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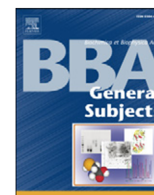

Lipoic acid prevents liver metabolic changes induced by administration of a fructose-rich diet☆

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ABSTRACT

Background: To evaluate whether co-administration of R/S-α-lipoic acid can prevent the development of oxidative stress and metabolic changes induced by a fructose-rich diet (F).
Methods: We assessed glycemia in the fasting state and during an oral glucose tolerance test, triglyceridemia and insulinemia in rats fed with standard diet (control) and fructose without or with R/S-α-lipoic acid. Insulin resistance and hepatic insulin sensitivity were also calculated. In liver, we measured reduced glutathione, protein carbonyl groups, antioxidant capacity by ABTS assay, antioxidant enzymes (catalase and superoxide dismutase 1 and 2), uncoupling protein 2, PPARδ and PPARγ protein expressions, SREBP-1c, fatty acid synthase and glycerol-3-phosphate acyltransferase-1 gene expression, and glucokinase activity.
Results: R/S-α-lipoic acid co-administration to F-fed rats a) prevented hyperinsulinemia, hypertriglyceridemia and insulin resistance, b) improved hepatic insulin sensitivity and glucose tolerance, c) decreased liver oxidative stress and increased antioxidant capacity and antioxidant enzymes expression, d) decreased uncoupling protein 2 and PPARδ protein expression and increased PPARγ levels, e) restored the basal gene expression of PPARδ, SREBP-1c and the lipogenic genes fatty acid synthase and glycerol-3-phosphate acyltransferase, and f) decreased the fructose-mediated enhancement of glucokinase activity.
Conclusions: Our results suggest that fructose-induced oxidative stress is an early phenomenon associated with compensatory hepatic metabolic mechanisms, and that treatment with an antioxidant prevented the development of such changes.
General significance: This knowledge would help to better understand the mechanisms involved in liver adaptation to fructose-induced oxidative stress and to develop effective strategies to prevent and treat, at early stages, obesity and type 2 diabetes mellitus.

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1. Introduction

The prevalence of diabetes and impaired glucose tolerance increases worldwide. It is estimated that in the USA up to 60 million people have some degree of glucose impairment and consequently an increased risk of developing type 2 diabetes (T2DM) [1]. This situation is associated with an increased consumption of unhealthy high calorie diets, a shift in the types of nutrient composition [2] and a sedentary behavior [3]. Several authors have suggested that the increased use of fructose-rich syrups have greatly contributed to the current epidemic of obesity and T2DM [4–7]. Although the underlying mechanism responsible for the detrimental effects of a fructose-rich diet (F) is not completely understood, it has been shown that it promotes increased glycoxidative stress [8–10]. In fact, we have previously demonstrated that short-term administration of fructose to normal rats induces changes in metabolic, endocrine and glycoxidative stress markers in several organs including the liver [11–14].

Mitochondrial function participates actively in this process producing reactive oxygen species (ROS) through the activity of the respiratory chain. As a counterpart, mitochondrial uncoupling proteins (UCPs) might play a key function in the antioxidant defense mechanism [15], as shown by their increased expression in F-fed rats [16]. Thus, if these changes were specifically linked to fructose-induced oxidative stress, the administration of an antioxidant agent should alleviate its development.

Lipoic acid (LA) is an antioxidant, a thiol-replenisher and a redox modulator [17]. Its administration to fatty Zucker rats improved insulin sensitivity [18] and hypertriglyceridemia in these animals by stimulating triacylglycerol clearance and down-regulating liver triacylglycerol

Abbreviations: T2DM, type 2 diabetes mellitus; F, fructose-rich diet; ROS, reactive oxygen species; UCP, uncoupling protein; LA, R/S-α-lipoic acid; FAS, fatty acid synthase; GPAT-1, glycerol-3-phosphate acyltransferase-1; HOMA-IR, homeostasis model assessment-insulin resistance; FPI, fasting plasma insulin; FPG, fasting plasma glucose; OGTT, oral glucose tolerance test; AUC, area under the glucose curve; GSH, reduced glutathione; SOD1, superoxide dismutase 1; SOD2, superoxide dismutase 2; ABTS, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonate)

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secretion [19]. In T2DM, LA may reduce oxidative stress by a simultaneous decrease in lipid peroxidation and increase in the activity of antioxidant enzymes [20,21], also improving insulin sensitivity [22–24]. In F-fed animals, co-administration of LA in combination with vitamin E [25] improved F-induced oxidative stress, hyperinsulinemia and insulin resistance. Finally, LA prevented the development of diabetes in obese diabetes-prone rats by reducing lipid accumulation in both non-adipose and adipose tissues [26], an effect accompanied by an AMPK-mediated increase of insulin sensitivity in skeletal muscle [27].

