-weeks-old male Wistar rats (Charles River, France) were divided in two groups of 24 animals and housed under controlled light and humidity conditions with free access to food and water. The control diet (#118754) and choline-deficient diet (#118753) were obtained from Dyets Inc., Bethlehem, PA, USA. Animals were sacrificed after 4, 8, 12 or 16 weeks of feeding with control or choline-deficient diet. The animals were weighted immediately before sacrifice and killed by cervical dislocation and decapitation.

### 2.2. Materials

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

### 2.3. Liver histology

Livers from the animals were quickly and immediately removed after the animal sacrifice, fixed in 10% buffered formaline and stained with haematoxylin and eosin (H&E) (Palmeira et al., 2003).

### 2.4. Preparation of liver mitochondria

Mitochondria were isolated from the liver of the Wistar rats by conventional methods (Gazotti et al., 1979), with slight modifications (Palmeira and Wallace, 1997). Protein content was determined by the biuret method (Gornall et al., 1949), calibrated with bovine serum albumin.

### 2.5. Evaluation of plasma biochemical parameters

Right after its sacrifice, a sample of the animal's blood was collected, centrifuged at 2300g for 3 min. Plasma was analyzed with commercial kits.

### 2.6. Determination of hepatic triglyceride content

The measurement of the hepatic triglyceride content was performed using a commercially available triglyceride detection kit (Linear Chemicals, Spain). Briefly, 50 mg of liquid nitrogen freeze-thawed hepatic tissue were homogenized in 1 mL isopropanol and immediately centrifuged for 5 min at 2300g. Supernatant

(10  $\mu$ L) was added to a 1 mL of a kit-supplied reagent and incubated at 37 °C for 5 min. The absorbance was measured at 500 nm and the quantification occurred by comparison with a kit-supplied standard.

### 2.7. Evaluation of mitochondrial oxygen consumption

Oxygen consumption of isolated mitochondria was polarographically determined with a Clark oxygen electrode (Estabrook, 1967), as previously described (Palmeira et al., 1994). 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<sub>2</sub>, 5 mM KH<sub>2</sub>PO<sub>4</sub>, 50  $\mu$ M 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  $\mu$ M rotenone, an inhibitor of complex I, were previously added. State 3 respiration was induced by adding ADP. Oxygen consumption was also measured in the presence of oligomycin (0.5  $\mu$ g/mg protein) and 1  $\mu$ M carbonylcyanide-*p*-trifluoromethoxyphenylhydrazon (FCCP).

### 2.8. Evaluation of mitochondrial membrane potential ( $\Delta\Psi$ )

$\Delta\Psi$  was estimated using an ion-selective electrode to measure the distribution of tetraphenylphosphonium (TPP<sup>+</sup>) according to previously established methods (Palmeira et al., 1994; Kamo et al., 1979). Mitochondria (1 mg) were suspended in 1 mL of standard respiratory medium (as in mitochondrial respiration) supplemented with 3  $\mu$ M TPP<sup>+</sup>. A matrix volume of 1.1  $\mu$ L/mg was assumed.

### 2.9. Determination of enzymatic activities

ATPase activity was determined 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<sub>2</sub> and 5 mM Hepes, pH 7.4). After the addition of freeze-thawed mitochondria (0.25 mg), the reaction was initiated by adding 2 mM Mg<sup>2+</sup>-ATP, in the presence or absence of oligomycin (1  $\mu$ g/mg protein). After 10 min, adding 1 mL of 40% trichloroacetic acid stopped the reaction. Samples were centrifuged for 5 min at 3000g and 2 mL of ammonium molybdate plus 2 mL dH<sub>2</sub>O were then added to 1 mL of supernatant. ATPase activity was calculated as the difference in total absorbance and absorbance in the presence of oligomycin.

Succinate-dehydrogenase activity was polarographically determined as previously described (Singer, 1994). The reaction was carried out at 25 °C in 1.3 mL of standard respiratory medium (as in mitochondrial respiration) supplemented with 5 mM succinate, 2  $\mu$ M rotenone, 0.1  $\mu$ g antimycin A, 1 mM KCN and 0.3 mg Triton X-100. After the addition of freeze-thawed mitochondria (0.25 mg), the reaction was initiated by adding 1 mM phenazine-metaphosphate (PMS).

Cytochrome *c* oxidase activity was polarographically determined, as previously described (Brautigan et al., 1978). The reaction was carried out at 25 °C in 1.3 mL of standard respiratory medium (as in mitochondrial respiration) supplemented with 2  $\mu$ M rotenone, 10  $\mu$ M oxidized cytochrome *c* and 0.3 mg Triton X-100. After the addition of freeze-thawed mitochondria (0.25 mg), the reaction was initiated by adding 5 mM ascorbate plus 0.25 mM tetramethylphenylene-diamine (TMPD).

### 2.10. Determination of carbonyl group content

Carbonyl group content in both isolated mitochondria and hepatic tissue was measured spectrophotometrically by using 2,4-dinitrophenyl-hydrazine (DNPH) (Levine et al., 1990; Schild et al.,

1997). Briefly, 100 mg of fresh tissue were homogenized in 1 mL of lysis buffer (250 mM sucrose, 0.5 mM EGTA and 10 mM Hepes, pH 7.4, supplemented with protease inhibitors). Ice-cold trichloroacetic acid (20% w/v, 500  $\mu$ L) was added to mitochondria (1 mg) or tissue homogenate (50  $\mu$ L) and allowed to stand for 15 min at 4 °C. The precipitated protein was collected by centrifugation at 6500g for 10 min, and the supernatant was discarded. The pellet was fully resuspended in 500  $\mu$ L of 0.1 M NaOH and 500  $\mu$ L of 10 mM DNPH dissolved in 2 M HCl were added to the samples. In parallel, a blank was prepared by addition of 500  $\mu$ L of 2 M HCl containing no DNPH to the protein sample. After incubation at room temperature, in the dark for 30 min, 500  $\mu$ L of trichloroacetic acid (20% w/v) were added to the samples. The precipitated protein was collected by centrifugation at 6500g for 10 min, and the supernatant was discarded. The pellet was washed three times with 1 mL of a 1:1 (vol/vol) mixture of ethanol and ethyl acetate and centrifuged at 6500g for 5 min to remove the free reagent. The final precipitated protein was redissolved in 200  $\mu$ L of 6 M guanidine hydrochloride solution. The amount of protein dinitrophenylhydrozone derivative was quantified by measuring the absorbance at 365 nm and converted to nmol of hydrazone by using a molar absorption coefficient of 22,000 M<sup>-1</sup> cm<sup>-1</sup>. Carbonyl content was expressed as nmol of DNPH incorporated per mg of protein.

