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Abstract: High consumption of fructose-sweetened beverages has been linked to a high prevalence of chronic metabolic diseases. We have previously shown that a short course of fructose supplementation as a liquid solution induces glucose intolerance in female rats. In the present work, we characterized the fructose-driven changes in the liver and the molecular pathways involved. To this end, female rats were supplemented or not with liquid fructose (10% w/v) for 7 or 14 days. Glucose and pyruvate tolerance tests were performed, and the expression of genes related to insulin signaling, gluconeogenesis and nutrient sensing pathways was evaluated.
Fructose-supplemented rats showed increased plasma glucose excursions in glucose and pyruvate tolerance tests and reduced hepatic expression of several genes related to insulin signaling, including insulin receptor substrate-2 (IRS-2). However, the expression of key gluconeogenic enzymes, glucose-6-phosphatase and phosphoenolpyruvate carboxykinase, was reduced. These effects were caused by an inactivation of hepatic forkhead box O1 (FoxO1) due to an increase in its acetylation state driven by a reduced expression and activity of sirtuin 1 (SIRT1). Further contributing to FoxO1 inactivation, fructose consumption elevated liver expression of the spliced form of X-box-binding-protein-1, as a consequence of an increase in the activity of the mammalian target of rapamycin 1 and protein 38-mitogen activated protein kinase (p38-MAPK). Liquid fructose affects both insulin signaling (IRS-2 and FoxO1) and nutrient sensing pathways (p38-MAPK, mTOR and SIRT1) thus disrupting hepatic insulin signaling without increasing the expression of key gluconeogenic enzymes.

Cover Letter

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Barcelona, October 22nd 2013

Dear Editor:

We are re-submitting to the *Journal of Nutritional Biochemistry* the manuscript entitled

"LIQUID FRUCTOSE DOWNREGULATES LIVER INSULIN RECEPTOR SUBSTRATE

2 AND GLUCONEOGENIC ENZYMES BY MODIFYING NUTRIENT SENSING FAC-

TORS IN RATS", authored by Alba Rebollo, Núria Roglans, Miguel Baena, Anna Pa-

drosa, Rosa M Sánchez, Manuel Merlos, Marta Alegret and Juan C Laguna. Following

your instructions, we have included points 1 and 4 of the "response to reviewer" docu-

ment in the first paragraph of the Discussion section.

Yours sincerely,

Dr. Juan C. Laguna

*Response to Reviewers

Dear Reviewer, following your suggestions, we have included responses to queries 1 and 4 in the first paragraph of the discussion section, as well as supplemental Table 2, corresponding to the estimated nutrient requirements for maintenance and growth of rats

LIQUID FRUCTOSE DOWNREGULATES LIVER INSULIN RECEPTOR

SUBSTRATE 2 AND GLUCONEOGENIC ENZYMES BY MODIFYING NUTRIENT

SENSING FACTORS IN RATS

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Running Title: FRUCTOSE REDUCES LIVER IRS2, G6Pc AND PEPCK

Abstract:

High consumption of fructose-sweetened beverages has been linked to a high prevalence of chronic metabolic diseases. We have previously shown that a short course of fructose supplementation as a liquid solution induces glucose intolerance in female rats. In the present work, we characterized the fructose-driven changes in the liver and the molecular pathways involved. To this end, female rats were supplemented or not with liquid fructose (10% w/v) for 7 or 14 days. Glucose and pyruvate tolerance tests were performed, and the expression of genes related to insulin signaling, gluconeogenesis and nutrient sensing pathways was evaluated. Fructose-supplemented rats showed increased plasma glucose excursions in glucose and pyruvate tolerance tests and reduced hepatic expression of several genes related to insulin signaling, including insulin receptor substrate-2 (IRS-2). However, the expression of key gluconeogenic enzymes, glucose-6-phosphatase and phosphoenolpyruvate carboxykinase, was reduced. These effects were caused by an inactivation of hepatic forkhead box O1 (FoxO1) due to an increase in its acetylation state driven by a reduced expression and activity of sirtuin 1 (SIRT1). Further contributing to FoxO1 inactivation, fructose consumption elevated liver expression of the spliced form of X-box-binding-protein-1, as a consequence of an increase in the activity of the mammalian target of rapamycin 1 and protein 38-mitogen activated protein kinase (p38-MAPK). Liquid fructose affects both insulin signaling (IRS-2 and FoxO1) and nutrient sensing pathways (p38-MAPK, mTOR and SIRT1) thus disrupting hepatic insulin signaling without increasing the expression of key gluconeogenic enzymes.

Introduction

During the last decade, there has been an increasing number of published epidemiological and interventional studies on human populations linking the high consumption of sugar-sweetened beverages, enriched in simple sugars such as fructose and glucose, to the high prevalence of chronic metabolic and related cardiovascular diseases [1-4]. These diseases include dyslipidemia [5,6], gout [7], hypertension [8,9], obesity [10,11], insulin resistance [12] and type 2 diabetes mellitus [10, 13-15]. High energy intake, lack of adequate energy compensation through a proportional decrease in the amount of energy ingested as solid foods, and the special metabolism of fructose, have been reported to contribute to this possible causal association [2,3].

Among the experimental animal models used, fructose-fed rats can reproduce metabolic alterations induced by fructose consumption in humans. Rats, like humans, cannot transform ingested fructose into glucose [16]. Moreover, in both rats and humans, continuous fructose ingestion induces the expression of fructokinase, an enzyme that drives fructose into liver metabolic pathways [17-19]. The changes associated with the human metabolic syndrome can be reproduced in rats by providing them fructose as a 10% w/v solution [20-22]. This mimics the level of dietary consumption of sweetened beverages (as a percentage of the daily caloric intake about 30%) that leads to clear metabolic disturbances in humans [23,24]. Using this model, we recently discovered a gender difference in the response to fructose-consumption, with clear signs of glucose intolerance after a short 14-day course of fructose supplementation in female, but not male, rats [17]. We also found

that fructose-fed female rats displayed a significantly reduced expression of liver insulin-related substrate-2 (IRS-2) [17], one of the key insulin-signaling proteins in the liver [25].

We therefore pursued the search for the molecular mechanism underlying the glucose intolerance observed after fructose supplementation in female rats. In the present work, we show that fructose induces a set of complex molecular alterations in the liver expression of nutrient sensing factors which reduces the expression of both liver IRS-2 and gluconeogenic enzymes in fructose-supplemented female rats.

