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Decreased ANT content in Zucker fatty rats: Relevance for altered hepatic mitochondrial bioenergetics in steatosis

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Abstract Mitochondria from Zucker fatty (ZF) rats (a model for fatty liver disease) showed a delay in the repolarization after a phosphorylative cycle and a decrease on state 3 respiration, suggesting alterations at the phosphorylative system level. The ATPase activity showed no differences between control and ZF rats, implying alterations in other components of the phosphorylative system. A pronounced depletion in the content of the adenine nucleotide translocator (ANT) was observed by Western blotting, while no alterations were found in the mitochondrial voltage-dependent anion channel content. These data suggest that hepatic accumulation of fat impairs mitochondrial function, reflecting the loss of oxidative phosphorylation capacity caused by a decrease in the ANT content.
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Keywords: Non-alcoholic fatty liver; Mitochondrion; ATPase; Oxidative phosphorylation; ANT; VDAC

1. Introduction

Non-alcoholic fatty liver disease (steatosis) is an increasing modern-world disease, recognized as the basis for inflammatory hepatitis and induction of liver cirrhosis [1]. It is marked by the accumulation of free fatty acids droplets inside the hepatocytes. This pathology maybe due to an increased consumption of fat on diet, a common situation in developed countries or due to deficiencies in any of the steps of lipid metabolism. A deregulation in fatty acids metabolism leads to overproduction of triglycerides with subsequent fat accumulation inside the liver cells. The excessive abundance of free fatty acids causes a shift in metabolism, turning these molecules into the main energy source of the cell. Such alteration causes augmentation of lipid peroxidation, destabilizing the inner cell redox environment [2]. The Zucker fatty (ZF) rat represents an established model for fatty liver disease. A mutation in the leptin receptor gene leads to a lack of response to leptin and consequent increased lipogenesis [3].

Being the main ATP production source for the cell, mitochondria play a key role in the majority of metabolic pathways. It is one of the most susceptible sites for damage, with a profound involvement in the onset of many pathologies [4]. 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 β-oxidation due to genetic and/or acquired causes [5].

We hypothesize that excessive free fatty acids accumulation in liver cells could causes impairment of mitochondrial bioenergetics that parallels the metabolic evolution of fatty liver disease and provides potential clues for the pathogenesis. By analyzing membrane potential, oxygen consumption and ATPase activity, we show that, in fact, hepatic mitochondrial bioenergetics is altered in ZF rats. Protein content of the adenine nucleotide translocator (ANT) was found to be decreased in ZF rats, which can be a possible cause for the observed loss of oxidative phosphorylation efficiency.

2. Materials and methods

2.1. Animals

Twelve weeks-old male ZF rats and their respective controls (Charles River, France) were divided in groups of five animals and housed under controlled light and humidity conditions with free access to powdered rodent chow (diet C.R.F. 20, Charles River) and water. The animals were killed by cervical dislocation and decapitation.

2.2. Materials

All compounds were purchased from Sigma 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 ZF and control rats were removed quickly and immediately after the animal sacrifice, fixed in 10% buffered formaline and stained with haematoxylin and eosin (H & E) [6].

2.4. Preparation of liver mitochondria

Mitochondria were isolated from the liver of ZF and control rats by conventional methods [7], with slight modifications [8]. Protein content was determined by the biuret method [9], calibrated with bovine serum albumine.

2.5. Mitochondrial respiration

Oxygen consumption of isolated mitochondria was polarographically determined with a Clark oxygen electrode [10], as previously described [8]. 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

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Abbreviations: ZF, Zucker fatty; ANT, adenine nucleotide translocator; VDAC, mitochondrial voltage-dependent anion channel

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rotenone, an inhibitor of complex I, were previously added. State 3 respiration was induced by adding ADP. The oxygen consumption was also measured in the presence of oligomycin (0.5 μg/mg protein) and 1 μM carbonylcyanide-*p*-trifluoromethoxyphenylhydrazon (FCCP).

2.6. Membrane potential (ΔΨ) measurements

ΔΨ was estimated using an ion-selective electrode to measure the distribution of tetraphenylphosphorium (TPP^+) according to previously established methods [11,12]. 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.

2.7. ATPase activity

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 (100 mM NaCl, 25 mM KCl, 5 mM $MgCl_2$, and 50 mM HEPES, pH 7.4). After the addition of freeze–thawed mitochondria (0.25 mg), the reaction was initiated by adding 2 mM Mg^{2+}-ATP, in the presence or absence of oligomycin (1 μg/mg protein). After 10 min, the reaction was stopped by adding 1 ml of 40% trichloroacetic acid and the samples centrifuged for 5 min at 3000 rpm. 2 ml of ammonium molybdate plus 2 ml dH_2O 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.