### 2.11. Western blotting analysis

Frozen mitochondrial pellets were homogenized in lysis buffer (10 mM Tris-HCl, pH 6.8, 0.8% w/v SDS, 4% v/v glycerol, 2% v/v 2-mercaptoethanol and 0.5% w/v bromophenol blue) at 100 °C, for 5 min. Aliquots were fractionated in 8% polyacrylamide gels, transferred to polyvinylidene difluoride membranes (Hybond<sup>TM</sup>-P) and blotted with goat anti-ANT (1:10,000 dilution, sc9299 from Santa Cruz, CA, USA), goat anti-UCP-2 (1:2500 dilution, sc-6526 from Santa Cruz, CA, USA), goat anti-F<sub>1</sub>-ATPase (1:5000 dilution, sc-16689 from Santa Cruz, CA, USA) or rabbit anti-VDAC (1:5000 dilution, Ab3434 from AbCam, UK). The immuno-reactive proteins were visualized with bovine anti-goat IgG alkaline phosphatase conjugated (1:20,000 dilution, sc-2351 from Santa Cruz, CA, USA) and goat anti-rabbit IgG alkaline phosphatase conjugated (1:20,000 dilution, sc-2007 from Santa Cruz, CA, USA). Membranes were reacted with the ECF detection system (Amersham) and read with the VersaDoc imaging system (Bio-Rad Laboratories, Inc., CA, USA). The densitometric analysis was performed with the Quantity One software (Bio-Rad Laboratories, Inc., CA, USA).

### 2.12. Detection of mitochondrial permeability transition induction

The induction of the mitochondrial permeability transition (MPT) was evaluated spectrophotometrically at 540 nm by the determination of the mitochondrial swelling and consequent light scattering diminishing by calcium stimuli (Palmeira and Wallace, 1997). Mitochondria (1 mg) was added to 2 mL of reaction medium (200 mM sucrose, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 5 mM succinate, 10  $\mu$ M EGTA, 3  $\mu$ M rotenone and 10 mM Tris-Mops, pH 7.4) to begin the reaction, at 25 °C. After a brief period, several calcium concentrations were added. In a selected tube, 1  $\mu$ M cyclosporin A (CyA) was added to serve as control.

### 2.13. Citrate synthase assay

Citrate synthase activity was determined spectrophotometrically at 412 nm at 25 °C, as previously described (Srere, 1969).

### 2.14. Statistical analysis

Data are presented as means  $\pm$  SEM of experiments with four different animals in each group. A Student's *t*-test was used for

the comparison between animals fed a control or choline-deficient diet for the same number of weeks. A *P* value <0.05 was considered statistically significant.

## 3. Results

### 3.1. Characterization of the animal model

Animals fed a choline-deficient diet (CDD animals) for 4, 8, 12 or 16 weeks presented an increase in body weight, when compared to control animals, except after 12 weeks of feeding. However, livers from CDD animals were heavier than control animals (Table 1) during all the time of the study, being statistically significant for 4 and 16 weeks. This is probably due to triglyceride accumulation, as shown in Fig. 1. The analysis of the biochemical plasma parameters did not reveal any alteration on the content on cholesterol, triglycerides or any of the evaluated liver enzymes in the blood of the analyzed animals (data not shown).

No specific histological alterations were observed in the livers from control animals. In contrast, under light microscope, sections stained with H&E in the livers from the CDD group showed steatosis (Fig. 2). While the 4, 8 and 12 weeks livers only presented severe macrovesicular steatosis, 16 weeks livers presented macro and microvesicular steatosis, which may suggest an impending change in the behaviour of the pathology. However, no inflammation markers were found, confirming the presence of steatosis alone.

No significant variation in the activity of citrate synthase, a mitochondrial enzymatic marker, was found between mitochondrial preparations from control and CDD animals (data not shown).

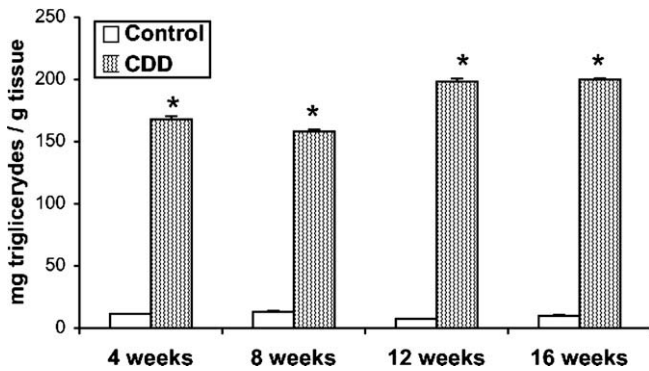
### 3.2. Effects of fat accumulation on mitochondrial transmembrane potential

Taking into account the fundamental role of mitochondrial transmembrane potential ( $\Delta\Psi$ ) for the phenomenon of oxidative phosphorylation,  $\Delta\Psi$  was evaluated in isolated liver mitochondria, after energization with glutamate/malate or succinate. Comparatively to mitochondria isolated from control animals, 4 weeks of feeding with CDD caused a decrease in  $\Delta\Psi$  in mitochondria energized with either glutamate/malate or succinate (Table 2). ADP-induced depolarization was decreased in succinate-energized mitochondria isolated from CDD animals. Furthermore, the mitochondrial capacity to establish  $\Delta\Psi$  after ADP phosphorylation (re-polarization) was also impaired in CDD animals, at 4 weeks. Interestingly, no significant changes were observed in liver mitochondria isolated from 8 weeks CDD-fed animals, suggesting a possible improvement of mitochondrial function (data not shown). In fact,  $\Delta\Psi$  developed by mitochondria isolated from 12 weeks CDD animals, as well as ADP-induced depolarization and  $\Delta\Psi$  after re-polarization, were similar to mitochondria from control animals (Table 2 and Fig. 3A). However, after 16 weeks of feeding with CDD, a decrease in  $\Delta\Psi$  in mitochondria energized with both glutamate/malate and succinate was observed (Table 2 and Fig. 3B). Also,

**Table 1**  
Liver weight in control and animals fed a choline-deficient diet (CDD)

	4 weeks	8 weeks	12 weeks	16 weeks
Control	15.5 $\pm$ 0.96	14.5 $\pm$ 0.65	16.3 $\pm$ 1.25	15.5 $\pm$ 0.5
CDD	24 $\pm$ 0.91*	21 $\pm$ 1.92	21.3 $\pm$ 3.01	21 $\pm$ 1.08*

Values are expressed as means  $\pm$  SEM (*n* = 4) from animals fed for 4, 8, 12 or 16 weeks with a normal (control) or a choline-deficient diet (CDD). Values statistically significantly different from control for each week: \**P* < 0.05.