Methods

Animals and experimental design

Female Sprague-Dawley rats purchased from Charles River (Barcelona, Spain) were maintained with water and food *ad libitum* at constant humidity and temperature with a light/dark cycle of 12 hours. The animals were randomly separated into a control and a fructose-supplemented group (8 and 12 rats per group, respectively). Fructose was supplied as a 10 % (weight/volume) solution in drinking water for 7 and 14 days. Control animals received no supplementary sugar. At the end of this period, animals were killed by decapitation under isoflurane anesthesia at 10 a.m. Food and fructose solution were removed at 8 a.m. To reduce the variability in plasma estrogen concentrations, female rats were killed during the diestrus period.

Subgroups of female rats were randomly separated into control and fructose-supplemented groups, as described above, for glucose and pyruvate tolerance tests.

All procedures were conducted in accordance with the guidelines established by the University of Barcelona's Bioethics Committee, as stated in the Autonomous Government of Catalonia's Law 5/1995 (21st July).

Sample preparations

Blood and liver tissue samples were collected and stored as described previously [21]. Total and nuclear extracts were isolated using the Helenius method [26]. Protein concentrations were determined by the Bradford method [27].

Lipids, glucose, insulin, adiponectin and leptin analysis

Plasma triglyceride, glucose, insulin, leptin, and adiponectin concentration, were measured as described previously [21].

Glucose Tolerance test

After a two hour fast, the rats were anesthetized, and following the collection of an unchallenged sample (*time 0*), a glucose solution of 2 g/kg body weight was administered into the peritoneal cavity. During the test, blood was collected from the saphenous vein at 15, 30, 60, 90, and 120 min after glucose administration. Glucose measurements were performed using a hand-held glucometer. Plasma insulin levels were measured at baseline, 15, 60, and 120 min post-glucose administration using a rat insulin ELISA kit (Millipore, Billerica, MA).

Pyruvate tolerance test

Rats were injected with sodium pyruvate (2 g/kg i.p.). Plasma glucose levels werre determined at 0, 15, 30, 60, 90, 120, and 150 minutes after injection with a hand-held glucometer as described above.

Cell culture

Rat hepatoma FaO cells were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). The cells were cultured in low glucose DMEM (Gibco, Life Technologies, Madrid, Spain) supplemented with 10% fetal bovine serum (FBS Gold, PAA, Pistcataway, NJ) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin, Gibco, Life Technologies, Madrid, Spain). When cells reached 80% confluence, the proportion of serum was reduced to 1% and the cells were incubated in the absence or presence of fructose (Sigma-Aldrich, St. Louis, MO) at a concentration of 25 mM for 24 hours.

RNA preparation and analysis

Total RNA was isolated using the TrizolR reagent (Invitrogen, Life Technologies, Madrid, Spain). The levels of specific mRNAs were assessed by real-time reverse transcription–polymerase chain reaction (RT–PCR) using Sybergreen PCR Master Mix, specific primers and the Applied Biosystems One-Step Plus sequence detection system (Applied Biosystems, Foster City,CA). For G6Pc, PEPCK and XBP-1, we used conventional RT-PCR as described previously [34]. Adenosyl phosphoribosyl transferase (APRT) or 18S was used as an internal control. The primer sequences, resulting PCR products and number of cycles are listed in Supporting Table 1.

Western-blot analysis

Ten to thirty micrograms of different protein fractions from the rat livers were subjected to SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to Immobilon polyvinylidene difluoride transfer membranes (Millipore, Billerica, MA), blocked for 1 h at room temperature with 5% non-fat milk solution in 0.1% Tween-20-Tris-buffered saline (TBS), and incubated as described previously [21]. Detection was performed with the ECL chemiluminescence kit for HRP (Amersham GE Healthcare Europe GmbH, Barcelona, Spain). To confirm the uniformity of protein loading, the blots were incubated with the β-actin or β-tubulin antibody (Sigma-Aldrich, St. Louis, MO) as a control. The size of the detected proteins was estimated using protein molecular-mass standards (Invitrogen, Life Technologies, Madrid, Spain). Antibodies were obtained from Santa Cruz Technologies (Santa Cruz, CA), except those for phospho- and total protein kinase B or Akt, phospho- and total p38-MAPK and PP2Ac, which were obtained from Cell Signaling (Danvers, MA), and phospho- and total mTOR, purchased from Millipore (Billerica, MA).

Co-immunoprecipitation

Two hundreds micrograms of protein extracts were precleared in preclearing matrix F (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at 4 ºC, and the resulting supernatant was immunoprecipitated for 4 h at 4 °C with 4 µg of anti-acetyl-lysine (Cell Signalling, Denvers, MA) in a final volume of 0.5 mL made up with buffer containing 10 mM phosphate-buffered saline (PBS) and 2% bovine serum albumin (BSA). Immunocomplexes were captured by incubating the samples with protein A-agarose suspension (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C on a rocker platform. Agarose beads were collected by centrifugation and washed three times with PBS-containing protease inhibitors. After microcentrifugation, the

pellet was re-suspended with 60 µL of SDS-PAGE sample buffer and boiled for 5 min at 100 °C. The supernatant was subjected to electrophoresis on 10% SDS-PAGE and immunoblotted with an antibody against FoxO1 (Santa Cruz Biotechnology, Santa Cruz, CA).

PCR-arrays

Total RNA was isolated from control and fructose-fed rats as described above, and then purified using RNeasy kit columns (Qiagen Iberia, Madrid, Spain). Single stranded cDNA and PCR arrays were performed using the *Rat Insulin Signaling Pathway RT^2 Profiler PCR Array* (PARN-030E) from SABiosciences (Quiagen Iberia, Madrid, Spain), following the manufacturer's guidelines. Array data processing and analysis were performed using a Web portal from SABiosciences.

Statistics

The results are expressed as the mean of *n* values $\pm$ standard deviation. Plasma samples were assayed in duplicate. Significant differences were established by the unpaired *t*-test, one-way ANOVA test (with *a posteriori* analysis) for cell-culture experiments, or the two-way ANOVA test (with *a posteriori* analysis) for plasma analytes, using the computer program GraphPad InStat (GraphPad Software V2.03). When the number of animals was too small or the variance was not homogeneous, a non-parametric Mann-Whitney test was performed for comparing two groups. * $P < 0.05$, ** $P < 0.01$; [#] $P < 0.001$ 14-day control *vs* 7-day control, in Tables and Figures.

