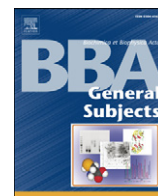


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Lipoic acid prevents fructose-induced changes in liver carbohydrate metabolism: Role of oxidative stress

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ABSTRACT

Background: Fructose administration rapidly induces oxidative stress that triggers compensatory hepatic metabolic changes. We evaluated the effect of an antioxidant, R/S- α -lipoic acid on fructose-induced oxidative stress and carbohydrate metabolism changes.

Methods: Wistar rats were fed a standard commercial diet, the same diet plus 10% fructose in drinking water, or injected with R/S- α -lipoic acid (35 mg/kg, i.p.) (control + L and fructose + L). Three weeks thereafter, blood samples were drawn to measure glucose, triglycerides, insulin, and the homeostasis model assessment-insulin resistance (HOMA-IR) and Matsuda indices. In the liver, we measured gene expression, protein content and activity of several enzymes, and metabolite concentration.

Results: Comparable body weight changes and calorie intake were recorded in all groups after the treatments. Fructose fed rats had hyperinsulinemia, hypertriglyceridemia, higher HOMA-IR and lower Matsuda indices compared to control animals. Fructose fed rats showed increased fructokinase gene expression, protein content and activity, glucokinase and glucose-6-phosphatase gene expression and activity, glycogen storage, glucose-6-phosphate dehydrogenase mRNA and enzyme activity, NAD(P)H oxidase subunits (gp91^{phox} and p22^{phox}) gene expression and protein concentration and phosphofructokinase-2 protein content than control rats. All these changes were prevented by R/S- α -lipoic acid co-administration.

Conclusions: Fructose induces hepatic metabolic changes that presumably begin with increased fructose phosphorylation by fructokinase, followed by adaptive changes that attempt to switch the substrate flow from mitochondrial metabolism to energy storage. These changes can be effectively prevented by R/S- α -lipoic acid co-administration.

General significance: Control of oxidative stress could be a useful strategy to prevent the transition from impaired glucose tolerance to type 2 diabetes.

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1. Introduction

Several authors have suggested that the increased use of refined carbohydrates such as fructose-rich syrups has greatly contributed to the epidemics of obesity and type 2 diabetes [1,2]. Additionally, many investigators have demonstrated that administration of fructose-rich diets to normal rats induces several metabolic and endocrine dysfunctions, affecting many tissues and organs [3–6]. Since the liver is primarily responsible for fructose uptake and metabolism, a number of studies have dealt with its effect on hepatic glucose metabolism [7,8]. Although the underlying mechanism of fructose-induced detrimental effects is not fully understood, experimental evidence suggests that oxidative stress could play a key role [9–12]. In this regard, we have previously

demonstrated that short-term fructose administration to normal rats induces a significant enhancement of oxidative stress markers in several organs including the liver [3,4], associated insulin resistance, a switch of hepatic carbohydrate and lipid metabolism towards its anabolic pathway and impaired glucose tolerance [5,6,13,14].

If the above-mentioned changes were specifically linked to fructose-induced oxidative stress, then the administration of an antioxidant agent should prevent/alleviate the development of oxidative stress. Supporting this assumption, we have previously shown that co-administration of an antioxidant, R/S- α -lipoic acid to fructose-fed rats prevents both oxidative stress and most of the endocrine-metabolic dysfunctions triggered by fructose [15]. We do not know, however, the potential molecular link between fructose-induced oxidative stress and the resultant impaired carbohydrate metabolism. In the series of experiments herein described we examined the effect of R/S- α -lipoic acid co-administration on carbohydrate metabolism in fructose-fed rats in order to clarify the adaptive mechanisms involved in fructose-induced oxidative stress that may be the basis for strategies for our understanding of obesity and type 2 diabetes mellitus frequently associated with high fructose consumption.

Abbreviations: HOMA-IR, homeostasis model assessment-insulin resistance; FPI, fasting plasma insulin; FPG, fasting plasma glucose; PFK-2, phosphofructokinase 2; NAFLD, non-alcoholic fatty liver disease

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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). An injectable solution (Megatioc®) of R/S- α -lipoic acid was purchased from John Martin S.R.L (Buenos Aires-Argentina).

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), the same diet plus 10% fructose in the drinking water (fructose), and two additional groups that were injected with R/S- α -lipoic acid (35 mg/kg, i.p) (control + L and fructose + L) during the last five days of treatment. Control and fructose animals were injected with the same volume of saline buffer. Water intake was measured daily, and 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 a portion of the median lobe of the liver was removed to perform all the assays. When the assays were not performed immediately, the lobe was quickly immersed in liquid nitrogen and thereafter stored in a deep freezer at –80 °C; all enzyme activities were measured within a week. 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 determined with a commercial kit (TG color GPO/PAP AA, Wiener lab, Argentina) and immunoreactive insulin levels were determined by radioimmunoassay (Linco Research Inc., IN, USA). Serum insulin and fasting glycemia values were used to estimate the homeostasis model assessment–insulin resistance (HOMA-IR) (serum insulin (μ J/ml) \times fasting blood glucose (mM)) / 22.5 [16]. The Matsuda index (hepatic insulin sensitivity) was calculated with the formula $k / \text{fasting plasma insulin (FPI)} \times \text{fasting plasma glucose (FPG)}$, where k : 22.5×18 [17].

2.4. Isolation of total RNA

Total liver RNA was isolated using TRIzol® Reagent (Gibco) [18]. Reverse transcription-PCR (RT-PCR) was performed using the SuperScript III (Invitrogen, USA) and total RNA from the rat livers as a template.

