TITLE: Lipoic acid prevents non-alcoholic steatosis by modulating mitochondrial function and lipid metabolism pathways

ABREVIATED TITLE: Lipoic acid prevents non-alcoholic steatosis

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Abreviatures: OXPHOS, oxidative-phosphorylation; LASY, lipoic acid synthase; HFD, high-fat diet; LA, α-Lipoic acid; ATP, adenosine triphosphate; AMPK, adenosine monophosphate-activated protein kinase.

Abstract

Non-alcoholic steatosis is an important hepatic complication of obesity linked to mitochondrial dysfunction. Lipoic acid (LA) is a medium chain fatty-acid that has been reported to be able to prevent body weight gain, ectopic lipid accumulation and modulate mitochondrial oxidative stress. The aim of this study was to explore the actions of this fatty-acid on lipid metabolism and mitochondrial function in the liver of rats fed on a high-fat diet (HFD). Wistar rats were fed with a standard diet (n=10), a HFD (n=10) and a HFD supplemented with LA (n=10). A HFD pair-fed group (n=6) was also included. LA prevented hepatic triglyceride accumulation and liver damage in rats fed with a HFD. This effect seems to be mediated by an inhibitory effect on DGAT2 and an increase of genes involved in β-oxidation and in PPARs and PGC-1s pathways. LA also induced an inhibitory action on oxidative-phosphorylation activity and in ATP synthesis and stimulates the activity of citric acid cycle and expression of UCP2. These results highlight that LA is able to preserve non-alcoholic steatosis by decreasing lipogenesis and increasing β-oxidation through the activation of compensatory mechanisms in the mitochondrial compartment to combat the excess of energy provided by a HFD.

Keywords: mitochondrial dysfunction; high-fat diet; oxidative-phosphorylation; energy efficiency; obesity; bioenergetics; fatty liver; triglyceride synthesis; β-oxidation

Introduction

Fatty liver or steatosis refers to a histopathological condition in which an excess accumulation of lipids, primarily triglycerides within hepatocytes occurs (1). The clinical significance of fatty liver is generally thought to be one of the main causes of hepatic dysfunction and an important manifestation of the metabolic syndrome and obesity (2). Because of the crucial importance of this organ in maintaining the overall metabolism, alterations in hepatic function have an impact on the whole health organism and could responsible for several complications derived from the consumption of rich fat diets including obesity.

Accumulating evidence indicates that an impaired mitochondrial function plays a central role in the development of fatty liver (3-4). In fact, mitochondria are involved in fatty acid β-oxidation, oxidative phosphorylation (OXPHOS) and lipogenesis. In this sense, it is well known that high-fat diet (HFD) cause alterations in hepatic mitochondrial compartment (5-6).

α-Lipoic acid (LA) is a natural compound derived from octanoic acid. It is widely distributed in plants and animals and synthesized through a reaction catalyzed by lipoic acid synthase (LASY) within the mitochondria (7). Also, LA acts as a cofactor of several mitochondrial bioenergetics enzymes (8) and in several process of aerobic metabolism. Apart from its role in the mitochondria, LA when supplemented in diets exerts beneficial physiological effects such as attenuation of oxidative stress (9), overcoming of ageing decay (10), modulation of glucose metabolism (11), prevention of body weight gain induce by HFD (12) as well as a reduction of energy efficiency (13). In addition, the ability of LA to reduce serum and tissue lipid levels has been reported by other investigators (14-15). Moreover, it has been demonstrated that LA decreases hepatic lipogenesis, although the underlying mechanisms are not completely understood (16).

In the light of the above considerations, we suggested that LA treatment may have a protective effect against the development of fatty liver associated with a long-term high fat diet feeding through the modulation of lipid metabolism pathways and mitochondrial function. To test this hypothesis, we evaluated several parameters of ectopic lipid storage in the liver, lipid metabolism and mitochondrial function in rats fed with HFD supplemented with LA.

Methods

Animals and diets

Male Wistar rats (n=36) aged six weeks were supplied from the Centre of Applied Pharmacology (CIFA, Pamplona, Spain). Animals were housed in cages in temperature-controlled room (22 ±2ºC) with a 12-hour light-dark cycle, fed a pelleted chow diet and given deionised water *ad libitum* for an adaptation period of 5 days. After this period, rats were assigned into 4 experimental groups for 8 weeks. The Control group (n=10) was fed with a standard diet (4.6% w/w of lipids), commercially available (Harlam Tekland Global Diets, USA). The Obese (n=10), obese supplementes with LA (OLIP) (n=10) and the pair-fed (PFO) (n=6) groups were fed with a high fat diet (60% w/w of lipids), externally supplied (OpenSource diets Research Diets Inc, USA). The diet of the OLIP subgroup was supplemented with LA in a proportion of 0.25 g LA/100 g of diet as previously described (13). The Pair-Fed group (PFO) received the same amount of food eaten by the OLIP group but without adding LA. Body weight and food intake were recorded every 2-3 days. At the end of the experimental period, rats were euthanized and blood and tissue samples were immediately collected, frozen in liquid nitrogen and kept at -80ºC. All experimental procedures were performed according to National and Institutional Guidelines for Animal Care and Use at the University of Navarra.

Tissue homogenization procedure and mitochondrial isolation

Livers were quickly excised after sacrificing the animals and either frozen in liquid nitrogen or placed in ice-cold buffer (250 mM sucrose, 1 mM EDTA and 5 mM NaTES, pH=7.4). Fresh tissue placed in buffer was used to prepare mitochondrial suspension according to modified method of Rickwood *et al* (1987) (17) with slightly modifications as previously described by Valdecantos *et al* (2010) (18).