Results

A 14-day course of fructose ingestion in liquid form is sufficient to induce glucose intolerance in female Sprague-Dawley rats. Fructose administration as a 10 % weight/volume liquid solution to female Sprague-Dawley rats increased energy intake by 26% and 29% after 7- and 14 days of supplementation, respectively, compared to the amount of energy consumed by control animals, despite fructose-supplemented rats reducing the ingestion of their solid diet by 22%. In agreement with our previous results [17], fructose consumption by female rats did not modify body weight, but increased plasma glucose and insulin concentrations only after the 14-day course of fructose-supplementation, thus significantly reducing the insulin sensitivity index (ISI) in these animals (Table 1). Furthermore, only the rats supplemented for 14 days displayed changes in their glucose tolerance test (GTT), with a significant 30% increase in the area under the curve (AUC) value for plasma glucose concentration (Table 1). Again, confirming our previous results [17], livers from 14-day fructose-supplemented rats showed a marked reduction in the expression of IRS-2 protein (Figure 1a), the main transducer of insulin signaling in liver tissue [28], and a significant increase (1.28-fold) in the amount of the active form of protein phosphatase 2 A (PP2Ac), a marker of increased fructose metabolism (Figure 1b). Moreover, in FaO rat hepatoma cells, incubation with 25 mM fructose for 24 hours blunted the increase in the IRS-2 protein levels induced by a short course (10 min) of insulin treatment (Supporting Figure 1a). Thus, to confirm fructose-mediated impairment of insulin signaling, we determined the expression of V-akt murine thymoma viral oncogene homolog-2 (Akt-2), one of the main transducers of insulin signaling downstream of IRS-2, *in vivo* and in FaO

cells. Despite the mild hyperinsulinemia present in Sprague-Dawley rats after 14-days of fructose-supplementation (see Table 1), neither the active, phosphorylated form of Akt, nor total Akt was not significantly affected in the livers of these rats (Figure 1c and d). Moreover, although insulin could significantly increase the amount of phosphorylated Akt in FaO cells (1.66-fold), this increase was partially blocked by incubation with 25 mM fructose for 24 hours (Supporting Figure 1b). At the same time, fructose markedly elevated the amount of total Akt in insulin-treated FaO cells, further decreasing the ratio of phosphorylated-Akt to total Akt with respect to insulin-treated cells (1.94 *vs* 0.88, 0.45-fold). Due to the decreased liver-insulin signaling in fructose-supplemented rats, the bolus-administration of a direct gluconeogenic precursor such as pyruvate, significantly increased (1.33-fold) the liver output of glucose in rats after 14-days of fructose (Figure 1e and 1f).

Fructose disrupts insulin signaling in the liver. To gain an overview of the effect of fructose on the expression of genes related to insulin signaling, we applied the Rat Insulin Signaling Pathway RT^2 Profiler PCR Array (see Experimental Procedures) to liver samples obtained from female rats supplemented with fructose for 7- and 14-days. As shown in Table 2, two clear patterns of changes in gene expression were detected: (1) an increase in the expression of genes related to lipid synthesis, such as *acc* (acetyl-CoA Carboxylase), *fas* (fatty acid synthase) and *l-pk* (liver-pyruvate kinase) at the two time-points studied; and (2) a decrease in the expression of genes related to insulin and insulin-growth factor signaling, such as *irs-2*, *cap1* (adenylate cyclase associated protein 1), and *dok1* and *3* (docking protein 1 and 3), confirming a deficit in the expression of key genes involved in insulin signal transduction. Surprisingly, the expression of two key gluconeogenic genes, *g6pc*

(glucose-6-phosphatase) *and pck* (phosphoenolpyruvate carboxykinase or PEPCK), was hardly affected in the livers of 14-day fructose-supplemented rats. Theoretically, disrupted insulin-signalling should trigger an increase in liver gluconeogenesis, as shown by the results obtained in the pyruvate-chalenge test (see Figure 1e and 1f), mainly through the increased expression and activity of G6Pc and PEPCK [29]. In fact, when we reassessed the mRNA levels fof G6Pc and PEPCK using gene-specific conditions, we detected significant reductions in the expression of G6Pc and PEPCK in the livers of 14-day fructose-supplemented rats (0.74- and 0.36-fold for G6Pc and PEPCK, respectively) (Supporting Figure 2a and b). The liver protein levels of both enzymes were also decreased (0.76- and 0.58-fold for G6Pc and PEPCK, respectively), although not significantly (Supporting Figure 2c and d).

To explain the increased hepatic glucose output despite a decreased expression of key gluconeogenic enzymes, we examined the expression of other enzymes controlling the substrate flux from cytosolic pyruvate to glucose formation (Figure 2). We found an increased expression of malate dehydrogenase (1.39-fold) and malic enzyme (2.90-fold) in the livers of 14-day fructose-supplemented rats (Figure 2e and 2f). In the case of very high concentrations of pyruvate, such as in the pyruvate tolerance test, the combined increase in the activities of malate dehydrogenase and malic enzyme could generate an amount of oxalacetate (substrate of PEPCK) sufficient to significantly increase the liver output of glucose in 14-day fructose-supplemented rats, despite the reduced expression of both G6Pc and PEPCK. Thus, we decided to explore the possible molecular mechanisms involved in the fructose-mediated reduction of liver gluconeogenic enzymes expression.

Fructose selectively modifies key liver transcription factors involved in the expression of the *g6pc and pck* genes. Peroxisome proliferator coactivator-1 alpha (PGC-1α) and FoxO1 transcription factors control the liver expression of the *g6pc and pck* genes [30,31]. Very recently, the spliced form of X-box-binding-protein-1 (XBP-1s) was directly implicated in the regulation of liver glucose homeostasis through an interaction with FoxO1 [32]. Hence, we were interested in assessing the expression of these transcription factors in liver samples from 7- and 14-day fructose-supplemented rats. Although the expression of PGC-1α was not modified (data not shown), fructose supplementation significantly increased XBP-1s expression, especially after 14-days (Figure 3). The effect of fructose on FoxO1 expression was very complex. Activation of the insulin signaling cascade, through the PIK3-IRS-2-Akt pathway, phophorylates FoxO1, which then exits the nucleus and translocates to the cytosol, leading to the suppression of gluconeogenic gene expression [31]. In liver samples from 14-day fructose-supplemented rats, the amount of the inactive, phosphorylated form of FoxO1 was increased (1.78-fold), while the total amount of FoxO1 protein was decreased (0.87-fold), further increasing the ratio of inactive phospho-FoxO1 to total FoxO1 (2.10-fold) (Figure 4a, 4b and 4c). Despite this, the amount of FoxO1 protein present in liver nuclear extracts rose by 2.08-fold in the livers of 14-day fructose-supplemented rats (Figure 4d). Thus, although the liver insulin- IRS-2-Akt signaling pathway was suppressed by fructose (see Figure 1), FoxO1 was phosphorylated (and inactivated), but remained in the nuclear compartment.