2.5. Gene expression by real-time PCR (qPCR) (quantitative PCR)

qPCR was performed with a Mini Opticon Real-Time PCR Detector Separate MJR (Bio-Rad Laboratories), using SYBR Green I as a fluorescent dye. Then 10 ng of cDNA was amplified in 25 μ l of the reaction mixture containing 0.6 μ M of each primer, 3 mM MgCl₂, 0.2 mM dNTPs and 0.15 μ l of platinum Taq DNA polymerase (6 units/ μ l; Invitrogen, USA). Samples were first denatured at 95 °C for 3 min followed by 40 PCR cycles (a melting step at 95 °C for 30 s, an annealing step at 62 °C for 45 s and an extension step at 72 °C for 30 s), followed by a final extension at 72 °C for 10 min. The oligonucleotide primers (forward and reverse) (Invitrogen) used are listed in Table 1. All amplicons were designed in

a size range of 90–288 bp. The specificity of the primers was double checked: a) performing an endpoint PCR and determining a single amplicon at the predicted size and b) measuring the melting curves in the qPCR system and controlling the existence of a single pick. The efficiency of the primers employed was calculated after performing calibration curves using different quantities of cDNA. Results are expressed as relative gene expression after normalization to the β -actin housekeeping gene using the Qgene96 and LineRegPCR software [19].

2.6. Western blot analysis

Immunodetection of glucokinase, phosphofructokinase 2 (PFK-2), fructokinase, p22^{phox} and β -actin was done in liver homogenates. Protein concentration was quantified by the Bio-Rad protein assay [20]. Thereafter, dithiothreitol and bromophenol blue were added to a final concentration of 100 mM and 0.1%, respectively. Nonspecific binding sites of the membranes were blocked by previous overnight incubation with non-fat dry milk at 4 °C. Enzyme identification and quantification were performed using specific primary antibodies (Table 2). Diaminobenzidine (Sigma Co.) or enhanced chemiluminescence (GE Healthcare, UK) was used for color development. Finally, the bands were quantified by densitometry using the Gel-Pro Analyser software. β -Actin density was used to normalize the protein content: the target protein relative content was divided by the relative β -actin protein level in each group.

2.7. Liver glycogen content

Pieces of fresh liver (400 mg) were placed in 1 ml of 33% KOH and incubated for 20 min at 100 °C. Then, 1.25 ml of ethanol was added to each tube and the mixture was incubated for 48 h at 4 °C and finally centrifuged at 700 \times g for 20 min. The pellets obtained were resuspended in 1 ml of distilled water plus 3 ml of Antrone solution (0.1% in 84% H₂SO₄) and incubated for 20 min at 100 °C. The absorbance was measured photometrically at 620 nm and the results expressed as μ mol of glycogen/mg of tissue [21].

2.8. Liver fructokinase activity

Pieces of liver were homogenized in buffer containing 25 mM HEPES (pH 7.1), 100 mM KCl, 1 mM DTT, and 0.1 mM EDTA; they were then spun at 10,000 \times g at 4 °C for 20 min and aliquots of the supernatant were frozen for further activity assay. To measure FK activity we used a coupled enzymatic assay based on existing methods [22]. Briefly, 10–20 μ l of the sample were added to 200 μ l of the reaction mixture containing of 25 mM HEPES (pH 7.1), 6 mM MgCl₂, 25 mM KCl, 10 mM NaF, 5 mM D-fructose, 0.2 mM NADH, 1 mM phosphoenolpyruvate,

Table 1
Primer sequences.

Gene	GenBank®	Sequences
Fructokinase	NM_031855.3	FW 5'-ACGGATCGCAGGTGCCTAT-3' RV 5'-AGCACAGTGCAGGAGTTGGA-3'
Glucose-6-phosphatase	NM_013098.2	FW 5'-GATCGCTGACCTCAGGAACGC-3' RV 5'-AGAGGCACGGAGCTGTGTGTG-3'
Glucose-6-phosphate dehydrogenase	NM_017006.2	FW 5'-TTCCGGATGGCCTTCTAC-3' RV 5'-TTTGGGATGTCATCCACTGT-3'
Glucokinase	NM_012565.1	FW 5'-GTGTACAAGCTGCACCCGA-3' RV 5'-CAGCATGCAAGCCTTCTTG-3'
PFK-2	NM_012621.4	FW 5'-CGATCTATCTACCTATGCCGCCAT-3' RV 5'-ACACCCGCATCAATCTCATTC-3'
gp91 ^{phox}	NM_023965.1	FW 5'-CCAGTGTGTCCGAATCTCCT-3' RV 5'-ACACCACTCCACGTTGAACA-3'
p22 ^{phox}	NM_024160.1	FW 5'-CAGCAGTGGTACTTTGGTG-3' RV 5'-CACGGACAGCAGTAAGTGG-3'
β -Actin	NM_031144.2	FW 5'-AGAGGGAATCTGTGCGTGAC-3' RV 5'-CGATAGTGATGACCTGACCT-3'

FW, forward primer. RV, reverse primer.

Table 2
Western blot protocol.

Protein (MW)	Whole protein (µg)	SDS-PAGE %	Primary antibody dilution	T (h)	Secondary antibody dilution	T (m)	D
Fructokinase (33)	100	12.5	Anti-fructokinase 1:1000	16	Peroxidase-conjugated anti-goat IgG 1:10,000	90	ECL
Glucokinase (60)	20	10	Anti-glucokinase 1:2000	1.5	Anti-sheep IgG 1:2000	30	DAB
					Streptavidin–peroxidase conjugate 1:2000	30	
PFK-2 (58)	200	10	Anti-PFK-2 1:10,000	2	Peroxidase-conjugated anti-chicken IgG 1:400,000	60	DAB
p22 ^{phox} (22)	100	12.5	Anti- p22 ^{phox} 1:200	2.5	Peroxidase-conjugated anti-rabbit IgG 1:10,000	90	DAB
β-actin (42)	20–200	10–12.5	Anti- β-actin 1:10,000	1	Anti-mouse IgG 1:2000	30	DAB
					Streptavidin–peroxidase conjugate 1:2000	30	

MW: molecular weight; T: time; h: hours; m: minutes; D: method used for developing.

