



Fructose-induced inflammation, insulin resistance and oxidative stress: A liver pathological triad effectively disrupted by lipoic acid



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ABSTRACT

Aims: Fructose administration induces hepatic oxidative stress, insulin resistance, inflammatory and metabolic changes. We tested their potential pathogenic relationship and whether these alterations can be prevented by R/S- α -lipoic acid.

Main methods: Wistar rats received during 21 days a commercial diet or the same diet supplemented with 10% fructose in drinking water without/with R/S- α -lipoic acid injection. After this period, we measured a) serum glucose, triglyceride, insulin, homeostasis model assessment-insulin resistance (HOMA-IR), insulin glucose ratio (IGR) and Matsuda indexes and b) liver oxidative stress, inflammatory markers and insulin signaling pathway components.

Key findings: Fructose fed rats had hyperinsulinemia, hypertriglyceridemia, higher HOMA-IR, IGR and lower Matsuda indices compared to control animals, together with increased oxidative stress markers, TNF α , IL1 β and PAI-1 gene expression, and TNF α and COX-2 protein content. Whereas insulin receptor level was higher in fructose fed rats, their tyrosine-residue phosphorylation was lower. IRS1/IRS2 protein levels and IRS1 tyrosine-phosphorylation rate were lower in fructose fed rats. All changes were prevented by R/S- α -lipoic acid co-administration.

Significance: Fructose-induced hepatic oxidative stress, insulin resistance and inflammation form a triad that constitutes a vicious pathogenic circle. This circle can be effectively disrupted by R/S- α -lipoic acid co-administration, thus suggesting mutual positive interaction among the triad components.

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1. Introduction

High calorie diet is one of the characteristics of modern society, frequently accompanied by marked changes in meal nutrient composition, such as fructose and refined carbohydrates. Consequently many authors have ascribed to the latter an important causal role for current obesity and type 2 diabetes epidemics [1].

Liver is the primary site of fructose extraction and metabolism; thus, its increased availability induces several local alterations such as glucose metabolic dysfunction [2–4], fat deposit [4,5], an inflammatory state [6, 7] and insulin resistance [8]. Also, several pro-inflammatory cytokines are critically involved in insulin resistance and in the development of fatty liver [9].

Abbreviations: LA, R/S- α -lipoic acid; HOMA-IR, homeostasis model assessment-insulin resistance; IGR, insulin glucose ratio; HIS, hepatic insulin sensitivity; GSH, reduced glutathione; IR, insulin receptor.

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Several authors have proposed glycooxidative stress as a key mechanism responsible for the deleterious effect of fructose [10–12]. Supporting this concept we have already demonstrated that three weeks of fructose administration to normal rats is associated with a state of oxidative stress due to an imbalance between reactive oxygen species production and antioxidant capacity [4,13].

On account of the mentioned results we postulated that those alterations were specifically linked to an oxidative stress state triggered by fructose overload, and consequently, they could be effectively prevented by an antioxidant co-administration. In this regard, we have shown that co-treatment with R/S- α -lipoic acid (LA) prevented all the metabolic and endocrine dysfunctions induced by fructose rich diet [14,15]. We have not yet found however, the potential molecular link joining fructose-induced oxidative stress, the resultant inflammatory response and impaired insulin signaling pathway.

In order to answer this question, we have now evaluated the effect of a fructose overload on liver inflammatory markers, together with the hepatic insulin signaling pathway and the possible preventive effect of co-administration of LA on these parameters. The outcomes of this study would provide a more comprehensive view of the complex

Table 1
Primer sequences.