Fructose significantly changes the activity and expression of key nutrient-sensing molecules in the liver. To decipher the complex changes induced by fructose in insulin signaling, we focused our attention on three key molecular transducers involved in nutrient sensing in liver cells: mTOR [33], the stress kinase p-38 MAPK [34], and SIRT1 [33]. As shown in Figure 5, fructose enhanced the phosphorylated, active form of mTOR (Figure 5a) (1.7-fold) and p-38 MAPK (Figure 5b) (2.7-fold) only in the livers of 14-day fructose-supplemented rats. On the contrary, SIRT1 protein levels were markedly reduced (Figure 6a) (0.51-fold), again only in the livers of 14-day fructose-supplemented rats. To confirm that both SIRT1 expression and activity were decreased by fructose supplementation, we assessed the presence of one of its substrates, acetylated-FoxO1 [35]. Indeed, the amount of acetylated-FoxO1 was markedly increased in liver samples from 14-day fructose-supplemented rats (Figure 6b), consistent with the decreased SIRT1 activity in these samples. When we assessed the effect of fructose on the expression of mTOR, p-38 MAPK and SIRT1 in FaO cells, only the phosphorylated, active form of p-38 MAPK was increased (1.23-fold, Supporting Figure 3b).

Discussion

We previously demonstrated that a short course (14 days) of fructose supplementation as a liquid solution was sufficient to induce glucose intolerance only in female, but not male, rats [17]. In the present work, we detected a clear rise in the liver glucose output after a bolus administration of a direct gluconeogenic precursor, such a pyruvate, to 14-day fructose-supplemented rats. Higher than usual plasma concentrations of glucose in control animals were due to the fact that rats were fasted only two hours before sacrifice (see Animals and experimental design). However, we were surprised to note a significant reduction in the expression of two key gluconeogenic enzymes, PEPCK and G6Pc, despite a clear impairment in insulin signaling in the livers of these rats. Thus, we searched for the molecular cues that could explain this situation. Our main findings indicate that through a complex chain of molecular changes affecting both insulin signalling (IRS-2 and FoxO1) and nutrient sensing pathways (p38-MAPK, mTOR and SIRT1), fructose metabolism in the liver disrupt insulin signalling without affecting, or even decreasing, the expression of key gluconeogenic enzymes. These results suggest that the path from fructose consumption to the production of insulin resistance is not as straightforward as previously thought. Moreover, these changes seem to be specifically related to fructose consumption, and not to general changes in the amount of solid food ingested or total energy intake. Although solid food consumption was reduced by 22% by fructose ingestion, the amount of macro- and micronutrients ingested daily by the fructose-supplemented animals was equally or above the estimated nutrient requirements for maintenance and growth of rats (see Supplemental Table 2). Furthermore, we have shown previously that rats supplemented with a 10 %

(weight/volume) liquid solution of glucose, despite ingesting the same amount of energy as rats supplemented with a 10% liquid solution of fructose, they did not show similar changes in main metabolic parameters, such as plasma and liver triglyceride, PPARα expression and β-oxidation activity [21].

p38-MAPK is a stress-related kinase [34] whose activity can be increased by the metabolic burden imposed by fructose metabolism in hepatocytes through two mechanisms: increased activity of protein phosphatase 2A [36] and the presence of bacterial toxins in blood, as a result of fructose-mediated changes in the intestinal barrier permeability [37,38]. Indeed, we could detect a clear increase in the phosphorylated, activated form of p38-MAPK only in the liver samples of 14-day fructose-supplemented rats, which coincided with an increased expression of the catalytic form of PP2A.

It has been described that increased p38-MAPK activity, by phosphorylating the tuberous sclerosis 2 gene product or tuberin, could release its inhibitory activity on mTORC1 (mammalian Target of Rapamicyn Complex 1) [39]. The mTOR signaling pathway transduces information from different signals, such as growth factors, amino acids, and energy overload in the cell [40] . In accordance with an increased energy load and p38-MAPK activity, liver samples from 14-day fructose-supplemented rats showed a clear increase in the phosphorylated, activated form of mTOR. As it has been shown that mTOR activation causes IRS-2 degradation [41], the increase in mTOR activity could be the final factor resulting in the decreased liver expression of IRS-2 induced by fructose-consumption. Furthermore, since p38-MAPK can phosphorylate FoxO1 [42], the increased p38-MAPK activity could

explain the elevated amount of phosphorylated, inactive FoxO1 in the livers of 14-day supplemented rats, despite the lack of insulin-mediated Akt activation.

FoxO1 presented two other anomalies in the livers of 14-day supplemented rats, namely a reduction in its total cellular amount and a nuclear localization, despite being hyperphosphorylated. A recent report demonstrated that XBP-1, an endoplasmic reticulum stress transcription factor, plays an essential role in maintaining plasma glucose concentration and glucose tolerance [32], interacting with FoxO1 and directing it toward proteasome degradation, as well as improving the control of plasma glucose concentrations even without enhancing insulin signalling. Moreover, it has been described that mTORC1 activity increases the splicing of XBP-1 [43], while p38-MAPK phosphorylates the splice-derived protein, facilitating its nuclear localization and activity [44]. Indeed, in accordance with the increased activity of mTOR and p38-MAPK, there was a marked increase in the spliced form of XBP-1 mRNA and nuclear protein in the livers of 14-day fructose-supplemented rats, thus giving a plausible explanation for the reduced amount of total FoxO1 protein in these samples.

FoxO1 activity can be regulated not only by phosphorylation, but also by acetylation. Liu et al. described that acetylated-FoxO1, although displaying reduced transcriptional activity, is retained mainly in the nucleus [45]. The NAD^+-dependent deacetylase SIRT1 controls metabolic processes in response to low nutrient availability [46], mediating the direct deacetylation of several transcription factors including FoxO1 [35]. Situations of energy overload, such as a high-fat diet, reduces SIRT1 expression and/or activity [47]. Consistently with the increased amount of

energy consumed by fructose-supplemented rats, SIRT1 expression was significantly reduced in the livers of 14-day fructose-supplemented rats. As a consequence of the reduced deacetylation activity, the amount of acetylated-FoxO1 was increased in the same samples, thus explaining the increased nuclear localization of FoxO1 in the livers of fructose supplemented rats.