40 U/ml pyruvate kinase, 40 U/ml lactate dehydrogenase, and 50 mM N-acetyl-D-glucosamine (to inhibit hexokinase but retain fructokinase activity). The reaction was started by adding 10 µl of ATP (5 mM final concentration) and quantitatively measured accordingly to a decrease of optical density at 340 nm during 30 min.

2.9. Liver glucokinase activity

Liver pieces removed from the animals were homogenized in ice-cold PBS containing 0.1 mM PMSF, 0.1 mM benzamidine, 2 mM DTT, 4 µg/ml aprotinin and 0.3 M sucrose, pH 7.5. Aliquots of these homogenates were centrifuged at 600 ×g to separate the nuclear fraction. The supernatant was centrifuged twice at 8000 and 100,000 ×g at 4 °C, and the resultant supernatant was collected and identified as the cytosolic fraction (c_f), which contains the active functional form of the enzyme. Thereafter, the nuclear fraction, containing the inactive form of glucokinase due to its binding with its regulatory protein, was re-suspended and incubated for 12 min at 20 °C in a permeabilizing medium containing 150 mmol/ml KCl, 3 mmol/l Hepes, 2 mmol/l DTT, and 0.04 mg/ml digitonin, pH 7.2. At the end of this incubation, samples were centrifuged (600 ×g) and the digitonin supernatant was removed and collected for further determinations (n_f). Digitonin treatment allows the release of glucokinase from its regulatory protein, rendering a soluble and active glucokinase. Glucokinase activity was finally measured in aliquots of both liver fractions and the activity ratio for c_f/n_f considered as an indirect measurement of nucleus/cytosol translocation [5]. Rates of glucose phosphorylation in both fractions were measured at 37 °C, pH 7.4, by recording the increase in absorbance at 340 nm in a well-established enzyme-coupled photometric assay [5,23]. Glucokinase activity was obtained by subtracting the activity measured at 1 mM glucose (hexokinase) from that measured at 100 mM glucose. Enzyme activity is expressed as m-units/mg of protein. One unit of enzyme activity was defined as 1 µmol of glucose-6-phosphate formed from glucose and ATP/min at 37 °C.

2.10. Liver glucose-6-phosphatase activity

Homogenization of liver samples and isolation of microsomes were carried out as described by Nordlie & Arion [24]. Homogenization medium was 0.25 M sucrose/5 mM Tris-acetate/0.5 mM EDTA, pH 7.4. Microsomes were washed once with 0.25 M sucrose/5 mM Tris-acetate, pH 7.4, and centrifuged at 100,000 ×g. Fully disrupted microsomes were prepared at 0 °C by adding 0.1 ml of 0.75% Triton X-100 to 0.9 ml of untreated microsomes (approximately 10 mg of protein) and allowed to stand on ice for 20 min. Then 50 µl of the mixture and 50 µl of glucose-6-phosphate sodium salt (0.8 M) were added and samples were incubated for 10 min at 30 °C. The reaction was stopped by adding 250 µl of 10% trichloroacetic acid; then, 2 ml of MoNH₄ (diluted in 1 M H₂SO₄) plus 320 µl of FeSO₄ (diluted in 0.15 M H₂SO₄) were added to 200 µl of each sample. The activity of glucose-6-phosphatase was determined by measuring the release of inorganic phosphate from glucose-6-phosphate. The absorbance was photometrically read against a reagent blank at 660 nm and results were expressed as

'latency', calculated according to the following formula: $100 \times (\text{activity in disrupted microsomes} - \text{activity measured in untreated microsomes}) / \text{activity measured in disrupted microsomes}$.

2.11. Liver glucose-6-phosphate dehydrogenase activity

Pieces of liver were homogenized in 0.1 M Tris/HCl and 1 mM EDTA, pH 7.6 (10 ml/g of tissue). The homogenate was centrifuged for 15 min at 10,000 ×g and enzyme activity was measured in the supernatants by recording the changes in absorbance at 340 nm in a well-established enzyme-coupled photometric assay as described by Beutler [25]. Briefly the sample was added to the buffer assay (Tris-HCl 1 M, EDTA 5 mM, pH 8, MgCl₂ 0.1 M and NADP⁺ 2 mM), and incubated for 10 min at 37 °C. Thereafter, the sample was divided into 4 aliquots (tubes 1 to 4): tube 1 without sample, tube 2 plus 6 mM G-6-P, tube 3 plus 6 mM 6-phosphogluconate and tube 4 plus both compounds. The glucose-6-phosphate dehydrogenase activity was calculated by subtracting the activity measured in tube 3 from that of tube 4 [25]. Glucose-6-phosphate dehydrogenase catalyzes the oxidation of glucose-6-phosphate to 6-phosphogluconolactone, which quickly and spontaneously hydrolyzes to 6-phosphogluconate. In the next step, 6-phosphogluconate dehydrogenase catalyzes the oxidation of 6-phosphogluconate to ribulose-5-phosphate and CO₂. These procedures allow the simultaneous calculation of glucose-6-phosphate dehydrogenase activity in the standard way as well as the glucose-6-phosphate dehydrogenase activity as corrected for 6-phosphogluconate dehydrogenase activity [25].

2.12. Statistical analysis

Results are expressed as means ± SEM for the indicated number of observations. Statistical analysis was performed using ANOVA followed by Dunnett's test for multiple comparisons using the Prism analysis program (GraphPad). The Bartlett's test was employed to assess variances homogeneity. 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 over the 3-week study period (Table 3). Fructose fed and fructose + L animals drank a larger volume of water than control and control + L (55 ± 11 and 47 ± 12 vs. 29 ± 2 and 28 ± 2 ml/day, respectively; $p < 0.05$). Conversely, control and control + L rats ate significantly more solid food than fructose and fructose + L 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 the experimental groups (carbohydrates/protein/lipids 45:43:12 for control and control + L compared to 59:32:9 and 57:34:9 for fructose and fructose + L respectively), their calorie intake was comparable (control: 58 ± 3; control + L: 65 ± 2; fructose: 66 ± 5; fructose + L: 66 ± 4 kcal/day). The daily calorie intake was calculated based on the amount of daily intake of nutrients (solid food plus the fructose in the

water) multiplied by either 4 (carbohydrates and protein) or 9 (fat) calories.