Gene	GenBank®	Sequences
IL1β	NM_031512.2	FW 5'-ACAAGGAGAGACAAGCAACGAC-3' RV 5'-TCTTCTTTGGGTATTGTTGGG-3'
PAI-1	NM_012620.1	FW 5'-CCACGGTAAGCAGTGGACT-3' RV 5'-TGCTGGCCTTAAGAAGGGG-3'
TNFα	NM_012675.3	FW 5'-GGCATGGATCTCAAGACAACC-3' RV 5'-CAAATCGGCTGACGGTGTG-3'
IRS1	NM_012969	FW 5'-TGTGCAAGCAACAGAAAG-3' RV 5'-ACGGTTTCAGAGCAGAGGAA-3'
IRS2	NM_001168633.1	FW 5'-CTACCCACTGAGCCCAAGAG-3' RV 5'-CCAGGGATGAAGCAGACTA-3'
β-Actin	NM_031144.2	FW 5'-AGAGGGAAATCGTGCCTGAC-3' RV 5'-CGATAGTGATGACCTGACCGT-3'

FW forward primer, RV reverse primer.

adaptive mechanisms put forward by the fructose-induced oxidative stress; additionally, they could facilitate implementation of evidence-based awareness against the consumption of unhealthy diets and for the prevention and treatment of type 2 diabetes.

2. Materials and methods

2.1. Chemicals and drugs

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

2.2. Animals

Adult male Wistar rats (150–180 g) were maintained at 23 °C on a fixed 12-h light–dark cycle (06:00–18:00 h) and divided into 3 different experimental groups: Control (C): standard commercial diet ad libitum and tap water, Fructose (F): commercial diet plus fructose 10% in the water, Fructose-LA (FL): F rats injected with LA (35 mg/kg, i.p.) during the last five days of diet. We measured and recorded daily water intake as well as weekly food consumption and individual body weight. The experiment was replicated 5 times (total: 20 animals per group). After three weeks of these treatments, blood samples from 4-h fasted animals were obtained from retroorbital plexus under light anesthesia (halothane) and collected to measure glucose, triglyceride and immunoreactive insulin levels. Rats were then immediately killed by decapitation and the liver median lobe was dissected to perform different proposed measurements. *Ethical Principles and Guidelines for Experimental Animals* (3rd Edition 2005) by the Swiss Academy of Medical Sciences was followed for animal care.

2.3. Serum measurements

In the blood samples obtained as explained above, glycemia was determined by the glucose-oxidase GOD-PAP method (Roche Diagnostics,

Table 2
Body weight and serum measurements at the end of the experiment.

Parameter	C	F	FL
Body weight change (g)	101 ± 4	96 ± 5	92 ± 6
Glucose (mg/dl)	113 ± 5	111 ± 5	115 ± 4
Triglyceride (g/l)	0.89 ± 0.05	1.71 ± 0.15*	0.73 ± 0.05 ^Δ
Insulin (ng/ml)	0.74 ± 0.04	1.15 ± 0.05*	0.72 ± 0.06 ^Δ
HOMA-IR	5.0 ± 0.2	8.2 ± 0.2*	5.3 ± 0.6 ^Δ
IGR	17.68 ± 0.7	27.96 ± 0.7*	16.9 ± 1.9 ^Δ
Matsuda index (k/FPI × FPG)	3.4 ± 0.2	2.2 ± 0.1*	3.5 ± 0.3 ^Δ

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

Mannheim, Germany); triglyceride levels were determined by an enzymatic reactions kit (TG color GPO/PAP AA, Wiener lab, Argentina). Immunoreactive insulin levels were measured by radioimmunoassay using an antibody against rat insulin, rat insulin standard (Linco Research Inc., IN, USA) and highly purified porcine ¹²⁵I-insulin. The homeostasis model assessment-insulin resistance (HOMA-IR) [Serum insulin (μU/ml) × fasting blood glucose (mM)/22.5] [16] and insulin-glucose ratio (IGR), were used to calculate insulin resistance. Both procedures have been shown to be valid to measure peripheral insulin sensitivity in rats [17,18]. Liver sensitivity to insulin (HIS) was also calculated as follows: k / (fasting plasma insulin) × fasting plasma glucose, where k: 22.5 × 18 [19].