The combined increases in the liver activities of malate dehydrogenase and malic enzyme could explain the increased liver output of glucose in 14-day fructose-supplemented rats submitted to the pyruvate tolerance test, despite the reduced expression of both G6Pc and PEPCK. Nevertheless, this situation indicates that in these animals, the altered ISI is not due to an increased output of liver glucose. New experiments should be performed in order to ascertain the existence of a state of skeletal muscle insulin resistance in 14-day fructose-supplemented rats. Decreased muscular consumption of glucose could explain the observed increase in the AUC for the glucose tolerance test in 14-day fructose-supplemented rats.

Further studies using chronic fructose supplementation should be performed in order to ascertain whether this situation persists in time or evolves to a typical liver insulin resistance with increased gluconeogenesis. This is an important issue, given the epidemiological evidence relating consumption of fructose sweetened beverages over time and insulin resistance and type 2 diabetes mellitus in human populations.

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Figure Legends

Figure 1: Expression of proteins involved in insulin signaling in the livers of fructose-supplemented female rats. Western-blot of IRS-2 (**a**), PP2Ac (**b**), and phospho- (**c**) and total Akt (**d**) proteins in the liver samples from control and fructose-supplemented female rats. For each protein, representative bands corresponding to three different rats in each treatment group are shown. Bar plots show the levels of each protein, expressed as the mean (a.u., arbitrary units) ± sd of the values obtained from 6 animals, in the hepatic samples from control (white bar) and fructose-supplemented (black bar) rats. The amount of protein loaded was confirmed by the Bradford method, and the uniformity of protein loading in each lane was assessed using β-actin as the loading control. (**e**) Plasma glucose concentrations after the injection of pyruvate (2 g/kg i.p.). (**f**) The mean value of the area under the curve, expressed as g of glucose/150 min/animal, for control and fructose-supplemented rats.

Figure 2: Expression of enzymes related to glucose metabolism in the livers of 14-day fructose-supplemented female rats. The mRNA levels of liver pyruvate dehydrogenase kinase 2 (PDK2) (**a**), pyruvate dehydrogenase kinase 4 (PDK4) (**b**), ATP citrate lyase (ACLY) (**c**), malate dehydrogenase (MD) (d), and malic enzyme (ME) (**e**) in the hepatic samples from control (white bar) and fructose-supplemented female (black bar) rats are given. Each bar represents the mean ± sd of the values obtained from 6 animals. Specific mRNA levels were determined by real-time PCR as described in the Experimental Procedures section.

Figure 3: X-box-binding-protein-1 (XBP-1) expression in the livers of control and fructose-supplemented rats. (**a**) Autoradiograph of an agarose gel showing bands corresponding to unspliced (u) and spliced (s) XBP-1 mRNA in liver samples corresponding to four control and four fructose-supplemented rats for each time point. Western-blot of the spliced form of XBP-1 (XBP-1s) (**b**) in the livers of control and fructose-supplemented female rats. A bar plot (**c**) shows the levels of the proteins, expressed as the mean (a.u., arbitrary units) ± sd of the values obtained from 6 animals, in the hepatic samples from control (white bar) and fructose-supplemented (black bar) rats. The amount of protein loaded was confirmed by the Bradford method, and the uniformity of protein loading in each lane was assessed using β-tubulin as loading control.

Figure 4: Forkhead box O1 (FoxO1) protein expression in the livers of control and fructose-supplemented rats. Western-blot of phospho-FoxO1 (**a**), and total FoxO1 levels in liver whole-homogenates (**b**) and nuclear extracts (**d**) from control and fructose-supplemented female rats. A bar plot shows the levels of the proteins, expressed as the mean (a.u., arbitrary units) ± sd of the values obtained from 6 animals, in the hepatic samples from control (white bar) and fructose-supplemented (black bar) rats. The amount of protein loaded was confirmed by the Bradford method, and the uniformity of protein loading in each lane was assessed using of β-actin as loading control. (**c**) A bar plot showing the ratio of phospho- to total FoxO1, expressed as the mean (a.u., arbitrary units) ± sd of the values obtained from 6 animals, in whole liver homogenates from control (white bar) and fructose-supplemented (black bar) rats.

Figure 5: Activity of nutrient-sensing kinases is increased in the livers of 14-day fructose-supplemented rats. Western-blot of phospho- and total mTOR (**a**), and phospho and total p38-MAPK (**b**) proteins in the livers of control and fructose-supplemented female rats. For each protein, representative bands corresponding to three different rats in each treatment group are shown. As a measure of the degree of kinase activation, bar plots show the ratio of the phospho- to the total contents of each kinase, expressed as the mean (a.u., arbitrary units) ± sd of the values obtained from 6 animals, in the hepatic samples from control (white bar) and fructose-supplemented (black bar) rats. The amount of protein loaded was confirmed by the Bradford method, and the uniformity of protein loading in each lane was assessed using β-actin as loading control.

Figure 6: Sirtuin 1 (SIRT1) expression and activity is decreased in the livers of 14-day fructose-supplemented rats. Western-blot of SIRT1 protein (**a**) in the livers of control and fructose-supplemented female rats. Representative bands corresponding to three different rats in each treatment group are shown. Bar plots show the protein level, expressed as the mean (a.u., arbitrary units) ± sd of the values obtained from 6 animals, in the hepatic samples from control (white bar) and fructose-supplemented (black bar) rats. The amount of protein loaded was confirmed by the Bradford method, and the uniformity of protein loading in each lane was assessed using β-actin as loading control. As an indirect measure of SIRT1 activity, the amount of the acetylated form of FoxO-1 (**b**) in the liver samples from control and fructose-supplemented animals was determined by co-immunoprecipitation, as described in the Experimental Procedures section.

