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TYPE OF SUPPLEMENTED SIMPLE SUGAR, NOT MERELY CALORIE INTAKE, DETERMINES ADVERSE EFFECTS ON METABOLISM AND AORTIC FUNCTION IN FEMALE RATS

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Running head: Metabolic & vascular effects of simple sugars in female rats

Abstract

High consumption of simple sugars causes adverse cardio-metabolic effects. We investigated the mechanisms underlying the metabolic and vascular effects of glucose or fructose intake and determined whether these effects are exclusively related to increased calorie consumption. Female Sprague-Dawley rats were supplemented with 20% w/v glucose or fructose for 2 months, and plasma analytes and aortic response to vasodilator and vasoconstrictor agents were determined. Expression of molecules associated with lipid metabolism, insulin signalling and vascular response were evaluated in hepatic and/or aortic tissues. Caloric intake was increased in both sugar-supplemented groups vs control, and in glucose- vs fructose-supplemented rats. Hepatic lipogenesis was induced in both groups. Plasma triglycerides were increased only in the fructose group, together with decreased expression of carnitine palmitoyltransferase-1A and increased microsomal triglyceride transfer protein expression in the liver. Plasma adiponectin and peroxisome proliferator-activated receptor (PPAR)α expression were increased only by glucose supplementation. Insulin signalling in liver and aorta were impaired in both sugar-supplemented groups, but the effect was more pronounced in the fructose group. Fructose supplementation attenuated aortic relaxation response to a nitric oxide (NO) donor, whereas glucose potentiated it. Phenylephrine-induced maximal contractions were reduced in the glucose group, which could be related to increased endothelial NO synthase (eNOS) phosphorylation and subsequent elevated basal NO in the glucose group. In conclusion, despite higher caloric intake in glucose-supplemented rats, fructose caused worse metabolic and vascular responses. This may be due to the elevated adiponectin level and the subsequent enhancement of PPARα and eNOS phosphorylation in glucose-supplemented rats.

NEW & NOTEWORTHY

This is the first study comparing the effects of glucose and fructose consumption on metabolic factors and aortic function in female rats. Our results show that although total caloric consumption was higher in glucose-supplemented rats, fructose ingestion had a greater impact in inducing metabolic and aortic dysfunction.

Keywords

fructose; glucose; liver; insulin resistance; adiponectin.

Insulin resistance, obesity and type 2 diabetes are metabolic disturbances leading to cardiovascular diseases (CVD). CVD are main causes of morbidity and mortality in diabetes, and both micro- and macro-vascular complications are thought to play a major role in the development of CVD in pre-diabetic and diabetic patients (14, 30).

In humans, an excessive intake of added sugars has been linked to the development of metabolic disturbances (22, 39), and therefore to an increase in the risk for CVD mortality (59). Despite the evidence generated from epidemiological studies, the molecular mechanisms linking diabetes and CVD in the population with excessive sugar intake are not fully understood. At the vascular level, insulin resistance (32, 34) along with dyslipidaemia, local inflammation and a decrease in the synthesis of endothelium derived relaxation factors such as nitric oxide (NO) may play a key role in the development of CVD (25).

The adverse cardio-metabolic effects of simple sugars seem to be worse when they are ingested in liquid than in solid form, because the level of food intake is not reduced enough to compensate the extra calories provided by beverages (53), leading to an increase in total caloric intake. Consumption of simple sugars in liquid form essentially occurs as sugar-sweetened beverages (SSB), which include sodas, colas, fruit punches, lemonade, and other fruit drinks with added sugars. The main compounds used by the food industry to sweeten these beverages are high fructose corn syrup (in USA) and sucrose (in Europe), both containing approximately equal amounts of fructose and glucose (48). At present, there is an intense debate in the scientific community about whether the adverse cardiovascular and metabolic effects of SSB are mostly attributable to specific effects of the simple sugars used as sweeteners, or are merely the consequence of the increase in caloric intake and weight gain in the population consuming large quantities of SSB (10, 26, 49, 52).

To study the effects of simple sugar consumption in liquid form on glucose and lipid metabolism, the rat is a good model which has been extensively used by us (7, 41–43, 54–56). In previous studies, we showed that female rats supplemented for short periods of time (14 days) with liquid fructose displayed a more detrimental response than male rats. Specifically, we reported that fructose induced hypertriglyceridemia and fatty liver in both sexes but only females showed glucose intolerance and hepatic insulin resistance (41, 56). Insulin resistance is a prominent feature of metabolic diseases such as obesity or type 2 diabetes mellitus, which is also a major risk factor for CVD. A relationship between insulin resistance and endothelial dysfunction has been proposed

as a link between cardiovascular and metabolic diseases (27). Endothelial dysfunction is defined as a reduced endothelium-dependent vasodilation (EDV) to vasodilators, such as acetylcholine (ACh) and bradykinin (BK), or flow-mediated vasodilation. Thus, EDV is generally used as a reproducible parameter to investigate endothelial function under various pathological conditions such as diabetes, obesity and dyslipidaemia. While past studies were performed largely on males, there is increasing awareness by the NIH that research should include females. Here, we sought to study the effects of 2-month supplementation with liquid fructose or glucose (20% w/v), on the metabolic response and vascular reactivity in aorta, a large conduit artery, in female rats. To measure the vascular function, EDV was assessed by examining the aortic relaxation responses to ACh and BK (receptor-mediated NO-dependent vasodilators). Aortic response to a NO donor was also determined by measuring the relaxation responses to sodium nitroprusside (SNP). Furthermore, vasoconstrictor responses to phenylephrine (PE) were studied. Our aims were to investigate the molecular mechanisms underlying the metabolic and vascular effects of these simple sugars and to determine whether these effects are exclusively related to increased calorie consumption.

Materials and methods

Animals and experimental design

Female Sprague-Dawley rats, aged 9–11 weeks (Simonsen Laboratories, Gilroy, CA, USA) were maintained with water and standard rodent chow food *ad libitum* at constant humidity and temperature, with a light/dark cycle of 12 h. After acclimation for 1 week, the animals were randomly assigned to a control group, a glucose-supplemented and a fructose-supplemented group (14 rats per group). Sugars were supplied as a 20 % (w/v) solution in drinking water for 8 weeks. Body weight, food and drink intake were monitored throughout the experiment. After 8 weeks, the rats were fasted for 12 h and euthanized using CO_2 according to the recommendations from the 2013 AVMA Guidelines on Euthanasia (28) and the NIH Guidelines for the Care and Use of Laboratory Animals: Eighth Edition (US National Institutes of Health 2011). All animal protocols were approved by the Animal Care Committee of the University of the Pacific and complied with the Guide for the Care and Use of Laboratory Animals: Eighth Edition (US National Institutes of Health 2011) and with ARRIVE guidelines. The 8-week duration of sugar supplementation was chosen in order to mimic a sub-

chronic regime, as this period of ingestion in rats is roughly equivalent to 6 years of consumption in humans (45).

Blood analysis

Glucose, triglycerides and cholesterol were measured in 12 h fasted rats using an Accutrend® Plus System glucometer and specific test strips (Roche Farma, Barcelona, Spain) with blood collected from the tail vein. Blood samples were obtained by intra-cardiac puncture and collected in tubes containing anticoagulant. Plasma was obtained by centrifugation at 10,000xg for 5 min at 4°C and stored at −80 °C until used. Leptin (Invitrogen, Camarillo, CA, USA), adiponectin (Adipogen, Liestal, Switzerland) and insulin (Spi Bio, Montigny Le Bretonneux France) levels were determined in plasma samples by ELISA kits according to the manufacturer's protocol.

Measurement of arterial tension

The thoracic aortae were isolated and cleaned of fatty and adhering connective tissues and then cut into 2 mm rings, exactly. To measure isometric tension, the rings were suspended horizontally between two stainless steel hooks in individual organ baths containing 20 ml of Krebs buffer (in mM: 119 NaCl, 4.7 KCl, 1.18 KH_2PO_4, 1.17 $MgSO_4$, 24.9 $NaHCO_3$, 0.023 EDTA, 1.6 $CaCl_2$, and 6.0 glucose) at 37°C bubbled with 95% O_2 and 5% CO_2 (40). Isometric tension was continuously monitored with a computer based data acquisition system (PowerLab, ADInstruments, Colorado Springs, USA). The rings were equilibrated for 40 min under a resting tension of 1 g to allow development of a stable basal tone. Stimulation of rings with 80 mM KCl was repeated two times every 20 min until maximum contraction was achieved. The ability of ACh (10 μM) to induce relaxation of phenylephrine (PE, 2 μM) pre-contracted vessels was taken as an evidence for the preservation of the intact endothelium.

Relaxation responses to ACh

Aortic rings were contracted with PE (2 μM), a concentration that produced 80% of the maximal effect (EC_{80}). Dilator response curves were obtained by the addition of increasing concentrations of ACh (10^{-8} to 10^{-5} M).

Relaxation responses to BK

The concentration response curves to BK, were measured following the addition of increasing concentrations of BK (10^{-9} to 10^{-5} M) in U46619 (100 nM) pre-contracted aortic rings taken from all groups.

Relaxation responses to SNP

Responses to SNP (10^{-9} to 10^{-5} M), a NO-donor, were generated in the aortic rings pre-contracted with PE (2 μM) from all groups.

Contractile responses to PE

The constrictor concentration response curves to PE (10^{-8} to 10^{-5} M) were generated before and after incubation with N^{ω}-Nitro-L-arginine methyl ester (L-NAME, 200 μM), a nitric oxide synthase (NOS) inhibitor, in the presence of indomethacin (indo, 10 μM, dissolved in DMSO), a cyclooxygenase (COX) inhibitor. Between each concentration response curve, tissues were washed with Krebs buffer to allow the rings to return to the basal tone. The use of this concentration of L-NAME was based on previous studies (18, 19). A vehicle only (no drugs present) study was performed simultaneously in aortic rings from the same animal.

Measurement of Nitrate and Nitrite in endothelial cells

The permanently established EA.hy926 endothelial cell line (passage 5-6) (American Type Culture Collection (ATCC), Manassas, VA, USA) were plated and cultured in low glucose (1000mg/l) Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (Thermo Scientific) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37 °C with 95% air and 5% CO_2. Upon reaching 80% confluence, cells were treated with Recombinant Human gAcrp30/Adipolean (Globular adiponectin, 5 and 15 μg/ml, Peprotech Inc., Rocky Hill, NJ, USA), glucose or fructose (25 mM, Sigma-Aldrich, St. Louis, MO, USA). The NO donor S-Nitroso-N-Acetyl-D,L-Penicillamine (SNAP, 100 μM, Tocris Biosciences, Minneapolis, MN, USA) was used as a positive control, and mannitol (25 mM, Sigma-Aldrich, St. Louis, MO, USA) as an osmotic control. After 30 min, cell culture supernatants were collected and NO levels were determined by measurement of the end products of its metabolism NO_x [nitrate (NO_3^-) plus nitrite (NO_2^-)] using the Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) according to manufacturer's suggestions. Five to six different samples for each condition were performed in duplicate.