In an attempt to provide a deeper insight into the knowledge of mediators involved in the adaptative mechanism of the liver to fructose-induced oxidative stress, we tested the effect of LA co-administration on the expression of UCP2 and other related genes (PPARδ, PPARγ, SREBP-1c, fatty acid synthase [FAS] and glycerol-3-phosphate acyltransferase-1 [GPAT-1]) as well as on glucokinase activity in the liver of F-fed rats.

2. Materials and methods

2.1. Chemicals and drugs

Reagents of the purest available grade were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Primary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, California, USA). The secondary antibody peroxidase-conjugated AffiniPure donkey anti-rabbit IgG was provided by Dianova (Hamburg, Germany).

2.2. Animals

Normal male Wistar rats (150–180 g) were maintained at 23 °C with a fixed 12-h light–dark cycle (06:00–18:00 h) and divided in 4 groups: standard commercial diet *ad libitum* and tap water (control, C), the same diet plus 10% fructose in the drinking water (F), and C and F injected with R/S-α-lipoic acid (35 mg/kg, i.p.) (CL and FL) during the last five days of treatment. Water intake was measured daily, while individual body weight was recorded weekly. This procedure was replicated 5 times (total, 20 animals per group). Twenty-one days after this treatment, blood samples from 4-h fasted animals were drawn from the retroorbital plexus under light halothane anesthesia and collected into heparinized tubes to measure blood glucose, serum triglyceride and immunoreactive insulin levels. Afterwards, the animals were killed by decapitation and the same portion of liver (median lobe) was removed to perform all the assays. Animal experiments and handling were performed according to the "Ethical principles and guidelines for experimental animals" (3rd Edition 2005) of the Swiss Academy of Medical Sciences.

2.3. Serum measurements

Glucose was measured with the glucose-oxidase GOD-PAP method (Roche Diagnostics, Mannheim, Germany); triglyceride levels were assayed enzymatically with a commercial kit (TG color GPO/PAP AA, Wiener lab, Argentina). Immunoreactive insulin levels were determined by radioimmunoassay using an antibody against rat insulin, rat insulin standard (Linco Research Inc., IN, USA) and highly-purified porcine insulin labeled with ^{125}I. Serum insulin and fasting glycemia values were used to estimate insulin resistance by the homeostasis model assessment-insulin resistance (HOMA-IR) (Serum insulin (μU/ml) × fasting blood glucose (mM))/22.5 [28]. Hepatic insulin sensitivity was calculated with the formula k/fasting plasma insulin (FPG) × fasting plasma glucose (FPG), where k:22.5 × 18 [29].

2.4. Oral glucose tolerance test (OGTT)

OGTT was performed in 12-h fasted rats from each experimental group 24 h prior to sacrifice. Glucose (1 g/kg in saline solution) was given through a gavage tube placed into the stomach and blood samples were obtained from the retro-orbital plexus under light pentobarbital anesthesia (48 mg/kg) at 0, 30, 60 and 120 min following the glucose load. In these samples, glucose concentration was measured with test strips (One touch Ultra, Lifescan, Milpitas, CA, USA). Results were expressed as the area under the glucose curve (AUC).

2.5. Liver measurements

2.5.1. Assessment of reduced glutathione (GSH) and protein carbonyl groups

Protein carbonyl content and GSH in liver were determined as described elsewhere [14]. Protein carbonyl content was measured spectrophotometrically at 366 nm and the results were expressed as nmol of carbonyl residues/mg protein based on the molar extinction coefficient of 21,000 M^{-1} cm^{-1}. The GSH content was measured spectrophotometrically at 414 nm and the results were expressed as μmol of −SH/g tissue.

2.5.2. Assessment of antioxidant capacity by ABTS assay

This assay is based on the inhibition of the absorbance of the radical cation of 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonate ($ABTS^{\bullet+}$), which has a characteristic long wavelength absorption spectrum showing maxima at 660, 734 and 820 nm by tested antioxidant. $ABTS^{\bullet+}$ radical is generated by reacting 7 mM ABTS solution in water with 2.45 mM potassium persulfate in the dark for 12–16 h. The antioxidant capacity of tissue homogenate was measured as described by Katalinic et al. [30] with some modifications. For our purpose, $ABTS^{\bullet+}$ solution was diluted with phosphate buffer saline pH 7.4 (PBS) to obtain an absorbance of 0.70 (±0.02) at 734 nm. After addition of 1.0 ml of diluted $ABTS^{\bullet+}$ solution to 10 μl of liver homogenate, or Trolox standard, the reaction mixture was incubated for 40 min at room temperature and thereafter the absorbance was measured at 734 nm. A Trolox calibration curve (0–200 μg) was settled and the results were expressed as μg of Trolox equivalents per milligram of protein; all measurements were performed in duplicate.