Blood samples and transminases serum levels

Blood was drawn into Vacutainer tubes containing gel without additives (Vacutainer Gel SST II). Serum samples were prepared after centrifugation for 15 min at 2200 *g* at 4ºC supernatant was collected and immediately frozen and kept at -80ºC. Transaminases serum levels were determined using a COBAS-Mira analyzer (Roche Diagnostics, Switzerland).

Hepatic triglyceride content

To determine the hepatic triglyceride (TG) content, 150 mg of these tissues were sonicated in a Branson Sonifier 250 equipment (Duty cycle 40%; Output control 4; Hold Continuous) for 40 seconds in 1.5 ml of buffer (150 mM NaCl, 0,1% Triton and 10 mM Tris (pH 8)) at 50ºC. After centrifugation at 12000*g* for 10 min, the obtained supernatant was used to measure the TG levels using a COBAS-Mira analyzer (Roche Diagnostics, Switzerland) as described elsewhere (19).

Morphological evaluation

For histopathological analysis, liver specimens fixed in 4% of paraformaldehyde was embedded in paraffin, sliced 10 µm thickness, and stained with hematoxylin and eosin staining for detection of hepatic steatosis, inflammation and morphological changes following routine procedures. Moreover, liver slices were stained with Red Sudan staining to determine collagen

fybers. The pathological changes were assessed and photographed under a Nikon eclipse E800 microscope (Nikon Corporation, Japan).

Real-time quantitative PCR

Total RNA was isolated from liver using Trizol® reagent (Invitrogen, USA) and according to the manufacturer´s instructions. RNA concentrations were determined by Nanodrop 1000 (Nanodrop, USA). To avoid contamination with genomic DNA, DNA digestion and inactivation was assessed using the DNAse kit (Ambion Inc, USA). Four micrograms of RNA were reverse transcribed to cDNA using the M-MLV kit (Invitrogen, USA) following instructions from the suppliers.

Twelve genes were analyzed (Table 1) using predesigned TaqMan® Assays-on- demand fixed on a low density array microplates (Applied Byosistems, USA). The reaction conditions were set up followed according to the manufacturer's instructions. Amplification and detection of specific products were performed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, USA). All the samples were analyzed in duplicate. C_T values were generated by the ABI software. Finally, the relative expression levels of each gene were calculated as $2^{-\Delta\Delta CT}$ previously reported (20). Hepatic expression levels of each gene were normalized by β-Actin.

Complex I, Complex II+III, succinate dehydrogenase and citrate synthase activities

Assays of the activities of these enzymes were carried out in frozen isolated mitochondria using a kinetic method in a Multiskan Spectrum spectrophotometer (Thermo Electron Corporation, USA), through standardized reproducible methods as described elsewhere (21-22). All activities were expressed in nmoles per minute and per milligram. Activity measurements were performed for both complexes using 50 µg of mitochondrial protein.

Complex IV activity and ATP synthase activity

Complex IV and ATP synthase activities were measured in frozen isolated rat liver mitochondria (25 µg and 75 µg of mitochondrial protein respectively) with a MitoProfile®Rapid Microplate Assay Kits according to the manufacturer´s instructions (Cat. nº MS447 and MS541, respectively. MitoScience, USA). Complex IV and ATP synthase were inmunocaptured within the wells of microplates, and the enzyme activity was measured by a kinetic colorimetric assay and activities were determined as previously described (23-24).

Liver mitochondrial ATP content

ATP was determined by the firefly luciferin-luciferase assay system accordingly to the method of Lemasters *et al (1976)* (25). Firstly, we extract the ATP from mitochondrial matrix according with a protocol (26), previously described by Dorta *et al* (2005) with minor modifications. Bioluminiscence was measured in the supernatant with a Proteinkinase sensitive assay kit (Proteinkinase, Biaffin GmbH & Co KG, Germany), according to the manufacturer´s instructions with slightly modifications and using a Luminoskan Ascent (Thermo Electron Corporation, USA).

Statistical analysis

Data are reported as mean ± S.E. Normal distribution was confirmed by two different test, Shapiro-Wilk and Kolmogorov-Smirnov. In order to determine the effects of LA treatment, one way ANOVA followed by a Bonferroni test were carried out. Relationships between variables were analyzed by calculating Pearson correlation coefficients. All statistical analysis were performed using the GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, USA). Differences were considered as statistically significant at $P<0.05$. Variables analyzed and presented in figures are unique and not required a multiple comparison correction.

Results

Effects of LA on body and liver weight and energy efficiency

As expected, a significant increase in body weight *(P<0.001)* was observed in rats fed a HFD. However, this increase was significantly prevented by LA treatment, but changes in liver weights were not observed. Energy intake was measured in order to find out if the differences in body weight evolution could be related to differences in food intake. In this sense, HFD fed rats showed a significant increase in their calorie intake *(P<0.001)*, which was also reduced by LA treatment *(P<0.001)*. Energy efficiency, calculated as a ratio between body weight gain and calorie intake, was significantly increased in the obese group *(P<0.05)*. Furthermore, LA induced a significant decrease in energy efficiency comparison with the obese rats as well as in comparison with the PFO group, suggesting that this reduction in energy efficiency is not secondary to calorie restriction *(P<0.01* OLIP *vs* PFO*)* (Table 2).