2.8. Western blotting analysis

Frozen mitochondrial pellets were homogenized in lysis buffer (1 M urea, 10 mM Tris, 2% SDS, pH 7.5, 60 °C). Aliquots were fractionated in 4–20% polyacrylamyde gels (pre-casted I-Gels, Gradipore, Australia), transferred to polyvinylidene difluoride membranes, and blotted with goat anti-ANT (1:10000, sc9299 from Santa Cruz, CA) or rabbit anti-voltage-dependent anion channel (VDAC) (1:5000 dilution, Ab3434 from AbCam, UK). The immunoreactive proteins were visualized with rabbit anti-goat IgG (ANT antibody) and goat anti-rabbit IgG (anti-VDAC antibody), both alkaline phosphatase-conjugated. Membranes were reacted with the ECF detection system (Amersham) and read with the Versa Doc imaging system (Bio-Rad).

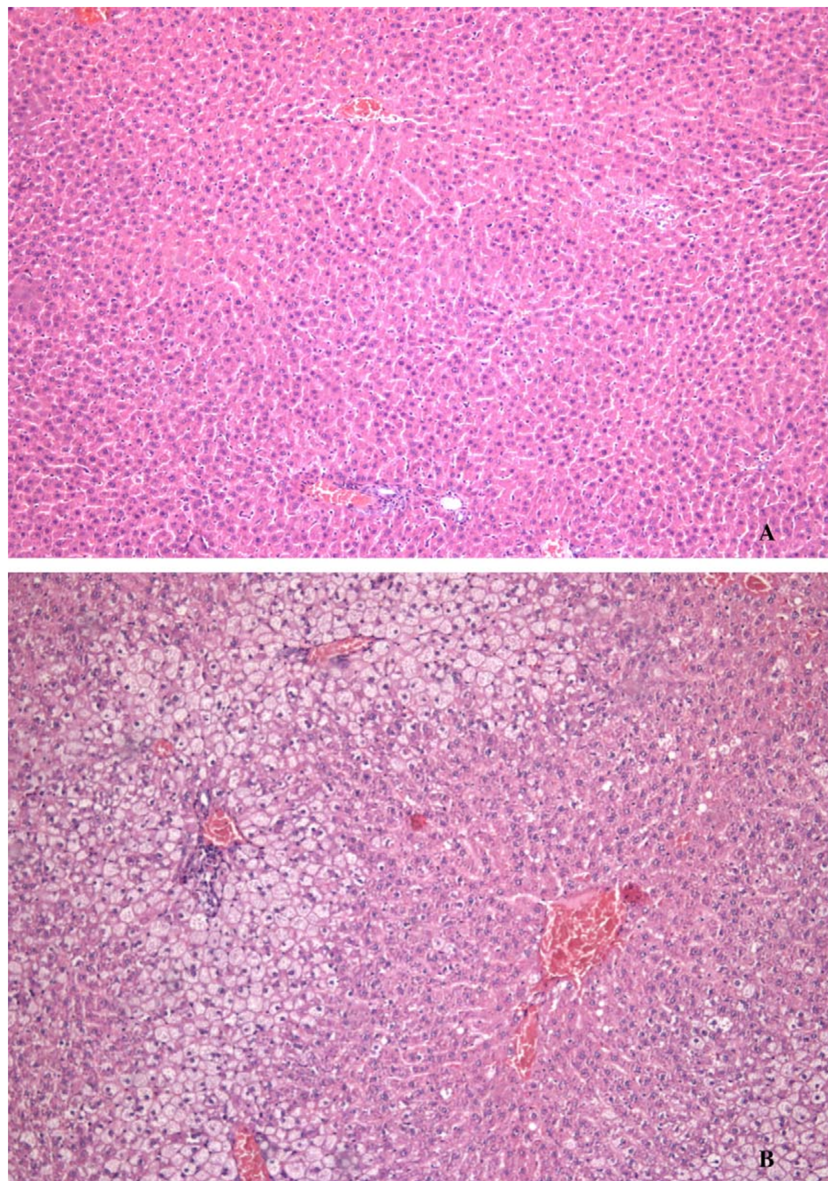

Fig. 1. Light microscopy for liver tissue of control (A) and ZF rats (B) stained with H & E (100×).