2.5.3. Total RNA

Total liver RNA from C, F, CL and FL rats was isolated using TRIzol Reagent (Gibco-BRL, Rockville, MD, USA) [31]. The integrity and quality of the isolated RNA were checked by agarose-formaldehyde gel electrophoresis and measuring the 260/280 nm absorbance ratio. DNA contamination was avoided using DNase I digestion (Gibco-BRL). Reverse transcription-PCR was performed using the SuperScript III (Gibco-BRL) and total RNA (50 ng) as a template.

2.5.4. Analysis of gene expression by real-time PCR (qPCR)

qPCR was performed with a Mini Opticon Real-Time PCR Detector Separate MJR (BioRad), using SYBR Green I as a fluorescent dye. Ten ng of cDNA was amplified in a qPCR reaction mixture containing 0.36 μM of each primer, 3 mM $MgCl_2$, 0.2 mM dNTPs and 0.15 μl Platinum Taq DNA polymerase 6 U/μl (Invitrogen). Samples were first denatured at 95 °C for 3 min followed by 40 PCR cycles. Each cycle comprised a melting step at 95 °C for 30 s, an annealing step at 65 °C for 30 s and an extension step at 72 °C for 45 s, followed by a final extension at 72 °C for 10 min. The oligonucleotide primers (Invitrogen) used are listed in Table 1. Amplicons were designed in a size range of 90 to 250 bp. β-Actin was used as housekeeping gene. The purity and specificity of the amplified PCR products were verified by performing melting curves generated at the end of each PCR. Data are expressed as relative gene expression after normalization to β-actin gene using the *Qgene96* and *LineRegPCR* software [13].

2.5.5. Western blot analysis

Catalase, superoxide dismutase (SOD) 1, SOD2, UCP2, PPARγ and PPARδ immunodetection was performed using liver homogenates from each experimental group. Protein concentration was quantified by the

Table 1
Primer sequences.

Gene	GenBank®	Sequences
UCP2	NM_019354.2	FW 5′-GGTAAAGGTCCGCTTCCAGG-3′ RV 5′-GCAAGGGAGGTCGTCTGTCA-3′
PPARα	NM_013196.2	FW 5′-CACCCTCTCTCCAGCTTCCA-3′ RV 5′-GCCTTGTCCCCACATATTCG-3′
PPARγ	NM_001145366.1	FW 5′-ATGGAGCCTAAGTTTGAGTTTGCT-3′ RV 5′-GGATGTCCTCGATGGGCTTCA-3′
PPARδ	NM_013141.2	FW 5′-AACGAGATCAGCGTGCATGTG-3′ RV 5′-TGAGGAAGAGGCTGCTGAAGTT-3′
SREBP-1c	XM_213329.6	FW 5′-TTTCTTCGTGGATGGGGACT-3′ RV 5′-CTGTAGATATCCAAGAGCATC-3′
FAS	NM_017332.1	FW 5′-GTCTGCAGCTACCCACCCGTG-3′ RV 5′-CTTCTCCAGGGTGGGGACCAG-3′
GPAT-1	NM_017274.1	FW 5′-GACGAAGCCTTCCGAAGGA-3′ RV 5′-GACGAAGCCTTCCGAAGGA-3′
Catalase	NM_012520.1	FW 5′-CCTCAGAAACCCGATGTCCTG- 3′ RV 5′-GTCAAAGTGTGCCATCTCGTCG-3′
SOD1	NM_017050.1	FW 5′-GTGCAGGGCGTCATTCACTTC- 3′ RV 5′-GCCTCTCTTCATCCGCTGGA-3′
SOD2	NM_017051.2	FW 5′-ACCGAGGAGAAGTACCACGA-3′ RV 5′-TAGGGCTCAGGTTTGTCCAG-3′
β-Actin	NM_031144.2	FW 5′-AGAGGGAAATCGTGCGTGAC-3′ RV 5′-CGATAGTGATGACCTGACCGT-3′

FW, forward primer and RV, reverse primer.

