Mitochondrial DNA copy number and citrate synthase activity in nonalcoholic fatty liver disease induced in rats by a high-fat plus fructose diet fortified or not with dehydroepiandrosterone (DHEA)

ANGELA MARIA LEZZA¹, PIERLUIGI CASSANO¹, RAFFAELLA MASTROCOLA², PESCE VITO¹, MANUELA ARAGNO², MARIA NICOLA GADALETA¹

¹ Dipartimento di Biochimica e Biologia Molecolare “Ernesto Quagliariello”
Università degli Studi di Bari
Via Orabona, 4 – 70125 Bari
ITALIA
E-mail: m.n.gadaleta@biologia.uniba.it
http://www.uniba.it

²Dipartimento di Medicina e Oncologia Sperimentale
Università degli Studi di Torino
Corso Raffaello, 30 - 10125 Torino
ITALIA
E-mail: manuela.aragno@unito.it
http://www.unito.it

Abstract: The mitochondrial DNA copy number and the citrate synthase activity have been studied in rat model of nonalcoholic fatty liver disease induced by a high-fat plus fructose (HF-F) diet which mimics a typical unhealthy Western diet. Rats treated by adding dehydroepiandrosterone (DHEA) to the diet were also studied to investigate on the protective effects of DHEA, a compound of physiological origin with multitargeted antioxidant properties. An HF-F diet, fortified or not with DHEA (0.01%, w/w), was administered for 15 weeks to male Wistar rats. After HF-F the rat liver showed a decrease of mtDNA content and of citrate synthase activity probably as results of oxidative stress. The addition of DHEA to the diet restored the activity of citrate synthase whereas it was unable to prevent the loss of mtDNA.

Key-Words: mtDNA copy number, citrate synthase activity, nonalcoholic fatty liver disease, high-fat diet plus fructose, dehydroepiandrosterone.

1 Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most widespread liver disease in the Western countries and its clinical relevance is due to the potential progression toward nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis and hepatocellular carcinoma.

The definition of NAFLD focuses on the accumulation of fat in the liver, that can be reversible or evolve to NASH, following the “two hits” hypothesis [1] highly in favour nowadays [see 2, review]. According to such hypothesis the first hit should lead to hepatic steatosis and the next to hepatocyte oxidative stress and inflammation of the liver. However, the primary metabolic cause for the lipid accumulation is still poorly understood. Growing evidence suggests that mitochondrial dysfunction might be crucial in the pathogenetic mechanism of NAFLD and that NASH might be a mitochondrial disease [3]. Inside mitochondria the reactions for the oxidation of fatty acids and pyruvate, for the citric acid cycle and for the oxidative phosphorylation take place and they make the organelles metabolically very relevant for the cell. Effectively, various mitochondrial alterations, including morphologic ultrastructural lesions, depletion of mitochondrial DNA (mtDNA), reduced activity of the respiratory complexes and impairment of β-oxidation, have been reported in liver of patients and animal models of NAFLD [2]. In order to explain such mitochondrial alterations, different molecular mechanisms have been proposed namely: 1) the excessive production of reactive oxygen species (ROS) leading to a condition of oxidative stress; 2) the increased expression of TNF-α, inducing dysfunction of some respiratory complexes; 3) the decreased expression of PGC-1 α and β, leading to derangement of β-oxidation and oxidative phosphorylation processes. Among the NAFLD animal models we have used the high-fat plus
fructose diet one which is known to induce rapid stimulation of lipogenesis with accumulation of triglycerides and to contribute to the development of hepatic insulin-resistance [4]. Under such diet, the sterol regulatory element binding protein-1c (SREBP-1c), very relevant in the regulation of the expression of genes involved in liver triglyceride synthesis, is strongly induced [5]. This increased expression of SREBP-1c can mediate the activation of the lipogenic genes transcription, likely cooperating, with the increased delivery of nutritional fatty acids to liver, to the hepatic lipid accumulation.