3.2. Serum measurements

Fructose-fed rats had higher serum insulin and triglyceride concentrations than control rats (Table 3). The high Matsuda index and HOMA-IR values measured in fructose-fed rats demonstrated the presence of a decrease in insulin sensitivity both in the liver and in the other peripheral tissues as well (Table 3). Co-administration of R/S- α -lipoic acid to these rats prevented the development of all the metabolic and endocrine changes as well as the decreased hepatic and general response to insulin; consequently, the different parameters tested attained values comparable to those recorded in control rats; this effect was even greater for triglycerides, in which triglyceride concentrations were lower than those recorded in control rats (Table 3).

3.3. Liver fructokinase gene expression, protein content and activity

Fructose-fed rats had increased fructokinase gene expression, protein content and activity, changes that were prevented by R/S- α -lipoic acid administration (Fig. 1A, B and C, respectively).

3.4. Liver glucokinase gene expression, protein content and activity

Glucokinase gene expression measured in liver homogenates was higher in fructose-fed compared to control rats, but the difference was not statistically significant (control, 1.36 ± 0.26 vs. fructose, 2.4 ± 0.69 , relative gene expression $\times 1000$). Nevertheless, its expression in fructose + L animals was similar to that seen in control rats (fructose + L, 1.58 ± 0.47 , relative gene expression $\times 1000$). Glucokinase protein content was comparable in all groups, suggesting that it was not significantly affected either by fructose or by R/S- α -lipoic acid co-administration (Fig. 2A).

On the other hand, when enzyme activity was measured independently in the cytosol (c_f) (active form of the enzyme) and in the nuclear (n_f) (inactive form) fractions of liver homogenates, it was higher in the n_f than in the c_f fraction of control, control + L and fructose + L animals. This was reversed in the fructose-fed animals (Fig. 2B–C). These results demonstrate a different nucleus/cytosolic translocation rate among the experimental groups.

3.5. Liver PFK-2 protein content

Fructose-fed animals had a significantly higher PFK-2 protein content than the controls. This increment was prevented by R/S- α -lipoic acid co-administration (Fig. 2D–E).

3.6. Liver glucose-6-phosphatase gene expression and activity

Rats fed fructose had a higher glucose-6-phosphatase gene expression and activity. This was prevented by R/S- α -lipoic acid co-administration (Fig. 3A and B).

Table 3

Body weight and serum measurements.

Parameter	C	CL	F	FL
Body weight change (g)	103 \pm 6	92 \pm 8	97 \pm 5	94 \pm 6
Insulin (ng/ml)	0.76 \pm 0.03	0.6 \pm 0.08	1.13 \pm 0.05*	0.74 \pm 0.08 ^Δ
Glucose (mg/dl)	114 \pm 5	104 \pm 4	110 \pm 4	115 \pm 3
Triglyceride (g/l)	0.965 \pm 0.052	0.45 \pm 0.09*	1.63 \pm 0.12*	0.71 \pm 0.06 ^Δ
HOMA-IR	5.1 \pm 0.2	3.9 \pm 0.6	8.0 \pm 0.1*	5.4 \pm 0.8 ^Δ
Matsuda index (k/FPI \times FPG)	3.37 \pm 0.2	4.68 \pm 0.3*	2.3 \pm 0.15*	3.43 \pm 0.2 ^Δ

Values are means \pm SEM (n = 20).

* p < 0.05 vs. C.

^Δ p < 0.05 vs. F.

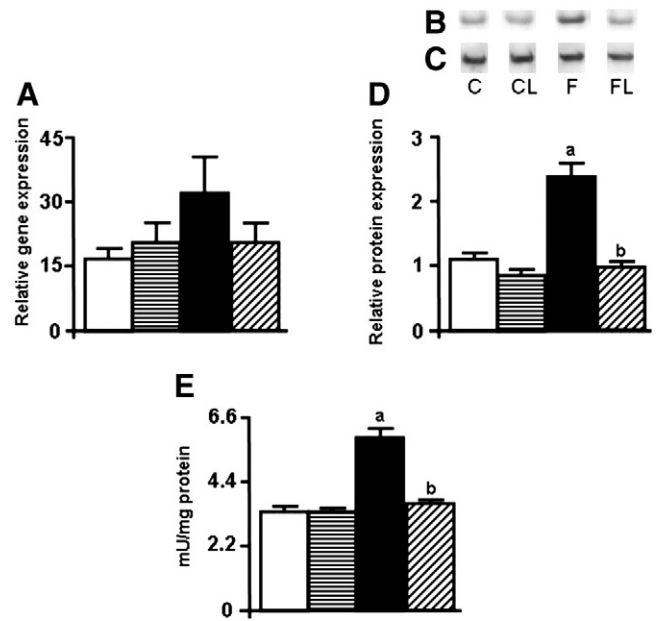


Fig. 1. Liver fructokinase expression and activity. Liver fructokinase gene expression (A), protein content (B and D) and activity (E). (B) Representative blots show the bands corresponding to fructokinase protein or β -actin protein (C) in control, control-L, fructose and fructose-L (C, CL, F and FL respectively). (D) Band intensities were measured in control (white bar), control-L (horizontal lines bar), fructose (black bar) and fructose-L (diagonal lines bar) animals. Results are means \pm SEM of 5 different experiments ran in triplicate. ^ap < 0.05 vs. C, and ^bp < 0.05 vs. F animals.