2.4. Liver measurements

2.4.1. Protein carbonyl groups and reduced glutathione (GSH)

Protein carbonyl content was assayed as previously described [4]. Briefly, supernatant of centrifuged hepatic homogenates was incubated for 1 h at 25 °C with 10 mM 2,4-dinitrophenylhydrazine in 2 M hydrochloric acid and thereafter precipitated with trichloroacetic acid (20%). In order to wash precipitated proteins, the pellet was suspended in ethanol/ethyl acetate (1:1) and centrifuged three times. Proteins were finally solubilized in guanidine hydrochloride (6 M) and centrifuged to remove insoluble material. Carbonyl level was assayed spectrophotometrically (366 nm) and expressed as nmol of carbonyl residues/mg protein. GSH content was measured as previously described [4]. Briefly, liver homogenates were centrifuged and 1 ml of the supernatant was mixed with 1 ml trichloroacetic acid 10% and incubated at 4 °C for 1 h. The mixture was centrifuged for 20 min at 3500 × g and 100 μl of supernatant mixed with 900 μl trichloroacetic acid 5%, 2 ml Tris-HCl buffer 0.01 M (pH 8.9) and 50 μl 5,5'-dithiobis 2-nitrobenzoic acid 0.4% in methanol. GSH level was assayed spectrophotometrically (414 nm) and expressed as μmol of GSH/g tissue.

2.4.2. Total RNA

Total RNA from C, F, and FL rat livers was isolated using TRIzol Reagent (Gibco-BRL, Rockville, MD, USA) [20]. Agarose-formaldehyde gel electrophoresis and the 260/280 nm absorbance ratio were used to evaluate integrity and quality of isolated RNA while DNase I (Gibco-BRL) digestion to avoid DNA contamination. Reverse transcription-PCR was done with SuperScript III (Gibco-BRL) and total RNA (50 ng) as a template.

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

We employed a Mini Opticon Real-Time PCR Detector Separate MJR (BioRad) and SYBR Green I as a fluorescent dye for qPCR reactions. Ten nanograms of cDNA was amplified using FastStart SYBR Green Master mix (Roche Diagnostics GmbH, Mannheim, Germany) employing 40 cycles (denaturation at 95 °C for 30 s, annealing at 65 °C for 30 s and extension at 72 °C for 45 s). Negative controls were performed replacing samples by the same volume of water. Oligonucleotide primers (Invitrogen) used are listed in Table 1. All amplicons included fragments with a 90 to 250 bp size range. Reaction specificity was checked by melting curve analysis. Data are expressed as relative gene expressions after normalization to the β-actin housekeeping gene using Qgene96 and LineRegPCR software [21,22].

2.4.4. Western blot analysis

Liver homogenates from C, F and FL animals were used for TNFα, COX2, insulin receptor (IR), IRS1, IRS2 and β-actin immunodetection. Protein content of the samples was evaluated by Bio-Rad protein assay [23] and afterwards homogenates were treated with dithiothreitol and bromophenol blue (final concentration of 100 mM and 0.1%, respectively). One hundred micrograms of whole protein from each homogenate was loaded into 10% SDS-PAGE and transferred by electroblotting to PVDF membranes. Nonspecific binding sites were blocked by overnight

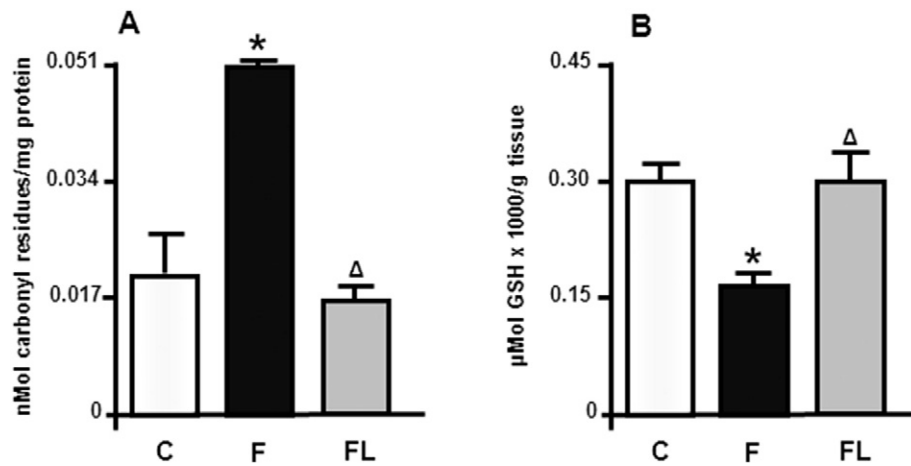


Fig. 1. Assessment of protein carbonyl groups (A) and reduced glutathione (GSH) (B) in liver tissue. C (white bars), F (black bars) and FL (gray bars) animals. Results are means \pm SEM of 5 different experiments run in triplicate. * $p < 0.05$ vs. C, and $^{\Delta}p < 0.05$ vs. F animals.