Effects of LA on hepatic triglyceride content and transaminases serum levels

HFD produced a very significant increase *(P<0.001)* in hepatic triglyceride content in liver compared with control rats (Fig.1.A). This effect was completely reversed by LA (*P<0.001 vs* OB). Moreover, the reduction observed after LA treatment seems to be a direct effect of this fatty-acid, as deduced from statistical differences (*P<0.001)* observed between OLIP and PFO groups (Fig.1.A). Furthermore, HFD induced a significant increase (*P<0.01*) in serum levels of both transaminases, and these increased were reversed by both LA treatment and calorie restriction (Fig.1.B).

Effects of LA in liver morphology

As revealed by a histological study, rats fed with a HFD developed macrovesicular and microvesicular steatosis with nuclear displacement, which was apparently reversed by LA

treatment. A microvesicular steatosis without nuclear displacement was observed in the PFO group. On the other hand, inflammatory infiltrates and higher macrophage presence were observed in obese rats but not in OLIP and PFO rats (Fig.1.C). Moreover, Red Sirius staining revealed a thickening of vascular walls and an increase in collagen fibers in obese (Fig.1.D), which was completely overturned by LA treatment (Fig.1.D).

Effects of LA on mRNA levels of several genes involved in lipogenesis, β-oxidation, PPARs and PGCs signalling pathways

In order to explore the potential mechanisms involved in the aforementioned reductory effects of LA on hepatic triglyceride content, the expression of several key enzymes and nuclear factors involved in lipogenesis and β-oxidation were evaluated.

A significant increase in SREBF1 (*P<0.001)* and DGAT2 (*P<0.01)* mRNA levels was observed in the obese group in comparison with the control animals. LA was able to reduce the expression of both genes (Table 3) in a statistical significant manner *(P<0.05-P<0.001,* respectively). Furthermore, the effects of LA on DGAT2 expression seem to be direct *(P<0.05 vs PFO).* Interestingly this value was strongly correlated with hepatic triglyceride content *(P<0.001;* r=0.6961*)* (data not shown).

As expected, rats fed with a HFD showed a marked decrease in CPT1A *(P<0.001),* ACADL *(P<0.001)* and ACOX1 *(P<0.05)* gene expression, all of them involved in mitochondrial and peroxisomal β-oxidation. Similarly as what was observed with lipogenic genes, LA was able to reverse these effects, reaching similar values than in the control group. Moreover, the effects of LA on ACADL expression seem to be direct *(P<0.05 vs PFO)* (Table 3). This value was negatively correlated with the hepatic triglyceride content *(P=0.0003;* r=-0.6278*)* (data not shown).

Furthermore, feeding a HFD induced a significant decrease (*P<0.01)* in the expression PPARα, however, did not modify the pattern of expression of PPARγ. Nevertheless, LA treatment induced a significant increase in the expression of PPARα (+86.5%, *P<0.001)* and PPARγ (+125.6%, *P<0.01)*, independently of calorie restriction. On the other hand, HFD produced a significant reduction of PGC1α *(*-76.2%, *P<0.001)* and PGC1β *(*-45.6%, *P<0.05)* gene expression, while LA was able to significantly increase *(P<0.01-P<0.05 vs* OB*)* the expression of these genes (Table 3).

Effects of LA on several key enzymes of citric acid cycle

The ingestion of a HFD induced a significant impairment *(P<0.001)* in the activities of citrate synthase and succinate dehydrogenase in hepatic isolated mitochondria. Nevertheless, rats fed with a HFD supplemented with LA increased the activity of both enzymes *(P<0.01* and *P<0.001)* and these stimulatory effects were independent of the calorie restriction, suggesting a direct role of LA on citric acid cycle (Fig.2).

Effects of LA on OXPHOS activity on liver mitochondria

LA treatment induced a strong inhibitory effect in the activity (-54.2%, *P<0.001 vs* control) of Complex I (Fig.3.A) despite consuming a high fat diet. Moreover, the statistical differences between OLIP and PFO group *(P<0.001)* could suggest that this inhibitory effect was independent of the reduction in food intake. On the other hand, HFD induced a stimulatory effect in the activity of Complex II+III (Fig.3.B) (+92.2%, *P<0.001)* and Complex IV (Fig. 3.C) (+103.2 %, *P<0.01)* of electron transport chain, being these effects reversed by LA treatment. Moreover, the reduction observed after LA treatment seems to be direct and not secondary to calorie restriction as suggested from the statistical differences observed between OLIP and PFO group in Complex II+III (*P<0.01*) and also in Complex IV (*P<0.05*) activities.

Actions of LA on ATP production and mitochondrial bioenergetics

HFD induced a significant increase in ATP synthase activity (+49.7%, *P<0.05)* (Fig.4.A), although, it did not exert any effect in ATP synthase gene expression (Fig.4.B). Supplementation with LA induced a significant decrease in the activity (-68.1%, *P<0.001 vs* obese group*)* and expression of ATP synthase *(P<0.01)*. Furthermore, LA treatment was able to directly reduce ATP production (-31.7%, *P<0.05)* (Fig.4.C). In fact, this outcome was a direct effect of LA and not secondary to caloric restriction *(P<0.05 vs* PFO*).* Interestingly, it was positively correlated to energy efficiency (Fig.4.D). Finally, HFD induced a significant decrease in mRNA expression of UCP2 (-39.3%;*P<0.05)* that was significantly reversed by LA treatment (+189.5%; *P<0.001)*. This finding appears as a direct effect of LA and not secondary to energy restriction *(P<0.05 vs* PFO*)* (Table 3).

Discussion

In the present study, we found that LA prevents ectopic fat storage in the liver induced by a high fat diet through the modulation of lipid metabolism pathways and mitochondrial bioenergetics. It has been demonstrated a strong reduction in hepatic triglyceride content in the OLIP group (animals fed a high-fat diet supplemented with LA), in comparison to its own control, the pair-fed group, suggesting direct lipid lowering effects of LA on liver despite a high-fat diet. Moreover, LA is able to reverse liver morphological changes and inflammatory effects induced by a HFD.

These physiological impairments were accompanied by changes in a number of genes within liver. In fact, our results revealed that the protective effects of LA treatment are related to its ability to decrease triglyceride synthesis and its stimulatory effect on fatty acid oxidation. Thus, high-fat feeding rats exhibited increased mRNA levels of DGAT2, an enzyme responsible for the final step in triacylglycerol synthesis (27) and involved in the development of hepatic steatosis (28). Interestingly, LA directly inhibited the expression of this gene which could contribute to

explain, at least in part, the lower triglyceride accumulation in the livers of these animals as demonstrated by the correlation found between DGAT2 mRNA levels and hepatic triglyceride content. In addition, several studies described the central role of SREBP1 in the activation of lipogenic pathway and in the development of fatty liver (29-30). Furthermore, Park *et al* (2008) observed that LA treatment reduced hepatic triglyceride content and mRNA levels of SREBP1 through AMPK dependent and independent mechanisms (16). We also observed an increase in SREBP1 mRNA levels after LA treatment, but it seems to be secondary to food restriction, suggesting that DGAT2, and not SREBP1, is a potential target of LA in the liver under our experimental conditions.

Other mechanism that could explain the development of fatty liver is the impairment of mitochondrial β-oxidation (31). Indeed, according with previous investigations (32), we also found that all genes involved in mitochondrial β-oxidation were down-regulated in high-fat feeding rats. Furthermore, this impairment observed on mitochondrial β-oxidation was partially improved by calorie restriction and completely reversed by LA treatment, mainly through its direct action on ACDL, an enzyme with a key role in the regulation of mitochondrial β-oxidation (33) and in the development of hepatic steatosis (34). Thus, we proposed that another potential mechanism that could explain the beneficial effects of LA on hepatic triglyceride content is its stimulatory effects on β-oxidation, mainly through the regulation of ACDL gene expression. Indeed, ACDL is a specific target of PPAR-α (35), a key transcriptional factor involved in fatty acid metabolism that is able to down-regulate fat accumulation by increasing fatty acid degradation (36). In addition, it is important to note the emerging and pivotal role of PGC-1β in the prevention of hepatic steatosits (37) as well as its ability to regulate transcriptional activity of PPAR-α (38). Thus, and based on the direct and stimulatory effects on PGC-1β mRNA levels observed after LA treatment, we suggest that there may be an association between the effects of LA on PGC-1β, PPAR-α and ACDL in liver. However, more

molecular studies are needed to deeply analyze what come first in this cascade of stimulatory effects.

We have also found very interesting effects of LA on mitochondrial energy efficiency as well as on the electron transport chain. Indeed, our results demonstrated that LA reduced the activity of the main complexes of the electron transport chain, which, in turn, leads to a lower efficiency of OXPHOS and therefore, lower ATP production. These results contrast with the increase observed in the activity of Krebs cycle, because this augment should enlarge the electron flow within the respiratory chain. In this sense, the stimulatory effects of LA in the activity of citric acid cycle were also described in other studies (39-40). However, we suggest that the reduction in energy efficiency could be a compensatory mechanism to combat the excess of energy provided by the high-fat diet through the Krebs cycle. In fact, our data evidenced that this reduction in ATP synthesis after LA treatment was strongly correlated with a decrease in body weight gain and total energy efficiency despite a high fat diet. These associations corroborate previous studies where a reduction in energy efficiency after LA treatment was also demonstrated (13, 41). Furthermore, the decrease in the activity of electron transport chain could produce an increase in mitochondrial membrane potential and expression of uncoupling proteins, such as UCP2, mainly expressed in liver and could therefore contribute to dissipate this membrane potential (42). In fact, we also found that LA increased through the stimulation of UCP2 gene expression and as accumulating data suggest that UCPs may play a pivotal role in mitochondrial function and contribute to energy expenditure (43). We hypothesized that this increase in UCP2 mRNA levels could also contribute to the reduction observed in energy efficiency as well as in body weight in rats fed a high fat diet supplemented with LA. Furthermore, recent data from our group showed the ability of LA to induce an uncoupling effect between electron transport chain and ATP synthesis in isolated rat liver mitochondria (18). Finally, it is well known that UCP2 is up-regulated through the PPAR-α

pathway (44). Thus, we suggest that the increased observed in UCP2 could be secondary to its stimulatory effects on PPAR-α.

The regulatory effects of PGC-1α in the mitochondrial function and metabolism as well as a role in the control of energy homeostasis have been widely reviewed (45-46). Some studies described that LA is able to stimulate the expression of PGC-1α in different tissues, such as skeletal muscle (47) or hepatocytes (10). We have also observed an stimulatory effect of LA on PGC-1α in liver of rats fed with a high fat diet, although this effect seem to be secondary to caloric restriction since no significant differences were observed between the OLIP and PFO groups. However, we demonstrated a direct stimulatory effect of LA on PGC-1β gene expression, independently of food restriction, suggesting that this transcription factor, instead of PGC-1α, is a direct target of LA in liver. In fact, different investigations established the key role of PGC-1β in the transcriptional control of mitochondrial metabolism and energy homeostasis (37, 48). Particularly, some studies highlight the ability of PGC-1β to stimulate similar pathways than PGC-1α with the exception of gluconeogenesis (49). Moreover, a higher ability of PGC1-β in comparison with PGC1-α to regulate mitochondrial function has been proposed in several studies (50-51) suggesting that LA actions on PGC1-β and not on PGC-1α could contribute, to some extent, to the energy dissipation observed in liver mitochondria after LA treatment.

In summary, this investigation demonstrated the ability of LA to prevent fatty liver induced by a HFD. The beneficial effects of LA in hepatic triglyceride content could be explained, at least in part, by a decrease in DGAT2 mRNA levels, accompanied by an increase in mitochondrial β-oxidation, mainly through the stimulation of PGC1-β, PPAR-α and ACDL genes expression. LA also reduced mitochondrial energy efficiency via the activation of several compensatory mechanisms to combat the excess of energy provided by a high-fat diet through the Krebs cycle. Thus, LA induced a significant decrease in the electron transport chain activity as well as

in ATP production, while, this antioxidant increased the expression of UCP2. This reduction in mitochondrial energy efficiency could also explain, at least in part, the beneficial effects of LA not only in fatty liver but also in preventing excessive body weight gain.

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Figure legends

Fig. 1: Effects of HFD and LA treatment in liver triglyceride content, transaminases serum levels and liver histopathology. (A) Hepatic triglyceride content in liver homogenate. (B)Transaminases serum levels. (C)Representative images of H&E-stained liver sections from different experimental groups; high-fat-fed rats displayed macrovesicular and microvesicular steatosis which displacing the nucleus (Picture 4, arrows); inflammatory infiltrate extended pericellularly in obese rats (Picture 3 lower right); focal hepatocyte necrosis was are engulfed by macrophages (Picture 3, lower right inset at 40X); increased of diploid hepatocytes(Picture 4, open arrows). PFO rat displayed microvesicular steatosis without disturbance of nucleus (Picture 8, white open arrowhead). (C)Red Sirius stain for collagen shows a thickening of vascular walls in obese rats (Picture 3, black arrow) and an increase in collagen fibers in hepatic section was observed in obese group (Picture 4). Values are mean±SE. ***P<0.01, ***P<0.001 vs* control group; $^{\#\#}$*P<0.01,* $^{\#\#\#}$*P<0.001 vs* obese group; ^{c}*P<0.001 vs* PFO group according to one way ANOVA followed by post-hoc multiple comparisons by Bonferroni test.

Fig. 2: Effects of HFD and LA on activities of citrate synthase (A) and succinate dehydrogenase (B). Values are mean±SE. ****P<0.001 vs* control group; $^{\#\#}$*P<0.01 vs* obese group; ^{a}*P<0.05 vs* PFO group according to one way ANOVA followed by post-hoc multiple comparisons by Bonferroni test.

Fig. 3: Activities of electron transport chain complexes. (A) Complex I; (B) Complex II+III; (C) Complex IV. Values are mean±SE. ***P<0.01, ***P<0.001 vs* control group; $^{\#}$*P<0.05,* $^{\#\#}$*P<0.01,* $^{\#\#\#}$*P<0.001 vs* obese group; ^{a}*P<0.05,* ^{b}*P<0.01,*^{c}*P<0.001 vs* PFO group according to one way ANOVA followed by post-hoc multiple comparisons by Bonferroni test.

Fig. 4: Changes in ATP synthase activity (A), mRNA levels of ATP5c1 (B), mitochondrial ATP levels (C) and correlation between liver mitochondrial ATP levels and metabolic

efficiency(D). Values are mean±SE. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ *vs* obese group; $^{a}P<0.05$, $^{b}P<0.01$ *vs* PFO group according to one way ANOVA followed by post-hoc multiple comparisons by Bonferroni test. r=Pearson correlation coefficient. mRNA level are expressed as fold changes ($2^{-\Delta\Delta CT}$) compared to control group considered as 1.

Tables

Table 1: Gene name and GenBank accession number used in qRT-PCR

Gen symbol	GenBank number	Gene name	Gene function
ATP5c1	NM_053825.1	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1	ATP synthesis
UCP2	NM_019354.2	Uncoupling protein 2	Energy expenditure
SREBF1	XM_213329.5	Sterol regulatory element binding transcription factor 1	Lipogenesis (regulation)
DGAT2	NM_001012345.1	Diacylglycerol O-acyltransferase homolog 2	Triglyceride synthesis
ACADL	NM_012819.1	Acyl-Coenzyme A dehydrogenase, long-chain	Mitochondrial β-oxidation
ACOX1	NM_017340.2	Acyl-Coenzyme A oxidase 1, palmitoyl	Peroxisomal β-oxidation
CPT1A	NM_031559.2	Carnitine palmitoyltransferase 1a	Mitochondrial β-oxidation
PPARA	NM_013196.1	Peroxisome proliferator activated receptor alpha	Lipid metabolism (regulation)
PPARG	NM_013124.3	Peroxisome proliferator-activated receptor gamma	Lipid metabolism (regulation)
PPARGC1A	NM_031347.1	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	Mitochondrial function (regulation)
PPARGC1B	NM_176075.2	Peroxisome proliferator-activated receptor gamma, coactivator 1 beta	Mitochondrial function (regulation)

Table 2: Body and liver weight, calorie intake and feed efficiency in lean and obese (high-fat fed) rats.

	CONTROL n=10	OBESE n=10	OLIP n=10	PFO n=6	ANOVA
Final body weight *(g)*	391.8±38.61	482.0±43.41***	363.7±42,97$^{\#\#\#, a}$	423.2±11.02*	*<0.001*
Liver weight *(g)*	9.4±1.37	10.3±0.88	9.3±1.60	9.0±1.08	*0.2995*
Liver/body weight *(%)*	2.48±0.457	2.13±0.284	2.59±0.386	2.13±0.258	*0.0386*
Calorie intake *(Kcal/day)*	69.9±2.16	99.8±4.47***	83.4±3.44$^{\#\#\#}$	81.8±2.92$^{\#\#\#}$	*<0.001*
Energy efficiency *(g/Kcal*100)*	4.2±0.57	4.9±0.31^{a}	3.2±0.60$^{\#\#,**, b}$	4.3±0.47	*<0.001*

Values are means±SE. **P<0.05, **P<0.01, ***P<0.001 vs* control group; $^{\#}$*P<0.05,* $^{\#\#}$*P<0.01,* $^{\#\#\#}$*P<0.001 vs* obese group; ^{a}*P<0.05,* ^{b}*P<0.01 vs* PFO group according to one way ANOVA followed by post-hoc multiple comparisons by Bonferroni test.

Table 3: Effects of LA on mRNA levels of several genes involved in liver lipogenesis, β-oxidation, PPARs and PGCs pathway

	CONTROL	OBESE	OLIP	PFO	ANOVA
	n=10	n=10	n=10	n=6	
LIPOGENESIS					
SREBF1	1.00±0.283	2.50±0.794***	1.73±0.417$^{\#,*}$	1.72±0.477	*0.0004*
DGAT2	1.00±0.233	1.51±0.403**	0.89±0.190$^{\#\#\#, a}$	1.31±0.212	*0.0008*
B-OXIDATION					
CPT1A	1.00±0.221	0.423±0.234***	0.992±0.311$^{\#\#\#}$	0.843±0.196$^{\#}$	*0.0003*
ACADL	1.00±0.189	0.59±0.348*	1.21±0.263$^{\#\#\#, a}$	0.87±0.170	*0.001*
ACOX1	1.00±0.222	0.36±0.215***	0.89±0.284$^{\#\#\#}$	0.67±0.110$^{\#,*}$	*<0.001*
PPARs PATHWAY					
PPAR-α	1.00±0.183	0.55±0.393*	1.85±0.493$^{\#\#\#,**, a}$	1.18±0.309$^{\#}$	*<0.001*
PPAR-γ	1.00±0.268	1.16±0.404	1.95±0.546$^{\#\#, a}$	1.22±0.168	*0.0011*
PGCs PATHWAY					
PGC1-α	1.00±0.383	0.22±0.131*	0.54±0.067$^{\#\#,**}$	0.47±0.152**	*0.0004*
PGC1-β	1.00±0.265	0.56±0.725**	1.37±0.385$^{\#\#\#, a}$	0.82±0.304	*<0.001*
UNCOUPLING PROTEINS					
UCP2	1.00±0.284	0.66±0.383*	1.13±0.167$^{\#\#}$	0.83±0.149^{a}	*0.0064*

Values are means±SE. **P<0.05, ***P<0.01, ***P<0.001 vs* control group; $^{\#}$*P<0.05,* $^{\#\#}$*P<0.01,* $^{\#\#\#}$*P<0.001 vs* obese group; ^{a}*P<0.05 vs* PFO group according to one way ANOVA followed by post-hoc multiple comparisons by Bonferroni test. Gene expression is expressed as fold changes ($2^{-\Delta\Delta CT}$) compared to the control group, which was considered as 1.

Figure 1

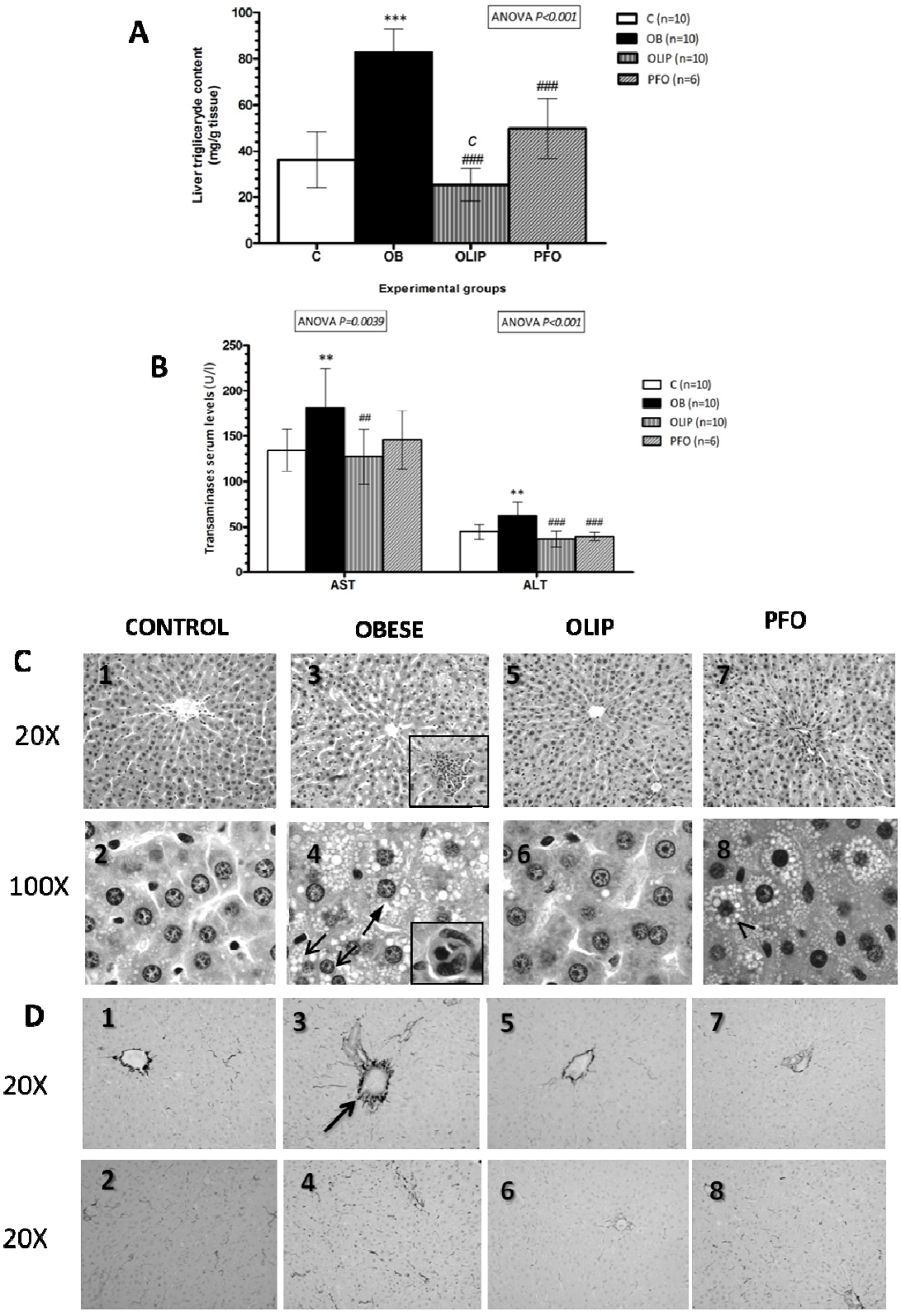

A
ANOVA P<0.001
C (n=10)
OB (n=10)
OLIP (n=10)
PFO (n=6)
Liver triglicerydе content (mg/g tissue)
Experimental groups
C
OB
OLIP
PFO
B
ANOVA P=0.0039
ANOVA P<0.001
Transaminases serum levels (U/l)
AST
ALT
CONTROL
OBESE
OLIP
PFO
C
20X
100X
D
20X
20X

Figure 2

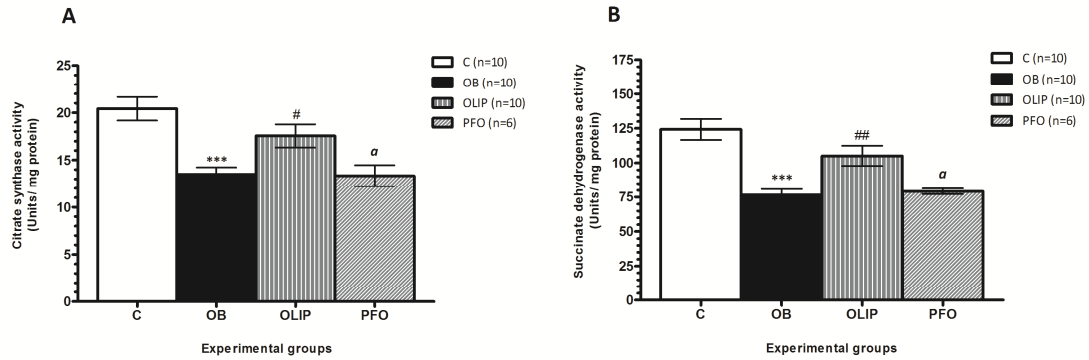

A
Citrate synthase activity (Units/ mg protein)
25
20
15
10
5
0
***
#
a
C
OB
OLIP
PFO
Experimental groups
C (n=10)
OB (n=10)
OLIP (n=10)
PFO (n=6)
B
Succinate dehydrogenase activity (Units/ mg protein)
175
150
125
100
75
50
25
0
***
##
a
C
OB
OLIP
PFO
Experimental groups
C (n=10)
OB (n=10)
OLIP (n=10)
PFO (n=6)

Figure 3

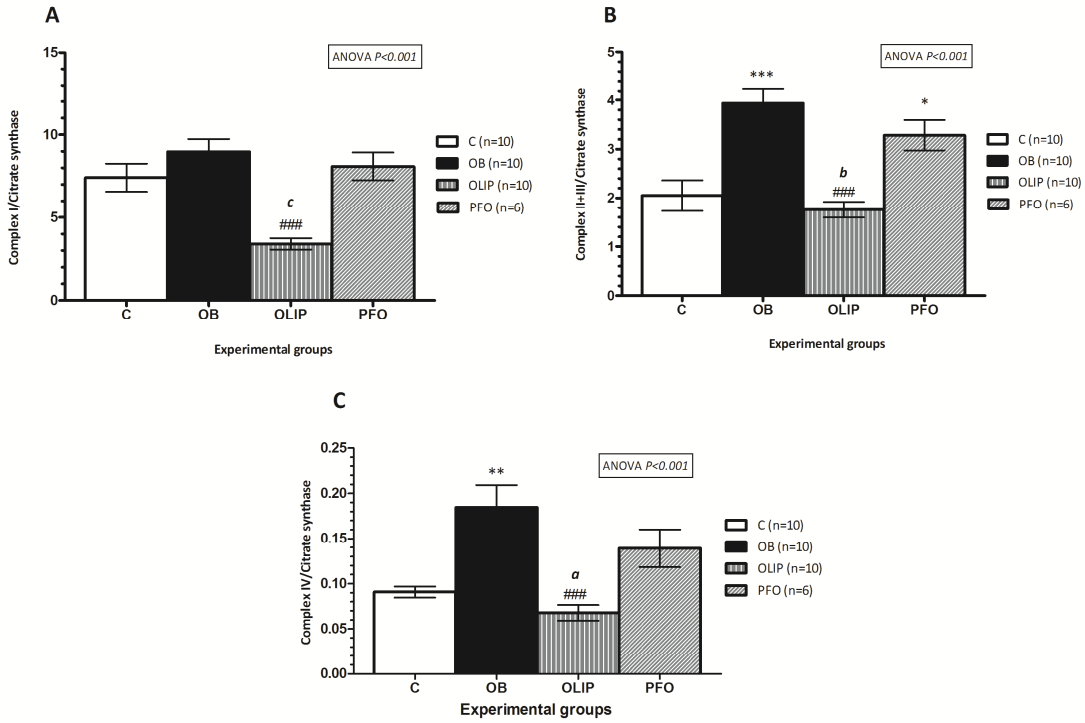
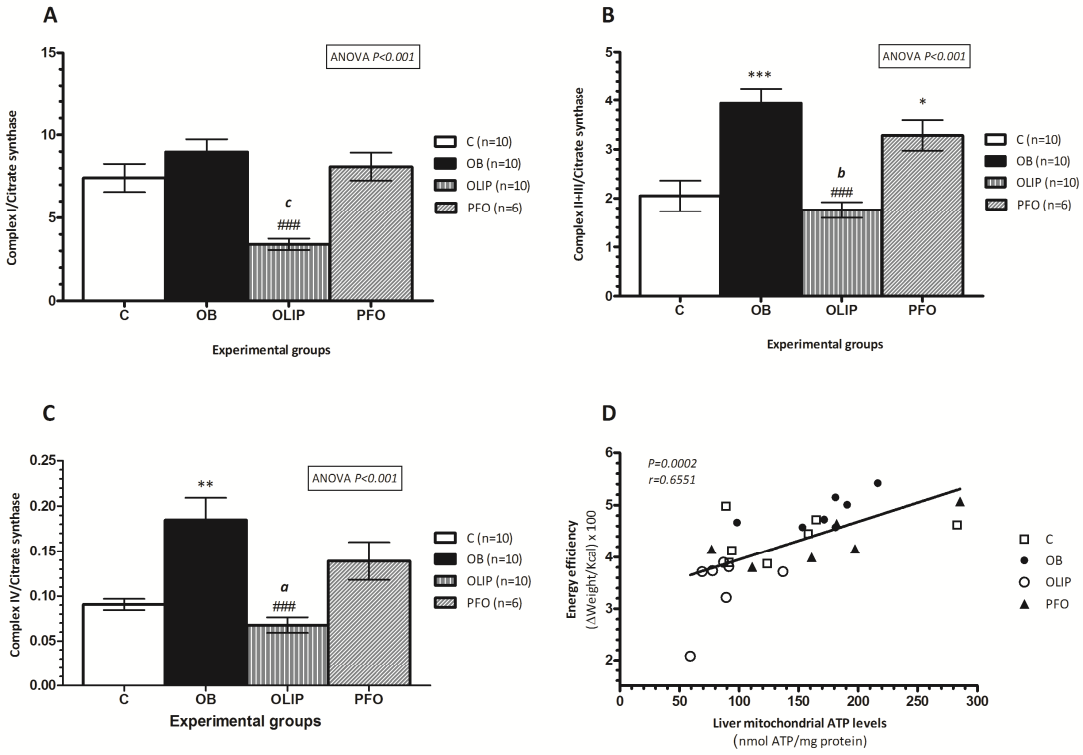

A
ANOVA P<0.001
Complex I/Citrate synthase
15
10
5
0
c
###
C
OB
OLIP
PFO
Experimental groups
C (n=10)
OB (n=10)
OLIP (n=10)
PFO (n=6)
B
ANOVA P<0.001
Complex II+III/Citrate synthase
5
4
3
2
1
0
***
*
b
###
C
OB
OLIP
PFO
Experimental groups
C (n=10)
OB (n=10)
OLIP (n=10)
PFO (n=6)
C
ANOVA P<0.001
Complex IV/Citrate synthase
0.25
0.20
0.15
0.10
0.05
0.00
**
a
###
C
OB
OLIP
PFO
Experimental groups
C (n=10)
OB (n=10)
OLIP (n=10)
PFO (n=6)

Figure 4


A
ANOVA P<0.001
Complex I/Citrate synthase
C (n=10)
OB (n=10)
OLIP (n=10)
PFO (n=6)
c
###
C
OB
OLIP
PFO
Experimental groups
B
ANOVA P<0.001
Complex II+III/Citrate synthase
***
*
b
###
C (n=10)
OB (n=10)
OLIP (n=10)
PFO (n=6)
C
OB
OLIP
PFO
Experimental groups
C
ANOVA P<0.001
Complex IV/Citrate synthase
**
a
###
C (n=10)
OB (n=10)
OLIP (n=10)
PFO (n=6)
C
OB
OLIP
PFO
Experimental groups
D
P=0.0002
r=0.6551
Energy efficiency
(ΔWeight/Kcal) x 100
C
OB
OLIP
PFO
Liver mitochondrial ATP levels
(nmol ATP/mg protein)