**Fig. 1.** Total amount of intrahepatic triglyceride content in control and animals fed a choline-deficient diet (CDD). Data expressed as means  $\pm$  SEM of four animals in each group. Values statistically significant different from control, for each week: \* $P < 0.05$ .

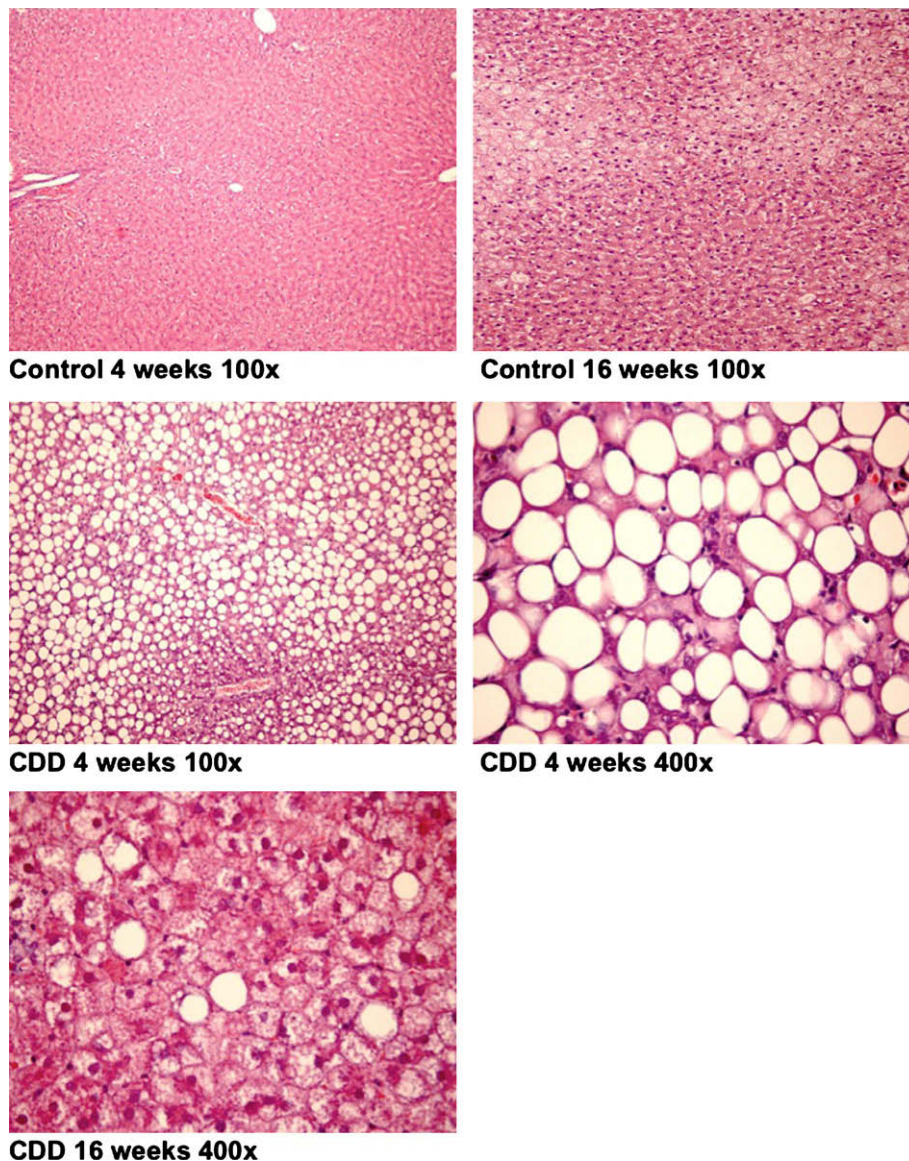
mitochondria from 16 weeks CDD-fed animals displayed impaired ability to recover membrane potential after ADP phosphorylation.

The lag phase (time necessary for ADP phosphorylation) was significantly affected by fat accumulation in 16 weeks CDD-fed animals (Fig. 3C), when compared with the lag phase evaluated in mitochondria from control animals. Interestingly, 12 weeks CDD animals presented a decreased lag phase, when compared to the same age control animals (Fig. 3C).

### 3.3. Effects of fat accumulation on mitochondrial respiration

Oxidative phosphorylation capacity of mitochondria isolated from control and steatotic animals was investigated by following oxygen consumption upon glutamate/malate or succinate oxidation.

Mitochondrial respiratory state 3 (consumption of oxygen in the presence of substrate and ADP) was decreased in mitochondria isolated from 4 and 16 weeks CDD-fed animals (Fig. 4A) irrespective of the substrate used. Similarly, oxygen consumption in the



**Fig. 2.** Light microscopy for liver tissue stained with H&E of control 4 weeks (A, 100 $\times$ ), control 16 weeks (B, 100 $\times$ ), CDD 4 weeks (C, 100 $\times$ ), CDD 4 weeks (D, 400 $\times$ ) and CDD 16 weeks (E, 100 $\times$ ).