It has been also demonstrated by some of the Authors [5] that the HF-F diet induces in liver an oxidative stress condition, by reporting the raised levels of H$_2$O$_2$ as well as of the oxidative derivative 4-hydroxynonenal (HNE). In the same study the effect of adding dehydroepiandrosterone (DHEA) to the HF-F diet as a means to counteract the onset of NAFLD through its antioxidant properties was evaluated. Effectively, low-dose DHEA supplementation prevented the SREBP-1c induction thus reducing lipid accumulation and proinflammatory progression. However, the mitochondrial involvement in the HF-F diet induction of NAFLD and in the DHEA delaying action needed to be thoroughly investigated and this was the aim of the present study.

2 Mitochondrial DNA copy number in rats fed an HF-F diet with and without DHEA supplementation.

In order to find out if the HF-F diet was responsible for an increased oxidative damage to mitochondria, finally affecting also the relative amount of mtDNA, we measured by real time PCR the ratio mtDNA/nDNA. The determination was also carried out in the liver samples from five groups of male Wistar rats.

Male Wistar rats (Harlan Laboratories, Udine, Italy) weighing 200–220 g were cared for in compliance with the Italian Ministry of Health Guidelines (No. 86/609/EEC) and with the Principles of Laboratory Animal Care (NIH No. 85–23, revised 1985). The scientific project, including animal care, was supervised and approved by the local ethical committee. The animals were divided into five groups: (group 1) rats maintained on standard lab chow, consisting of 24% protein, 11% fat, and 65% carbohydrate (percentages by weight) and water ad libitum (control group, C); (group 2) rats maintained on standard lab chow fortified with 0.01% (w/w) DHEA (DHEA); (group 3) rats maintained on standard lab chow plus 10% (w/v) fructose dissolved in the drinking water (F); (group 4) rats maintained on a high-fat diet plus 4% cholesterol plus 10% (w/v) fructose dissolved in the drinking water (HF-F group); and (group 5) rats maintained on the HF-F diet fortified with 0.01% (w/w) DHEA (HF-F-DHEA group). The high-fat diet consisted of 17% protein, 26% fat plus 4% cholesterol and 53% carbohydrate. Rats were fed the standard or HF-F diet with or without DHEA for 15 weeks. Body weight and water and food intake were recorded weekly. Rats were then anesthetized with 20 mg/kg bw of Zoletil 100 (Virbac S.r.l., Carros, France) and killed by aortic exsanguination. The liver was isolated, weighed, cut into a number of portions, and stored at −80°C. The mtDNA content was evaluated by quantitative Real-Time PCR (qRT-PCR) as the mtDNA/nuclear DNA ratio in liver from five groups of male Wistar rats.

Total DNA was prepared from about 40–50 mg of liver according to Wizard Genomic DNA purification kit manual instructions (Promega) The measurement of the mtDNA content was carried out using specific primers and TaqMan probes, accurately designed by Primer Express software (Applied Biosystems, Foster City, CA) for the mitochondrial D-loop region (D-loopFor: 5’-GGTTCTTACTTCAGGGCCATA-3’; D-loopProbe: 5’-6FAM-TTGGTTTCATCGTCCATACGTCCCTTATTAMRA-3’; D-loopRev: 5’-GATTAGACCGTTACCCATCGAGAT-3’) and for the nuclear beta-actin gene (beta-actinFor: 5’-GGATGGTTGCTCAAACCAGTAC-3’; beta-actinRev: 5’-GGGATGTGGCTCAAACCAGTAC-3’; beta-actinProbe: 5’-VIC-CGGCTTTGGACTCAAGGGATTAA-3’; beta-actinProbe: 5’-VIC-CGGCTTTGGACTCAAGGGATTAA-3’). The method has been validated by primer limiting experiments to determine the proper primers concentrations (200 nM for each primer pair and 100 nM for both probes) and by evaluating the equal reaction efficiency of the two amplicons. Each sample was analyzed in triplicate and fluorescence spectra were monitored by the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, CA). The difference in
threshold cycle values (DeltaCt, namely Ct D-loop – Ct beta-actin) was used as a measure of the relative abundance of the mitochondrial genome. To compare the mtDNA amount between groups of rats, the control animals class was taken as the reference one and the relative amount of mtDNA to nuclear DNA was calculated by the following equation: $R = 2^{-\Delta\Delta Ct}$, where $R$ is the calculated ratio and $\Delta\Delta Ct$ is the $\Delta Ct_{analyzed\ class} - \Delta Ct_{reference\ class}$ value. Data were submitted to analysis of variance (ANOVA) with Tukeys HSD post hoc test using SPSS Base 11.5 software (SPSS Inc., Chicago, USA) to assess the significance of the differences observed between groups.