RNA isolation and Real-Time PCR

Samples of liver, visceral white adipose and aortic tissues were weighed, immediately frozen in dry ice and stored at −80 °C until used for RNA extraction. Total RNA was isolated from liver and adipose tissue samples by using TrizolR reagent (Invitrogen™, Carlsbad, CA, USA) and from aortic samples using RNeasy mini kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's instructions. cDNA was synthesized by reverse transcription using the Omniscript reverse

transcriptase kit (Qiagen, Valencia, CA, USA). Samples were incubated at 37°C for 60 min in MJ Mini™ Personal Thermal Cycler (Bio-Rad, Hércules, CA, USA). The PCR reaction was carried out in StepOnePlus™ Real-Time PCR System Thermal Cycling Block (Applied Biosystems, Foster City, CA, USA) using SYBR® Green PCR Master Mix (Applied Biosystems), 100 nM of each specific primer and 10 - 50 ng of cDNA for each gene. PCR reactions were performed in duplicate and normalized to a housekeeping gene using the $2^{-\Delta\Delta Ct}$ method. The TATA box binding protein (*tbp*) was used as a control for liver and β-actin (*actb*) was used for adipose tissue and aorta. Primer sequences and PCR product length are listed in Table 1.

Preparation of total protein and nuclear extracts

Aortic and visceral adipose tissue samples were micronized through freezing with liquid nitrogen and grinding with a mortar as previously described (7). Liver samples were homogenized with a Dounce homogenizer. For total protein extraction, lysis buffer with proteases, phosphatases and acetylases inhibitors (in mM: 50 Tris–HCl pH=8, 150 NaCl, 10 NaF, 1 EDTA, 1 EGTA, 2 Nappi, 1 PMSF, 1 Na_3VO_4, 10 NaM, 0.001 TSA) plus 1% Igepal, 2 µg/mL leupeptin and 2 µg/mL aprotinin was used. Samples were incubated with this buffer for 1.5 h at 4°C, centrifuged at 15,000×g for 15 min at 4°C and supernatants were collected. To obtain hepatic nuclear extracts for the determination of the transcription factors PPARα, ChREBP and SREBP-1C, homogenization buffer (in mM: 10 Tris–HCl pH=8, 1.5 $MgCl_2$, 10 KCl, 2 Nappi, 10 NaF, 0.5 DTT, 1 PMSF, 1 Na_3VO_4, 10 NaM, 10^{-6} TSA) plus 2 µg/mL leupeptin and 2 µg/mL aprotinin was used. The homogenates were kept on ice for 10 min and centrifuged at 1000xg for 10 min at 4°C. Lysis buffer was added to the pellet obtained and samples were incubated for 1.5 h at 4°C, centrifuged at 25,000×g for 30 min at 4°C and supernatants were collected. Protein concentrations were determined by the Bradford method (9).

Western blot analysis

20-30 µg of protein was subjected to SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to Immobilon polyvinylidene difluoride transfer membranes (Millipore, Billerica, MA, USA), blocked for 1 h at room temperature with 5% non-fat milk solution in 0.1% Tween-20-Tris-buffered saline (TBS), and incubated overnight at 4°C with primary antibodies. Primary antibodies for p-ACC (Ser79), total ACC, p-Akt (Ser473), total Akt, p-eNOS (Ser1177), iNOS, PKG-1 (recognizing both α and βisoforms) p-VASP (Ser157), p-VASP (Ser239) and total VASP were supplied by

Cell Signaling (Danvers, MA, USA). Antibodies against IRS-1, IRS-2, ChREBP, SREBP-1c, MTP and FAS were obtained from Santa Cruz Biotechnologies (Dallas, TX, USA). PPARα, PDE4 and SCD1 antibodies were obtained from AbCam (Cambridge, UK). Antibody against rat liver CPT-1A was kindly provided by L. Herrero and D. Serra (Department of Biochemistry and Physiology, School of Pharmacy, University of Barcelona) (21, 37). Detection was performed using the Pierce® ECL Western Blotting Substrate (Thermo Scientific) and Immobilion™ Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA). To confirm the uniformity of protein loading, blots were incubated with β-actin or β-tubulin antibodies (Sigma-Aldrich, St. Louis, MO, USA) as a control.

Data presentation and statistical analysis

Results are expressed as the mean of *n* values ± SEM, where n represents data from one rat. Relaxation responses to ACh, BK, and SNP were calculated as the percentage of relaxation from maximum PE or U46619 contraction. Similarly, the recorded increase in the force of contraction was calculated as the percentage of maximum contraction obtained with PE at the highest dose. EC_{50}, the concentration of the agonist which produces half of the maximum effect (E_{max}), was calculated by a sigmoidal dose-response model (for variable slope) using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA). The sensitivity of the agonists was expressed as pD_2 values (-log [EC_{50}]). In general, statistical analyses were performed by one-way ANOVA test, after gaussian distribution of the data was verified by the Kolmogorov-Smirnov normality test and equality of variance by Bartlett's test. When the ANOVA test returned $P<0.05$, *post-hoc* analysis using Bonferroni's test was performed. Comparison of concentration response curves between groups was done using two-way ANOVA, with one factor being concentration and the other being groups. To compare the effect of L-NAME on the PE response, the PE results were expressed as differences of area under the concentration response curve (ΔAUC) in control (absence of L-NAME) and experimental (presence of L-NAME) condition. The level of statistical significance was set at $P<0.05$.

Results

Only fructose supplementation causes hypertriglyceridemia and increases body and liver weight

As it is shown in Table 2, rats supplemented with 20% glucose or fructose increased their liquid consumption (by 2-fold and 1.5-fold, respectively). Further, they partially compensated the increase in caloric intake by reducing the consumption of solid food (by 0.3-fold in glucose group and by 0.5-fold in fructose group). As a result, the total amount of ingested calories was increased in both groups. However, total caloric intake in glucose-supplemented rats was significantly higher than that of fructose-supplemented rats (1.15-fold). Despite this difference, only the fructose group exhibited a significant increase in final body weight (by 1.1-fold). Due to the increase in body weight, organ weights were normalized to femur length, which was not altered by sugar supplementation. Liver weight was increased only by fructose supplementation (by 1.4-fold vs control, and 1.3-fold vs glucose-fed rats), whereas adipose tissue weight was significantly increased in both sugar-supplemented groups (by 5.6-fold in the glucose group and 5.2-fold in the fructose group). Consistent with the increase in adiposity, plasma leptin levels and adipose tissue *leptin* mRNA expression were also elevated in both experimental groups (Table 2).

Blood lipid analysis revealed that the cholesterol concentration was not modified in either glucose- or fructose-supplemented rats (Table 2). However, the levels of triglycerides were significantly increased in blood taken from fructose-supplemented rats (by 1.2-fold, Table 2).

Both sugars induce hepatic lipogenesis but only fructose increases microsomal triglyceride transfer protein expression

The hypertriglyceridemia and increased liver weight observed in fructose-supplemented rats prompted us to examine the mRNA/protein expression of lipogenesis-related enzymes. The mRNA expression of stearoyl-CoA desaturase-1 (*scd1*) was increased by glucose (3.4-fold) and fructose (4.2-fold) (Figure 1A). Accordingly, SCD1 protein levels were increased after glucose and fructose supplementation (by 1.4 and 1.7-fold, respectively), but the increase in the glucose group did not reach statistical significance (Figure 1B). Although the mRNA expression of fatty acid synthase (*fas*) showed a non-significant tendency to increase in both groups (Figure 1C), FAS protein levels were significantly increased by 2-fold in glucose-supplemented and by 2.4-fold in the fructose-supplemented group (Figure 1D). The amount of total and phosphorylated acetyl-CoA carboxylase (ACC) was significantly increased in the liver by both sugars (Figure 1E, F), suggesting that the degree of ACC activation was not modified. The expression of these lipogenic genes is mainly

controlled by two transcription factors, sterol response element binding protein-1c (SREBP-1c) and carbohydrate response element binding protein (ChREBP). Although the amount of ChREBP in nuclear extracts remained unaffected (Figure 1G), the protein expression of the mature form of SREBP-1c was significantly increased only in hepatic nuclear extracts taken from fructose-supplemented rats (Figure 1H).

As shown in Table 2, only glucose-supplemented animals displayed higher plasma adiponectin levels than controls (2.6-fold increase), which correlates with increased mRNA expression of the adiponectin gene in adipose tissue of this group (by 2.6-fold, Table 2). It has been shown that stimulation of adiponectin signalling activates peroxisome proliferator-activated receptor α (PPARα), a key factor in the transcriptional control of genes encoding fatty acid β-oxidation enzymes (29). Western blot analysis revealed that the expression of hepatic PPARα was significantly increased only in the nuclear extracts from glucose-supplemented rats (by 1.3-fold) (Figure 2A). Accordingly, the mRNA levels of PPARα target genes, such as liver carnitine palmitoyl-CoA transferase-I (*l-cpt-1a*) and acyl-CoA oxidase (*aco*), were significantly increased only in the liver of glucose-supplemented animals (Figure 2B, C). When we determined L-CPT-1A protein levels, we found no significant difference between the glucose-supplemented and the control group. However, the protein level of L-CPT-1A was significantly reduced in liver samples taken from fructose-supplemented rats (by 0.5-fold, Figure 2D). Since the amount of plasma triglycerides not only depends on the balance between the synthesis and catabolism of fatty acids in the liver, but also on the export of triglycerides from hepatocytes, we also determined the protein expression of microsomal triglyceride transfer protein (MTP). This protein is essential for the assembly and secretion of very-low density lipoproteins (VLDL) by the liver. As shown in Figure 2E, MTP protein expression was increased only in fructose-supplemented rats.

Supplementation with fructose and glucose impairs insulin signalling in aorta and liver

Plasma glucose concentrations were not altered after simple sugar supplementation (Table 2). On the other hand, fructose increased plasma insulin concentration (by 2.4-fold) and the glucose-supplemented group showed a tendency (P=0.07) to higher plasma insulin level. Thus, the insulin sensitivity index (ISI) was significantly reduced in both glucose- and fructose-supplemented rats (Table 2), suggesting that insulin signalling could be impaired in these groups. Therefore, we analyzed the expression of insulin receptor substrate (IRS)-1 and IRS-2, the main

insulin signal transducers in the liver and in aortic tissue. Our results show that the protein expression of hepatic IRS-1 was not altered by sugar supplementation (Figure 3A), but IRS-2 expression was significantly reduced in both groups (by 0.5-fold in glucose-supplemented and by 0.4-fold in fructose-supplemented animals, Figure 3B). On the other hand, both IRS-1 and IRS-2 protein levels were reduced in aortic tissue from fructose- and glucose-supplemented rats (Figure 3C, D). We also determined the expression of V-akt murine thymoma viral oncogene homolog-2 (Akt), one of the main transducers of insulin signalling downstream of IRS in both tissues. The amount of phosphorylated Akt (p-Akt) was significantly reduced only in the livers of fructose-supplemented rats in comparison with the control group, whereas total Akt protein remained unaffected by both sugars (Figure 3E). Similarly, in aortic tissues the expression of p-Akt was significantly reduced only in fructose-fed animals and total Akt expression was not significantly modified by sugar supplementation (Figure 3F).

Supplementation with glucose and fructose alters relaxation responses of aortic rings to a NO donor

Relaxation to ACh was used to examine the effect of simple sugars on the receptor-mediated endothelium-dependent release of NO. No significant differences in responses to ACh (10^{-8}-10^{-5} M) occurred between aortic rings from the control, glucose- and fructose-supplemented groups (Figure 4A). Both the sensitivity of aortic rings to ACh as assessed by -log[EC_{50}] (pD_2) and the E_{max} to ACh were similar in all groups (Figure 4A, Table 3). Similar to the findings for ACh, the sensitivity and E_{max} to BK, another receptor-mediated endothelium-dependent vasodilator agent were not affected by sugar supplementation (Figure 4B, Table 3). However, there was a significant rightward shift of BK response curve in aortic rings from fructose-supplemented rats relative to control animals (Figure 4B).

Aortic relaxation responses to a NO donor were also investigated by performing concentration response curves to SNP (10^{-9}-10^{-5} M). Although the E_{max} to SNP was similar in all groups (Table 3), supplementation with glucose significantly augmented the responsiveness of aortic rings to SNP. On the other hand, fructose supplementation significantly attenuated the sensitivity of aortic rings to SNP (Figure 4C, Table 3).

Glucose (but not fructose) supplementation reduces the contractile response of aortic rings

Eight week fructose supplementation did not affect vasoconstrictor responses of rat aorta to PE, as shown in Figure 5A and Table 3. However, PE-induced maximal

contractions were significantly reduced in aortic rings from glucose-supplemented rats compared with controls (Figure 5A, Table 3). Despite PE contractile responses were affected by glucose supplementation, the mRNA expression of α1-adrenergic receptors was unchanged (Table 5). To study the possible role of basal NO, PE response curves were performed in aortic rings before and after pre-treatment with the NO synthase inhibitor L-NAME (200 μM, 20 min) in the presence of indomethacin (10 μM). The difference in the contractile level to PE after addition of L-NAME would indicate the extent of endothelial NO release (13, 20). Incubation of the aortic rings with L-NAME resulted in a significant increase of the contractile responses to PE in all groups (Figure 5C-E). However, the ΔAUC, defined as difference in the area under the curve between PE concentration response curve before and after L-NAME were only higher in aortae of the rats supplemented with glucose compared to the control group (Table 4).

To investigate a mechanism by which NO release might have been increased in glucose-supplemented rats, endothelial NO synthase (eNOS) activation by phosphorylation was investigated. As shown in Figure 6A, the phosphorylation of eNOS at Ser1177 was increased in aortic tissue from glucose- but not fructose-supplemented rats, whereas in the expression of total eNOS showed no significant difference between the simple sugar supplemented groups and the control group.

On the other hand, protein levels of inducible NOS (iNOS) in aorta were significantly increased only in the fructose-supplemented animals (by 1.8-fold) (Figure 6B).

In vitro adiponectin (but not glucose or fructose) treatment increases NO level in EA.hy926

It is well known that adiponectin stimulates the synthesis of NO in endothelial cells. Thus, the increase in adiponectin levels observed in glucose-supplemented rats could be responsible for the elevated basal NO in this group. To test this hypothesis, we determined the effects of adiponectin or high glucose or fructose on NO level in endothelial EA.hy926 cells. As shown in Figure 7, incubation of Eahy926 cells with 25 mM glucose or fructose for 30 min did not alter cellular NO production, whereas incubation with 5 and 15 μg/ml adiponectin, dose dependently (and significantly for the highest concentration) increased NO levels in the cell culture supernatant.

Differential effects of fructose and glucose supplementation on intracellular pathways related to aortic relaxation

To study the molecular mechanisms that could explain the differences in the responses of aortic rings from rats supplemented with glucose or fructose, the expression of several molecular mediators of vascular relaxation were examined. The NO-dependent relaxation is mediated by cGMP signalling which leads to the stimulation of a specific cGMP-dependent protein kinase (PKG), resulting in the phosphorylation of vasodilator-stimulated phosphoprotein (VASP) in Ser239 (44). Our results show that the mRNA expression of soluble guanylate cyclase (*gcsa1*) in aortae from fructose-supplemented rats was reduced by 0.8-fold compared to controls (Figure 8A). Neither the mRNA levels of the phosphodiesterase that hydrolyses cGMP (*pde5*) (Figure 8A), nor the protein expression of protein kinase G (PKG) (Figure 8B) were significantly modified by sugar supplementation. However, phosphorylation of VASP in Ser239 was reduced in both sugar-supplemented groups vs control (Figure 8C), and the amount of total VASP showed also a reduction, which was significant only in fructose-supplemented rats (Figure 8D).

The NO-independent vasodilatory pathway in large conduit arteries is mainly controlled by cAMP levels resulting in the phosphorylation of VASP in Ser157 (44). Aortic tissue adenylyl cyclase (*AC6*) mRNA levels and phosphorylation of protein kinase A (PKAc) in Thr197 were not significantly modified by sugar supplementation compared to control rats, although fructose-supplemented rats showed reduced values compared to the glucose group (Figure 8A, F). Fructose supplementation significantly increased the mRNA and protein expression (by 1.9-fold and 1.5-fold, respectively) of phosphodiesterase 4 (PDE4), which specifically catalyzes the hydrolysis of cAMP (Figure 8A, G). Thus, the combined reduction of total VASP and the putative decrease of cAMP levels may explain the significant reduction of VASP Ser157 phosphorylation (by 1.46-fold) which was observed only in fructose-supplemented rats (Figure 8E).

Finally, in order to rapidly identify possible changes in other pathways involved in the vascular effects of simple sugars, we measured the mRNA level of a broad range of genes associated with vascular function. As shown in Table 5, the mRNA expression of genes and enzymes such as angiotensin II and BK receptors, cyclooxygenases, prostacyclin synthase or thromboxane synthase, which have been reported to be involved in aortic reactivity, remained unaffected by sugar supplementation. Although we are aware that mRNA expression and protein abundance are not always well correlated, the lack of effect of sugar supplementation on the mRNAs analyzed

426 discouraged us from further studying the involvement of these pathways on the
427 observed vascular effects.
428
429 **Discussion**
430 The adverse effects of simple sugar consumption on metabolic function are well
431 documented in human and animal studies (3, 49, 51), and there are also reports on the
432 effects of diets supplemented with fructose or high fructose corn syrup on vascular
433 reactivity in rodents (2, 5, 16, 31, 38). Most of these studies were performed on males,
434 but there is increasing awareness to include females in research studies. To our
435 knowledge we are the first to compare the effects of simple sugars (glucose or fructose)
436 consumption on female rat metabolic factors and aortic function. Here, we identify
437 some key molecular targets responsible for metabolic and vascular disturbances induced
438 by simple sugar supplementation in liquid form to female rats. We also provide
439 evidences that these effects are not merely a direct result of the amount of ingested
440 calories.
441 We previously reported that the ingestion of liquid fructose elicits an incomplete
442 compensatory reduction of solid food consumption in rats, leading to an increase in total
443 caloric intake (7, 41–43, 54, 55). Our current data show that when rats are supplemented
444 with a glucose solution, the compensatory response is even worse compared with
445 fructose supplementation, as liquid intake is higher and comparatively solid food
446 consumption is reduced to a lesser extent. As a result, the total caloric intake is higher in
447 rats receiving glucose than in rats with fructose supplementation (Table 2). It has been
448 suggested that if simple sugars cause any adverse effects on health, these effects are
449 exclusively due to the calories that the sugars provide (26). According to this
450 hypothesis, the metabolic and vascular alterations in the present study should have been
451 equal or more intense in glucose-supplemented rats. On the contrary, our results show
452 that compared with the fructose group, rats supplemented with liquid glucose exhibit
453 less adverse metabolic and vascular effects. Analysis of the results shown in Table 2
454 and Figure 3 indicates that both sugars impair insulin signalling in the liver and aortic
455 tissue, but the effect is far more intense in fructose- than in glucose-supplemented rats.
456 Although hepatic and aortic IRS-1/2 were reduced to a similar extent by glucose and
457 fructose, p-Akt was significantly decreased in the liver and aorta of fructose-
458 supplemented animals only. This is in accordance with our previous results showing an
459 abnormal glucose tolerance test and reduced p-Akt in female rats supplemented with

10% liquid fructose, but not glucose, for 2 months (6). In the current study, insulin resistance is observed not only in the liver but also in aortic tissues of fructose-supplemented animals, suggesting a common origin for both metabolic and vascular dysfunction.

Moreover, our data shows that the hypertriglyceridemia appears only in fructose-supplemented rats. This observation cannot be explained solely by increased hepatic lipogenesis, as both sugars induced the expression of major lipogenic genes. However, fructose supplementation induced a more prominent effect (Figure 1). Hepatic lipogenesis is controlled by two transcription factors, SREBP-1c and ChREBP (57). As ChREBP is activated by phosphorylated intermediates derived from the direct metabolism of dietary carbohydrates (1), the 12-h fasting period in our study explains the lack of activation of this transcription factor (Figure 1G). On the contrary, activation of SREBP-1c by carbohydrates is not a direct effect but it is mediated by an increase in plasma insulin levels (57), a mechanism that is maintained in insulin resistance states (24). In the present study, plasma insulin level was markedly elevated only in fructose-supplemented animals (Table 2), driving the increased expression of SREBP-1c in hepatic nuclear extracts from this group (Figure 1H).

Regarding lipid catabolism, fructose supplementation did not affect hepatic PPARα nuclear content or the mRNA expression of *l-cpt-1a*, a PPARα target gene. However, when we determined the protein expression of L-CPT-1A, we observed a significant decrease in the liver of the fructose-supplemented rats (Figure 2D). L-CPT-1A catalyzes the rate-limiting step of the hepatic mitochondrial β-oxidation of fatty acids, suggesting that this process may be inhibited in the liver by fructose supplementation. On the contrary, fructose increased the hepatic expression of MTP, a protein which is essential for the assembly of triglycerides into VLDL and for the secretion of these lipoproteins (17). This effect had been already reported in fructose-fed hamsters, and was correlated to hepatic insulin resistance (11, 50). Taking these results together, the hypertriglyceridemia observed in the fructose-supplemented rats may arise from the combined effects of increased lipogenesis, reduced fatty acid catabolism and enhanced triglyceride export from liver to plasma in the form of VLDL through MTP induction.

On the other hand, plasma adiponectin level is increased in glucose-supplemented rats, which correlates with increased mRNA expression of adiponectin in adipose tissue of this group (Table 2). Although the mechanism responsible for this

inductive effect is unclear, our results suggest that the overexpression of hepatic PPARα in this group may be due to the elevated level of adiponectin. This theory is supported by other reports showing that adiponectin up-regulates PPARα expression (58). It should be noted that despite PPARα induction and elevated *l-cpt-1a* mRNA in the glucose-supplemented group, we did not observe increased hepatic CPT-1A protein expression, suggesting that fatty acid oxidation is not induced by glucose supplementation. Overall, our results on the decreased L-CPT-1A protein levels in fructose-supplemented rats suggest that simple sugars might affect L-CPT-1A protein stability, causing a reduction in its hepatic content. However, this effect is counteracted in glucose-supplemented rats, possibly via the control of its expression by increased PPARα.

It has been shown that adiponectin stimulates NO production in vascular endothelial cells (12), and therefore the enhanced basal NO in aortae of glucose-supplemented rats (Table 4) may result from an increased adiponectin in this group. In the current study, we assessed the *in vitro* effects of adiponectin or high glucose or fructose on NO level by measuring NO metabolites. Treatment of EA.hy926 with adiponectin but not high glucose or fructose caused a significant increased in NO level in these cells (Figure 7). These results exclude a direct effect of glucose on NO production and suggest that elevated adiponectin is the possible cause of increased NO in aortae of glucose-supplemented rats. Moreover, it has been reported that adiponectin stimulates the synthesis of NO in endothelial cells by phosphorylation of eNOS at Ser1177 (12, 15), and our results show that eNOS phosphorylation at this position is increased in aortic tissues from glucose-supplemented rats (Figure 6A). Taken together, these results suggest that the increase in basal NO after glucose supplementation could be mainly attributed to the hyperadiponectinaemia observed in this group.

Elevated basal NO in aortae from glucose-supplemented rats may also, in part, explain decreased vasoconstrictor response to PE in this group (Figure 5B). In addition, the fact that the SNP-induced relaxation was increased in the glucose-supplemented group suggests that the increased sensitivity of intact aorta to NO may also contribute to the decreased PE contractile responsiveness. It is important to note that glucose supplementation enhanced aortic responses to SNP despite a decrease in phosphorylation of VASP at Ser239 and preserved PKG expression, suggesting that VASP phosphorylation or upstream cGMP-dependent phosphorylation of VASP is not a mechanism for the increased relaxation response to SNP. Along similar lines, Aszódi et

al. observed an intact relaxation after exposure to cGMP and cAMP in aortic rings of VASP-null mice (4). Furthermore, Yousif et al. (60) reported that SNP induces cGMP-independent vasodilator responses in the perfused rabbit ovarian vascular bed. Unlike the glucose-supplemented group, our data show that SNP-induced relaxation is significantly reduced in aortic rings of the fructose-supplemented rats. Assessments of vasodilatory responses in the presence of a soluble guanylate cyclase inhibitor or a PKG inhibitor/activator could help to determine how fructose and glucose differentially influence the NO/cGMP/PKG relaxation pathways.

EDV is used as a reproducible parameter to investigate endothelial function under various pathological conditions. In the present study, we showed a preserved EDV of the aorta in the sugar-supplemented rats despite the altered relaxation responses to SNP. Accordingly, Mourmoura et al. (33) reported that the EDV of coronary arteries was fully maintained while the response to a NO donor was even enhanced in type 2 diabetic rats. Both impairment (16, 31, 38) or no change (23, 36) of EDV in vessels from fructose-supplemented rats have been reported. The varied vasodilatory responses after sugar supplementation may be attributed to differences in the type of the agents used, animal's sex and strain, vascular beds, and duration of sugar supplementation.

NO has been generally considered as the principal mediator of EDV in normal state in large arteries, and impaired EDV is often associated with reduced bioavailability of NO. However prostaglandin I_2 (PGI_2), and endothelium-derived hyperpolarizing factor (EDHF) may also be important regulators of vascular tone and reactivity in diabetes. There is an established negative regulatory effect of NO on EDHF synthesis (8). On the other hand, an augmented EDHF response was shown to compensate for the loss of NO-mediated vasorelaxation in arteries in diabetic rats (46). In agreement with those studies that demonstrate compensatory interactions between pathways, the preserved ACh response (regardless of altered SNP responses) suggest that other molecules besides NO (e.g. EDHF or PGI2) may be involved in ACh relaxation. The fact that *AC6* mRNA and phosphorylated PKA protein levels were significantly reduced in aorta taken from fructose rats (Figure 8A, F) suggests that the preserved ACh responses in the arteries of this group is likely due to the elevated EDHF (rather than increased PGI_2). Furthermore, we demonstrated that fructose- but not glucose-supplementation enhances PDE4 mRNA and protein expression (Figure 8A, G). The elevated PDE4 expression and subsequent reduction in cAMP levels may specifically hinder phosphorylation of VASP at Ser157 (Figure 8E). This effect combined with

562 reduced VASP expression (Fig 8D) possibly leads to an impairment of NO-independent
563 relaxation in aortae of fructose-supplemented rats.
564 Another mechanism that could account for the difference between the response
565 to ACh and SNP could be oxidative stress. Reactive oxygen species (ROS), particularly
566 superoxide anions, inactivate NO which could lead to a reduction of SNP potency. In
567 order to examine this possibility, we measured the mRNA expression of the catalytic
568 subunits of NADPH oxidase Nox1 and Nox4, major source of ROS in vascular cells
569 (19). However, we did not see significant differences in the mRNA expression for Nox1
570 and Nox4 in the aorta of experimental animals (Table 5).
571 It has been shown that under metabolic stress conditions iNOS is able to produce
572 an abnormal amount of NO leading to ROS production and decreased bioavailability of
573 NO. Although we did not measure ROS production and NO bioavailability, we
574 observed a significant induction of iNOS protein expression in aortic tissues from
575 fructose-supplemented rats (Figure 6B), which could in part lead to vascular
576 dysfunction in this group (35). Vascular dysfunction in metabolic syndrome may also be
577 associated with increased vasoconstrictor sensitivity (47). Here, however, we showed
578 that fructose supplementation did not affect vasoconstrictor responses to PE. Along
579 similar lines, no differences were reported in the vasoconstrictor responses to PE in
580 mesenteric arteries (36) or aortic rings (23) from fructose-fed rats.
581 In conclusion, we have shown that dietary supplementation with liquid glucose
582 or fructose causes metabolic and vascular alterations in female rats. Despite higher
583 caloric intake in glucose-supplemented rats, fructose caused worse metabolic and
584 vascular responses. This may be due to the elevated adiponectin level and the
585 subsequent enhancement of PPARα and eNOS phosphorylation in glucose-
586 supplemented rats, a mechanism which is absent in the fructose group. Clearly,
587 additional studies will be needed to document the direction and magnitude of these
588 interactions in sugar-supplemented rats along with the relative importance of elevated
589 adiponectin level to metabolic and vascular function in the glucose-supplemented rats.
590

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595

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Author contributions

G.S. and S.S. were in charge of all experiments. F.A. contributed to vascular reactivity experiments. NR contributed to PCR/western blot experiments and prepared the figures. J.C.L helped in data interpretation and reviewed the manuscript. R.R and M.A designed the experiments, analyzed the data and wrote the manuscript.

Disclosures

The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript. The authors have no financial or other conflicts of interest to disclose.

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Figure Legends

Figure 1. Supplementation with glucose and fructose induces hepatic lipogenesis. mRNA levels of *scd1* (A) and *fas* (C) in the liver from control, glucose- and fructose-supplemented rats. Bars represent the mean±SEM of values obtained from n=8 animals. Protein levels of SCD1 (B), FAS (D), p-ACC (E), total ACC (F), ChREBP (G) and mature SREBP-1 (F) in liver samples from control, glucose- and fructose-supplemented rats. Each bar represents the mean±SEM of values obtained from n=5 animals. To show representative bands corresponding to 3 different rats per treatment group, images from different parts of the same gel have been juxtaposed, which is indicated by white

821 dividing lines in the figure.; $^{*}P$ <0.05, $^{**}P$ <0.01 and $^{***}P$ <0.001 versus control. One-
822 way ANOVA followed by Bonferroni's *post hoc* test.
823
824 **Figure 2. Differential effects of glucose and fructose on PPARα, PPARα target**
825 **genes and MTP.** (A) Protein levels of PPARα (A), L-CPT-1A (D) and MTP (E) in
826 hepatic samples from control, glucose- and fructose-supplemented rats. Each bar
827 represents the mean±SEM of values obtained from n=5 animals. To show representative
828 bands corresponding to 3 different rats per treatment group, images from different parts
829 of the same gel have been juxtaposed, which is indicated by white dividing lines in the
830 figure. mRNA levels of *l-cpt-1a* (B) and *aco* (C) in hepatic samples from control,
831 glucose- and fructose-supplemented rats. Each bar represents the mean±SEM of values
832 obtained from n=8 animals; $^{*}P$ <0.05 and $^{**}P$ <0.01 versus control; $^{\#\#}P$ <0.01 versus
833 glucose. One-way ANOVA followed by Bonferroni's *post hoc* test.
834
835 **Figure 3. Effects of glucose and fructose supplementation on the expression of**
836 **proteins involved in insulin signalling in liver and aortic tissues.** Protein levels of
837 hepatic IRS-1 (A), and IRS-2 (B), aortic IRS-1 (C) and IRS-2 (D), and hepatic (E) and
838 aortic (FD) phosphorylated and total Akt in samples from control, glucose- and
839 fructose-supplemented rats. Each bar represents the mean±SEM of values obtained from
840 n=5 animals. To show representative bands corresponding to 3 different rats per
841 treatment group, images from different parts of the same gel have been juxtaposed,
842 which is indicated by white dividing lines in the figure. $^{*}P$ <0.05, $^{**}P$ <0.01 and $^{***}P$
843 <0.001 versus control. One-way ANOVA followed by Bonferroni's *post hoc* test.
844
845 **Figure 4. Effects of supplementation with glucose and fructose on the responses of**
846 **aortic rings to relaxation agents.** Relaxation responses to cumulative concentrations of
847 (A) acetylcholine (ACh), (B) bradykinin (BK) and (C) sodium nitroprusside (SNP) in
848 intact aortic rings from female rats after 2 months of supplementation with 20% w/v
849 liquid fructose or glucose. Aortic rings were pre-contracted with phenylephrine (2 μM)
850 (A, B) or U46619 (100 nM) (C). Data are expressed as mean±SEM of values obtained
851 from n=5-8 animals; $^{*}P$ <0.05 and $^{***}P$ <0.001 versus control, analyzed using two-way
852 ANOVA followed by Bonferroni's post hoc test.
853

Figure 5. Supplementation with glucose reduces the contractile responses of aortic rings. (A) Contractile responses to cumulative concentrations of phenylephrine (PE) in intact aortic rings from female rats after 2 months of supplementation with 20% w/v liquid fructose or glucose. (B-D) Contraction to PE was measured in aortae from control, glucose- and fructose-supplemented rats before and after incubation with Nω-Nitro-L-arginine methylester (L-NAME, 200 μM). Responses were performed in the presence of indomethacin (10 μM). Data are expressed as mean±SEM of vàlues obtained from n=7-8 animals; $^{*}P < 0.05$ and $^{***}P < 0.001$ versus control condition (control rats in A, before L-NAME in B-D), analyzed using two-way ANOVA followed by Bonferroni's post hoc test.

Figure 6. Supplementation with glucose enhances eNOS phosphorylation and supplementation with fructose increases iNOS expression in aortic tissue. Western blots of phosphorylated and total eNOS (A) and iNOS (B) in aortic samples from control, glucose- and fructose-supplemented rats. Each bar represents the mean±SEM of values obtained from n=5 animals. To show representative bands corresponding to 3 different rats per treatment group, images from different parts of the same gel have been juxtaposed, which is indicated by white dividing lines in the figure. $^{*}P < 0.05$ versus control, $^{\#\#}P < 0.01$ versus glucose. One-way ANOVA followed by Bonferroni's *post hoc* test.

Figure 7. In vitro adiponectin (but not glucose or fructose) increases NO level in EA.hy926 cells. Levels of nitrate and nitrite in EA.hy926 medium after incubation with vehicle (CT), adiponectin (APN, 5 and 15 μg/ml), 25 mM glucose, 25 mM fructose, 25 mM mannitol (MAN) and the NO donor SNAP (100 μM). Each bar represents the mean±SEM of five to six different assays performed in duplicate. $^{***}P < 0.001$ and $^{****}P < 0.0001$ versus control. One-way ANOVA followed by Bonferroni's *post hoc* test.

Figure 8. Differential effects of fructose and glucose supplementation on intracellular pathways related to aortic relaxation. (A) mRNA levels of *ac6, gcsa1* , *pde4d*and *pde5* in aortic tissue samples from control, glucose- and fructose-supplemented rats. Each bar represents the mean±SEM of values obtained from n=8 animals. Protein levels of PKG (B), VASP phosphorylated in Ser239 (C), total VASP

888 (D), and VASP phosphorylated in Ser157 (E), phosphorylated PKAc (F) and PDE4 (G)
889 in aortic samples from control, glucose- and fructose-supplemented rats. Each bar
890 represents the mean±SEM of values obtained from n=5 animals. To show representative
891 bands corresponding to 3 different rats per treatment group, images from different parts
892 of the same gel have been juxtaposed, which is indicated by white dividing lines in the
893 figure. $^{*}P$ <0.05 and $^{***}P$ <0.001 versus control, $^{\#\#}P$ <0.01 versus glucose. One-way
894 ANOVA followed by Bonferroni's *post hoc* test.
895
896
897

Table 1: Primers used for RT-PCR

Gen	GenBankTM nº	Primer sequences	PCR product
ac6	NM_001270785.1	Forward: 5'-FCTTTGCCACCAGTTCTCTGC-3' Reverse: 5'-GCCTTGGCTAATTAAGCGCC-3'	107 bp
ace	NM_012544.1	Forward: 5'-GAGCCATCCTTCCCTTTTTC-3' Reverse: 5'-GGCTGCAGCTCCTGGTATAG-3'	154 bp
aco	NM_017340.2	Forward: 5'-GTGAGGCGCCAGTCTGAAA-3' Reverse: 5'-ACTGCTGGGTTTGAAAATCCA-3'	70 bp
actb	NM_031144.3	Forward: 5'-CTAAGGCCAACCGTGAAAAG-3' Reverse: 5'-GGGGTGTTGAAGGTCTCAAA-3'	55 bp
adipoq	NM_144744.3	Forward: 5'-GAGACGCAGGTGTTCTTG-3' Reverse: 5'-CCTACGCTGAATGCTGAG-3'	148 bp
adra1b	NM_016991.2	Forward: 5'-AGCGGTAGATGTCCTGTGCT-3' Reverse: 5'-AGATGACCGTGGACAAGACC-3'	164 bp
aldob	NM_012496.2	Forward: 5'-ACAGCCTCCTACACCTACT-3' Reverse: 5'-GCTCATACTCGCACTTCA-3'	198 bp
agtr1a	NM_030985.4	Forward: 5'-CACAGTGTGCGCGTTTCATT-3' Reverse: 5'-GTAAGGCCCAGCCCTATGG-3'	63 bp
agtr2	NM_012494.3	Forward: 5'-TTGTGTTGGCATTCATCATTTG-3' Reverse: 5'-ATACCCATCCAGGTCAGAGCAT-3'	76 bp
bdkrb1	NM_030851.1	Forward: 5'-CAGCGCTTAACCATAGCGGAAAT-3' Reverse: 5'-CCAGTTGAAACGGTTCCCGATGTT-3'	112 bp
bdkrb2	NM_173100.2	Forward: 5'-TTTGTCCTCAGCGTGTTCTG-3' Reverse: 5'-TCACAAGCATCAGGAAGCAG-3'	226 bp
cox1	NM_017043.4	Forward: 5'-AAGTACTCATGCGCCTGGTACTC-3' Reverse: 5'-CATGTGCTGTGTTGTAGGTTGGA-3'	75 bp
cox2	NM_017232.3	Forward: 5'-TCGACTTTTCCAGGATGGAAA-3' Reverse: 5'-GAGTGTCTTTGACTGTGGGAGGAT-3'	77 bp
enos	NM_021838.2	Forward: 5'-GTGACCCTCACCGATACAACATAC-3' Reverse: 5'-GATGAGGTTGTCCGGGTGTCT-3'	73 bp
fas	NM_017332	Forward: 5'-GGCTCTATGGGTTGCCTAAGC-3' Reverse: 5'-GGTGGACCCCAAAAAAGGA-3'	78 bp
gcsa1	NM_017090.2	Forward: 5'-CAGTGTGGAGAGCTGGATGTCT-3' Reverse: 5'-AATCCCCCTGCCACACAAT-3'	68 bp
l-cpt1a	NM_031559.2	Forward: 5'-TGCAGAACACGGCAAAATGA-3' Reverse: 5'-CCGACCTGAGAGGACCTTGA-3'	70 bp
leptin	NM_013076.3	Forward: 5'-GGTCACCGGTTTGGACTTCA-3' Reverse: 5'-GGTCTGGTCCATCTTGGACAA-3'	67 bp
nox1	NM_053683.1	Forward: 5'-ATACACATCACCTTTTCATCATCTATATCA-3' Reverse: 5'-GTTTGACCCCGGACAATCC-3'	76 bp
nox4	NM_053524.1	Forward: 5'-CGCACAGTCCTGGCTTACCT-3' Reverse: 5'-GCTTTTGTCCAACAATCTTCTTGTT-3'	75 bp
pde4d	NM_001113328.1	Forward: 5'-GCCAGCCTTCGAACTGTAAG-3' Reverse: 5'-ATGGATGGTTGGTTGCACAT-3'	98 bp

pde5	NM_133584.1	Forward: 5'-CCCTTTGGAGACAAAACGAGAG-3' Reverse: 5'-AGGACTTTGAGGCAGAGAGC-3'	129 bp
ptgis	NM_031557.2	Forward: 5'-GCAGGAGAAAGGTCTGCTTGA-3' Reverse: 5'-TCCACTCCATACAGGGTCAGGTA-3'	75 bp
scd1	NM_139192.2	Forward: 5'- CAGAGCCAGGTGCCACTTTT-3' Reverse: 5'-TGCTAGAGGGTGTACCAAGCTTT-3'	104 bp
tbp	NM_001004198.1	Forward: 5'-TGGGATTGTACCACAGCTCCA-3' Reverse: 5'-CTCATGATGACTGCAGCAAACC-3'	132 bp
tbxas	NM_012687.1	Forward: 5'-GAGCTCCGAGAGCGATATGG-3' Reverse: 5'-CTGGGTCTGAAATGACAATGTACAT-3'	76 bp

ac: adenylyl cyclase; ace: angiotensin I converting enzyme; aco: acyl-CoA oxidase; actb: actin beta; adipoQ: adiponectin, C1Q and collagen domain containing; adra: adrenergic receptor, α; agtr: angiotensin II receptor; aldob: aldolase B; bdkr: bradikinin recpetor; cox: cyclooxigenase; eNOS: endothelial nitric oxide synthase; fas: fatty acid synthase; gcsa: soluble guanylate cyclase; l-cpt: liver carnitine palmitoyltransferase; nox: NADPH oxidase; pde: phosphodiesterase; ptgis: prostaglandin I2 (prostacyclin) synthase; scd1: stearoyl-CoA desaturase; tbp: TATA box binding protein; tbxas: thromboxane synthase.

Table 2. Zoometric parameters, plasma/blood analytes and adipokine mRNA levels in adipose tissue of female rats supplemented with 20% w/v liquid fructose or glucose for 2 months

	Control	Glucose	Fructose
AUC liquid intake (ml/[rat x 2 months])	2378 ± 127	4778 ± 185*	3577 ± 182$^{*,\#}$
AUC solid food intake (g/[rat x 2 months])	874,8 ± 19,5	302,6 ± 24,4*	415,4 ± 21,1$^{*,\#}$
Kcal ingested from solid (rat x 2 months)	2642 ± 59	914 ± 74*	1255 ± 634$^{*,\#}$
Kcal ingested from liquid (rat x 2 months)	0	3822 ± 148	2862 ± 146
Total ingested kcal (2 rats x 2 months)[a]	2642 ± 59	4736 ± 158*	4116 ± 129$^{*,\#}$
Final body weight	239.1 ± 3.9	245.1 ± 5.5	254.9 ± 5.9*
Femur length (cm)	3.32 ± 0.10	3.35 ± 0.05	3.38 ± 0.12
Adipose tissue weight (g)/femur length (cm)	0.37 ± 0.08	2.06 ± 0.29*	1.91 ± 0.38*
Liver weight (g)/femur length (cm)	2.92 ± 0.10	3.16 ± 0.19*	4.00 ± 0.09$^{*,\#}$
Plasma leptin (ng/ml)	2.46 ± 0.08	4.51 ± 0.09*	3.89 ± 0.08$^{*,\#}$
Adipose tissue *leptin* mRNA levels (a.u)	100 ± 22	189 ± 28*	193 ± 26*
Plasma adiponectin (µg/ml)	22.58 ± 2,4	58.42 ± 5,0*	26.48 ± 2.5
Adipose tissue *AdipoQ* mRNA levels (a.u)	100 ± 23	263 ± 48*	140 ± 32
Plasma insulin (mg/dl)	0.67 ± 0.13	1.24 ±0.24	1.64 ± 0.35*
Blood glucose (mg/dl)	101.7 ± 5.1	112.7 ± 5.9	109.7 ± 6.0
Blood triglycerides (mg/dl)	116.1 ± 3.9	125.7 ± 2.8	144.4 ± 13.1*
ISI	1.23 ± 0.02	0.88 ± 0.03*	0.76 ± 0.02*
Blood cholesterol (mg/dl)	166.0 ± 1.4	166.6 ± 0.9	165.5 ± 0.8

ISI: Insulin Sensitivity Index, calculated as [2/(blood insulin (nM) x blood glucose (µM) + 1]. Values are expressed as mean ± SEM (n= 14 rats/group for plasma / blood analytes and

zoometric parameters; n=8 rats/group for mRNA expression). $^{*}P<0.05$ vs control; $^{\#}P<0.05$ vs glucose-supplemented rats. One-way ANOVA test followed by Bonferroni's post hoc test.

Table 3. pD_2 and E_{max} or $tension_{max}$ to vasodilator and vasoconstrictor agents in the aortae from female rats supplemented with 20% w/v liquid fructose or glucose for 2 months

		n	pD2	E max (%)
Ach	Control	8	6.75 ± 0.15	85.21 ± 2.63
	Glucose	8	6.86 ± 0.10	85.59 ± 2.12
	Fructose	8	6.88 ± 0.15	87.59 ± 2.46
BK	Control	5	6.27 ± 0.51	90.51 ± 4.26
	Glucose	6	5.00 ± 0.80	62.48 ± 14.22
	Fructose	5	4.58 ± 0.69	54.38 ± 16.60
SNP	Control	8	8.97 ± 0.07	100.23 ± 0.28
	Glucose	8	9.51 ± 0.10*	100.01 ± 0.19
	Fructose	8	8.64 ± 0.14	100.01 ± 0.19
		n	pD2	Tension max (g)
PE	Control	7	7.15 ± 0.08	1.60 ± 0.15
	Glucose	8	7.02 ± 0.08	1.10 ± 0.10*
	Fructose	7	7.32 ± 0.10	1.54 ± 0.14

Data are expressed as mean ± SEM. *$P<0.05$, one-way ANOVA test followed by Bonferroni's post hoc test.

Table 4. E_{max}, $tension_{max}$ pD2 and ΔAUC to phenylephrine in the aortae from female rats supplemented with 20% w/v liquid fructose or glucose for 2 months

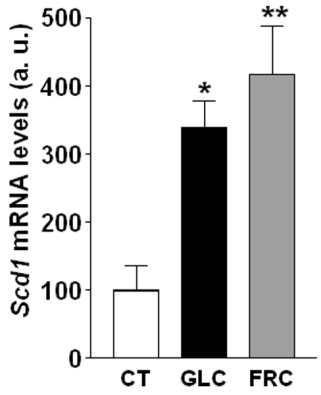
	n	E_{max} (%)	$Tension_{max}$ (g)	pD_2	ΔAUC
Control	7				
Before L-NAME		91.10±9.19	1.48±0.24	7.05±0.09	-
After L-NAME		139.7±4.00**	2.21±0.18*	7.30±0.07	116.90±21.49
Glucose	8				
Before L-NAME		92.05±8.15	0.97±0.07	6.98±0.06	-
After L-NAME		179.4±16.07$^{*, \#}$	1.86±0.08**	7.20±0.06	197.24±30.78$^{\#}$
Fructose	7				
Before L-NAME		81.63±5.32	1.26±0.16	7.05±0.05	-
After L-NAME		143.6±11.62*	2.16±0.17*	7.38±0.08	158.90±19.75

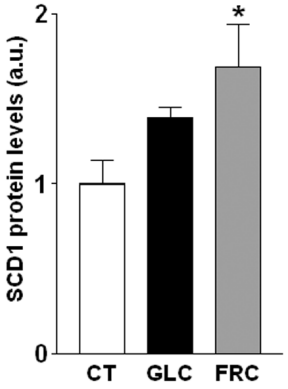
Data are expressed as mean ± SEM. * P<0.05 (vs. before L-NAME), paired Student's t test; $^{\#}$ P<0.05 (vs. control), one way ANOVA followed by Bonferroni's post hoc test.

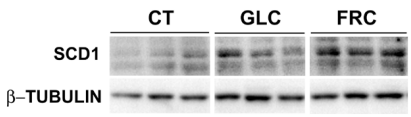
Table 5. mRNA expression of genes related to vascular reactivity in aortic tissue from female rats supplemented or not with 20% w/v liquid fructose or glucose for 2 months

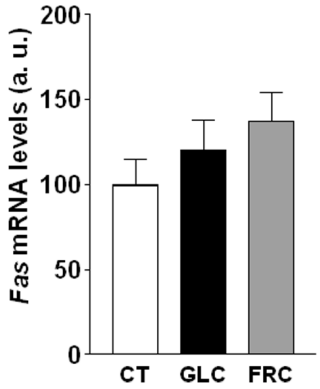
	Control	Glucose	Fructose
Cox-1	100±14	132±6	115±11
Cox-2	100±7	121±29	77±18
Ptgis	100±9	95±5	89±5
Tbxas	100±17	85±10	92±6
Ace	100±12	87±7	78±7
Agtr1a	100±25	100±6	91±12
Agtr2	100±34	94±22	85±25
Aldob	104±25	149±51	140±39
Adra1a	100±40	194±65	102±14
Adra1b	100±8	129±22	144±18
Adra1d	100±8	114±22	113±18
Bdkrb1	100±39	93±28	102±45
Bdkrb2	100±19	142±33	118±25
Nox1	100±38	123±36	102±36
Nox4	100±3	130±9	124±7

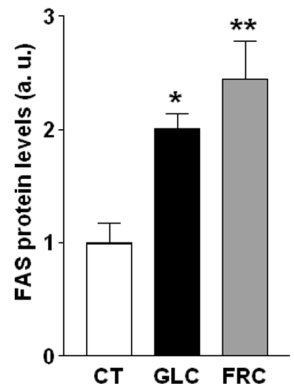
Ace: angiotensin I converting enzyme; Adra: adrenergic receptor, α; Agtr: angiotensin II receptor; Aldob: aldolase B; Bdkr: bradikinin recpetor; Cox: cyclooxigenase; Nox: NADPH oxidase; Ptgis: Prostaglandin I2 (Prostacyclin) Synthase; Tbxas: Thromboxane synthase


Scd1 mRNA levels (a. u.)
500
400
300
200
100
0
*
**
CT
GLC
FRC


SCD1 protein levels (a.u.)
2
1
0
*
CT
GLC
FRC


CT
GLC
FRC
SCD1
β–TUBULIN


Fas mRNA levels (a. u.)
200
150
100
50
0
CT
GLC
FRC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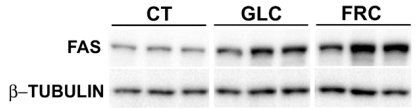
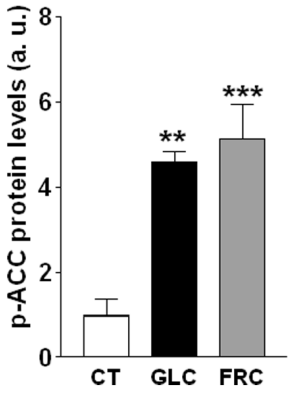
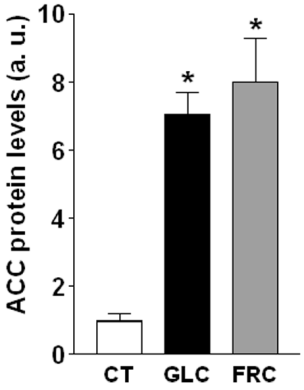
3
2
1
0
*
**
CT
GLC
FRC


CT
GLC
FRC
FAS
β–TUBULIN


p-ACC protein levels (a. u.)
8
6
4
2
0
**
***
CT
GLC
FRC


ACC protein levels (a. u.)
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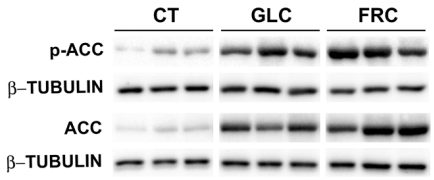
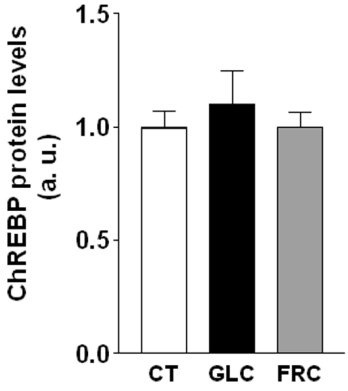
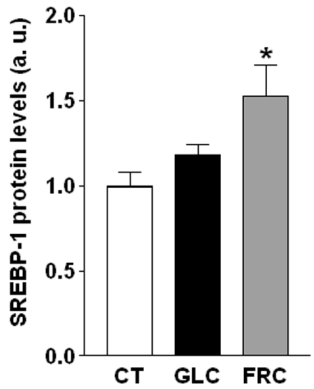
8
6
4
2
0
*
*
CT
GLC
FRC


CT
GLC
FRC
p-ACC
β–TUBULIN
ACC
β–TUBULIN


ChREBP protein levels (a. u.)
1.5
1.0
0.5
0.0
CT
GLC
FRC


SREBP-1 protein levels (a. u.)
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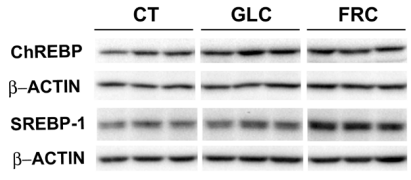
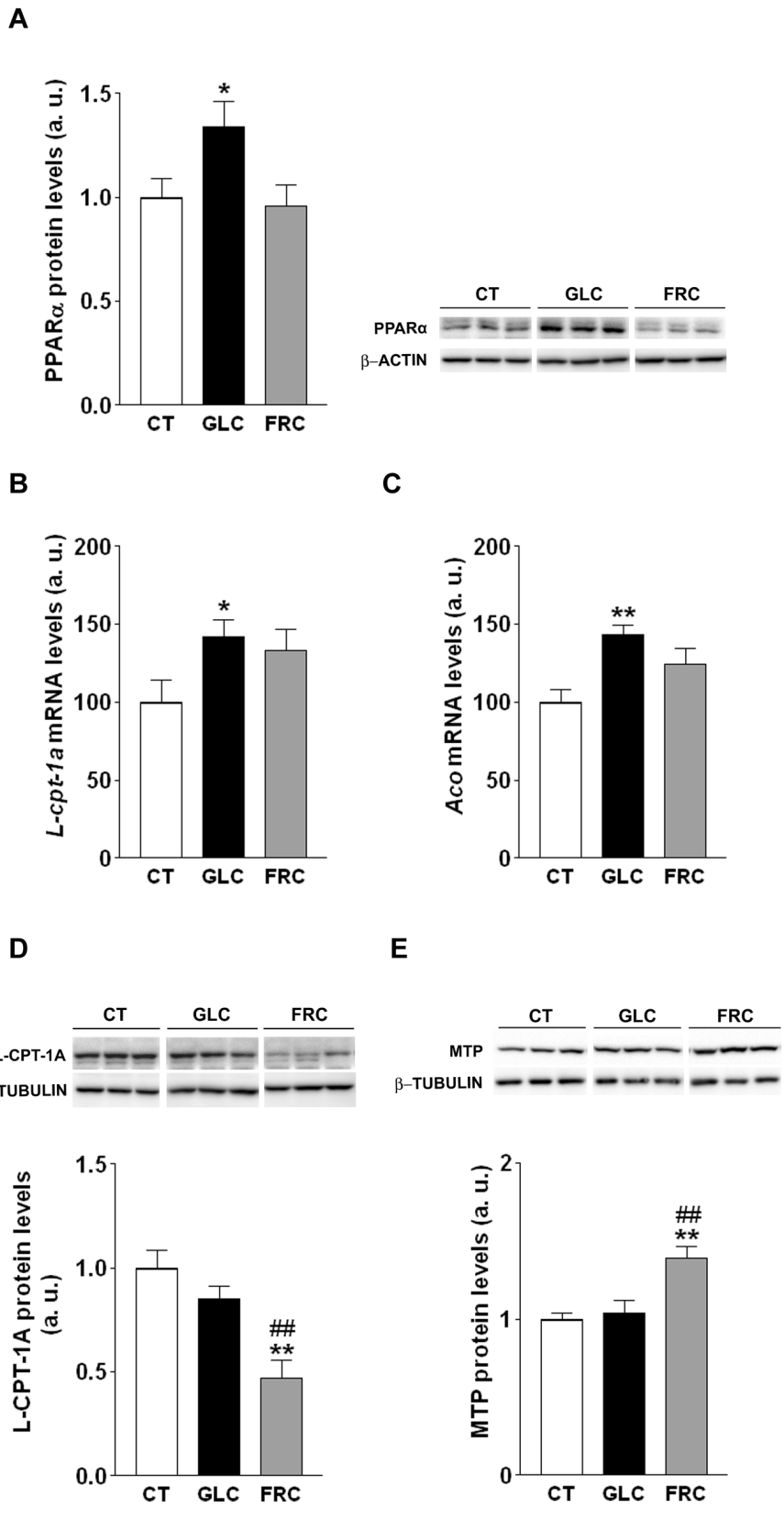
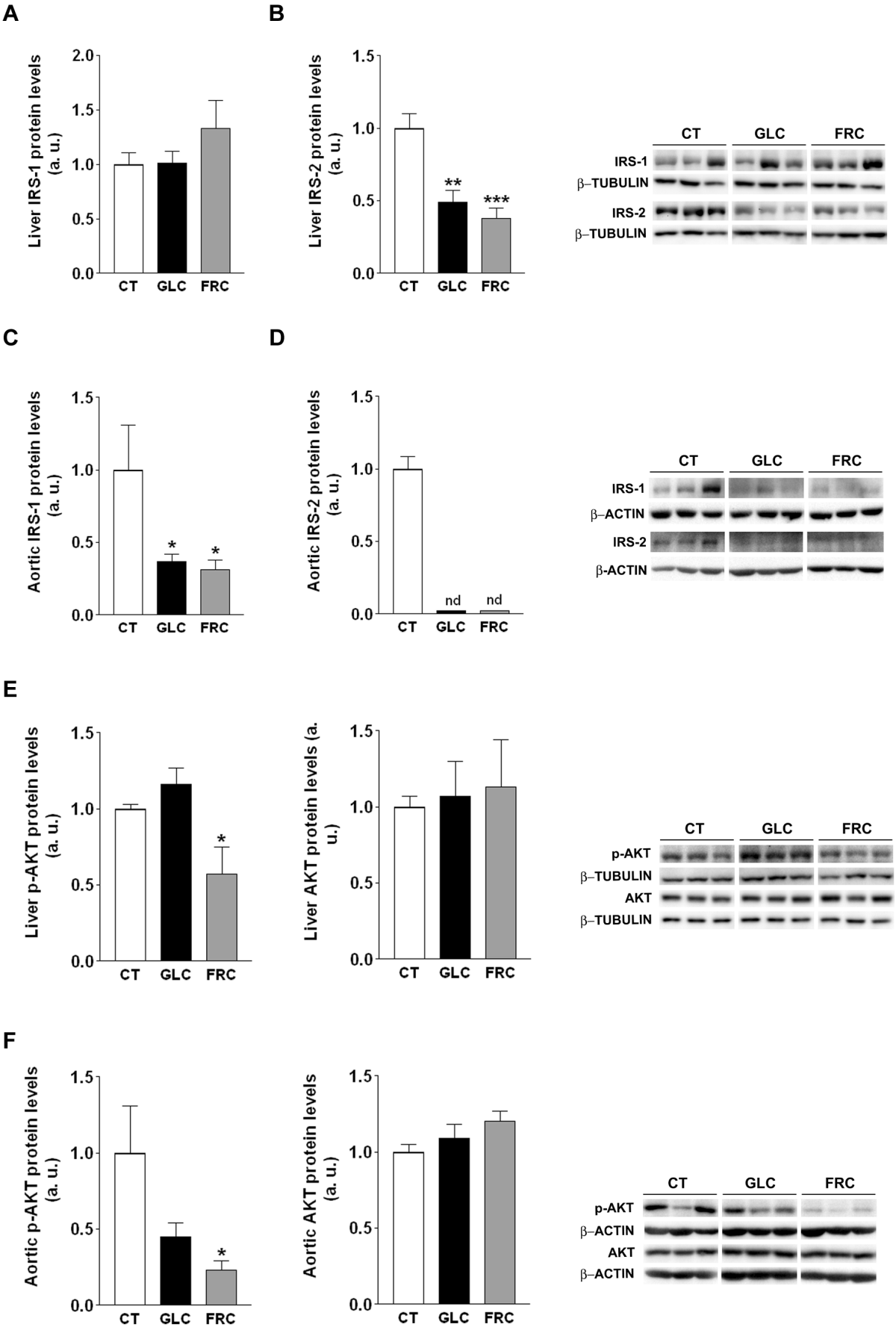
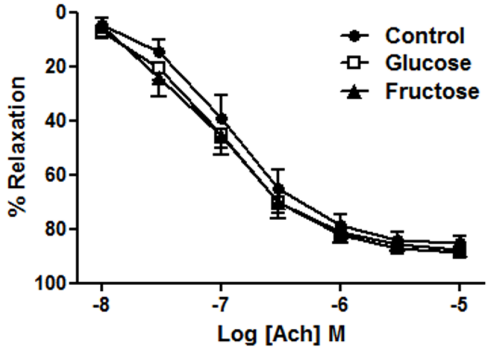
1.5
1.0
0.5
0.0
*
CT
GLC
FRC


CT
GLC
FRC
ChREBP
β–ACTIN
SREBP-1
β–ACTIN

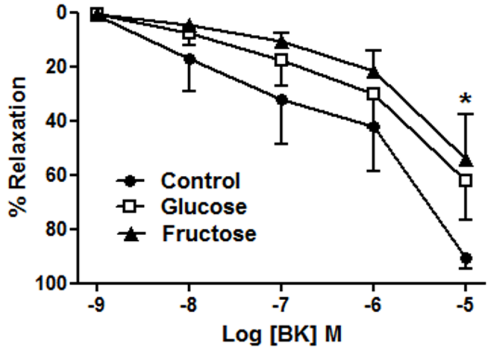

A
PPARα protein levels (a. u.)
1.5
1.0
0.5
0.0
*
CT
GLC
FRC
CT
GLC
FRC
PPARα
β–ACTIN
B
L-cpt-1a mRNA levels (a. u.)
200
150
100
50
0
*
CT
GLC
FRC
C
Aco mRNA levels (a. u.)
200
150
100
50
0
**
CT
GLC
FRC
D
CT
GLC
FRC
L-CPT-1A
β–TUBULIN
L-CPT-1A protein levels (a. u.)
1.5
1.0
0.5
0.0
##
**
CT
GLC
FRC
E
CT
GLC
FRC
MTP
β–TUBULIN
MTP protein levels (a. u.)
2
1
0
##
**
CT
GLC
FRC


A
Liver IRS-1 protein levels (a. u.)
2.0
1.5
1.0
0.5
0.0
CT
GLC
FRC
B
Liver IRS-2 protein levels (a. u.)
1.5
1.0
0.5
0.0
**
***
CT
GLC
FRC
CT
GLC
FRC
IRS-1
β–TUBULIN
IRS-2
β–TUBULIN
C
Aortic IRS-1 protein levels (a. u.)
1.5
1.0
0.5
0.0
*
*
CT
GLC
FRC
D
Aortic IRS-2 protein levels (a. u.)
1.5
1.0
0.5
0.0
nd
nd
CT
GLC
FRC
CT
GLC
FRC
IRS-1
β–ACTIN
IRS-2
β-ACTIN
E
Liver p-AKT protein levels (a. u.)
1.5
1.0
0.5
0.0
*
CT
GLC
FRC
Liver AKT protein levels (a. u.)
1.5
1.0
0.5
0.0
CT
GLC
FRC
CT
GLC
FRC
p-AKT
β–TUBULIN
AKT
β–TUBULIN
F
Aortic p-AKT protein levels (a. u.)
1.5
1.0
0.5
0.0
*
CT
GLC
FRC
Aortic AKT protein levels (a. u.)
1.5
1.0
0.5
0.0
CT
GLC
FRC
CT
GLC
FRC
p-AKT
β–ACTIN
AKT
β–ACTIN

A


Control
Glucose
Fructose
% Relaxation
0
20
40
60
80
100
-8
-7
-6
-5
Log [Ach] M

B


Control
Glucose
Fructose
% Relaxation
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20
40
60
80
100
*
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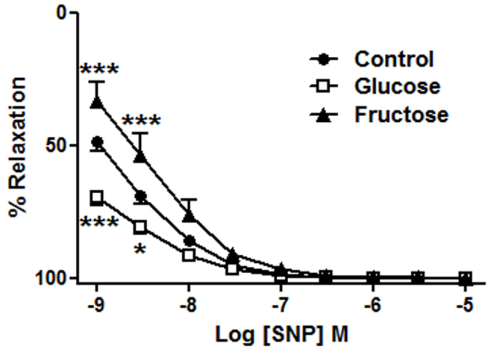
-8
-7
-6
-5
Log [BK] M