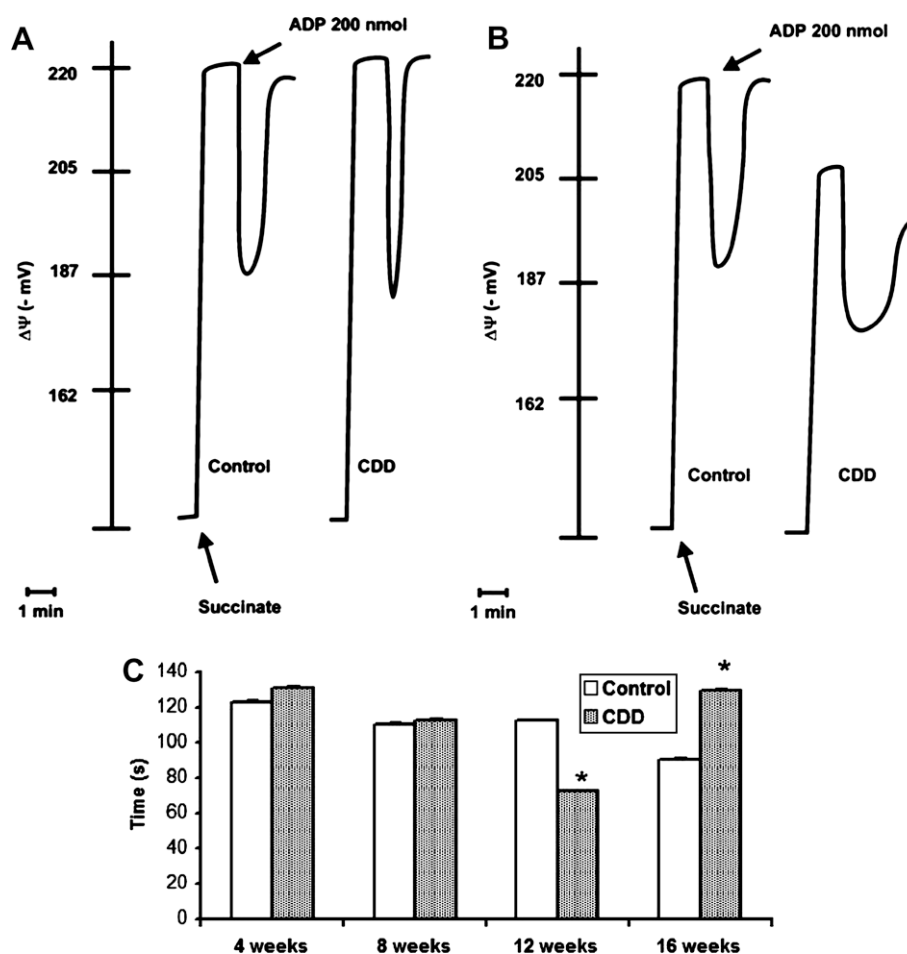
**Table 2**  
Mitochondrial membrane potential ( $\Delta\Psi$ ) in control and animals fed for 4, 12 and 16 weeks a choline-deficient diet (CDD)

		Glutamate/malate		Succinate	
		Control	CDD	Control	CDD
4 weeks	$\Delta\Psi$ (–mV)	206.67 ± 0.41	201 ± 0.51 <sup>†</sup>	206.62 ± 0.38	198.21 ± 0.49 <sup>†</sup>
	Depolarization (–mV)	25.2 ± 0.53	23.31 ± 0.64	39.98 ± 0.43	32.6 ± 0.53 <sup>†</sup>
	Repolarization (–mV)	202.96 ± 0.4	195.38 ± 0.52 <sup>†</sup>	204.15 ± 0.44	194.28 ± 0.53 <sup>†</sup>
	Lag phase (s)	57 ± 0.48	50.5 ± 0.48	123 ± 0.83	131 ± 0.71
12 weeks	$\Delta\Psi$ (–mV)	213.61 ± 0.29	215.02 ± 0.23	215.8 ± 0.23	216.75 ± 0.18
	Depolarization (–mV)	25.49 ± 0.26	23.39 ± 0.28	36.26 ± 0.26	31.96 ± 0.19
	Repolarization (–mV)	209.72 ± 0.4	212.36 ± 0.34	213.73 ± 0.26	215.70 ± 0.2
	Lag phase (s)	44.25 ± 0.91	36.75 ± 0.57	112 ± 0.67	72 ± 0.57
16 weeks	$\Delta\Psi$ (–mV)	216.76 ± 0.14	209.54 ± 0.28 <sup>†</sup>	215.18 ± 0.15	209.24 ± 0.21 <sup>†</sup>
	Depolarization (–mV)	25.13 ± 0.36	17.45 ± 0.33 <sup>†</sup>	32.17 ± 0.26	31.85 ± 0.24
	Repolarization (–mV)	213.97 ± 0.16	199.88 ± 0.42 <sup>†</sup>	213.86 ± 0.18	201.93 ± 0.29 <sup>†</sup>
	Lag phase (s)	40.13 ± 0.22	55.5 ± 0.43 <sup>†</sup>	90.5 ± 0.48	129.5 ± 0.56 <sup>†</sup>

$\Delta\Psi$  was determined in mitochondria isolated from the livers of animals fed during 4 weeks a control or a choline-deficient diet. Glutamate/malate or succinate were used as respiratory substrate as described in Section 2. Phosphorylation was induced by adding 200 nmol ADP. Data expressed as means ± SEM of four different experiments. Values statistically significant different from control for each week: <sup>†</sup> $P < 0.05$ .

presence of FCCP, a well-known respiratory chain uncoupler, was also decreased in CDD animals after 4 and 16 weeks of feeding. No statistical significant alterations were observed in 8 weeks

CDD-fed animals. However, after 12 weeks, CDD animals exhibited increased state 3 and FCCP-induced respiratory rates (Fig. 4A). The ADP/O ratio, an indicator of oxidative phosphorylation efficiency,



**Fig. 3.** (A) Mitochondrial transmembrane potential ( $\Delta\Psi$ ) in succinate-energized liver mitochondria isolated from control and animals fed for 12 weeks a choline-deficient diet (CDD).  $\Delta\Psi$  was measured with a TPP<sup>+</sup>-selective electrode. Reactions were carried out in 1 mL of reaction medium, supplemented with 2  $\mu$ M rotenone and 1 mg of freshly isolated mitochondria, as described in Section 2. Energization was achieved with 5 mM succinate and phosphorylation 200 nmol ADP. The traces represent typical direct recordings. (B) Mitochondrial transmembrane potential ( $\Delta\Psi$ ) in succinate-energized liver mitochondria isolated from control and animals fed for 16 weeks a choline-deficient diet (CDD).  $\Delta\Psi$  was measured with a TPP<sup>+</sup>-selective electrode. Reactions were carried out in 1 mL of reaction medium, supplemented with 2  $\mu$ M rotenone and 1 mg of freshly isolated mitochondria, as described in Section 2. Energization was achieved with 5 mM succinate and phosphorylation induced by 200 nmol ADP. The traces represent typical direct recordings. (C) Lag phase in succinate-energized liver mitochondria isolated from control and animals fed for 4, 8, 12 or 16 weeks a choline-deficient diet (CDD).  $\Delta\Psi$  was measured with a TPP<sup>+</sup>-selective electrode as described in Section 2. Phosphorylation was induced by 200 nmol ADP. Data expressed as means ± SEM of four different experiments. Values statistically significant different from control for each week: <sup>†</sup> $P < 0.05$ .

was decreased in CDD animals after 4, 8 and 16 weeks of feeding, irrespective of the substrate used (data not shown), but it was similar to controls in mitochondria isolated from 12 weeks CDD-fed animals.