3.7. Liver glucose-6-phosphatase dehydrogenase gene expression and activity

The amount of glucose-6-phosphatase dehydrogenase mRNA and the enzyme's activity was significantly higher in fructose-fed compared with control animals. Once again, R/S- α -lipoic acid co-administration prevented these changes (Fig. 3C and D, respectively).

3.8. Liver glycogen content

Supporting our previous report, fructose induced a significant increase in glycogen storage that was prevented by R/S- α -lipoic acid co-administration (Fig. 4).

3.9. NAD(P)H oxidase subunits gene and protein

Fructose-fed rats had a higher relative gene expression of gp91^{phox} and p22^{phox} than control animals. Co-administration of R/S- α -lipoic acid prevented this increase (Fig. 5A and B).

A significant increase in p22^{phox} protein concentration was recorded in fructose-fed animals (a single band in the Western blot of about 22 kDa, compatible with the molecular weight of this enzyme). Once more, this increase was prevented by R/S- α -lipoic acid treatment (Fig. 5C).

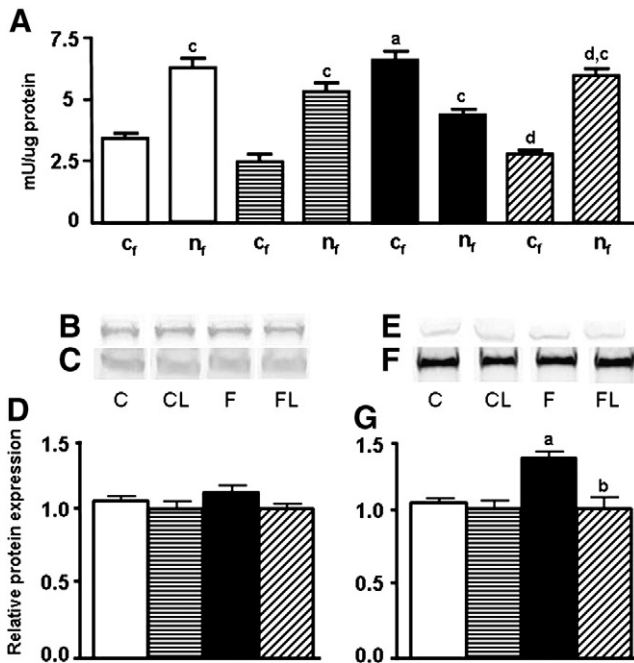


Fig. 2. Glucokinase and PFK-2 in rat livers. Glucokinase activity in the cytosolic (c_f) and digitonin permeabilized nuclear fraction (n_f) (A), ^a $p < 0.05$ vs. C c_f , ^b $p < 0.05$ vs. F c_f , ^c $p < 0.05$ n_f vs. c_f , and ^d $p < 0.05$ vs. F n_f ; glucokinase (B and D) and PFK-2 (E and G) protein content in control, control-L, fructose and fructose-L animals (C, CL, F and FL respectively). (B and E) Representative blots show the bands corresponding to glucokinase and PFK-2 protein in control, control-L, fructose and fructose-L animals (C, CL, F and FL respectively). (C and F) Representative blots show the bands corresponding to β -actin. (D and G) Band intensities were measured in control (white bar), control-L (horizontal lines bar), fructose (black bar) and fructose-L (diagonal lines bar) animals. Results are means \pm SEM of 5 different experiments ran in triplicate. ^a $p < 0.05$ vs. C, and ^b $p < 0.05$ vs. F animals.

4. Discussion

We have previously shown that normal Wistar rats fed fructose for 21 days developed several generalized metabolic and endocrine disorders [6]. These changes were accompanied by increased hepatic concentration of oxidative stress markers and significant changes in

carbohydrate and lipid metabolism that would channel liver metabolites preferentially to energy storage rather than to mitochondrial oxidation [5,6,14]. Additionally, using the same animal model, we recently demonstrated that R/S- α -lipoic acid co-administration prevented the development of high triglyceride serum concentrations and hepatic content, probably by decreasing PPAR δ and its target lipogenic gene expression [15]. Thus, these changes would be part of the mechanisms by which liver compensates the overload of lipid substrates [26], such as it occurs in the fructose-rich model.

As we have earlier reported [5], fructose also induces an increase in hepatic glucokinase activity. Since the enhanced activity was not accompanied by significant changes in glucokinase gene expression/protein concentration, the effect primarily resulted from a significant increase in the glucokinase nucleus/cytosol translocation as well as an increase in PFK2 concentration, a cytosolic glucokinase activator [5]. The current data support those results and demonstrated that co-administration of R/S- α -lipoic acid to fructose-fed rats drove the values of nuclear-cytosol ratio and PFK2 content to those recorded in control animals. We speculate that the fructose-induced increased production of reactive oxygen species plays an active modulatory role on liver glucokinase activity. Since insulin stimulates glucokinase gene expression via PI3K [27–29] and fructose-fed rats have hyperinsulinemia, it could be argued that hyperinsulinemia also contributes to increased glucokinase activity. However, since these rats demonstrated decreased insulin sensitivity (higher HOMA-IR and lower Matsuda indices in fructose-fed as compared to control rats), this possibility seems less likely. While the normal range of those indices is unclear in rodents, the significant difference between the two groups strongly suggests that the fructose-treated animals have a decreased insulin responsiveness as compared to control rats not only at the liver (Matsuda index) but also overall (HOMA-IR).