Bio-Rad protein assay [32]; thereafter, dithiothreitol and bromophenol blue were added to the samples to a final concentration of 100 mM and 0.1%, respectively. Aliquots of homogenates containing 100 μg of whole protein were placed in reducing 12.5% SDS-PAGE and electroblotted to PVDF membranes. The uniformity of protein loading in each lane was assessed by staining the blot with Ponceau S. Nonspecific binding sites of the membranes were blocked by overnight incubation with non-fat dry milk at 4 °C. Enzyme identification and quantification were performed using specific primary antibodies against catalase (1:5000), SOD1 (1:3000), SOD2 (1:3000), UCP2 (1:500), PPARγ (1:1000) and PPARδ (1:1000) overnight. At the end of the incubation period, the membranes were further incubated for 75 min with the following secondary antibodies: peroxidase-conjugated AffiniPure donkey anti-rabbit IgG (final dilution, 1:10,000) for catalase, SOD1, PPARδ and PPARγ detection; anti-rabbit antibody (final dilution, 1:2000) for SOD2 detection; and anti-goat IgG biotinyl antibody (final dilution, 1:1000) for UCP2 detection. In the case of SOD2 and UCP2, the membranes were finally incubated for 75 min with the streptavidin-peroxidase complex (1:2500). Diaminobenzidine (DAB, Sigma Co.) was used for color development. The bands were quantified by densitometry using the Gel-Pro Analyser software.

2.5.6. Glucokinase activity

Liver pieces were removed from the sacrificed animals, immediately homogenized in a hand-held homogenizer (20 times) and suspended in ice cold phosphate saline buffer containing 0.1 mM PMSF, 0.1 mM benzamidin, 2 mM DTT, 4 μg/ml aprotinin and 0.3 M sucrose; pH 7.5. The homogenate was then passed through a 23-gauge needle syringe (5 times) to ensure appropriate sample mixing. Aliquots of these homogenates were centrifuged at 600 ×*g* to separate and discard the nuclear fraction. The supernatant was centrifuged twice at 8000 ×*g* and 100,000 ×*g* at 4 °C, and the resultant supernatant was collected and identified as the cytosolic fraction (CF). Phosphorylation in the 100,000 ×*g* soluble CF was measured at 37 °C, pH 7.4, by recording the increase in absorbance at 340 nm in a well-established enzyme-coupled photometric assay containing glucose-6-phosphate dehydrogenase (G-6-P DH), ATP and NADP [14]; samples obtained from five different experiments were assayed in triplicate. Glucokinase activity was obtained by subtracting the activity measured at 1 mM glucose (hexokinase) from that measured at 100 mM glucose [13]. Enzyme activity was expressed as mU per milligram of protein. One unit of enzyme activity was defined as 1 μmol glucose-6-phosphate formed from glucose and ATP per minute at 37 °C.

2.6. Statistical analysis

Data are expressed as means ± SEM. Statistical analyses were performed using ANOVA followed by Dunnett's test for multiple comparisons using the Prism analysis program (Graphpad, San Diego, USA). Differences were considered significant when $p<0.05$.

3. Results

3.1. Body weight and water intake

Comparable body weight changes were recorded in all groups after the 3-week study period (Table 2). F and FL animals drank a larger volume of water than C and CL, respectively (55 ± 11 and 47 ± 12 *vs.* 29 ± 2 and 28 ± 2 ml/day; $p<0.05$). Conversely, C and CL rats ate a significantly larger amount of solid food than F and FL rats (21 ± 1 and 22 ± 1 *vs.* 16 ± 1 and 17 ± 1 g/animal/day; $p<0.05$). Consequently, while the daily intake of nutrients (expressed as percentage) was different in both experimental groups, calorie intake was comparable (C: 58 ± 3; CL: 65 ± 2; F: 66 ± 5; FL: 66 ± 4 kcal/day).

3.2. Serum measurements

F rats had higher serum insulin and triglyceride concentrations than C rats (Table 2). The high HOMA-IR values demonstrated the existence of an insulin resistance state in F rats (Table 2). Co-administration of R/S-α-lipoic acid to these rats prevented the development of the above mentioned changes; comparable values to those recorded in C rats were obtained, except for triglyceride levels that became even lower (Table 2). The low hepatic insulin sensitivity index value measured in F rats, indicative of hepatic insulin resistance, also returned to C values after LA administration (Table 2).

Table 2
Body weight and serum measurements.