Table 1
Oxygen consumption measurements as described in Section 2

	Control		Fatty	
	Glutamate/malate	Succinate	Glutamate/malate	Succinate
State 3 (natomsO/min/mg protein)	57.1 ± 6.2	77.7 ± 7.7	57.1 ± 6.9	57.1 ± 6.7*
State 4 (natomsO/min/mg protein)	12.1 ± 1.1	16.9 ± 0.6	12.3 ± 1.0	21.0 ± 3.2
RCR	4.9 ± 0.5	4.7 ± 0.5	5.3 ± 1.2	3.0 ± 0.3*
ADP/O	2.5 ± 0.2	1.7 ± 0.1	2.6 ± 0.1	1.5 ± 0.1
Oligomycin (natomsO/min/mg protein)	4.5 ± 0.0	5.0 ± 0.4	3.4 ± 1.2	9.3 ± 2.4*
FCCP (natomsO/min/mg protein)	50.3 ± 14.3	130.4 ± 13.0	43.7 ± 6.5	112.1 ± 19.0

Data are means ± S.E.M. of five different experiments.
*Values statistically different from control, with the same substrate.

2.9. Statistical analysis

Results are presented as means ± S.E.M. of the number of indicated experiments. Statistical evaluation was performed using the two-tailed Student's *t* test (GraphPad Prism 4 for Windows, GraphPad Software Inc). A *P* value <0.05 was considered statistically significant.

3. Results

Twelve weeks-old ZF rats were 20% heavier than the respective controls (432 ± 20 g and 358.9 ± 17 g, respectively). No specific histologic alterations were observed in the control livers. In contrast, under light microscope, sections stained with H & E in the ZF group showed steatosis, with macrovesicular and microvesicular fatty infiltration into hepatocytes (Fig. 1).

Oxidative phosphorylation capacity of mitochondria isolated from fatty and control animals was investigated by following oxygen consumption upon glutamate/malate or succinate oxidation (Table 1). The results show that both state 3 respiration and respiratory control ratio (RCR) were decreased in fatty animals with succinate as oxidative substrate, while mitochondrial respiration in the presence of oligomycin was increased in ZF rats. Additionally, oxidative phosphorylation efficiency (ADP/O), state 4 respiration and mitochondrial respiration in the presence of FCCP showed no differences between both experimental groups, irrespective of the substrate used.

Analysis of energization and phosphorylation capacities showed that $\Delta\Psi$ developed in liver of ZF rats upon energization with succinate (Fig. 2), was significantly decreased as compared to control animals. Depolarization induced by the addition of ADP was decreased in mitochondria from ZF rats as well as repolarization. Interestingly, the lag phase preceding repolarization was enlarged in ZF rats (Fig. 2). With glutamate/malate as substrate, although not statistically significant, $\Delta\Psi$ fluctuations associated with the phosphorylation cycle were similar to the ones obtained with succinate (data not shown).

To further explore and understand these findings ATPase activity was evaluated. No differences were found between hepatic mitochondria from ZF and control animals (29.1 ± 4.9 nmol Pi/min/mg protein and 25.3 ± 2.4 nmol Pi/min/mg protein, respectively). Since the decreased performance of phosphorylation in ZF rats, as reflected by an increased lag phase, was not correlated with a less efficient ATPase, we analyzed other components of the phosphorylation system. By Western blotting we evaluated mitochondrial content of ANT protein. ZF rats exhibited a significant decrease in ANT content, comparatively to control animals. No significant differences were found in VDAC content between both groups (Fig. 3).

	Control	Fatty
Initial ΔΨ (- mV)	224.6 ± 1.28	217.8 ± 2.02*
ΔΨ Depolarization (- mV)	23.12 ± 0.83	20.99 ± 0.74*
ΔΨ Repolarization (- mV)	221.9 ± 1.63	212.5 ± 2.02*
Lag phase (s)	65 ± 5.18	98.5 ± 9.66*

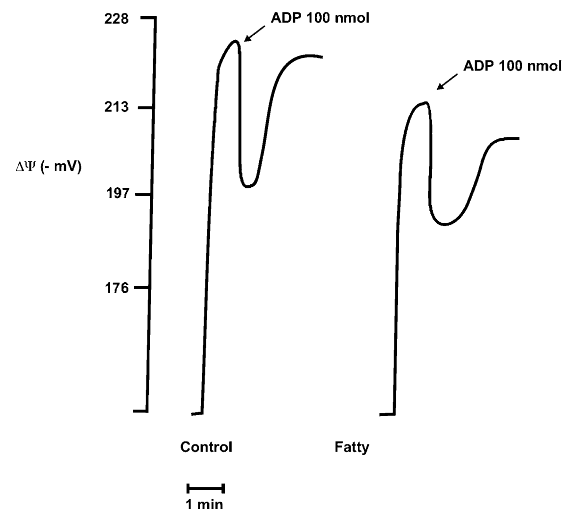

Fig. 2. $\Delta\Psi$ determined in the presence of succinate as respiratory substrate as described in Section 2. Phosphorylation was induced by adding 100 nmol ADP. The traces represent typical direct recordings and data are means ± S.E.M. of five different experiments. Values statistically different from control: $^{*}P < 0.05$.