It has been claimed that timely and rapid metabolic adaptation to changes in carbohydrate supply is critical to maintain energy homeostasis and that liver glucose futile cycling plays an important role in such process [30]. It has also been shown that in mice, hepatic glucose/glucose-6-phosphate recycling compensates for peripheral glucose disposal, in order to preserve glucose homeostasis [31]. A comparable increase in glucokinase and glucose-6-phosphatase activity was measured in our fructose-fed rats (107 and 141% over the control values, respectively), thus suggesting that this futile cycle could be actively operating in our model, decreasing the glucose metabolism and the liver substrates flow to the mitochondria for reactive oxygen species production. Similarly, the increased activity of glucose-6-phosphate dehydrogenase activity (limiting enzyme of the pentose phosphate shuttle) and of glycogen and fat deposit in the liver would be part of the adaptive process because it simultaneously diminishes the overload of substrates to the mitochondria and enhances the redox power by

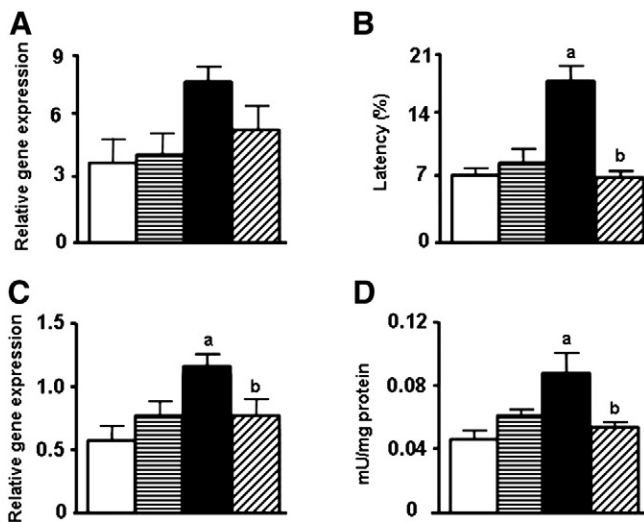


Fig. 3. Liver glucose-6-phosphatase and glucose-6-phosphate dehydrogenase expression and activity. Liver glucose-6-phosphatase and glucose-6-phosphate dehydrogenase gene expression (A and C) and activity (B and D) measured in control (white bar), control-L (horizontal lines bar), fructose (black bar) and fructose-L (diagonal lines bar) animals. Results are means \pm SEM of 5 different experiments ran in triplicate. ^a $p < 0.05$ vs. C, and ^b $p < 0.05$ vs. F animals.

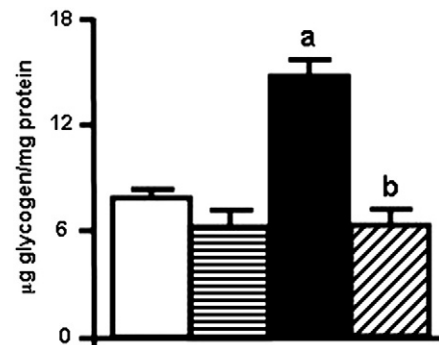


Fig. 4. Liver glycogen content. Glycogen content measured in control (white bar), control-L (horizontal lines bar), fructose (black bar) and fructose-L (diagonal lines bar) animals. Results are means \pm SEM of 5 different experiments ran in triplicate. ^a $p < 0.05$ vs. C, and ^b $p < 0.05$ vs. F animals.

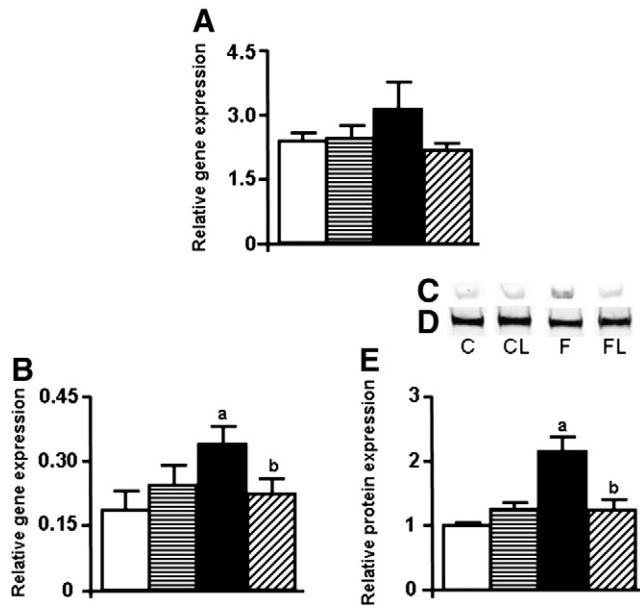


Fig. 5. NAD(P)H oxidase subunit expression. gp91^{phox} and p22^{phox} gene expression (A and B) and p22^{phox} protein expression. (C and E). (C and D) Representative blots show the bands corresponding to p22^{phox} and β -actin proteins in control, control-L, fructose and fructose-L animals (C, CL, F and FL respectively). (E) Band intensities were measured in control (white bar), control-L (horizontal lines bar), fructose (black bar) and fructose-L (diagonal lines bar) animals. Results are means \pm SEM of 5 different experiments ran in triplicate. ^a $p < 0.05$ vs. C, and ^b $p < 0.05$ vs. F animals.

increasing NAD(P)H production [32]. NAD(P)H plays an important antioxidant role by a complex mechanism that includes the reduction of oxidized glutathione to reduced glutathione through the enzyme glutathione-reductase, acting as coenzyme for peroxidases (via glutathione) and been required for catalase stabilization [32–34]. The fact that R/S- α -lipoic acid co-administration prevented all these changes reinforces our original interpretation.

The fact that R/S- α -lipoic acid co-administration blunted the fructose-induced increased gene expression and protein level of NAD(P)H oxidase subunit p22^{phox} (there was a simultaneous non-significant reduction in gp91^{phox}) also suggests the existence of a link between mitochondrial and cytosolic reactive oxygen species production. This possibility merits further research.