Parameter	C	CL	F	FL
Body weight change (g)	103 ± 6	92 ± 8	97 ± 5	94 ± 6
Insulin (ng/ml)	0.76 ± 0.03	0.6 ± 0.08	1.13 ± 0.05*	0.74 ± 0.08**
Glucose (mg/dl)	114 ± 5	104 ± 4	110 ± 4	115 ± 3
Triglyceride (g/l)	0.965 ± 0.052	0.45 ± 0.09*	1.63 ± 0.12*	0.71 ± 0.06**
HOMA-IR	5.1 ± 0.2	3.9 ± 0.6	8.0 ± 0.1*	5.4 ± 0.8**
Hepatic insulin sensitivity (k/FPI × FPG)	3.37 ± 0.2	4.68 ± 0.3*	2.3 ± 0.15*	3.43 ± 0.2**
OGTT (AUC) (mM glucose/120 min)	4258 ± 249	4635 ± 188	6191 ± 249*	4647 ± 271**

Values are means ± SEM (n = 20).
* $p<0.05$ *vs.* C.
** $p<0.05$ *vs.* F.

3.3. OGTT

The AUC during the OGTT was significantly higher in F than in C animals and it decreased significantly to almost control values in FL rats (Table 2).

3.4. Assessment of GSH and protein carbonyl groups

Protein carbonyl content was significantly higher in F than in C rats (Fig. 1), while GSH content was significantly lower in the former (Fig. 1). These abnormalities were prevented by LA administration to F rats (Fig. 1).

3.5. Assessment of antioxidant capacity by ABTS assay

LA administration to FL rats increased significantly their *in vitro* assayed antioxidant capacity compared with F animals (FL, 171.7 ± 19.7 *vs.* F, 125.6 ± 9.4 μg of Trolox equivalents/mg protein, $p<0.05$).

3.6. q-PCR

A significantly lower relative gene expression of catalase and SOD1 was recorded in F compared to C animals (Fig. 2A and D). LA administration also prevented such decreased gene expression. Conversely, there were no statistical differences in SOD2 gene expression among groups (Fig. 2G).

Whereas PPARδ gene expression was significantly higher and that of PPARγ was significantly lower in F compared to C rats (Fig. 3D and G, respectively), the one of PPARα remained unchanged (data not shown). LA administration restored values to the levels measured in C rats.

While UCP2 gene expression was significantly higher in F compared to C rats, LA administration induced an even higher increase (Fig. 3A).

The increase in PPARδ expression in F rats was accompanied by a parallel increase in the expression of SREBP-1c and the lipogenic genes FAS and GPAT-1 (Fig. 4). These higher expression levels returned to C values in FL animals (Fig. 4).

3.7. Western blot analyses

The protein expression of catalase, SOD1 and SOD2 in F samples was lower than that of C animals (Fig. 2B–C, E–F and H–I). Conversely, UCP2 expression increased in liver homogenates of F rats compared to C animals (Fig. 3B–C). In F rats, PPARδ and PPARγ protein expressions followed the same pattern as that of their corresponding gene expression, being significantly higher and lower, respectively, than in samples from C rats (Fig. 3E–F and H–I). LA administration to F rats prevented the development of all these changes.

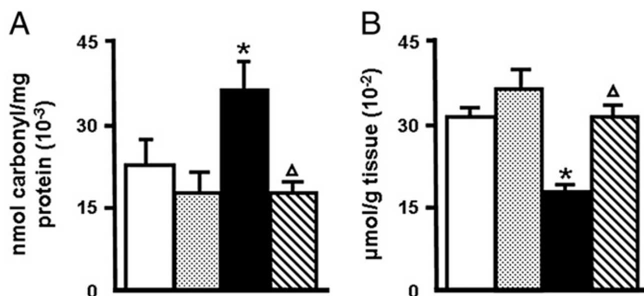

Fig. 1. \Assessment of protein carbonyl groups (A) and GSH (B). C (white bars), CL (dotted bars), F (black bars) and FL (lined bars) animals. Results are means ± SEM (n = 20). *$p<0.05$ *vs.* C and $^{\Delta}p<0.05$ *vs.* F animals.