Table 1

Table 1: Fructose effects on líquid and food ingestions, plasma analytes, insulin sensitivity inndex and glucose tolerance test

	7 days		14 days	
	Control	**Fructose**	**Control**	**Fructose**
AUC ingested líquid (ml/days/2 rats)	360 ± 8	845 ± 198*	706 ± 91	1995 ± 430*
AUC consumed diet (g/days/2 rats)	227 ± 2	177 ± 13**	503 ± 12	392 ± 44*
Body Weight (g)	249 ± 7	257 ± 19	264 ± 26	278 ± 21
Adiponectin µg/ml	2.7 ± 0.7	3.9 ± 1.5	2.3 ± 0.7	4.0 ± 0.7*
Leptin ng/ml	2.0 ± 1.0	3.2 ± 2.3	2,8 ± 2,7	4.7 ± 2.2
Insulin µg/ml	0.46 ± 0.07	0.65 ± 0.26	0.51 ± 0.12	0.94 ± 0.43*
Glucose mg/dl	151 ± 7	158 ± 11	190 ± 12[#]	210 ± 16*
ISI	1.21 ± 0.07	1.09 ± 0.14	1.06 ± 0.14[#]	0.76 ± 0.21*
AUC glucose concentration for glucose tolerance test (% versus control)	100±15	100±12	100±23	132±25*

Table 2

Table 2: Change in gene expression after 7 or 14 days of fructose feeding for a selection of genes analyzed using real time PCR arrays (Rat Insulin Signaling Pathway RT2 Profiler)

| Description	Symbol	7 days		14 days	
		Fold change	P value	Fold change	P value
CAP adenylate cyclase-associated protein-1	Cap-1	0.883	0.340	0.822	0.029
Docking protein-1	Dok1	0.933	0.479	0.537	0.052
Docking protein-3	Dok3	1.165	0.602	0.485	0.044
GRB2-associated binding protein1	Gab1	0.918	0.624	0.737	0.043
Insulin-like growth factor1 receptor	Igf1r	1.009	0.896	0.551	0.009
Insulin receptor substrate 1	IRS-1	1.662	0.024	0.640	0.063
Insulin receptor substrate 2	IRS-2	1.197	0.451	0.545	0.013
Mitogen activated protein kinase 1	Mapk1	1.012	0.961	0.770	0.032
Protein kinase C, zeta	PrKcz	1.721	0.004	0.349	0.059
Solute carrier family 27 (fatty acid transportar) membre 4	Slc27a4	0.783	0.189	0.597	0.025
Son of sevenless homolog 1	Sos1	1.009	0.377	0.781	0.033
Phosphoenolpyruvate carboxykinase	PEPCK	1.197	0.403	1.313	0.018
Glucose-6-phosphatase	G6Pc	1.598	0.317	0.412	0.19