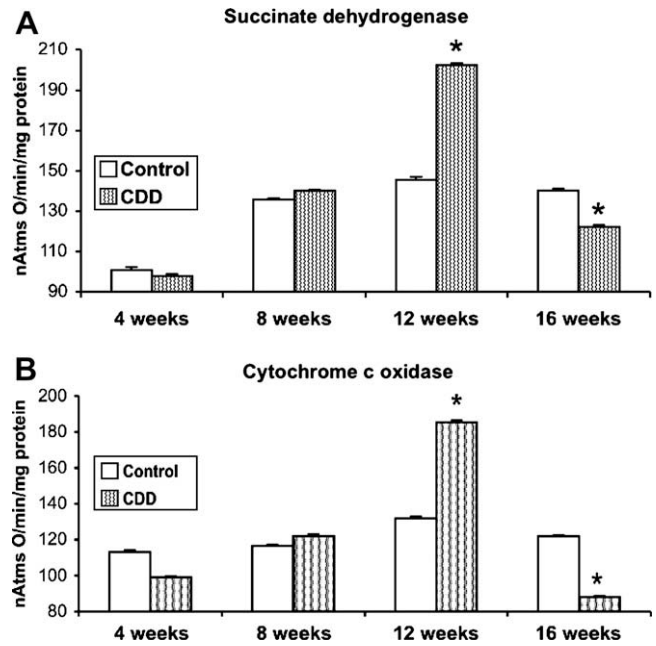
Fat accumulation induced a significant increase in mitochondrial respiratory state 4 (consumption of oxygen after ADP phosphorylation) in animals fed with CDD for 4, 8, 12 or 16 weeks, when compared with state 4 of control animals, irrespective of the substrate used (Fig. 4B). The ratio between mitochondrial respiratory state 3 and state 4 (RCR) was significantly decreased in mitochondria isolated from 4, 8 and 16 weeks animals fed with CDD, but not in 12 weeks CDD animals (data not shown).

Mitochondrial respiration in the presence of oligomycin, a known inhibitor of the mitochondrial  $F_1F_0$ -ATP synthase, was also increased in CDD animals at 4, 8, 12 and 16 weeks of feeding with CDD (Fig. 4B), suggesting an increased protonic leak (the passage of protons from the intermembrane space to the matrix without ATP synthesis).

### 3.4. Effects of fat accumulation on mitochondrial enzyme activities and protein carbonyl content

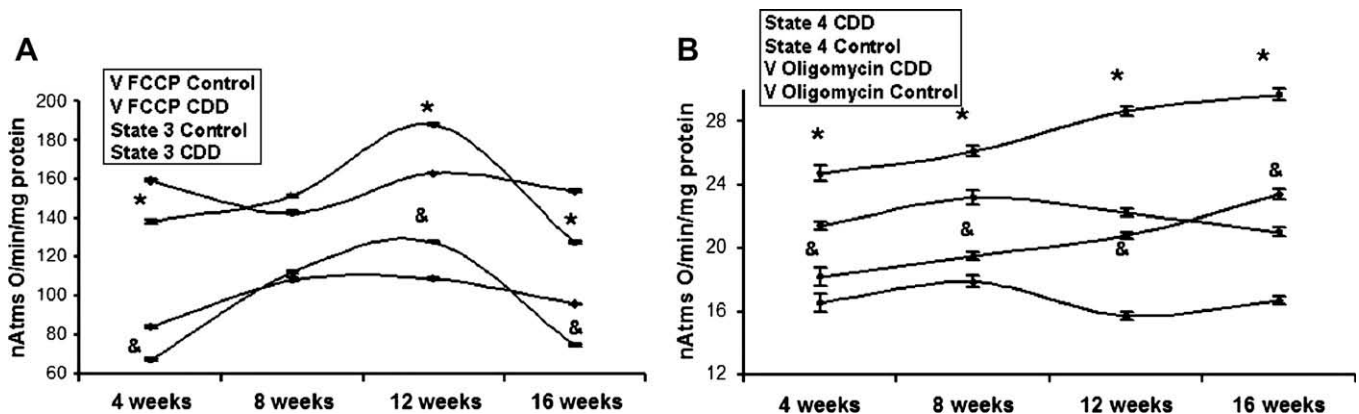
The decreased rate of oxygen consumption induced by FCCP in mitochondria isolated from 4 and 16 weeks CDD-fed animals, suggested alterations in the mitochondrial electron transport chain complexes. In view of this, succinate-dehydrogenase and cytochrome *c* oxidase activities were evaluated. Mitochondria isolated from 16 weeks CDD-fed animals exhibited decreased activities in both of these enzymes, while at 12 weeks, activities of succinate-dehydrogenase and cytochrome *c* oxidase were increased, comparatively to the same age controls (Fig. 5A and 5B, respectively). No statistical significant differences were observed at 4 and 8 weeks. Interestingly, the analysis of protein carbonyl content, a marker of protein oxidative damage, in both liver tissue and mitochondria, revealed an increased content of protein carbonyls in 16 weeks CDD-fed animals, but decreased in 12 weeks CDD animals, comparatively to controls (Table 3).