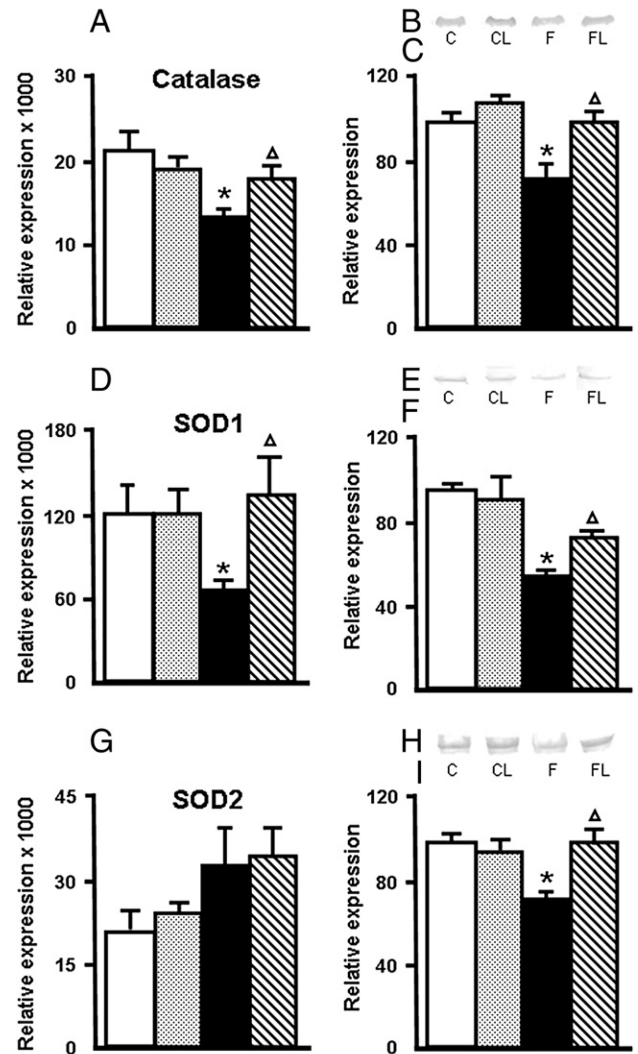

Fig. 2. Catalase, SOD1 and SOD2 relative gene (A, D and G) and protein (B–C, E–F and H–I) expressions. (A, D and G) Catalase, SOD1 and SOD2 relative gene expressions in C (white bars), CL (dotted bars), F (black bars) and FL (lined bars) animals. (B, E and H) Representative blots show the bands corresponding to catalase, SOD1 and SOD2 proteins in C (line 1), CA (line 2), F (lines 3) and FL (line 4), respectively. (C, F and I) Band intensities were measured in C, CL, F and FL animals. Results are means ± SEM (n = 20). *$p<0.05$ *vs.* C, and $^{\Delta}p<0.05$ *vs.* F animals.

3.8. Glucokinase activity

The three week administration of the F-rich diet increased significantly the liver glucokinase activity (C, 3.26 ± 0.1 *vs.* F, 6.72 ± 0.21 mU/mg of protein, $p<0.001$). However, after LA administration to FL animals, glucokinase activity was comparable to that recorded in C rats (FL, 2.69 ± 0.1 *vs* F, 6.72 ± 0.21 mU/mg of protein, $p<0.001$). Glucokinase activity in CL rats was similar to that recorded in C animals (CL, 2.62 ± 0.12 mU/mg of protein).

4. Discussion

As previously reported our F-fed rats presented insulin resistance, hypertriglyceridemia and oxidative stress markers as well as impaired glucose tolerance [11–14]. These changes are also accompanied by an increase in glucokinase activity, the so called hepatic "glucose sensor" [13]. Additionally, the F-induced oxidative stress triggered an adaptive response in the liver that involved an enhanced UCP2 expression, probably *via* PPARδ induction [16].