Our fructose-fed rats had a significant increase of fructokinase mRNA, protein level and activity supporting the findings of others using either fructose- or sucrose-rich diets [35–37]. On the other hand, fructose induced increases in fructokinase gene expression, protein level and activity in cultured hepatocytes [22]. Additionally, it has been demonstrated that fructose stimulates its own metabolism by inducing liver fructokinase expression [38,39] and establishing a vicious cycle, i.e., increased phosphorylation and increased metabolism, thus potentiating the deleterious effect of fructose upon hepatic metabolism [40]. Consistent with these data, patients with non-alcoholic fatty liver disease (NAFLD) portray twice as high fructokinase activity than the general population associated with a significantly higher consumption of sweetened beverages (rich in fructose) [22]. The fact that R/S- α -lipoic acid co-administration to our fructose-fed rats prevented the fructokinase changes strongly suggests that the enzyme activity not only depends on fructose overload but additionally on the positive feedback effect of some down-stream metabolite or signal. In this regard, the redox state of the hepatocyte could be a potential candidate: compelling evidence indicates that enhanced levels of radical oxygen species can modify protein activity or even alter its conformation [41]. However, an oxidative stress-mediated specific alteration in fructokinase structure is only hypothetical and needs further experimental support.

5. Conclusion

In brief, our results show that fructose induces many metabolic changes in the liver that likely begin with an increase in fructose phosphorylation by fructokinase followed by adaptive changes that attempt to switch the substrate flow from mitochondrial metabolism to energy storage. All these changes are essentially prevented by co-administration of R/S- α -lipoic acid. Therefore, oxidative stress together with fructokinase appear to be key mediators of the metabolic changes induced by fructose. The reduction of fructokinase activity by R/S- α -lipoic acid suggests that some oxidative stress compound or a metabolite produced subsequently acts as a positive feedback to maintain its high levels in fructose-fed rats. Identification of such an active link, and of the effect of antioxidants in the prevention of the transition from impaired glucose tolerance to diabetes merits further studies.

Conflict of interest

The authors manifest no conflict of interest.

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References

- [1] G.A. Bray, S.J. Nielsen, B.M. Popkin, Consumption of high-fructose corn syrup in beverages may play a role in the epidemic of obesity, *Am. J. Clin. Nutr.* 79 (2004) 537–543.
- [2] F.B. Hu, V.S. Malik, Sugar-sweetened beverages and risk of obesity and type 2 diabetes: epidemiologic evidence, *Physiol. Behav.* 100 (2010) 47–54.
- [3] O.R. Rebolledo, C.A. Marra, A. Raschia, S. Rodríguez, J.J. Gagliardino, Abdominal adipose tissue: early metabolic dysfunction associated to insulin resistance and oxidative stress induced by an unbalanced diet, *Horm. Metab. Res.* 40 (2008) 794–800.
- [4] A. Alzamendi, A. Giovambattista, A. Raschia, V. Madrid, R.C. Gaillard, O. Rebolledo, J.J. Gagliardino, E. Spinedi, Fructose-rich diet-induced abdominal adipose tissue endocrine dysfunction in normal male rats, *Endocrine* 35 (2009) 227–232.
- [5] F. Francini, M.C. Castro, J.J. Gagliardino, M.L. Massa, Regulation of liver glucokinase activity in rats with fructose-induced insulin resistance and impaired glucose and lipid metabolism, *Can. J. Physiol. Pharmacol.* 87 (2009) 702–710.
- [6] F. Francini, C. Castro, G. Schinella, M.E. Garcia, B. Maiztegui, M.A. Raschia, J.J. Gagliardino, M.L. Massa, Changes induced by a fructose-rich diet on hepatic metabolism and the antioxidant system, *Life Sci.* 86 (2010) 965–971.
- [7] M.E. Bizeau, M.J. Pagliassotti, Hepatic adaptations to sucrose and fructose, *Metabolism* 54 (2005) 1189–1201.
- [8] M. Pagliassotti, T. Horton, Sucrose, insulin action and biologic complexity, *Recent Res. Devel. Physiol.* 2 (2004) 337–353.
- [9] J. Busserolles, W. Zimowska, E. Rock, Y. Rayssiguier, A. Mazur, Rats fed a high sucrose diet have altered heart antioxidant enzyme activity and gene expression, *Life Sci.* 71 (2002) 1303–1312.
- [10] A. Girard, S. Madani, F. Boukourt, M. Cherkaoui-Maiki, J. Belleville, J. Prost, Fructose-enriched diet modifies antioxidant status and lipid metabolism in spontaneously hypertensive rats, *Nutrition* 22 (2006) 758–766.
- [11] J. Lange, W.J. Arion, A.L. Beaudet, Type Ib glycogen storage disease is caused by a defect in the glucose-6-phosphate translocase of the microsomal glucose-6-phosphatase system, *J. Biol. Chem.* 255 (1980) 8381–8384.
- [12] V. Thirunavukkarasu, C.V. Anuradha, Influence of α -lipoic acid on lipid peroxidation and antioxidant defense system in blood of insulin-resistant rats, *Diabetes Obes. Metab.* 6 (2004) 200–207.
- [13] M.C. Castro, M.L. Massa, H. Del Zotto, J.J. Gagliardino, F. Francini, Changes in rat liver mitochondrial uncoupling protein 2 induced by a fructose-rich diet, *Life Sci.* 89 (2011) 609–614.
- [14] M.C. Castro, F. Francini, G. Schinella, C.I. Caldiz, M.G. Zubiría, J.J. Gagliardino, M.L. Massa, Apocynin administration prevents the changes induced by a fructose-rich diet on rat liver metabolism and the antioxidant system, *Clin. Sci.* 123 (2012) 681–692.
- [15] M.C. Castro, M.L. Massa, G. Schinella, J.J. Gagliardino, F. Francini, Lipoic acid prevents liver metabolic changes induced by administration of a fructose-rich diet, *Biochim. Biophys. Acta* 1830 (2013) 2226–2232.
- [16] D.R. Matthews, J.P. Hosker, A.S. Rudenski, B.A. Naylor, D.F. Treacher, R.C. Turner, Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man, *Diabetologia* 28 (1985) 412–419.
- [17] M. Matsuda, R.A. DeFronzo, Insulin sensitivity indexes obtained from oral glucose tolerance testing, *Diabetes Care* 22 (1999) 1462–1479.