The decrease in the performance of phosphorylation of mitochondria isolated from CDD-fed animals for 4, 8 and 16 weeks, as reflected by an increased lag phase, and decreased ADP/O, suggested alterations at the level of the mitochondrial  $F_1F_0$ -ATP synthase, a key component of the phosphorylative system. The evaluation of mitochondrial ATPase activity revealed a less efficient ATPase in



**Fig. 5.** (A) Succinate-dehydrogenase activity in control and animals fed a choline-deficient diet (CDD) for 4, 8, 12 or 16 weeks. Enzyme activity was polarographically determined with a Clark oxygen electrode. Mitochondria (0.25 mg) were suspended under constant stirring, at 25 °C, in 1 mL of standard respiratory medium. The reaction media was supplemented with 5 mM succinate, 2  $\mu$ M rotenone, 0.1  $\mu$ g antimycin A, 1 mM KCN, 0.3 mg Triton X-100 and the reaction initiated by adding 1 mM PMS. Data expressed as means  $\pm$  SEM of four different experiments. Values statistically significant different from control for each week: \* $P < 0.05$ . (B) Cytochrome *c* oxidase activity in animals fed a choline-deficient diet (CDD) for 4, 8, 12 or 16 weeks. Enzyme activity was polarographically determined with a Clark oxygen electrode. Mitochondria (0.25 mg) were suspended under constant stirring, at 25 °C, in 1 mL of standard respiratory medium. The reaction media was supplemented with 2  $\mu$ M rotenone, 10  $\mu$ M cytochrome *c*, 1 mM KCN, 0.3 mg Triton X-100 and the reaction initiated by adding 0.25 mM ascorbate/TMPD. Data expressed as means  $\pm$  SEM of four different experiments. Values statistically significant different from control for each week: \* $P < 0.05$ .

mitochondria isolated from 8 weeks CDD-fed animals, when compared to mitochondria from control animals (Fig. 6). No differences statistically significant were observed at 4 and 16 weeks. However, mitochondria from 12 weeks CDD-fed animals exhibited an increase



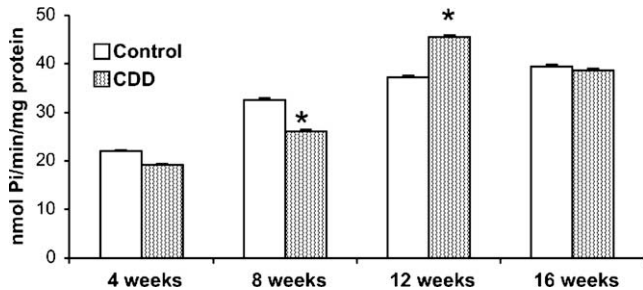
**Fig. 4.** (A) State 3 respiration and FCCP-stimulated oxygen consumption in succinate-energized liver mitochondria isolated from control and animals fed for 4, 8, 12 or 16 weeks a choline-deficient diet (CDD). Reactions were carried out in 1 mL of reaction medium, supplemented with 2  $\mu$ M rotenone and 1 mg of freshly isolated mitochondria, as described in Section 2. Energization was achieved with 5 mM succinate and phosphorylation induced by 200 nmol ADP. Data expressed as means  $\pm$  SEM of four different experiments. Values statistically significant different from control: \* $P < 0.05$  (FCCP);  $\&$  $P < 0.05$  (State 3). (B) State 4 respiration and oligomycin-inhibited oxygen consumption in succinate-energized liver mitochondria isolated from control and animals fed for 4, 8, 12 or 16 weeks a choline-deficient diet (CDD). Reactions were carried out in 1 mL of reaction medium, supplemented with 2  $\mu$ M rotenone and 1 mg of freshly isolated mitochondria, as described in Section 2. Energization was achieved with 5 mM succinate and phosphorylation induced by 200 nmol ADP. Data expressed as means  $\pm$  SEM of four different experiments. Values statistically significant different from control for each week: \* $P < 0.05$  (State 4);  $\&$  $P < 0.05$  (oligomycin).

**Table 3**

Carbonyl content in hepatic tissue and mitochondria from control and animals fed for 12 or 16 weeks a choline-deficient diet (CDD)

Sample	Control	CDD
Tissue 12 weeks	3.6 ± 0.75	2.4 ± 1.18*
Tissue 16 weeks	2.3 ± 0.31	3.6 ± 0.56*
Mitochondria 12 weeks	2.57 ± 0.27	1.15 ± 0.65*
Mitochondria 16 weeks	1 ± 0.34	2.6 ± 0.57*

Carbonyl group content in both isolated mitochondria and hepatic tissue was measured spectrophotometrically by using 2,4-dinitrophenyl-hydrazine (DNPH) as described in Section 2. Data expressed as means ± SEM of four different experiments. Values statistically significant different from control, for each week: \*P < 0.05.



**Fig. 6.** ATPase activity in liver mitochondria isolated from control and animals fed for 4, 8, 12 or 16 weeks a choline-deficient diet (CDD). 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 Section 2. Data expressed as means ± SEM of four different experiments. Values statistically significant different from control for each week: \*P < 0.05.

in ATPase activity, when compared to control animals (Fig. 6), comparatively to the respective controls.

**3.5. Effects of fat accumulation on mitochondrial protein content**

Since the decrease in the phosphorylative efficiency induced by 16 weeks of feeding was not associated with impaired activity of

the ATP synthase, the content on adenine nucleotide translocator (ANT), another component of the phosphorylative system, was evaluated. The content on ANT was found to be decreased after 16 weeks, but tending to be increased after 12 weeks of feeding (Fig. 7). Additionally, the increase in ATPase activity observed at 12 weeks was not associated with increased content, since no changes were observed comparatively to the respective control animals (Fig. 7).

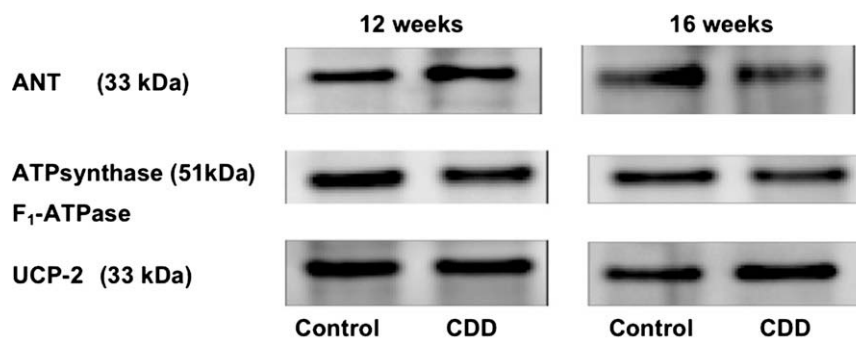
The mitochondrial content in uncoupling protein UCP-2 was also evaluated, since CDD animals exhibited increased state 4 respiratory rates that were not caused by an increased leak through the ATP synthase, as indicated by the experiments with oligomycin. However, no changes were observed on the UCP-2 content (Fig. 7).