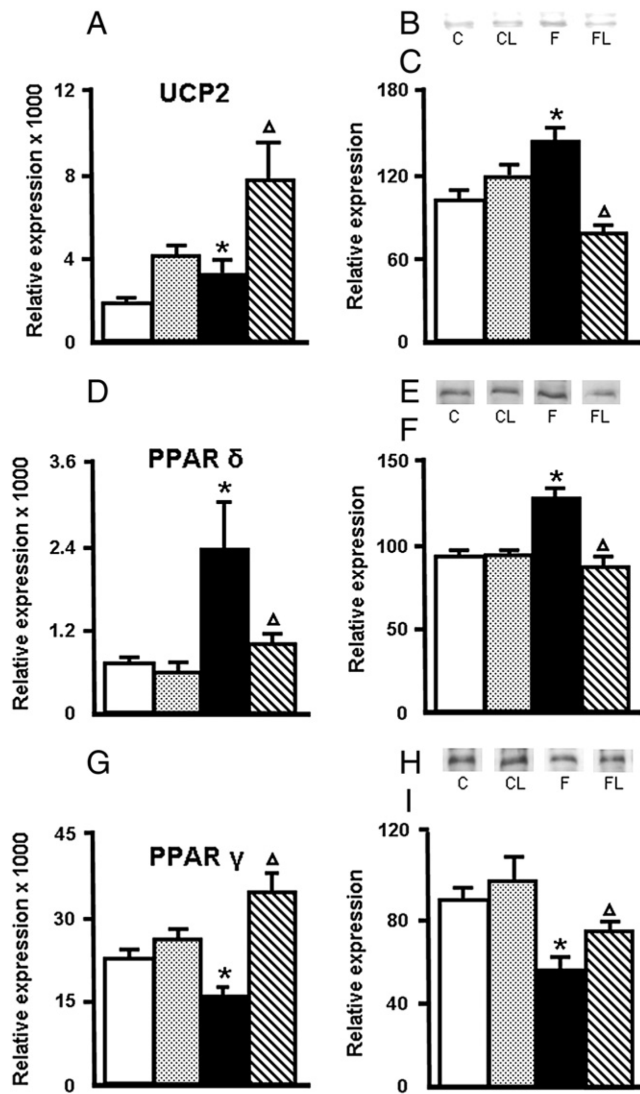

Fig. 3. F-induced changes in UCP2, PPARγ and PPARδ relative gene (A, D and G) and protein (B–C, E–F and H–I) expressions. (A, D and G) UCP2, PPARγ and PPARδ relative gene expressions in C (white bars), CL (dotted bars), F (black bars) and FL (lined bars) animals. (B, E and H) Representative blots show the bands corresponding to UCP2, PPARγ and PPARδ proteins in C (line 1), CL (line 2), F (lines 3) and FL (line 4), respectively. (C, F and I) Band intensities were measured in C, CL, F and FL animals. Results are means ± SEM (n = 20). $^{*}p<0.05$ *vs.* C, and $^{\Delta}p<0.05$ *vs.* F animals.

The presence of hepatic oxidative stress in our F rats is supported by the increase in protein carbonyl groups and the reduction in GSH content as well as in the expression of antioxidant enzymes. These data suggest

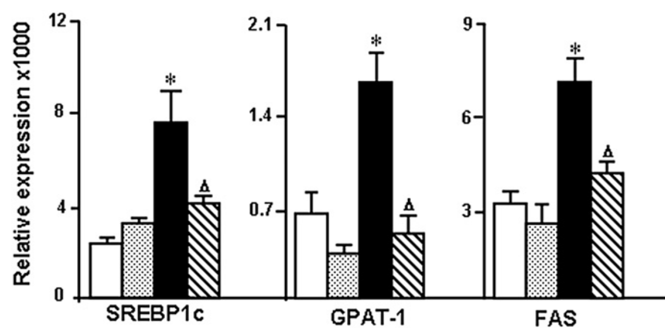

Fig. 4. F-induced changes in SREBP-1c, GPAT-1 and FAS relative gene expression. SREBP-1c, GPAT-1 and FAS relative gene expressions in C (white bars), CL (dotted bars), F (black bars) and FL (lined bars) animals. Results are means ± SEM (n = 20). $^{*}p<0.05$ *vs.* C, and $^{\Delta}p<0.05$ *vs.* F animals.

that F-induced oxidative stress is the consequence of a combination of two opposite effects: an increment of ROS production and a reduction of the antioxidant enzymes' availability. LA administration reduced the liver oxidative stress burden by preventing all these changes and by increasing its antioxidant capacity. These results are in agreement with those reporting the use of a 60% fructose load in the diet and LA treatment [22–24].

Since LA administration to our F rats corrected hypertriglyceridemia, we can assume that its development was linked to the effect of F upon oxidative stress. This correction was associated to a reduced expression of SREBP-1c and the lipogenic enzymes (FAS and GPAT-1), suggesting that LA partly produces its effect by normalizing the hepatic *de novo* triglyceride synthesis. The fact that LA reduced FAS and SREBP-1c gene expression in acyl-CoA synthase transgenic mice [33] and the activity and the gene expression of lipogenic enzymes in the rat liver [34] supports this assumption. Further, Butler et al. [19] demonstrated that the LA-induced improvement of hypertriglyceridemia in ZDF rats was the consequence of a higher triacylglycerol clearance and a down-regulation of liver triglyceride secretion, being the latter effect partly due to the inhibition of *de novo* triglyceride synthesis.

Interestingly, our data show for the first time that LA also induced a significant reduction in F-enhanced glucokinase activity. Since it has been shown that glucose metabolism exerts a synergistic effect on the expression of lipogenic genes *via* glucokinase and SREBP-1c [35], the protective effect of LA upon liver lipogenesis could be linked to its inhibitory action on both glucokinase activity and SREBP-1c gene expression.

The uneven effect of F on the liver expression of PPARδ (increase) and PPARγ (decrease) we have currently measured, correlates well with the enhanced expression of the two lipogenic genes FAS and GPAT-1 that, according to Lee et al. [36], could be also controlled by PPARδ. Since LA administration to F-fed animals brought the PPAR values to levels attained in C animals, it can be assumed that the changes in the expression of these receptors could also actively participate in the mechanism by which LA decreased triglyceridemia.

Insulin resistance plays a crucial role in the pathogenesis of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis, reason why insulin sensitizers have been used for their treatment [37]. It has been suggested that oxidative stress could induce insulin resistance by impairing insulin action [38] as well as late T2DM complications through an increased production of ROS [39,40]. The improvement of insulin resistance induced by LA administration in our F-fed animals lends further support to this hypothesis. Interestingly, it has been recently shown that PPARδ regulates insulin sensitivity by gearing glucose metabolism towards fat production in the liver and increasing fat burning by the muscle to reduce lipid burden [36]. However, evidence suggesting that changes in PPARδ expression are effectively involved in the beneficial effect of LA on fructose-induced insulin resistance and impaired glucose tolerance should be further investigated.

It has been demonstrated that LA administration prevents nonalcoholic steatosis related to high-fat feeding by improving [41] and modulating [42] mitochondrial function. In this regard, we have previously shown that in F-fed animals, PPARδ enhanced expression is involved in UCP2 expression [16], while its protein expression has been associated to metabolic stress in adult rat liver [43]. Accordingly, an increase in liver UCP2 has been recorded in response to starvation, in obese, leptin-deficient animals and in rodents treated with a high-fat diet [44–46]. Consistently, the high protein expression level of liver UCP2 measured in our F rats was reverted and accompanied by a reduction in oxidative stress after LA treatment. These data lend further support to our assumption that the increased UCP2 protein expression would represent an adaptive mechanism to the metabolic overload caused by F administration.

The results show that down-regulation of UCP2 protein occurs despite an increase in mRNA expression, with the consequent disconnect between both levels in F rats treated with LA. The time course of these regulations, however, is unclear. Even when it could be argued that

the up-regulation of mRNA occurs relatively early in the progression of LA treatment whereas protein down-regulation is a late-stage phenomenon, our current experimental design only allows us to mention that this compound affects UCP2 gene and protein expression through uneven mechanisms.

The lower expression of PPARγ depicted in our F rats merits some comments. It is known that its activation by thiazolidinediones improves insulin action and decreases intracellular triglyceride accumulation in several tissues, included the liver [47–49]. Furthermore, it has been demonstrated that PPARγ reduces TG synthesis in rat hepatoma cells by reducing the nuclear SREBP-1 [50]. It has also been shown that: a) activators of PPARγ increase SOD1 expression in endothelial cells, suggesting a PPARγ role as radical scavenger [51], and b) PPARγ expression is affected by oxidative stress in patients with metabolic syndrome, thus suggesting an association of a fall in PPARγ expression with oxidative stress and hypertriglyceridemia [52]. This association could be mediated by changes in SREBP-1c gene expression and the related lipogenic genes [50]. Such association of PPARγ expression and insulin resistance, plasma SOD1 activity and serum triglyceride and free fatty acid changes has been observed in obese persons given a high-fat meal [53]. On account of these data and the fact that in our study LA administration prevented the development of all these alterations, we could postulate that the multiple metabolic dysfunctions induced by F administration could be related to an increase in oxidative stress that was followed by a decrease of PPARγ expression and by an impairment of triglyceride and carbohydrate metabolism and thereby insulin sensitivity. In this context, the simultaneous increased protein expression of PPARδ and UCP2 could be part of a compensatory mechanism by which the liver attempts to reduce the enhanced ROS production.

5. Conclusion

In conclusion, our data provide objective evidence of the role of the expression of PPARδ and PPARγ as well as of UCP2 in the mechanism by which F affects oxidative stress, triglyceride and carbohydrate metabolism. They also contribute to better understand the mechanisms involved in the liver adaptation to fructose-induced oxidative stress. Additionally, these data would help to develop appropriate strategies to prevent and treat early clinical conditions with abnormal oxidative stress levels such as obesity and T2DM.

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