Figure 1 reports the changes of mtDNA content, measured as mtDNA/ nuclear DNA ratio, in liver from rats fed with HF-F diet fortified or not with DHEA.

**Figure 1.** Determination of the mitochondrial DNA content, measured as mtDNA/ nuclear DNA ratio, in liver of control (C), DHEA, F, HF-F, HF-F-DHEA fed rats. Data are given as mean value ± SE of three different experiments. * = statistically significant difference vs. C (p<0.05). Data were submitted to ANOVA with Tukeys HSD post-hoc test using SPSS Base 11.5 software.

The analysis reveals a significant decrease (-32%; p<0.05) of mtDNA content in liver of HF-F-fed animals with respect to the control group. DHEA supplementation to the HF-F diet does not influence the reduced liver mtDNA content of these animals. No effect of DHEA supplementation alone or fructose diet alone on mtDNA content is detectable.

### 3 Citrate synthase activity in rats fed an HF-F diet with and without DHEA supplementation.

To verify if HF-F diet would affect mitochondrial matrix enzyme Citrate Synthase (CS) its activity in the hepatic homogenate was determined. Total proteins were extracted from ≈20 mg of liver by homogenization in buffer (pH 7.4) containing 100 mM mannitol, 1 mM ATP, 0.2% BSA, 100 mM KCl, 3 mM MgCl2, 5 mM Tris, 1 mM EDTA and centrifugation at 700xg for 10 minutes at 4°C. Protein concentration was determined by the Bradford’s method (Bio-Rad Laboratories, Hercules, CA) according to the supplier’s instructions. Citrate synthase activity (μmol/min/mg total proteins) was determined spectrophotometrically in liver homogenates according to the method of Srere [6].

Figure 2 shows that HF-F diet induces a significant decrease of citrate synthase activity of about 35% (p<0.05). Interestingly, DHEA supplementation to HF-F diet is able to fully prevent such a decrease, restoring the CS activity value back to the control group one.

**Figure 2.** Determination of citrate synthase activity (μmol/min/mg total proteins) in the liver homogenate of control (C), DHEA, F, HF-F, HF-F-DHEA fed rats. Data are expressed as mean values ± SE. * = statistically significant difference vs. C (p<0.05); # = statistically significant difference vs. HF-F (p<0.01). Data were submitted to ANOVA with Tukeys HSD post-hoc test using SPSS Base 11.5 software.

### 4 Discussion and Concluding Remarks

As reported in the previous study dealing with the same groups of animals [5] the HF-F diet, besides
causing insulin resistance is able to induce a derangement of cell lipid metabolism, pinpointed by all the increased lipid parameters (triglyceride, total cholesterol, LDL and NEFA levels) versus the decreased HDL content.

The final consequence of these synergistic adverse metabolic alterations is the establishment of an oxidative stress condition, verified through the determination of ROS, oxidative derivatives and antioxidant defences levels, carried out in the same previous study [5]. The mtDNA content decrease here reported in HF-F fed rats might be a consequence of this oxidative stress condition and explain the already reported decreased respiratory complexes activities in the liver of patients [7] and animal models with NAFLD [8].

As for the citrate synthase activity, the HF-F diet causes a derangement of β-oxidation which probably reduces the availability of the acetyl CoA for the synthase reaction, leading to the decreased enzyme activity here reported. It is noteworthy that the diet with only high F and not HF is characterized by a citrate synthase activity comparable to that of the control animals suggesting the absence of any dysfunctional effect, when the oxidative metabolism is well-supplied by glycidic substrates. The chronic DHEA supplementation, at a low levels to avoid hormonal interferences, is likely able to wipe off the deranging effect of the HF diet on lipid catabolism preventing the decrease of citrate synthase activity and maintaining it at the same level of controls and fructose-fed animals. Viceversa, the effect of DHEA is not probably sufficient to prevent the loss of mtDNA, here demonstrated, induced by the HF-F diet and persisting with DHEA too.

References: