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Lipoic Acid Improves Mitochondrial Function in Nonalcoholic Steatosis Through the Stimulation of Sirtuin 1 and Sirtuin 3

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Nonalcoholic steatosis is an important hepatic complication of obesity linked to mitochondrial dysfunction and oxidative stress. Lipoic acid (LA) has been reported to have beneficial effects on mitochondrial function and to attenuate oxidative stress. The sirtuin (SIRT) family has been demonstrated to play an important role in the regulation of mitochondrial function and in the activation of antioxidant defenses. In this study, we analyzed the potential protective effect of LA supplementation, via the modulation of mitochondrial defenses through the SIRT pathway, against oxidative stress associated with high-fat feeding. Wistar rats were fed a standard diet (control group (C), $n = 10$), a high-fat diet (obese group (OB), $n = 10$) and a high-fat diet supplemented with LA (OLIP, $n = 10$). A group pair-fed to the latter group (pair-fed OLIP group (PFO), $n = 6$) was also included. LA prevented hepatic triglyceride (TG) accumulation (–68.2%) and liver oxidative damage ($P < 0.01$) through the inhibition of hydroperoxide (H_2O_2) production ($P < 0.001$) and the stimulation of mitochondrial antioxidant defenses. LA treatment upregulated manganese superoxide dismutase (SOD2) (60.6%) and glutathione peroxidase (GPx) (100.2%) activities, and increased the reduced glutathione (GSH): oxidized glutathione (GSSG) ratio and UCP2 mRNA levels ($P < 0.001$–$P < 0.01$). Moreover, this molecule reduced oxidative damage in mitochondrial DNA (mtDNA) and increased mitochondrial copy number ($P < 0.001$–$P < 0.01$). LA treatment decreased the acetylation levels of Forkhead transcription factor 3a (Foxo3a) and PGC1β ($P < 0.001$–$P < 0.01$) through the stimulation of SIRT3 and SIRT1 ($P < 0.001$). In summary, our results demonstrate that the beneficial effects of LA supplementation on hepatic steatosis could be mediated by its ability to restore the oxidative balance by increasing antioxidant defenses through the deacetylation of Foxo3a and PGC1β by SIRT1 and SIRT3.

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INTRODUCTION

Nonalcoholic fatty liver disease, which is also considered the major hepatic representation of metabolic syndrome is a critical complication of obesity (1,2). Oxidative stress together with insulin resistance contributes to the transition from simple steatosis to nonalcoholic steatohepatitis (3). In this regard, not only is the mitochondrion a major cellular site involved in energy metabolism, but it is also the main source of reactive oxygen species (ROS). Furthermore and in order to counterbalance the negative effects of ROS, mitochondria also contain enzymatic antioxidant defenses, such as manganese superoxide dismutase (SOD2), glutathione peroxidase (GPx) and mitochondrial glutathione. When the balance between mitochondrial ROS generation and the antioxidants defenses was displace to the pro-oxidant side an increase in mitochondrial oxidative stress is observed, leading to impaired mitochondrial function. Moreover, several studies have described that impaired mitochondrial function plays a central role in the development of nonalcoholic steatohepatitis (4–6).

Research into the attenuation or complete suppression of oxidative stress as a means of combating several diseases has flourished and has become a major challenge in recent years. Several approaches have been carried out in order to either decrease the high levels of ROS generated or boost the endogenous levels of antioxidants. α-Lipoic acid (LA) is a natural antioxidant synthesized through a reaction catalyzed by LA synthase within the mitochondria (7). Furthermore, LA acts as a cofactor of several mitochondrial bioenergetic enzymes (8) and in several processes of aerobic metabolism. Apart from its role in the mitochondria, when supplemented in diets LA exerts beneficial physiological effects

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such as: attenuation of oxidative stress, prevention of body weight gain induced by HFD, as well as reduction of energy efficiency (9,10).

The sirtuins (SIRTs) are a conserved family of proteins with NAD^+-dependent deacetylase activity, distinct form class I and II histone deacetylases (11). SIRTs have been demonstrated to play important roles in many physiological and pathophysiological situations, including metabolic syndrome, cell survival, aging, and calorie restriction-mediated longevity through deacetylation of numerous substrates (12). Furthermore, the role of SIRT 1 (SIRT1) in the regulation of mitochondrial biogenesis and energy homeostasis through the activation of peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α) has been widely studied (13,14). Sirtuin 3 (SIRT3), another member of the SIRT family, which is expressed in mitochondria, plays a role in regulating *in vivo* energy homeostasis (15). Forkhead transcription factor 3a (Foxo3a) is a substrate of SIRT3 (16) and in its deacetylated form has been demonstrated to stimulate several antioxidant defenses (16,17).

In the light of the above considerations, we suggest that LA treatment may have protective effects against the oxidative stress associated with a high-fat feeding via the modulation of mitochondrial defenses through the SIRT pathway. To test this hypothesis,several parameters of oxidative stress and antioxidant defenses in the mitochondrial hepatic compartment as well as mitochondrial function and SIRT pathway in rats fed with high-fat diet supplemented with LA were assessed.

METHODS AND PROCEDURES

Animals and diets

Six-week-old male Wistar rats ($n = 36$) were obtained from the Research Centre of Applied Pharmacology (Pamplona, Spain). Animals were housed in cages in a temperature-controlled room (22 ± 2 °C) with a 12-h light-dark cycle, fed a pelleted chow diet and given deionizer water *ad libitum* for an adaptation period of 5 days. After this period, rats were assigned to 4 experimental groups for 8 weeks. The control (C) group ($n = 10$), was fed a standard commercially available (Harlan Tekland Iberica, Barcelona, Spain) diet (4.6% wt/wt of energy as lipids). The obese group (OB) ($n = 10$) was fed *ad libitum* a high-fat commercially available (OpenSource Diets; Research Diets, New Brunswick, NJ) diet (60% wt/wt of energy as lipids); the OLIP (obese + LA) group ($n = 10$) was fed *ad libitum* a HFD supplemented with racemic α-LA (Sigma-Aldrich, St Louis, MO) in a proportion of 0.25 g LA/100 g of diet, as previously described (9); the pair-fed OLIP group (PFO) ($n = 6$) was fed the same amount of HFD provided to the OLIP group but without the addition of LA. This group will be used to determine if LA actions are independent of the LA effect on food intake. 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 −80 °C. 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 the animals had been sacrificed and either frozen in liquid nitrogen or placed in ice-cold buffer (250 mmol/l sucrose, 1 mmol/l EDTA, and 5 mmol/l NaTES, pH = 7.4). Fresh tissue placed in buffer was used to prepare mitochondrial suspensions according to the modified method of Rickwood *et al.* with minor modifications (18). Antioxidant defenses, total and cleaved poly ADP ribose polymerase (PARP) protein levels and mitochondrial DNA (mtDNA) damage were measured in isolated rat liver mitochondria. Triglyceride content, lipid peroxidation, and protein levels were assayed in total liver homogenates.

Hepatic TG content

To determine the hepatic triglyceride (TG) content, 150 mg of frozen liver were sonicated in a Branson Sonifier 250 for 40 s in 1.5 ml of buffer (150 mmol/l NaCl, 0,1% Triton, and 10 mmol/l Tris (pH 8)) at 50 °C. After centrifugation at 12,000*g* for 10 min, the supernatant obtained was used to measure the TG levels using a COBAS-Mira analyzer (Roche Diagnostics, Barcelona, Spain).

Lipid peroxidation

Malondialdehyde levels were quantified following the controlled reaction with thiobarbituric acid. Colorimetric changes were measured in liver homogenates with a commercial kit (Cayman Chemical, Ann Arbor, MI) following the manufacturer's instructions with minor modifications and using a Luminoskan Ascent (Thermo Electron, Foster City, CA). Results were corrected by the amount of tissue in mg.

Chemiluminescent measurement of superoxide and H_2O_2 production

Superoxide ($O_2^{-\cdot}$) and hydroperoxide (H_2O_2) production was quantified in isolated mitochondria by measuring the lucigenine-derived and luminol-derived chemiluminescence as described previously (18).

Measurement of antioxidant defenses

SOD2 and GPx activities were measured in isolated rat liver mitochondria using enzymatic colorimetric activity kits (Assay Designs, Ann Arbor, MI) with slight modifications as previously described (19). Total reduced glutathione (GSH) and oxidized (GSSG) glutathione levels were measured in the same samples using a colorimetric assay (Assay Designs). The ratio between GSH and GSSG was also calculated as a marker of antioxidant status in both erythrocytes and the hepatic mitochondrial compartment.

Mitochondrial oxidative damage estimation

mtDNA oxidative damage was estimated as the ratio of 80-bp fragment (260–339 position), compared to a 162-bp fragment (260–421 position) of the same sample. For real-time PCR reactions of both fragments, we used SYBR Green qPCR Master Mix with ROX as reference dye (Invitrogen, Carlsbad, CA) in an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). The primers used were HVII-FOR260 (5′-GCCACTTTCCACACAGACATCATA-3′), HVII-L421 (5′-AGTGCATACCGCCAAAAGATAAA-3′) and HVII-C339 (5′-TGTTTAAGTGCTGTGGCCAGA-3′) from Sigma-Aldrich. Both fragments were amplified in a total volume of 10 µl with 15 ng of total DNA, 5 µl SYBR Green Mix, and 10 µmol/l of HVII-FOR260 and HVII-L421 for large fragments and 2.5 µmol/l HVII-FOR260 and HVII-C339 for short fragments. The PCR conditions were 2 min at 50 °C, 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min.

Real-time quantitative PCR and mitochondrial copy number

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

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

Gen symbol	GenBank number	Gene name	Gene function
SOD2	NM_017051.2	Superoxide dismutase 2, mitochondrial	Antioxidant defenses
GpX1	NM_ 030826.3	Glutathione peroxidase 1, hepatic	Antioxidant defenses
UCP2	NM_019354.2	Uncoupling protein 2	Antioxidant defenses
SIRT3	NM_001106313.2	Sirtuin (silent mating type information regulation 2 homologue) 3	NAD^+-dependent deacetylase, mitochondrial
SIRT1	NM_001159590.1	Sirtuin (silent mating type information regulation 2 homologue) 1	NAD^+-dependent deacetylase 1
Foxo3a	NM_001106395.1	Forkhead box O3a	Transcription factor
PPARGC1A	NM_031347.1	Peroxisome proliferator-activated receptor γ, coactivator 1α (PGC1α)	Mitochondrial biogenesis
PPARGC1B	NM_176075.2	Peroxisome proliferator-activated receptor γ, coactivator 1β (PGC1β)	Mitochondrial biogenesis
NRF1	NM_001100708.1	Nuclear respiratory factor 1	Mitochondrial biogenesis
MTCO2	MIM_516040	Cytochrome c oxidase subunit 2	Mitochondrial-encoded gene
18S	X03205.1	Eukaryotic 18S rRNA	House keeping
ACTB	NM_031144.2	β-Actin	House keeping
Ppil3	NM_175707.3	Peptidylprolyl isomerase (cyclophilin)-like 3	House keeping

qRT-PCR, quantitative reverse transcription-PCR.

Twelve genes were analyzed (**Table 1**) using predesigned TaqMan Assays-on-demand (Applied Biosystems). The reaction conditions were set-up according to the manufacturer's instructions. Amplification and detection of specific products were performed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). 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 fold-change ($2^{-\Delta\Delta CT}$). Hepatic expression levels of each gene were normalized by β-actin or cyclophilin. The ratio between mitochondrial-encoded gene and nuclear endogenous gene (18s) was used to calculate the mitochondrial copy number.

Western blotting

Total proteins were extracted by treating 100 mg of frozen liver with 500 µl of RIPA buffer containing protease inhibitor (Sigma-Aldrich) and phosphatase inhibitors (2 mmol/l $NaVO_3$, 1 mmol/l NaF). Samples were sonicated in Branson Sonifier 250 equipment, then were incubated at 4 °C for 30 min and then centrifuged at 12,000*g* for 1 h at 4 °C, and the supernatant was collected for analysis. Mitochondrial proteins were extracted by treating isolated rat liver mitochondria with lysis buffer (1:1) (20 mmol/l Tris–HCl pH 8, 137 mmol/l NaCl, 10% glycerol, and 1% Triton) with proteases and phosphatases inhibitors in same proportion than in RIPA buffer. Samples were incubated at 4 °C for 30 min and then centrifuged at 13,000*g* for 15 min. The supernatant was collected for analysis. Proteins were quantified with the BCA method (Bio Rad Laboratories, Madrid, Spain) according to the supplier's instructions. Total proteins were resolved in SDS-PAGE minigels and electroblotted onto polyvinylidene fluoride membranes (GE Healthcare Europe, Barcelona, Spain). The membranes were blocked and incubated with specific antibodies against PARP 95425 (Cell Signaling Technology, Boston, MA), SIRT1 SC95404, SIRT3 SC99143, and actin A5396 (Santa Cruz Biotechnology, Heidelberg, Germany). Secondary antibody was horseradish peroxidase goat anti-rabbit IgG-HRP (Bio Rad Laboratories). The immunoreactive proteins were detected with enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL). Band intensities were quantified using a GS-800 calibrated densitometer (Bio Rad Laboratories).

Immunoprecipitation

For acetylation analysis, 2 µg of anti-Foxo3a SC11351 (Santa Cruz Biotechnology) or anti-PGC1β SC13067 (Santa Cruz Biotechnology) antibody were added to protein extracts and incubated for 2 h at 4 °C. After the addition of 30 µg of protein A/G PLUS-Agarose (Santa Cruz Biotechnology), incubation at 4 °C with shaking was carried out overnight. The A/G PLUS-Agarose-beads were pelleted by centrifugation at 12,000*g* for 2 min at 4 °C and washed four times with RIPA buffer at 4 °C to remove nonadsorbed proteins. After the final wash, protein was released from the beads by treatment at 95 °C for 7 min in sample buffer and electrophoresed on 10% SDS-PAGE gels and transfered to polyvinylidene fluoride membranes. The membranes were blocked and incubated with specific antibodies against Foxo3a or PGC1β (Santa Cruz Biotechnology) to determine total Foxo3a or PGC1β and anti-acetylated Lys 9441 (Cell Signaling) to analyze acetylation of Foxo3a and PGC1β.

Statistical analysis

Data are reported as mean ± SE. Normal distribution was confirmed by two different methods, Shapiro–Wilk and Kolmogorov–Smirnov. In order to determine the effects of LA treatment, one-way ANOVA followed by Bonferroni *post-hoc* analysis or Kruskal–Wallis followed by Dunns *post-hoc* analysis were carried out depending on the distribution of the data. Relationship between variables was analyzed by calculating Pearson correlation coefficients. Variables analyzed and presented in figures are unique and did not require a multiple comparison correction. All statistical analyses were performed using the GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA). Differences were considered as statistically significant at $P < 0.05$.

RESULTS

Effects of LA on body and liver weights and hepatic TG content

As expected, an increase in body weight ($P < 0.001$) was observed in rats fed a HFD. Interestingly, this increase was significantly prevented by LA treatment ($P < 0.001$ vs. OB). Furthermore, HFD increased white adipose tissue accumulation ($P < 0.001$) which was completely reversed by LA treatment ($P < 0.001$ vs. OB) (**Table 2**). Moreover, HFD produced a very significant increase ($P < 0.001$) in hepatic TG content in the liver as compared with control rats (**Table 2**). Calorie restriction was also able to reduce this increase ($P < 0.001$ vs. OB), but the LA-lowering effects were a major magnitude and the

Table 2 Body weight gain, body composition, liver triglyceride content, calorie intake, and feeding efficiency in lean and obese rats

	Control	Obese	OLIP	PFO	
	n = 10	*n* = 10	*n* = 10	*n* = 6	ANOVA
Initial body weight (g)	215.1 ± 6.43	218.1 ± 5.61	214.7 ± 6.01	209.8 ± 4.01	n.s.
Body weight gain (g)	166.7 ± 8.55	275.9 ± 5.77^{***,b}	153.7 ± 10.23$^{###,b}$	200.1 ± 6.86	<0.001
Energy intake (kJ/day)	297 ± 2.9	424 ± 6.0^{***,c}	354 ± 4.2$^{###,***}$	347 ± 5.1***	<0.001
WAT (%)	7.2 ± 0.39	13.3 ± 0.58^{***,b}	7.2 ± 0.38$^{###,b}$	9.4 ± 0.39*	<0.001
Liver weight (%)	2.48 ± 0.162	2.13 ± 0.101	2.59 ± 0.137	2.13 ± 0.105	0.0386
Liver triglyceride content (%)	3.6 ± 0.42	9.2 ± 0.31^{***,c}	2.5 ± 0.25$^{###,c}$	5.0 ± 0.53*	<0.001
Plasma triglyceride (mg/dl)	69.9 ± 7.16	63.7 ± 4.62^{a}	47.2 ± 2.02$^{\#}$	45.9 ± 3.96	0.0024
Plasma cholesterol (mg/dl)	68.8 ± 2.63	68.4 ± 3.85	66.94 ± 4.76	58.7 ± 2.81	0.2182
HDL cholesterol (mg/dl)	23.7 ± 0.96	19.6 ± 1.03*	26.0 ± 0.95$^{###,c}$	19.9 ± 0.81*	<0.001
LDL cholesterol (mg/dl)	52.7 ± 4.75	51.4 ± 3.76	59.9 ± 3.64	46.1 ± 2.89	n.s.

Values are mean ± SE.
PFO, pair-fed OLIP group; WAT, white adipose tissue.
$^{*}P < 0.05$, $^{***}P < 0.001$ 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.

statistically significant differences between the OLIP and the PFO group ($P < 0.001$) suggest a direct lowering effect of LA on liver TG content. Furthermore, histological analysis previously reported by our group demonstrated that LA treatment in these animals was able to prevent the development of steatosis induced by a HFD (19).

Effects of LA on lipid peroxidation

As shown in **Figure 1**, HFD induced an important increase in lipid peroxidation in the liver ($P < 0.001$). This effect was reversed by LA treatment, which induces a lower malondialdehyde content in liver in comparison with the OB ($P < 0.01$) but also in relation with C ($P < 0.01$). Furthermore, this effect can be directly attributed to LA, because a significant difference was observed between OLIP and PFO groups ($P < 0.001$).

Effects of HFD and LA treatment on ROS production in IRLM

Oxidative stress has been described as an imbalance between ROS production and antioxidant defenses. Taking into account that mitochondria are the main source of ROS production in cells, we checked the role of mitochondrial oxidative stress in the development of obesity. We measured the production of superoxide anion ($O_2^{-\cdot}$) the firstly ROS produced in the mitochondrial electron transport chain. LA treatment induced a significant increase in $O_2^{-\cdot}$ production ($P < 0.001$), with no effects observed by dietary treatment (**Figure 1b**). Preliminary results of our group showed that LA treatment induced a strong inhibition on complex I and complex II + III. Interestingly, a higher correlation between superoxide production and the activity of complex I ($r = -0.6226$; $P = 0.0012$) and complex II+III ($r = -0.5687$; $P = 0.0058$) was found (20). Surprisingly, the opposite effect was observed on H_2O_2 production (**Figure 1c**). Thus, HFD induced a strong increase in the production of H_2O_2 that was completely reversed by LA treatment ($P < 0.001$). The differences observed between OLIP and PFO group ($P < 0.001$) in H_2O_2 production suggested that LA may activate other mechanisms independent of calorie restriction to counteract the increase observed in the levels of this ROS.

LA treatment increase antioxidant defenses on rat liver mitochondria

To gain a better insight into the mechanisms that could explain the effects of LA against mitochondrial oxidative damage, gene expression, and activity of several antioxidant enzymes were investigated. The analysis of gene expression showed that HFD induced a reduction in mRNA level of SOD2 (0.6 ± 0.13-fold changes; $P < 0.01$ vs. C) that overturned to control levels after LA treatment (1.2 ± 0.53-fold changes; $P < 0.001$ vs. OB). However, pair-fed rats present the same expression levels as obese animals (0.7 ± 0.11 fold changes; $P < 0.05$ vs. OLIP). Conversely, GPx gene expression was stimulated by HFD (1.8 ± 0.50-fold changes; $P < 0.001$ vs. C) and this effect was reversed by LA treatment (1.1 ± 0.34- fold changes; $P < 0.01$ vs. OB) and calorie restriction (1.2 ± 0.22- fold changes). As expected, animals with liver steatosis exhibited an important decrease in SOD2 ($P < 0.01$) and GPx ($P < 0.05$) activities in liver mitochondria (**Figure 2a** and **b**). Interestingly, LA treatment stimulates the activity of both enzymes and these effects were not secondary to a reduction of food intake, which highlights that LA was able to improve mitochondrial antioxidant defenses by different mechanisms than calorie restriction.

As for the effects of HFD and LA treatment on non enzymatic antioxidant defenses, HFD induced a significant decrease in GSH:GSSG ratio in rat liver mitochondria ($P < 0.05$), a robust marker of antioxidant status. LA treatment (**Figure 2c**) was able to counteract the decrease and to increase this ratio to levels even higher than in the C ($P < 0.001$ vs. C, OB and

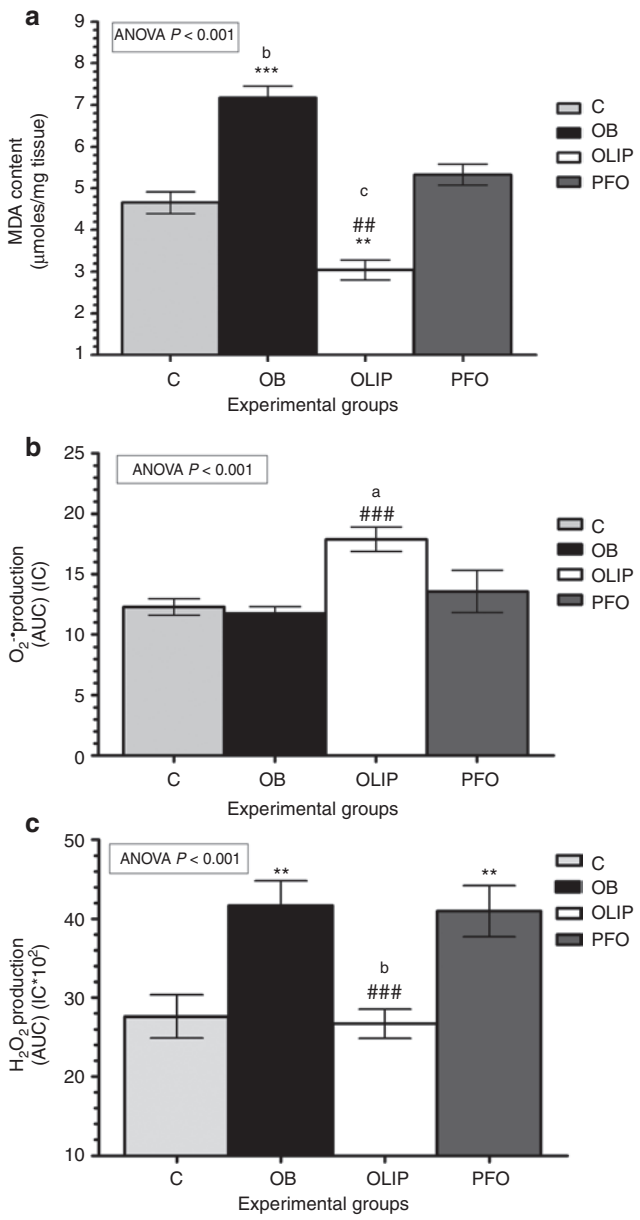

Figure 1 Effects of lipoic acid (LA) on liver oxidative damage and reactive oxygen species. (**a**) Malondialdehyde levels; (**b**) superoxide production, (**c**) hydroperoxide production. Values are mean ± SE (n = 10–6). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control group; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. obese group; $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ vs. *pair-fed* OLIP group (PFO) group according to one-way ANOVA followed by *post-hoc* multiple comparisons by Bonferroni test. H_2O_2, hydroperoxide; MDA, malondialdehyde; $O_2^{-\bullet}$, superoxide anion.

PFO groups). Moreover, the analysis of mRNA levels of UCP2, which has an important role in the regulation of mitochondrial redox status, revealed that animals with liver steatosis showed lower gene expression levels than the control animals ($P < 0.05$), whereas LA treatment was able to overturn this effect (**Figure 2d**). Similarly, as in other antioxidant defenses this effect did not seem secondary to calorie restriction as statistical differences between OLIP and PFO groups were found ($P < 0.05$).

Changes on mitochondrial biogenesis induced by LA treatment

Regarding mitochondrial copy number, HFD produced an important reduction (49.6%; $P < 0.001$) as compared to control animals. Interestingly, LA reversed the effect of high-fat feeding on mitochondrial biogenesis and also induced a significant increase in the mitochondrial copy number to higher levels than those observed in the C (151.1%; $P < 0.05$) (**Figure 3a**). Moreover, a negative correlation between mitochondrial copy number and mtDNA oxidative damage ($r = 0.6052$; $P < 0.001$) was found. HFD also down regulated PGC1α mRNA levels ($P < 0.01$) and this effect was partially reversed by LA although no differences were observed in comparison with the PFO group (**Figure 3b**). HFD also induced a reduction in NRF1 gene expression ($P < 0.05$) and LA was able to counteract these effect ($P < 0.01$) and, as deduced from statistical differences between groups, this stimulatory effect was not secondary to changes in food intake.

Effects of LA on mtDNA oxidative damage and PARP

The aforementioned data suggested that liver steatosis induced a pro-oxidant state in the mitochondrial liver compartment. This state may lead to oxidative injury in different mitochondrial structures such as membrane, proteins of electron transport chain or mtDNA. Taking into account this hypothesis, we analyzed mtDNA oxidative damage by quantitative reverse transcription-PCR and observed that, as expected, high-fat intake induced oxidative damage in mtDNA (**Figure 3c**) which was completely reversed by LA treatment. This protective effect was independent of a reduction in energy intake, as significant differences between OLIP and PFO group were found ($P < 0.001$). Moreover, mtDNA oxidative injury were highly correlated with H_2O_2 production ($r = 0.7523$; $P < 0.001$).

Next, we investigated the role of LA on mtDNA repair by the measurement of PARP cleavage in our experimental animal groups. Obese animals showed highest levels of PARP cleavage (**Figure 3d** and **e**). This effect of HFD was reversed in part by calorie restriction and completely overturned by LA treatment. These data could suggest that LA stimulates mtDNA repair by the regulation of PARP cleavage. In fact, a highly significant correlation between mtDNA damage and PARP cleavage was observed ($r = 0.6315$; $P < 0.001$).

Changes on Foxo3a mRNA levels and protein acetylation levels

To determine the potential role of Foxo3a in the stimulating actions of LA on the antioxidant defenses previously described, protein levels and acetylation of Foxo3a were measured. As observed in **Figure 4a**. HFD induced a significant decrease in gene expression of this transcription factor. Calorie restriction was able to partially reverse the effect of HFD on Foxo3a gene expression although it did not reach statistical significance. However, LA up regulated the mRNA levels of this gene to levels even higher than those observed in the C (**Figure 4a**). Taking into account that protein acetylation of Foxo3a inhibits the activity of this transcription factor

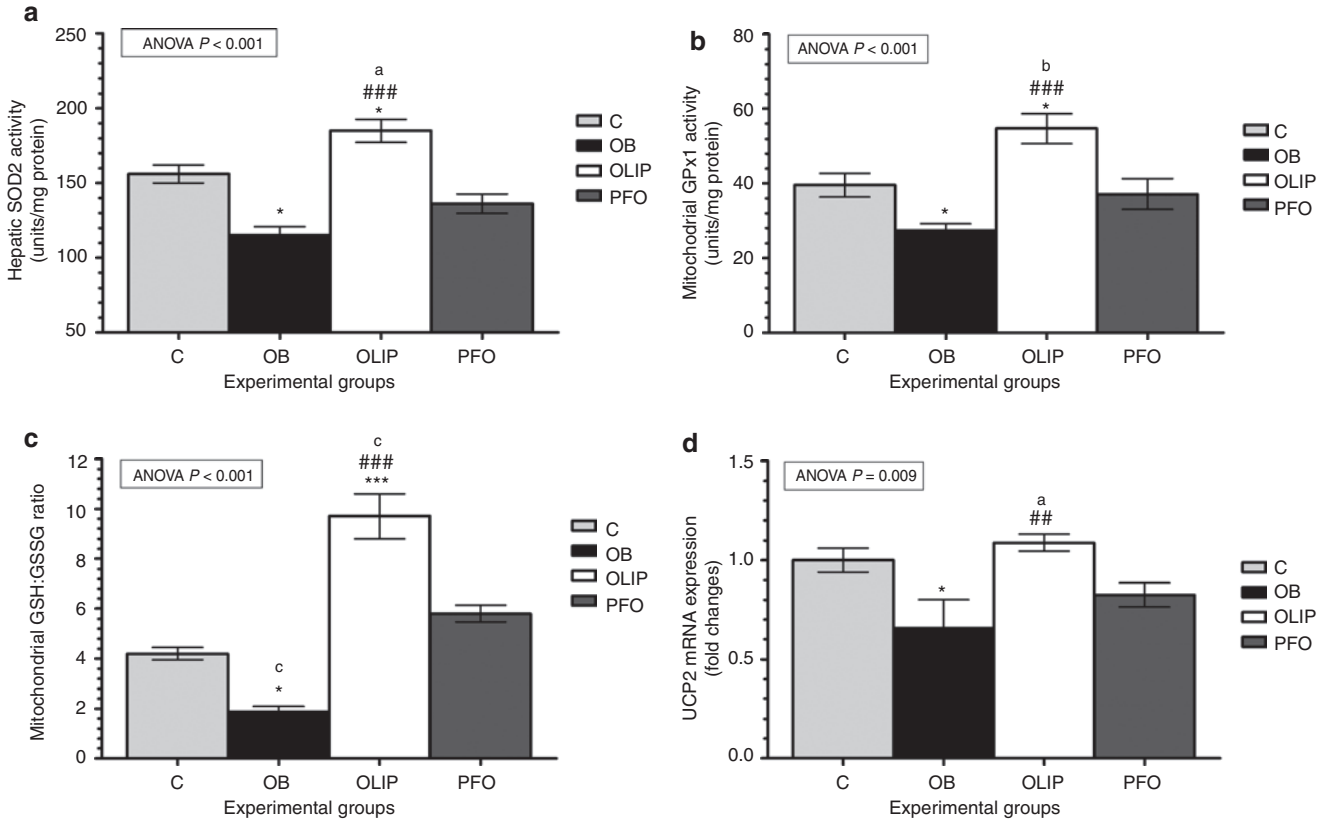

Figure 2 Changes on mitochondrial antioxidant defenses in different experimental group. (n = 10–6). (**a**) Manganese superoxide dismutase activity, (**b**) glutathione peroxidase mRNA activity, (**c**) mitochondrial GSH:GSSG ratio (**d**) UCP2 mRNA levels. Values are mean ± SE. $^{*}P < 0.05$, $^{***}P < 0.001$ vs. control group; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. obese group; $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ vs. pair-fed OLIP group (PFO) group according to one-way ANOVA followed by *post-hoc* multiple comparisons by Bonferroni test. GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; SOD2, manganese superoxide dismutase; UCP2, uncoupling protein 2.

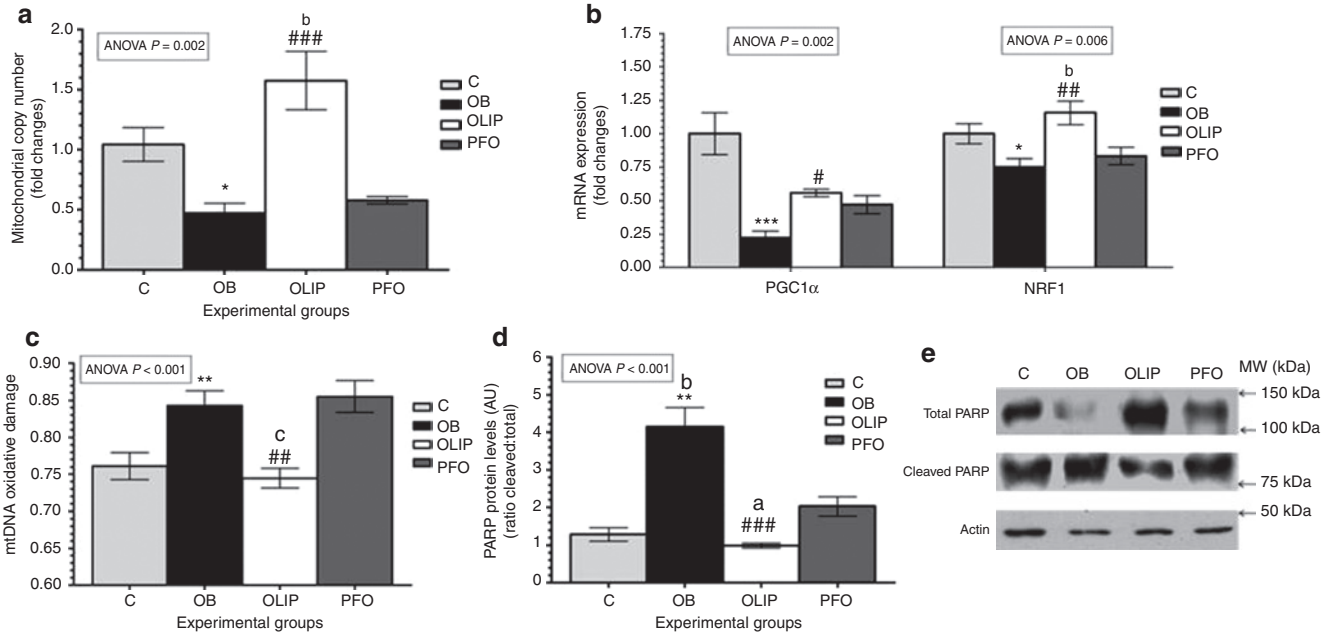

Figure 3 Effect of lipoic acid (LA) on mitochondrial biogenesis and mitochondrial DNA damage (n = 10–6). (**a**) Mitochondrial copy number, (**b**) mRNA levels of genes involved in transcriptional control, (**c**) mtDNA oxidative damage, (**d**) quantification of PARP western blot, (**e**) western blot analysis of PARP cleavage. Values are mean ± 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; $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ vs. pair-fed OLIP group (PFO) group according to one-way ANOVA followed by *post-hoc* multiple comparisons by Bonferroni test. mtDNA, mitochondrial DNA; PARP, poly ADP ribose polymerase.

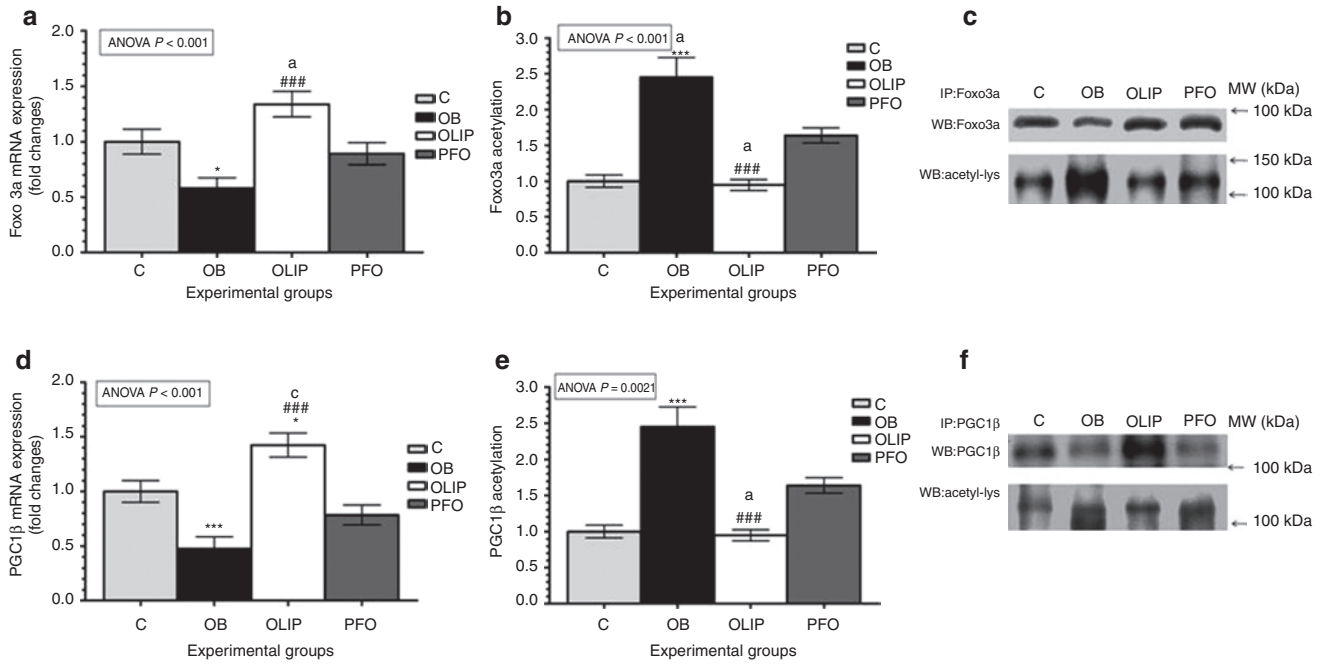

Figure 4 Effects of lipoic acid (LA) on Forkhead transcription factor 3a Foxo3a and PGC1β mRNA levels and protein acetylation (n = 10–6). (**a**) mRNA levels of Foxo3a, (**b**) lysine acetylation of Foxo3a expressed in fold changes vs. control group, (**c**) immunoprecipitation and western blot analysis of total and acetylated Foxo3a, (**d**) mRNA levels of PGC1β, (**b**) lysine acetylation of PGC1β expressed in fold changes vs. control group, (C) Inmunoprecipitation and western blot analysis of total and acetylated PGC1β. Values are mean ± 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; $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ vs. pair-fed OLIP group (PFO) group according to one-way ANOVA followed by *post-hoc* multiple comparisons by Bonferroni test. Foxo3a, Forkhead transcription factor; PGC1β, peroxisome proliferator-activated receptor γ, coactivator 1β.

on stimulation of antioxidant defenses; we measured the acetylation levels by immunoprecipitation. We found that high-fat feeding animals had higher Foxo3a acetylation levels than the C (**Figure 4b**). Interestingly, LA treatment returned acetylation levels to control values. Moreover, the Foxo3a acetylation percentage of OLIP animals (51.2 ± 11.63%) was lower than PFO animals (88.5 ± 14.05%). These data suggest that the regulation of Foxo3a gene expression and its deacetylation could be mediated by the effects of LA on antioxidant defenses.

Changes on PGC1β gene expression and acetylation levels

To determine the potential role of PGC1β in the previously described stimulating actions of LA on mitochondrial biogenesis, gene expression and protein acetylation of this coactivator were measured. HFD induced a significant decrease in the mRNA levels of this transcription factor (**Figure 4d**). LA upregulated the expression of this gene in comparison to both obese and PFO groups ($P < 0.001$). Some studies propose that protein acetylation of PGC1β could modulate its activity. Thus, we measured the acetylation levels by immunoprecipitation. As was the case with Foxo3a, HFD induced an intensification of acetylation of PGC1β (**Figure 4e**) and LA was able to decrease these acetylation levels ($P < 0.001$ vs. OB). Furthermore, the acetylation levels of PGC1β were negatively associated with protein level of SIRT1 ($r = -0.7559$; $P < 0.001$) as well as mitochondrial copy number ($r = -0.7168$; $P < 0.001$).

Effects of LA on SIRTs

To determine the role of SIRTs in LA actions on hepatic mitochondrial oxidative status, we analyzed the mRNA and protein levels of SIRT1 and SIRT3 in our experimental groups. Regarding to SIRT1, HFD downregulated the mRNA levels of this gene ($P < 0.01$) whereas LA was able to up regulate the mRNA levels up to control values (**Figure 5a**). However, this effect may be due to calorie restriction as deduced from the same values observed in the PFO group. By contrast, LA induced a strong stimulatory effect on protein level of SIRT1 (6.5 fold; $P < 0.001$ vs. C and OB group) whereas dietary patterns (obesity and calorie restriction) had no effect (**Figure 5b** and **c**). In addition, a highly significant correlation between protein levels of SIRT1 and mitochondrial copy number was found ($r = 0.7800$; $P < 0.001$). On the other hand, and similarly to what was observed with SIRT1, HFD induced a reduction in SIRT3 gene expression and this effect was completely reversed by LA treatment ($P < 0.001$). This upregulation was not secondary to calorie restriction as inferred from differences with PFO group ($P < 0.05$) (**Figure 5d**). Furthermore, LA also increases SIRT3 protein levels ($P < 0.001$) and independently of reduction in food intake (**Figure 5e** and **5f**). Additionally, the protein levels of SIRT3 were negatively associated with acetylation of Foxo3a ($r = -0.6108$; $P = 0.002$) suggesting that SIRT3 could stimulate the activity of Foxo3a by deacetylation and, therefore, could mediate the effects of LA.

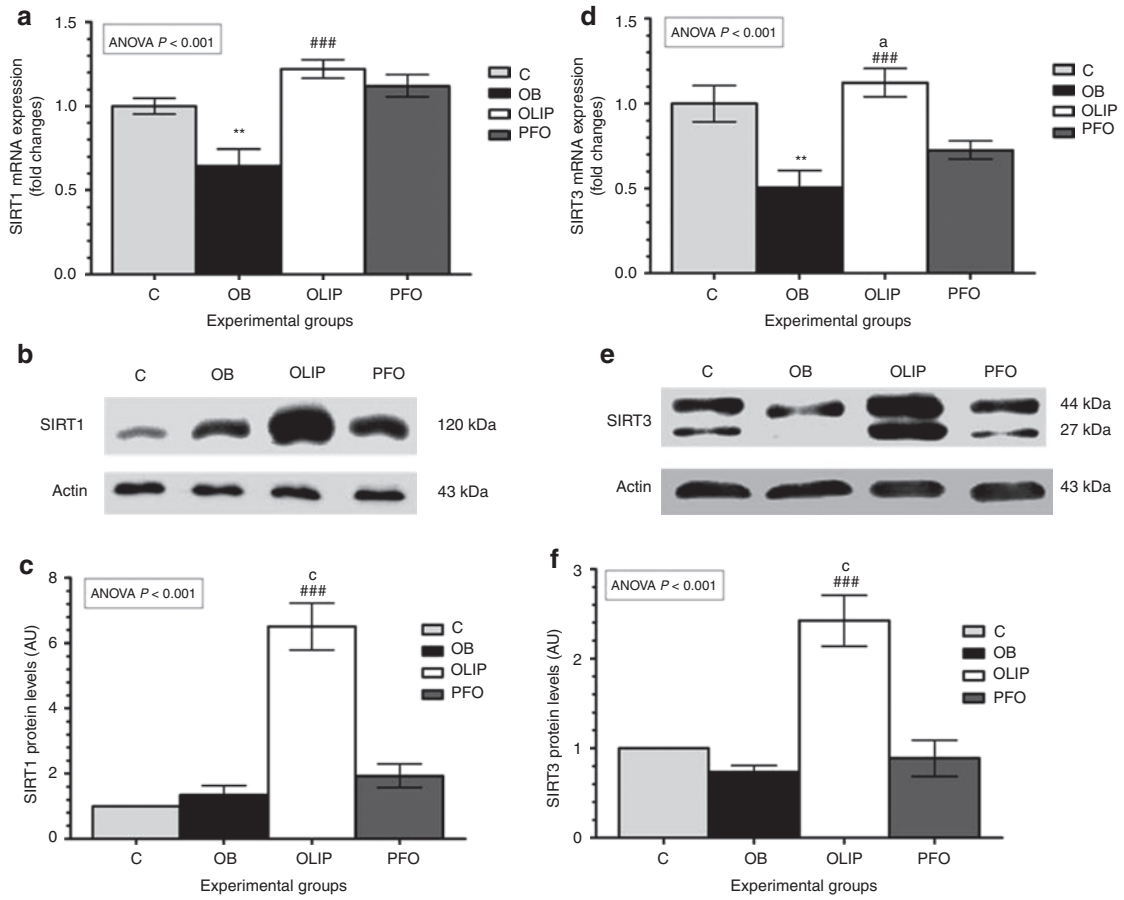

Figure 5 Effects of lipoic acid (LA) on the sirtuins pathway (n = 10–6). (**a**) SIRT1 mRNA levels, (b) western blot analysis of SIRT1, (**c**) quantification of SIRT1 western blot, (**d**) SIRT3 mRNA levels, (e) western blot analysis of SIRT3, (**f**) quantification of SIRT3 western blot. Values are mean ± SE. $^{**}P < 0.01$ vs. control group; $^{###}P < 0.001$ vs. obese group; $^{a}P < 0.05$, $^{c}P < 0.001$ vs. pair-fed OLIP group (PFO) group according to one-way ANOVA followed by post-hoc multiple comparisons by Bonferroni test. SIRT1, sirtuin 1; SIRT3, sirtuin 3.

DISCUSSION

To our knowledge, this is the first study where the effects of LA on SIRTs have been studied. We have found that the beneficial effects of LA on fatty liver could be mediated through its actions on mitochondria and more specifically due to its ability to increase the antioxidant defenses in this compartment which seems to be due to the increase observed in SIRT1 and SIRT3 proteins.

Nonalcoholic fatty liver disease is now considered the main hepatic manifestation of obesity and metabolic syndrome (1,21,22). In fact, our data have shown that high-fat feeding increased body weight gain and subsequently induced liver steatosis. Further events in the liver include oxidative stress and the decrease in antioxidant defenses induced by early mitochondrial dysfunction (23,24). Moreover, some researchers described the ability of LA, a compound with antioxidant properties, to reverse the effects of HFD on liver steatosis (25,26). In this context, our data confirm these protective effects of LA on hepatic TG accumulation in an animal model of diet induced obesity.

The central role of mammalian SIRTs in the regulation of mitochondrial function and oxidative stress has been reviewed in detail over recent years (12,27–29). In fact, several studies have proposed that SIRTs are important targets to prevent several disorders involving mitochondrial dysfunction and an increase in oxidative stress (5,30). Accordingly, in the present study we observed that the increase in SIRTs 1 and 3 induced after LA treatment was accompanied by an enhancement in mitochondrial copy number which could be associated with the improvement observed in the antioxidant status of hepatic mitochondrial compartment and could contribute to the beneficial effects of LA on fatty liver after the ingestion of a HFD.

Several lines of evidence described that SIRT3 stimulates antioxidant defenses by different ways (29,31). One of the most important mechanisms is the deacetylation of mammalian Foxo factors that control various biological functions, including detoxification of ROS and repair of DNA damage. Particularly, the ability of Foxo3a to stimulate mitochondrial antioxidant defenses such as SOD2 has been described in different studies (17,32). Moreover, Jacobs *et al.* (2008) suggested that Foxo3a is a mitochondrial protein, whose function could be regulated through the interaction with SIRT3 in the mitochondria (16). In agreement with this hypothesis, other research described that activation of Foxo3a through deacetylation is specifically stimulated in response to oxidative stress stimuli (33). In this

context, our results demonstrate for the first time that acetylation levels of Foxo3a are strongly increased in steatotic livers whereas LA treatment completely reversed this effect.

In this sense, some studies have indicated that the full-length form of SIRT3 is located in the nucleus whereas a shorter form, that is more active, was found in mitochondria (34). Our data suggest that the increase in Foxo3a acetylation in the OB could be mediated by a strong reduction in the shorter-active form of SIRT3 in these animals. Moreover, our study demonstrated for the first time that LA treatment is able to induce a strong stimulation of SIRT3 protein and this stimulatory effect could mediate the increase in antioxidant defenses through the deacetylation of Foxo3a. In fact, some studies have described that SIRT3 stimulates mitochondrial antioxidant defenses by the activation of SOD2 through acetylation (35).

In this context, our data revealed an important decrease in antioxidant defenses in obese animals in agreement with previous studies (6,26). In such a situation, LA is highly effective in reducing ROS levels as observed with H_2O_2 but also in stimulating other antioxidant systems. Manganese dependent superoxide dismutase, acts as the first line of defense against ROS production in electron transport chain by catalyzing the dismutation of superoxide anion to H_2O_2 which is further remove by GPx (36). Moreover, several studies suggest that one of the main mechanisms by which LA modulates redox balance is its ability to participate in thiol/disulfide exchange (18). Accordingly, our data revealed that LA treatment increases GSH:GSSG ratio. These findings suggest that deacetylation of Foxo3a could mediate the stimulatory effects of LA on mitochondrial antioxidant defenses.

Moreover, our data showed that LA treatment is able to reverse the deleterious effect of HFD in mtDNA oxidative damage, corroborating its beneficial effects on oxidative stress of hepatic mitochondrion after the ingestion of a high-fat diet. In this sense, PARP is a nuclear and mitochondrial enzyme that exerts numerous functions in the maintenance of DNA stability to transcriptional regulation (36). Increase of oxidative stress activates caspase 3, which inhibits PARP by cleavage into two fragments and produces damage accumulation in mtDNA (37). Consistently, we observed that in obese animals, PARP is completely cleaved. However, we found a balance between cleaved-inactive and total-active PARP forms in control and LA-treated groups. Interestingly, levels of mtDNA damage and cleaved ratio of PARP are strongly associated and support the hypothesis that this enzyme contributes to mtDNA repair.

On the other hand, the stimulatory role of mammalian SIRT1 in mitochondrial biogenesis has been reviewed in detail over recent years (12,28). Accordingly, in the present study we observed a strong activation of SIRT1 protein levels as a response to LA treatment, which are correlated with an increase of mitochondrial copy number. Regarding the transcriptional control of mitochondrial biogenesis, several studies have highlighted the central role of PGC1α and its activation by SIRT1 (28). Interestingly, we observed that LA treatment is able to upregulate PGC1α gene expression but this increase seems to be secondary to calorie restriction, as stated in previous studies (38). However, the stimulatory effect of LA on PGC-1β gene expression, independently of food restriction, suggests that this transcription factor, instead of PGC-1α, is a direct target of LA in the liver. In fact, different studies have established the key role of PGC-1β in the transcriptional control of mitochondrial metabolism and energy homeostasis (39). Moreover, a higher ability of PGC1-β in comparison with PGC1-α to regulate mitochondrial function has been proposed in several studies (39,40), suggesting that LA actions on PGC1-β and not on PGC-1α could contribute, to some extent, to the increase in mitochondrial biogenesis. Moreover, Kelly *et al.* reported the ability of SIRT1 to regulate PGC1β activity by acetylation (39). Confirming this finding, our data showed a marked correlation between SIRT1 protein levels and PGC1β acetylation. Interestingly, PGC1β acetylation levels were also correlated with mitochondrial copy number suggesting that the deacetylation of PGC1β could mediate the increase observed in mitochondrial biogenesis. Moreover, based on the aforementioned stimulatory effects of LA, which seem not to be mediated by calorie restriction, we propose that this molecule exerts its actions on mitochondrial biogenesis through the stimulation of SIRT1 and consequently of deacetylation of PGC1β. Regarding the effects of HFD and/or LA treatment in NRF1 expression levels, another transcriptional regulator of mitochondrial biogenesis (40), the pattern of effects was similar to those observed with PGC1β according to previous investigations.

In summary, our results demonstrate for the first time that the beneficial effects of LA supplementation on fatty liver resulting from the ingestion of a high-fat diet and associated with an obese state, could be mediated, at least in part, by its effects on oxidative status of hepatic mitochondria. Thus, LA seems to restore the oxidative balance by increasing antioxidant defenses through the activation of SIRT1 and SIRT3 proteins. More specifically, SIRT1 seems to induce an increase in the mitochondrial copy number through the deacetylation of PGC1β, whereas SIRT3 seems to exert a direct effect on antioxidant defenses such as SOD2 through Foxo3a deacetylation.

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DISCLOSURE

The authors declared no conflict of interest.
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