incubation with non-fat dry milk. Each protein was identified and quantified using specific primary antibodies against TNF α (1:1000), COX2 (1:1000), IR (1:2000), IRS1 (1:2000), and IRS2 (1:2000) overnight and β -actin (1:10,000) for 1 h. Thereafter membranes were incubated (75 min), with the corresponding secondary antibodies: peroxidase-conjugated AffiniPure donkey anti-rabbit IgG (final dilution, 1:10,000) for COX2, IR, IRS1 and IRS2 identification; anti-goat IgG biotinyl antibody (final dilution, 1:1000) for TNF α identification and anti-mouse IgG biotinyl antibody (final dilution, 1:20,000) for β -actin. Intensity of specific bands was determined by densitometry using Gel-Pro Analyser software. β -Actin density was used to normalize protein content: target protein relative content was divided by relative β -actin protein level in each group.

2.5. Statistical analysis

We used ANOVA and Dunnett's post-test for multiple comparisons and Bartlett's test to assess variance homogeneity. Results are expressed as means \pm SEM for the indicated number of observations. Differences between groups were considered significant when $p < 0.05$.

3. Results

3.1. Body weight and water intake

After the 21 day treatment, similar body weight changes were observed in all experimental groups (Table 2). Fructose treated rats (F and FL) drank a significantly larger volume of water than control animals (55 ± 11 and 47 ± 12 vs. 29 ± 2 ml/day; $p < 0.05$). By contrast, control rats had eaten a larger volume of solid food than fructose-fed rats (F and FL) (21 ± 1 vs. 16 ± 1 and 17 ± 1 g/animal/day; $p < 0.05$). Therefore, although differences were recorded in daily intake of food, calorie intake was comparable in all groups (C: 58 ± 3 ; F: 66 ± 5 ; FL: 66 ± 4 kcal/day).

Table 3
Relative to β -actin gene expression at the end of the experiment.

Gene	C	F	FL
TNF α	0.19 ± 0.05	$0.42 \pm 0.06^*$	$0.26 \pm 0.01^{\Delta}$
IL1 β	0.61 ± 0.04	$1.50 \pm 0.24^*$	$0.55 \pm 0.13^{\Delta}$
PAI-1	0.08 ± 0.02	$0.15 \pm 0.02^*$	$0.07 \pm 0.01^{\Delta}$

Values are means \pm SEM (n = 20). * $p < 0.05$ vs. C and $^{\Delta}p < 0.05$ vs. F.

3.2. Serum measurements

F rats had significantly higher triglyceride and serum insulin concentration than C rats with comparable glucose levels in all groups (Table 2). Consequently, higher HOMA-IR and IGR and lower HIS values were recorded in F rats demonstrating that they portray lower insulin sensitivity both in peripheral tissues and in the liver (insulin resistance state) (Table 2). Co-administration of LA to F animals drove the mentioned values to those recorded in C rats and, in the case of triglyceride, to even lower levels (Table 2). LA co-administration to F rats also prevented impairment of systemic and hepatic insulin sensitivity (Table 2).

3.3. Protein carbonyl groups and reduced glutathione (GSH)

Protein carbonyl content was significantly higher while GSH content was significantly lower in F compared to C rats. In LA-treated rats both marker values were comparable to those measured in C animals (Fig. 1).

3.4. Inflammatory markers

F rats evinced a significantly higher TNF α , IL1 β and PAI-1 relative to β -actin gene expression compared to control animals (Table 3). Relative protein levels of TNF α and COX2 were also higher in F than in C rats, thus showing increased inflammatory reaction in F rats. LA administration to F rats fully prevented the increase in gene expression but only partially that of protein expression (Fig. 2).