**3.6. Effects of fat accumulation on the susceptibility to calcium-induced mitochondrial membrane permeability (MPT)**

Mitochondria possess a finite capacity for accumulating calcium before undergoing the calcium-dependent mitochondrial permeability transition (MPT). Mitochondria isolated from 4, 8, 12 or 16 weeks CDD-fed animals, were more susceptible to undergo the calcium-dependent mitochondrial swelling, in comparison with the respective control animals (Fig. 8). Pretreatment with CyA completely prevented calcium-dependent mitochondrial swelling.

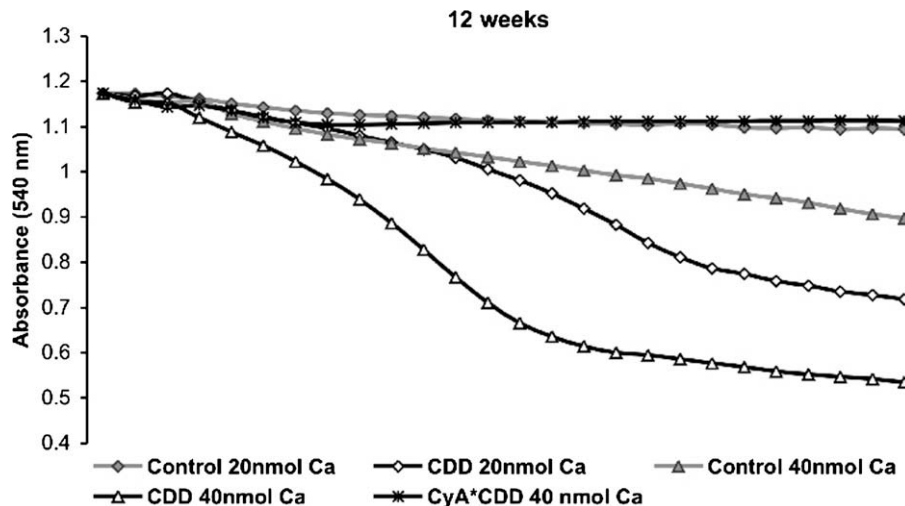
**4. Discussion**

The loss of mitochondrial functionality is undoubtedly associated with the emergence of several aggressive human pathologies (Mahler et al., 1997; Jacobsen et al., 2001; Brealey et al., 2002; Sayeed, 2002; DiMauro and Schon, 2003; Duchon, 2004). In the fatty liver, the accumulation of free fatty acids due to disturbances in lipid metabolism or due to diets with high-fat content (Haque and Sanyal, 2002) alters the energetic metabolism of the cell, with high rates of fatty acid oxidation that lead to mitochondrial impairment (Browning and Horton, 2004). Altered mitochondrial morphology was described early in an animal model of fatty liver



Sample	ANT/VDAC (% Control)
CDD 12 weeks	125.75 ± 12.22
CDD 16 weeks	71.68 ± 7.28*

**Fig. 7.** ANT, ATP synthase and UCP-2 content in hepatic mitochondria isolated from control or animals fed a choline-deficient diet (CDD), evaluated by Western blotting. Mitochondrial pellets were fractionated in 8% polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and blotted with anti-ANT, ATP synthase, UCP-2 or VDAC. The table indicates the ratio of the densitometry ANT/VDAC. Data expressed as means ± SEM of four different experiments. Values statistically significant different from control for each week: \*P < 0.05.



**Fig. 8.** Typical recording of mitochondrial swelling induced by calcium in liver mitochondria isolated from control and animals fed for 12 weeks a choline-deficient diet (CDD). The incubation medium (final volume 2 mL at 25 °C) contained 200 mM sucrose, 10 mM Tris–Mops (pH 7.4), 1 mM  $\text{KH}_2\text{PO}_4$ , and 10  $\mu\text{M}$  EGTA supplemented with 3  $\mu\text{M}$  rotenone, and 5 mM succinate. The experiments were started by the addition of 1 mg of CDD or control mitochondria (not shown). After a basal line was established, calcium was added (20 or 40 nmol). Where indicated, cyclosporine A (CyA, 1  $\mu\text{M}$ ) was added prior to calcium. The traces are typical of four independent experiments.

(Danzani, 1957). From then on, several studies were conducted on diverse models of steatosis, where mitochondrial dysfunction was found and studied. However, the mechanisms underlying the link between impaired mitochondrial function and NAFLD onset still eludes the scientific community.

This paper further recognizes that fat accumulation in the liver is associated with decreased efficiency of the phosphorylative system, in concordance with our previous studies in another animal model of fatty liver (Teodoro et al., 2006). Although other studies have been conducted with the choline-deficient model of fatty liver disease (Vendemiale et al., 2001; Oliveira et al., 2002; Grattagliano et al., 2003; Zafrani, 2004), this work proved invaluable since it is the first of its kind to apply a timeline approach. The mitochondrial function, mainly at the bioenergetic level, was found to be impaired due to fat accumulation. Interestingly, these data showed that, apparently, there is an attempt, by the liver, to cope and recover from the fat insult, as shown by the data regarding 12 weeks CDD feeding.