Figure 1
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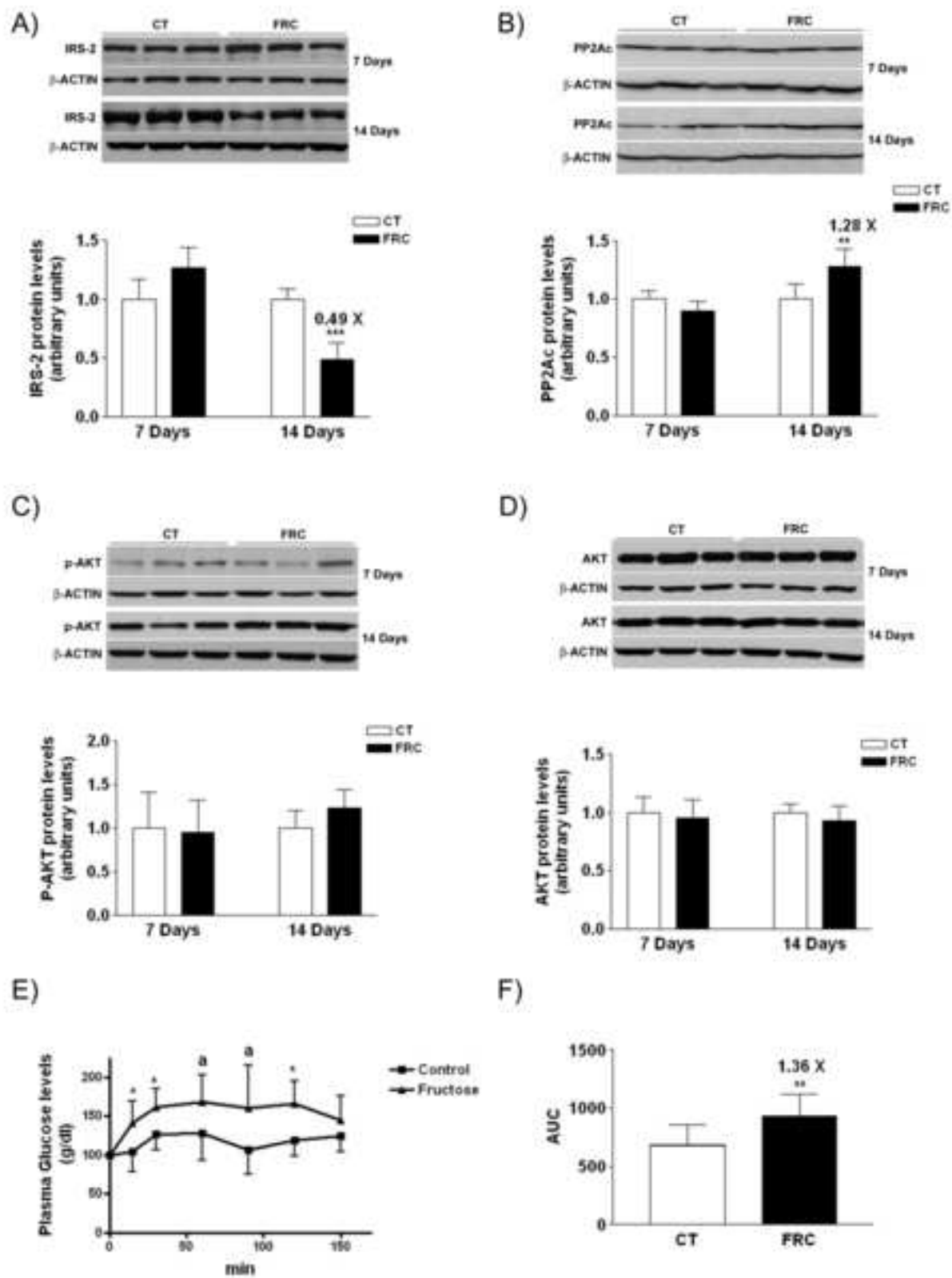
Figure 2
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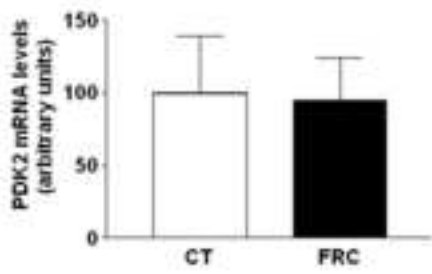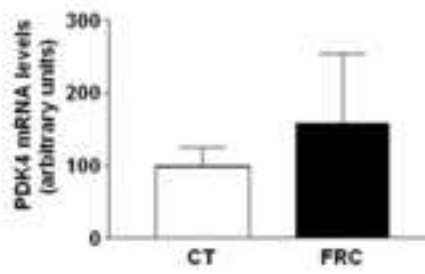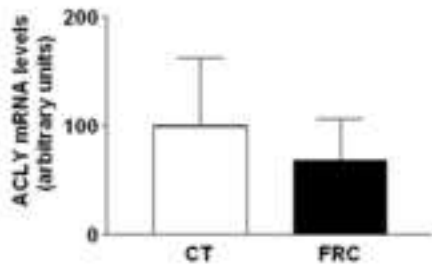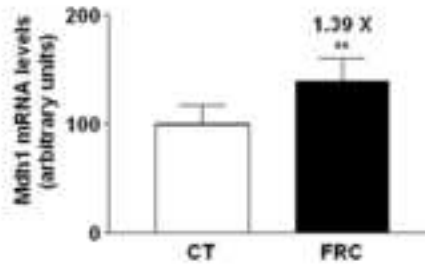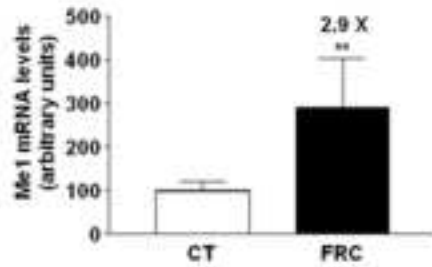
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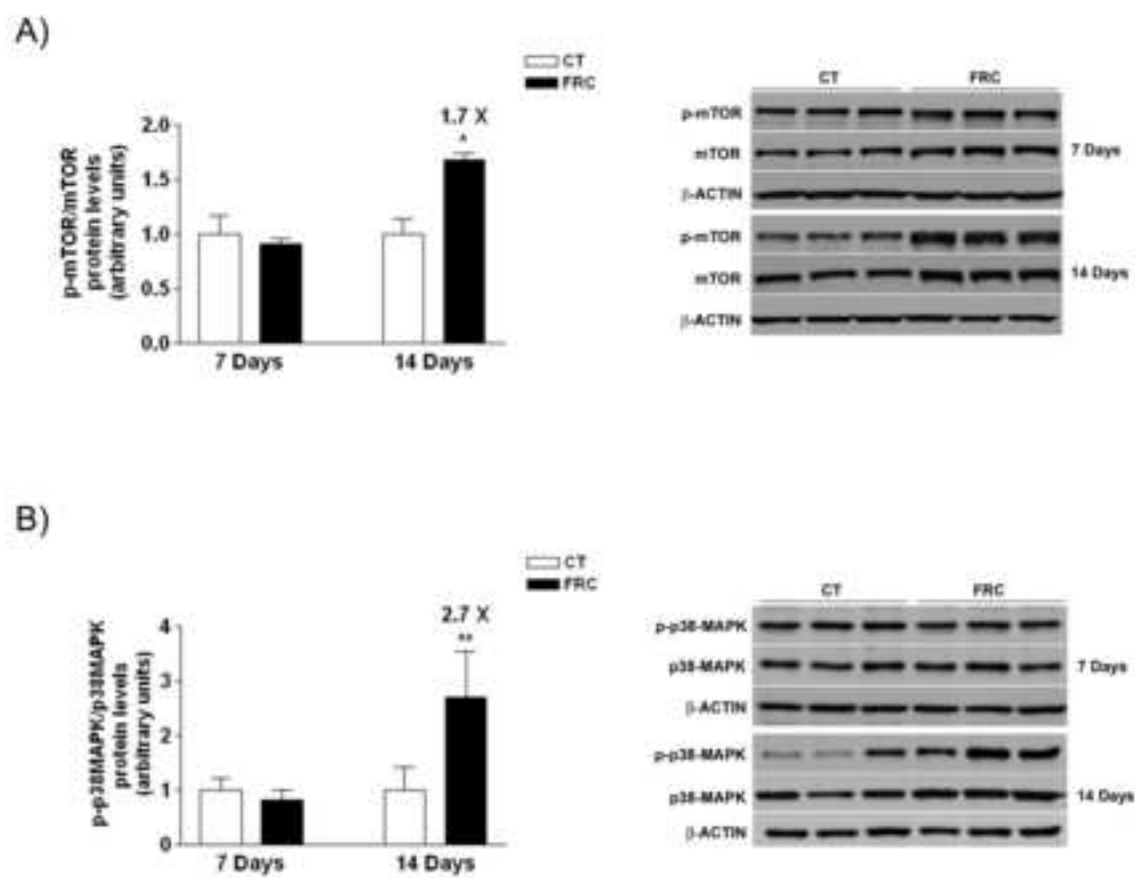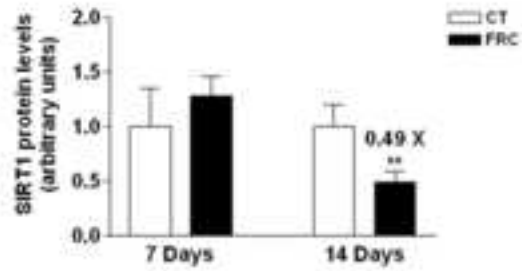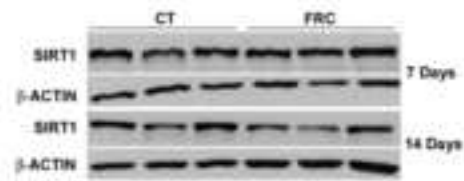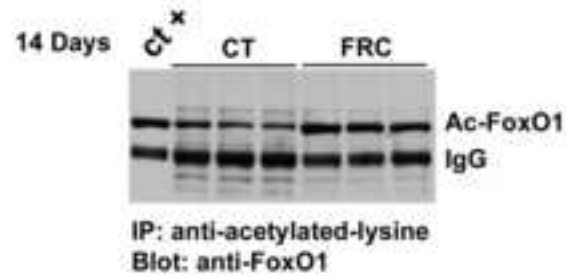**Click here to download high resolution image**
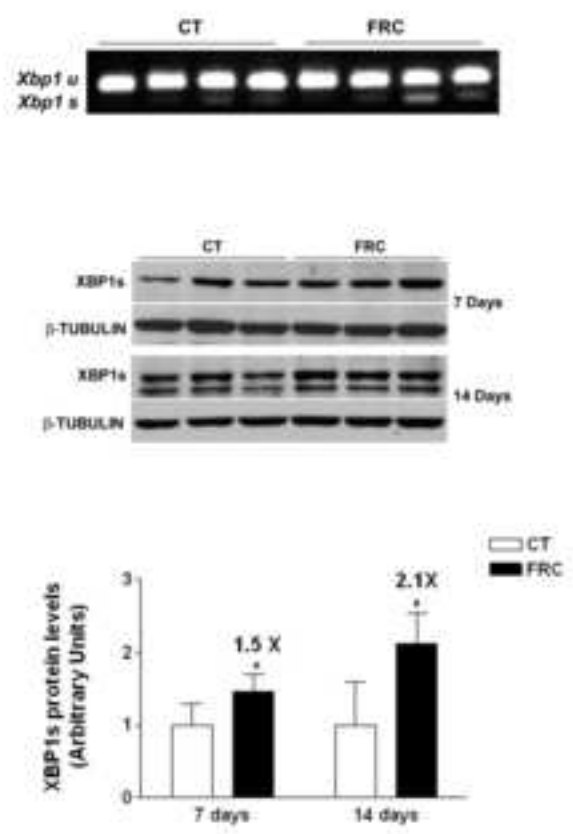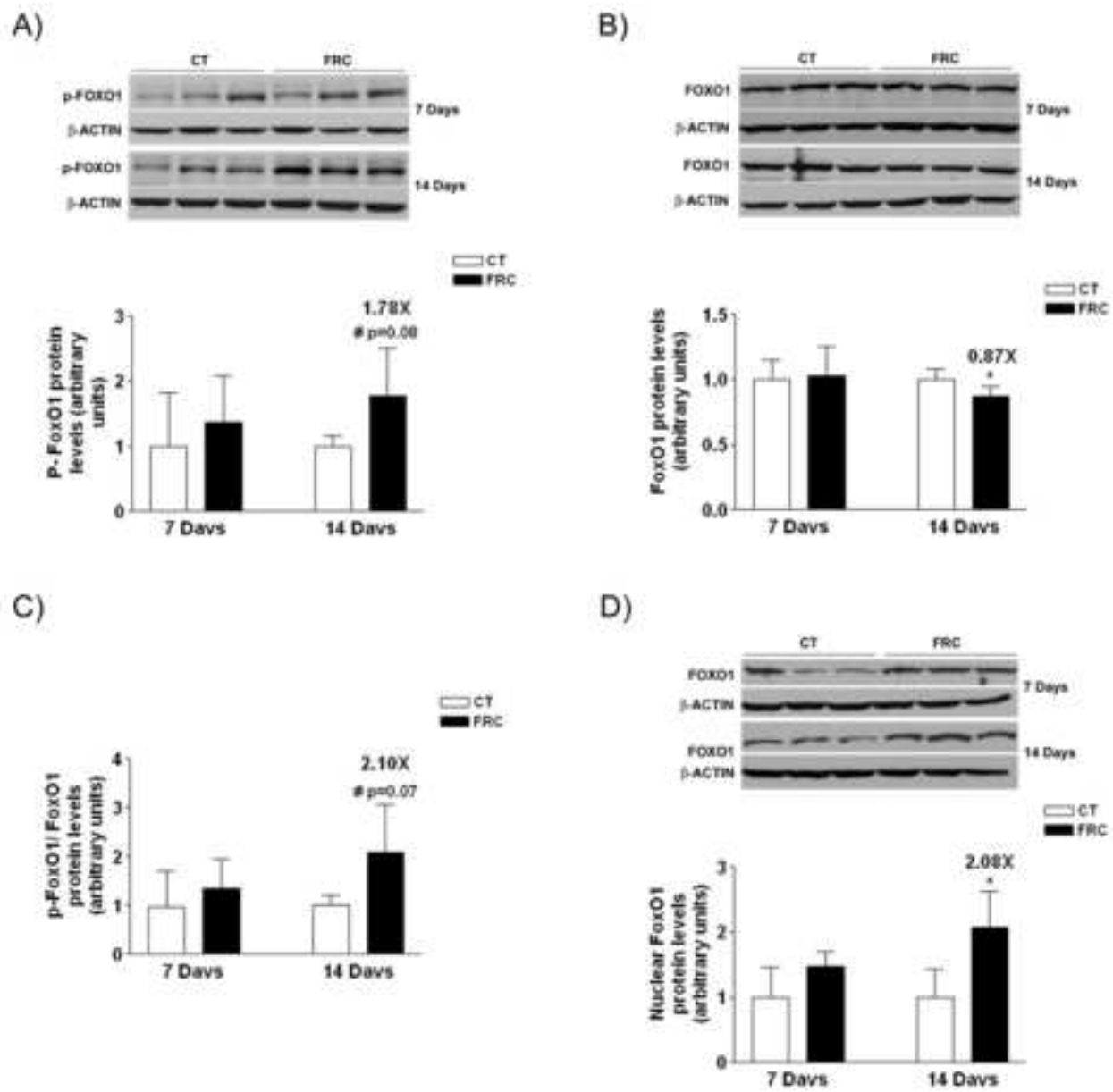
Figure 5

Figure 6
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A)

B)
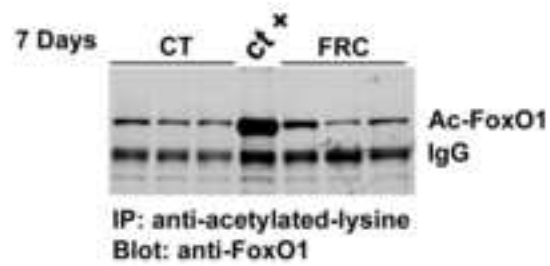
IP: anti-acetylated-lysine
Blot: anti-FoxO1

Supplemental Table 1

Supplemental Table 2

Supplemental Figure legends

Supplemental Figure 1

Supplemental Figure 2

Supplemental Figure 3