3.5. Insulin pathway

Whereas relative to β -actin IR protein level was significantly higher in F compared to C rats, its tyrosine residue phosphorylation rate was lower. On the other hand, the relative IRS1 and IRS2 protein levels and the IRS1 phosphorylation rate were lower in F than in control rats (Fig. 3). LA administration rendered IR, pTyr-IR, IRS1 and pTyr-IRS1 values comparable to those measured in C rats (Fig. 3). There were no significant differences in IRS1 and IRS2 relative gene expression levels among groups (IRS1: C = 0.35 ± 0.05 ; F = 0.36 ± 0.04 and FL = 0.38 ± 0.05 ; IRS2: C = 3.4 ± 0.7 ; F = 4.4 ± 1.1 and FL = 4.3 ± 1.3).

4. Discussion

As previously shown by our group, we are currently reporting that animals fed for three weeks with a fructose rich diet present high

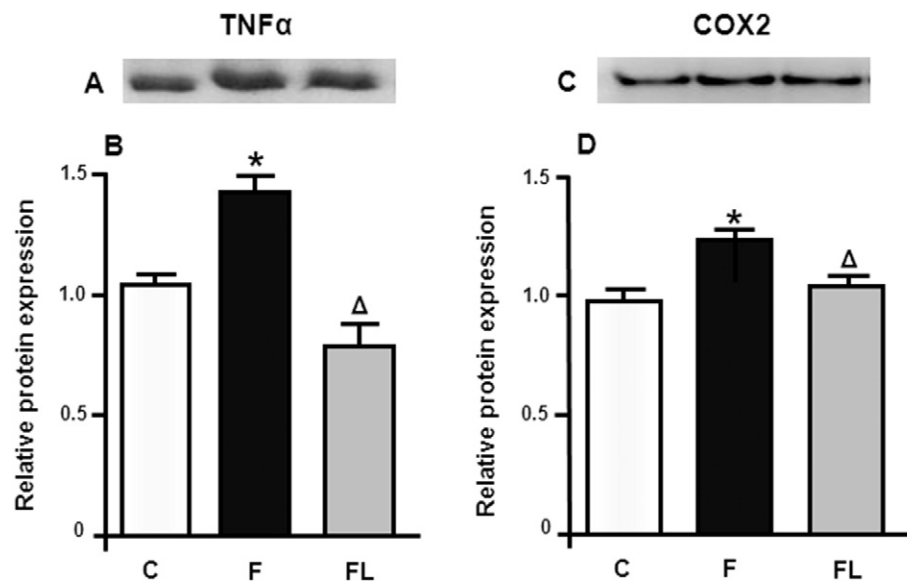


Fig. 2. TNF α and COX2 protein level in liver tissue. Band intensity for TNF α (B) and COX2 (D) in C (white bars), F (black bars) and FL (gray bars) animals. Representative blots show the bands corresponding to TNF α (A) and COX2 (C). β -Actin density was used to normalize protein content: target protein relative content was divided by relative β -actin protein level in each group. Results are means \pm SEM of 5 different experiments run in triplicate. * p < 0.05 vs. C, and Δp < 0.05 vs. F animals.

serum levels of triglyceride and insulin as well as higher HOMA-IR values together with normal glucose and an increased oxidative stress [3,4,13,14]. In these rats we have also currently measured an increase in TNF α , IL1 β and PAI-1 gene expression together with a rise in TNF α and COX2 protein level; the latter represents a key enzyme involved in prostaglandin production, compounds closely related to inflammatory and oxidative stress processes [24]. Thus all these markers demonstrate the presence in F rats of an inflammatory state and its potential link with oxidative stress.

F rats simultaneously portray a decrease in IRS1 and IRS2 protein levels as well as in tyrosine-residue phosphorylation of insulin receptor and of IRS1. Conversely, these animals show a higher insulin-receptor protein level. These data demonstrate that the liver insulin resistance and general insulin resistance depicted in F rats (low HIS and high IGR plus high HOMA-IR values), might be ascribed to an impaired intracellular insulin mediator cascade rather than to a decrease in its receptor availability. We could thus assume that the impaired tyrosine phosphorylation must be the consequence of increased oxidative stress and inflammatory processes triggered by high fructose intake.