- [18] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, *Anal. Biochem.* 162 (1987) 156–159.
- [19] P.Y. Muller, H. Janovjak, A.R. Miserez, Z. Dobbie, Processing of gene expression data generated by quantitative real-time RT-PCR, *Biotechniques* 32 (2002) 1372–1379.
- [20] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.* 72 (1976) 248–254.
- [21] Y. Chun, Z.D. Yin, Z.D. Glycogen assay for diagnosis of female genital *Chlamydia trachomatis* infection, *J. Clin. Microbiol.* 36 (1998) 1081–1082.
- [22] X. Ouyang, P. Cirillo, Y. Sautin, S. McCall, J.L. Bruchette, A. Mae Diehl, R.J. Johnson, M.F. Abdelmalek, Fructose consumption as a risk factor for non-alcoholic fatty liver disease, *J. Hepatol.* 48 (2008) 993–999.
- [23] L. Massa, S. Baltrusch, D.A. Okar, A.J. Lange, S. Lenzen, M. Tiedge, Interaction of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) with glucokinase activates glucose phosphorylation and glucose metabolism in insulin-producing cells, *Diabetes* 53 (2004) 1020–1029.
- [24] R.C. Nordlie, W.J. Arion, Glucose-6-phosphatase, in: W.A. Wood (Ed.), *Methods in Enzymology IX*, Academic Press, New York, 1966, pp. 619–625.
- [25] E. Beutler, Red cell Metabolism. A Manual of Biochemical Methods, Grune Stroutan, NY, 1975, 67–69.
- [26] L. Hue, H. Taegtmeyer, The Randle cycle revisited: a new head for an old hat, *Am. J. Physiol. Endocrinol. Metab.* 297 (2009) E578–E591.
- [27] M.L. Massa, J.J. Gagliardino, F. Francini, Liver glucokinase: an overview on the regulatory mechanisms of its activity, *IUBMB Life* 63 (2011) 1–6.
- [28] P.B. Iynedjian, Mammalian glucokinase and its gene, *Biochem. J.* 293 (1993) 1–13.
- [29] P.B. Iynedjian, Molecular physiology of mammalian glucokinase, *Cell. Mol. Life Sci.* 66 (2009) 27–42.
- [30] K. Xu, K.T. Morgan, A. Todd Gehris, T.C. Elston, S.M. Gomez, A whole-body model for glycogen regulation reveals a critical role for substrate cycling in maintaining blood glucose homeostasis, *PLoS Comput. Biol.* 7 (2011) e1002272, <http://dx.doi.org/10.1371/journal.pcbi.1002272>.
- [31] J. Xu, L. Gowen, C. Raphaelides, K.K. Hoyer, J.G. Weigner, M. Renard, J.J. Troke, B. Vaitheeswaran, W.N.P. Lee, M.F. Saad, M.W. Sleeman, M.A. Teitell, I.J. Kurland, Decreased hepatic futile cycling compensates for increased glucose disposal in the *Pten* heterodeficient mouse, *Diabetes* 55 (2006) 3372–3380.
- [32] Z. Spolarics, M. Meyenhofer, Augmented resistance to oxidative stress in fatty rats livers induced by a short-term sucrose rich diet, *Biochim. Biophys. Acta* 1487 (2000) 190–200.
- [33] R.F. Kletzien, Glucose-6-phosphate dehydrogenase: a housekeeping enzyme subject to issue-specific regulation by hormones, nutrients, and oxidant stress, *FASEB J.* 8 (1994) 174–181.
- [34] H.N. Kirkman, S. Galiano, G.F. Gaetani, The function of catalase-bound NADPH, *J. Biol. Chem.* 262 (1987) 660–666.
- [35] C.F. Burant, M. Saxena, Rapid reversible substrate regulation of fructose transporter expression in rat small intestine and kidney, *Am. J. Physiol.* 267 (1994) G71–G79.
- [36] H.Y. Koo, M.A. Wallig, B.H. Chung, T.Y. Nara, B.H.S. Cho, M.T. Nakamura, Dietary fructose induced a wide range of genes with distinct shift in carbohydrate and lipid metabolism in fed and fasted rat liver, *Biochim. Biophys. Acta* 1782 (2008) 341–348.
- [37] C.A. Roncal-Jimenez, M.A. Lanasa, C.J. Rivard, T. Nakagawa, L.G. Sanchez-Lozada, D. Jalal, A. Andres-Hernando, K. Tanabe, M. Madero, N. Li, C. Cicerchi, K. Mc Fann, Y.Y. Sautin, R.J. Johnson, Sucrose induces fatty liver and pancreatic inflammation in male breeder rats independent of excess energy intake, *Metabolism* 60 (2011) 1259–1270.
- [38] A. Korieh, G. Crouzoulon, Dietary regulation of fructose metabolism in the intestine and in the liver of the rat. Duration of the effects of a high fructose diet after the return to the standard diet, *Arch. Int. Physiol. Biochim. Biophys.* 99 (1991) 455–460.
- [39] L. Vilà, N. Roglans, V. Perna, R.M. Sánchez, M. Vázquez-Carrera, M. Alegret, J.C. Laguna, Liver AMP/ATP ratio and fructokinase expression are related to gender differences in AMPK activity and glucose intolerance in rats ingesting liquid fructose, *J. Nutr. Biochem.* 22 (2011) 741–751.
- [40] L. Vilà, A. Rebollo, G.S. Adalsteisson, M. Alegret, M. Merlos, N. Roglans, J.C. Laguna, Reduction of liver fructokinase expression and improved hepatic inflammation and metabolism in liquid fructose-fed rats after atorvastatin treatment, *Toxicol. Appl. Pharmacol.* 251 (2011) 32–40.
- [41] Y. Wang, J. Yang, J. Yi, Redox sensing by proteins: oxidative modifications on cysteines and the consequent events, *Antioxid. Redox Signal.* 16 (2012) 649–657.