C


Control
Glucose
Fructose
% Relaxation
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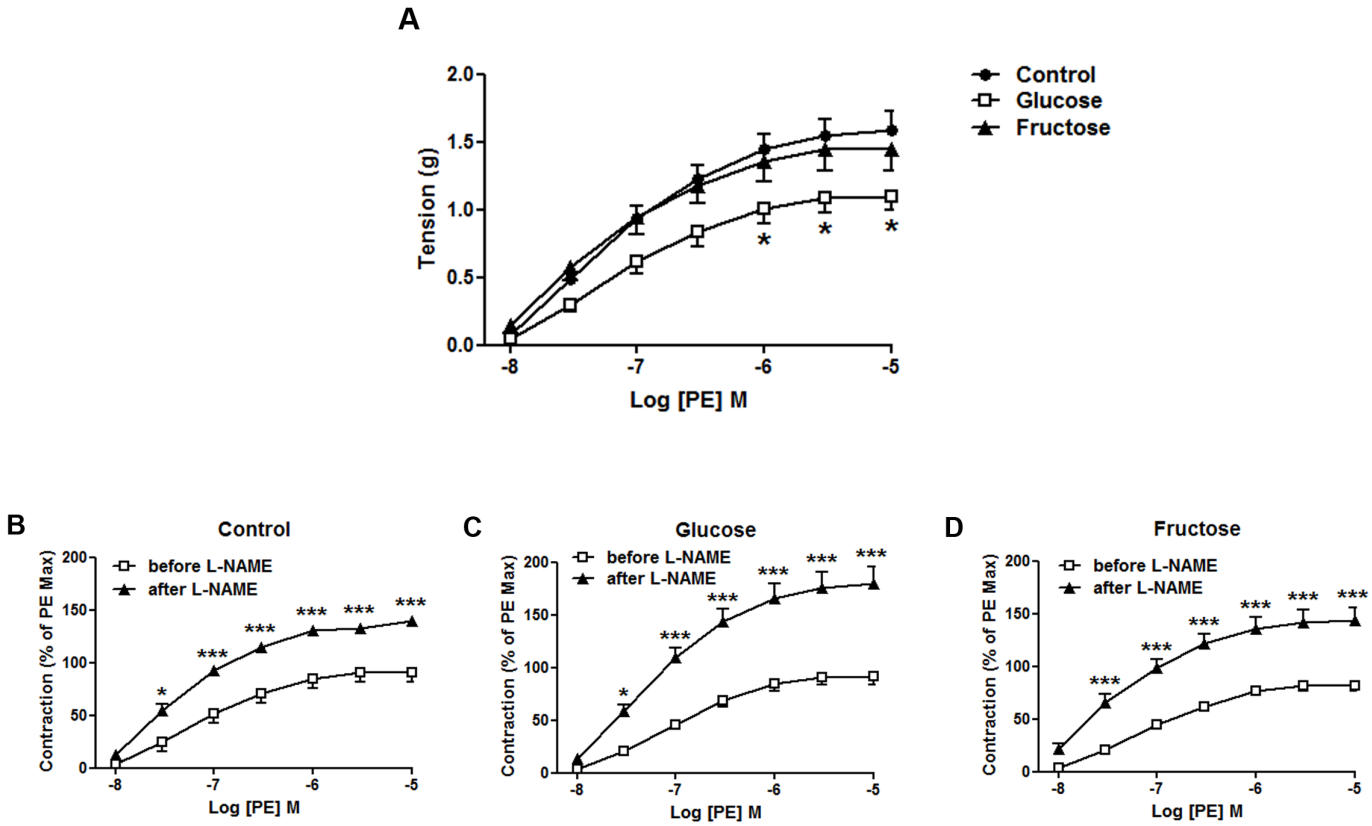
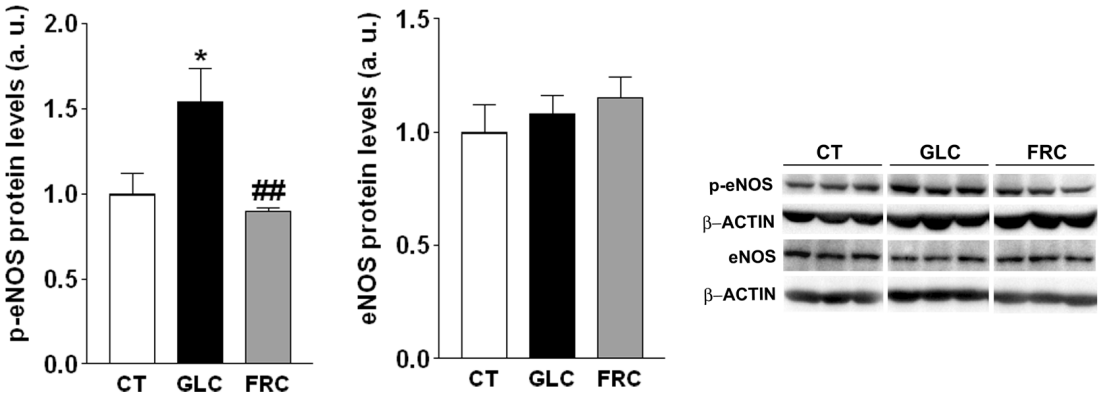
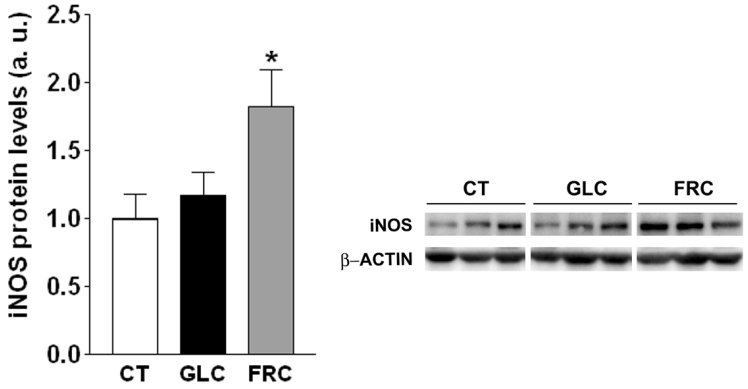
50
100
***
***
***
*
-9
-8
-7
-6
-5
Log [SNP] M


A
Control
Glucose
Fructose
Tension (g)
2.0
1.5
1.0
0.5
0.0
-8
-7
-6
-5
Log [PE] M
*
*
*
B
Control
before L-NAME
after L-NAME
Contraction (% of PE Max)
200
150
100
50
0
*
***
***
***
***
***
Log [PE] M
C
Glucose
before L-NAME
after L-NAME
*
***
***
***
***
***
D
Fructose
before L-NAME
after L-NAME
***
***
***
***
***

A


p-eNOS protein levels (a. u.)
2.0
1.5
1.0
0.5
0.0
*
##
CT
GLC
FRC
eNOS protein levels (a. u.)
1.5
1.0
0.5
0.0
CT
GLC
FRC
CT
GLC
FRC
p-eNOS
β–ACTIN
eNOS
β–ACTIN

B


iNOS protein levels (a. u.)
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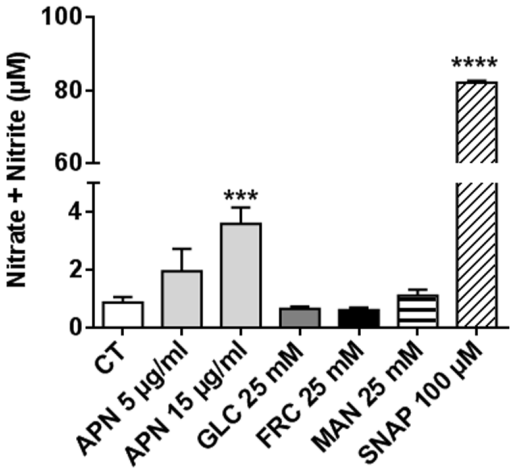
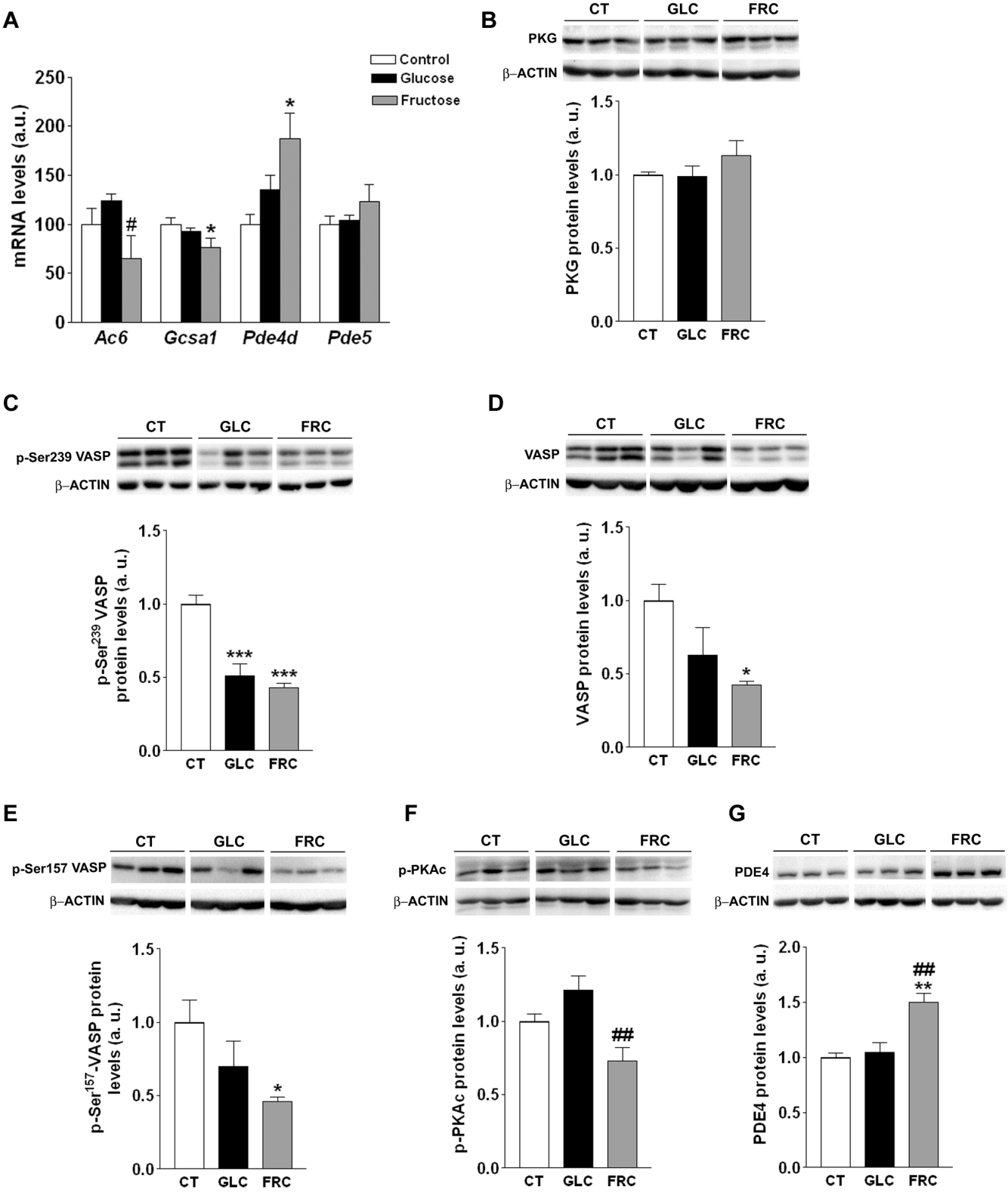
2.0
1.5
1.0
0.5
0.0
*
CT
GLC
FRC
CT
GLC
FRC
iNOS
β–ACTIN


Nitrate + Nitrite (µM)
100
80
60
4
2
0
***
****
CT
APN 5 µg/ml
APN 15 µg/ml
GLC 25 mM
FRC 25 mM
MAN 25 mM
SNAP 100 µM


A
Control
Glucose
Fructose
mRNA levels (a.u.)
250
200
150
100
50
0
Ac6
Gcsa1
Pde4d
Pde5
#
*
*
B
CT
GLC
FRC
PKG
β–ACTIN
PKG protein levels (a. u.)
1.5
1.0
0.5
0.0
C
p-Ser239 VASP
β–ACTIN
p-Ser239 VASP protein levels (a. u.)
***
***
D
VASP
β–ACTIN
VASP protein levels (a. u.)
*
E
p-Ser157 VASP
β–ACTIN
p-Ser157-VASP protein levels (a. u.)
*
F
p-PKAc
β–ACTIN
p-PKAc protein levels (a. u.)
##
G
PDE4
β–ACTIN
PDE4 protein levels (a. u.)
2.0
##
**