How can we put together all these metabolic, endocrine and inflammatory dysfunctions in a reasonable pathogenic sequence? To answer this question we have earlier studied the chronological sequence of events triggered by fructose administration to normal rats. We found that increased serum levels and liver content of triglyceride together with an increase in liver GK activity were the earliest alteration recorded in these animals (one week after fructose administration), followed by an increase in liver carbonyl/decrease in GSH content at two weeks, while increased serum insulin levels and HOMA β (β cell reaction), HOMA IR and G6Pase activity only appeared after the third week of treatment [25]. These data suggest that the overload of metabolic substrate (triglyceride) would trigger liver (and also other tissues) oxidative stress with a later β -cell compensatory response to this metabolic impairment. On time, oxidative stress would favor inflammatory reaction (showed in our case by increase in TNF α , IL1 β and PAI-1 RNA levels and a rise in TNF α and COX2 protein level), data largely supported by other authors' reports [26–29]. Thus, these two processes would settle a positive feedback pathological mechanism.

The negative impact of oxidative stress on tyrosine phosphorylation was early described by Brownlee and later confirmed by several authors [30–32].

The report of Furukawa et al. [33] that H₂O₂ induces expression of PAI-1, TNF α and IL6, lends support to the possible link between oxidative stress, inflammation and insulin sensitivity; the recent report of Renaud et al. [28] also reinforces the relationship between intake of imbalanced diet, oxidative stress and liver inflammation processes.

Regarding the negative effect of inflammatory process on insulin sensitivity, it has been shown that a) fructose down-regulates liver insulin receptor substrate 2 [34], b) TNF α switches tyrosine to serine phosphorylation of IRS1 [35,36], while ob/ob obese mouse and fa/fa rats without functional TNF α or without TNF α receptor have higher insulin sensitivity [37], c) C-reactive protein induces phosphorylation of insulin receptor substrate-1 on Ser307 and Ser612 rather than in Tyr residues decreasing its activity [38] and d) IL1 β reduces RNA and protein level of IRS1 [39] while knock-down of IL-1Ra ameliorates hepatic inflammation and insulin sensitivity in obese mice [40].

Supporting all these sequential pathogenic events, our data shows that LA co-administration decreased hepatic oxidative stress burden as well as all the metabolic, endocrine and inflammatory changes induced by fructose overload.

On account of our results and the above-mentioned evidence, we could postulate that high fructose administration, even for a relative short period, sets in motion two initial processes, oxidative stress and inflammation, that working in complementary way decrease liver insulin sensitivity. Thereafter, this pathological triad establishes an active vicious circle that self-sustains the development of wider liver dysfunction. The effective preventive action of LA co-administration to rats fed with fructose rich diet supports this assumption.

As previously shown, LA has anti-inflammatory properties [41,42], decreasing transcription levels of IL-1 β , IL-6 and TNF α [43], an effect ascribed to its interaction with PI3K signaling pathway [44]. However, LA effect might play a more specific and active role disrupting the mechanism by which fructose induces development of this pathological triad. Endogenous lipoic acid is synthesized by lipoic acid synthase and its deficient activity impairs the antioxidant defense system [45]. In that condition, Padmalayam et al. observed an exacerbation of the inflammatory state, mitochondrial dysfunction and insulin resistance. Consequently, they postulated that the increase of lipoic acid synthase expression to enhance mitochondrial levels of LA would be a promising strategy to potentially improve mitochondrial function, thus reducing oxidative stress, inflammation and insulin resistance [46]. Further studies are necessary to prove this challenging hypothesis.