4. Discussion

Impairment of mitochondrial function is undoubtedly associated with the emergence of severe human disorders [4]. Alterations in mitochondrial morphology were early described in an experimental model of fatty liver [13]. Since then, mitochondrial dysfunction has been studied in a variety of models of fatty liver disease, describing increased mitochondrial lipid peroxides and decreased ATP content [2,14]. However, uncertainty still remains regarding the relationship between impairment of mitochondrial function in fatty liver and the onset of the disease.

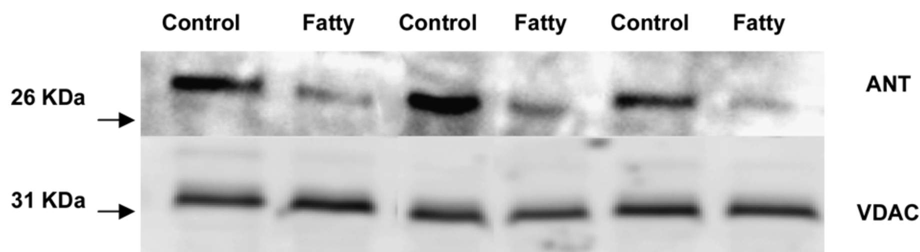

Fig. 3. ANT and VDAC content in liver mitochondria from ZF and control rats evaluated by Western blotting. Arrows indicate the position of the molecular weight marker.

The present paper clearly indicates that fat accumulation in the liver is associated with decreased efficiency of the phosphorylative system. The capabilities to generate membrane potential, to recover from depolarization and maintain a potential similar to the initial after repolarization, are affected in ZF rats. The decrease in mitochondrial membrane potential in ZF rats is probably due to an increased proton leak, as suggested by the increased rate of mitochondrial respiration in the presence of oligomycin, an inhibitor of proton translocation by the mitochondrial ATPase. Thus, in the presence of oligomycin, mitochondrial respiration is reduced to a residual rate that is due to a proton leak. In fatty livers, the increase in free fatty acids probably induces the back flow of protons, justifying the reported decrease in membrane potential in ZF rats.

The differences found in the lag phases demonstrate that mitochondria from ZF rats have decreased capability to repolarize after ADP addition. FCCP is a protonophore, which permeabilizes the mitochondrial membrane to protons and leads to maximal respiratory rates. The observation that mitochondrial respiration, in the presence of FCCP, was not different between control and ZF rats indicates no alterations on the enzymes of the electron transport chain. This added to the fact that oxidative phosphorylation efficiency, as indicated by ADP/O, was not affected by fat accumulation, such as ATPase or ANT. Interestingly, we uncovered a specific change in the protein levels of the ANT. ANT, a crucial component of oxidative phosphorylation, is the most abundant mitochondrial inner membrane protein and catalyses the exchange of ADP to ATP, thus supplying the cytoplasm with newly synthesized ATP in oxidative phosphorylation. ANT2 is expressed in liver and in cells capable of proliferation. There is some evidence that ANT2 could favor the mitochondrial uptake of cytosolic ATP in situations where oxidative phosphorylation is inhibited [15]. The ANT is also a component of the mitochondrial permeability transition (MPT), a large conductance pore formed apparently through a conformational change of several constituent proteins of the mitochondrial membrane. Other elements of the MPT structure seem to include VDAC and cyclophilin D, which confers sensitivity to cyclosporine A, a specific MPT inhibitor [16]. MPT induction is associated with pathological conditions and is triggered by high Ca^{2+}, oxidative stress, ATP depletion, high inorganic phosphate and mitochondrial depolarization.

Previous studies have shown higher content of oxidative products of proteins and lipids and the lower content of glutathione in mitochondria isolated from fatty liver, associated with increased oxidative stress [17]. Mitochondria are indeed a major potential source of reactive oxygen species in fatty liver disease [2,14]. However, these alterations in protein content appear to be specific alterations in the components of the phosphorylative system and not just the result of general oxidative damage since no differences were observed in VDAC content. A possible explanation could be alterations in oxidative metabolism induced by a dysregulation in fat metabolism.

Further studies focused on understanding the upstream molecular mechanisms by which metabolic pathways are dysregulated in fatty liver disease may ultimately help to develop therapeutic strategies and to prevent the onset of the disease.

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