CDD animals, at 4 and 16 weeks, exhibited decreased  $\Delta\Psi$ , when compared to control mitochondria. Respiratory state 4 was higher at 4, 8, 12 and 16 weeks in mitochondria from CDD animals. Respiration, in the presence of oligomycin, an ATP synthase inhibitor, was stimulated in CDD animals, when compared to control. This suggested an increase in mitochondrial internal membrane permeability to protons, which was not associated with higher content of UCP-2 content. Respiratory state 3 was decreased in CDD animals, at 4 and 16 weeks, but stimulated at 12 weeks, when compared to control animals. Similar results were observed for uncoupled respiration, suggesting alterations at the level of the electron transport chain at 16 weeks. Respiratory control ratio was decreased in mitochondria from CDD animals, at 4 and 16 weeks. The lag phase was lower in CDD animals at 12 weeks, but higher at 16 weeks in CDD animals, while ADP/O ratio was decreased at 8 and 16 weeks, when compared to the respective controls. These observations suggest that at 12 weeks, there is a mechanism to stimulate the efficiency of the phosphorylative system, capacity that is abolished at 16 weeks. Mitochondrial ATPase activity was lower in CDD animals, at 8 weeks than in controls, but higher at 12 weeks, associated with decreased ATP/ADP ratio at 8 weeks and decreased ATP content at 16 weeks, but higher ATP/ADP ratio at 12 weeks. CDD animals at 16 weeks had a significant reduction in adenine nucleotide translocase

(ANT), probably the cause for the decreased efficiency of the phosphorylative system. In CDD animals, succinate-dehydrogenase and cytochrome *c* oxidase activities were higher at 12 weeks, but lower at 16 weeks. Additionally, at 12 weeks, there was a decrease in carbonyl content in both hepatic tissue and mitochondria but at 16 weeks carbonyl content was higher in CDD animals than in control.

The analysis of the mitochondrial transmembrane potential ( $\Delta\Psi$ ) revealed that the capability of creating and maintaining a potential was compromised on mitochondria from CDD animals, after 4 and 16 weeks of feeding with a choline-deficient diet. This decreased  $\Delta\Psi$  observed in CDD-fed animals could be the result of an increase in the permeability of the mitochondrial inner membrane to protons (proton leak). In fact, the resting mitochondrial oxygen consumption, the state 4 respiratory rate, was found to be significantly increased in CDD animals at 4, 8, 12 and 16 weeks, as well the respiratory velocity in the presence of oligomycin, an ATP synthase inhibitor. These data suggest an increased protonic leak, which can be in part attributed to membrane alterations caused by phosphatidylcholine deficiency, the main phospholipid of eukaryotic membranes (Zeisel and Blusztajn, 1994). No alterations in UCP-2 content were observed, thus ruling out the role of uncoupling proteins on the increased resting oxygen consumption. A causal link between UCP-2 induction and fatty liver disease is still not established. In fact, high-fat feeding of UCP-2 knockout animals had no effect on fatty liver disease progression (Baffy et al., 2002), suggesting that UCP-2 expression has no protective or deleterious effect on hepatic injury associated to fatty liver. Additionally, and similarly to our results, a recent study has shown that animals fed diets deficient in choline or methionine had no alterations in hepatic UCP-2 content (Donthamsetty et al., 2007).

The decrease in both state 3 respiratory rate and FCCP-induced oxygen consumption indicated a loss of oxidative capacity in mitochondria from CDD animals. Such impairment was probably caused by decreased efficiency of the mitochondrial electron transport chain, as demonstrated by the decrease in the activities of both succinate-dehydrogenase and cytochrome *c* oxidase, in mitochondria from 16 weeks CDD-fed animals. Such alteration could be caused by oxidative damage, as the content in protein carbonyls was increased in both hepatic tissue and mitochondria of these animals. One of the biochemical mechanisms indicated as



responsible for the evolution of NAFLD to more aggressive pathologies is the increased production of reactive oxygen species (ROS) (Pessayre et al., 2002; Begriche et al., 2006; Day and James, 1998; Chitturi and Farrell, 2001), due to increased rates of fatty acid oxidation in both mitochondria and peroxisomes, as consequence of fat accumulation.

The increase in the lag phase and the decrease in the ADP/O ratio after 16 weeks of CDD feeding indicated an uncoupling between the phosphorylative and oxidative mitochondrial subsystems. However, the decrease in the phosphorylative efficiency in 16 weeks CDD-fed animals, was not due to decreased activity or content of ATP synthase, but was caused by a depletion in ANT, as previously reported on a genetic model of NAFLD (Teodoro et al., 2006). ANT, the most abundant protein in the mitochondrial inner membrane, is essential to oxidative phosphorylation by exchanging ADP and ATP, thus maintaining ATP cytosolic content.

The analysis of the hepatic mitochondrial function 12 weeks CDD-fed animals revealed an extremely interesting response, trying to revert the deleterious effects of fat accumulation. Despite some of the data demonstrate the continuity of the aggression of the fat on the mitochondrial functionality (maintenance of the macrovesicular steatosis associated with increased state 4, increased respiratory rate in the presence of oligomycin and increased susceptibility to MPT induction), most of the evaluated parameters totally reverted their tendency towards impairment of mitochondrial function. In fact, the increased state 3, FCCP-induced oxygen consumption and enzymatic activities (succinate dehydrogenase, cytochrome *c* oxidase, and ATP synthase), when compared to control animals, suggest a recovery on most of the mitochondrial functionality that was lost, as demonstrated by the data from the 4 and 8 weeks CDD-fed animals. The decreased lag phase and the unaltered ADP/O demonstrated that mitochondrial oxidative and phosphorylative subsystems were perfectly coupled by 12 weeks of feeding CDD diet. Furthermore, the analysis of the ANT content on the CDD 12 weeks revealed a tendency, although not statistically significant, to an increased ANT content on these animals; no alterations were found on the content on ATP synthase. Curiously, the data from the CDD animals after 12 weeks of feeding demonstrated a probable increased redox capability, as suggested by the decrease in protein carbonyl content, comparatively to the same age control animals.

These data clearly suggest that fat accumulation causes a severe impairment of mitochondrial function, but more importantly, it also triggers a response mechanism, designed to withstand (and probably overcome) the challenge created by the steatosis, on the bioenergetic level. While this manuscript was in preparation, work conducted by Petrosillo and co-workers demonstrated that impairment of complex I activity after 30 weeks of feeding with a choline-deficient diet is caused by a ROS dependent cardiolipin peroxidation (Petrosillo et al., 2007). Our study confirms that the mitochondrion plays a central role in the development of the non-alcoholic steatosis, particularly on the oxidative phosphorylation efficiency level. Moreover, by studying the progression of the pathology through 16 weeks of choline-deficient diet, it unveiled an extremely interesting response designed to prevent and revert the mitochondrial damage associated with this pathology. Further studies on the development and physiological mechanisms of non-alcoholic fatty liver, specially aimed at the regulation of mitochondrial function, are required in order to fully understand this disease and unravel possible therapeutic targets.

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