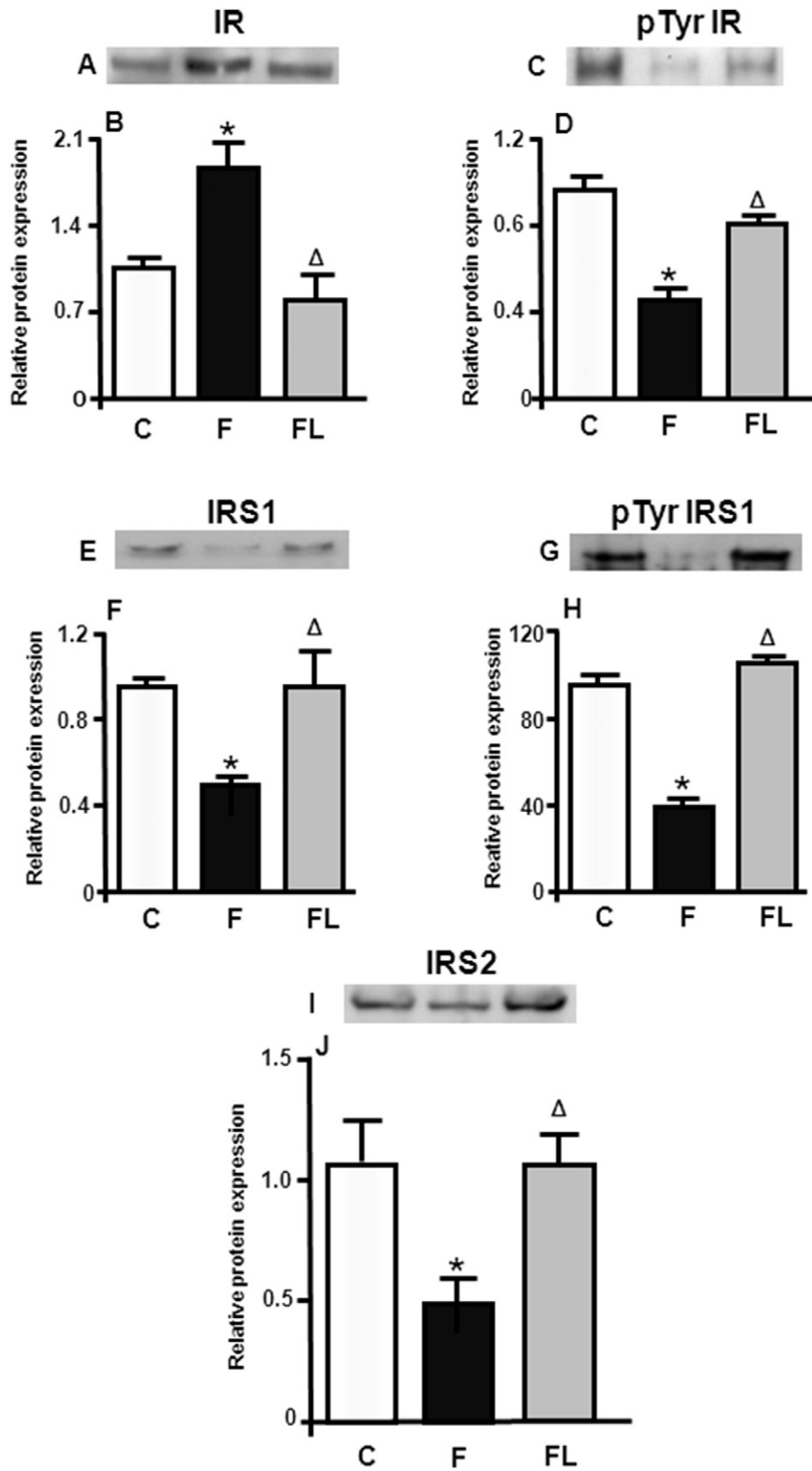


Fig. 3. Proteins involved in insulin signaling pathway in liver tissue. IR (B), pTyrIR (D), IRS1 (F), pTyrIRS1 (H) and IRS2 (J) protein level in C (white bars), F (black bars) and FL (gray bars) animals. Representative blots show the bands corresponding to IR (A), pTyrIR (C), IRS1 (E), pTyrIRS1 (G) and IRS2 (I). β -Actin density was used to normalize protein content: target protein relative content was divided by relative β -actin protein level in each group. Results are means \pm SEM of 5 different experiments run in triplicate. * $p < 0.05$ vs. C, and $^{\Delta}p < 0.05$ vs. F animals.

5. Conclusions

Our data suggest that fructose induces development of a pathological triad and triggers a vicious pathogenic circle that seriously affects liver function; this circle could be effectively disrupted by LA co-administration. All together our results open new avenues to explore potential strategies to prevent and treat diet-induced liver dysfunction.

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