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ORIGINAL PAPER

Fructose-rich diet-induced abdominal adipose tissue endocrine dysfunction in normal male rats

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Abstract We have currently studied the changes induced by administration of a fructose-rich diet (FRD) to normal rats in the mass and the endocrine function of abdominal (omental) adipose tissue (AAT). Rats were fed ad libitum a standard commercial chow and tap water, either alone (control diet, CD) or containing fructose (10%, w/vol) (FRD). Three weeks after treatment, circulating metabolic markers and leptin release from adipocytes of AAT were measured. Plasma free fatty acids (FFAs), leptin, adiponectin, and plasminogen activator inhibitor-1 (PAI-1) levels were significantly higher in FRD than in CD rats. AAT mass was greater in FRD than in CD rats and their adipocytes were larger, they secreted more leptin and showed impaired insulin sensitivity. While leptin mRNA expression increased in AAT from FRD rats, gene expression of insulin receptor substrate, IRS1 and IRS2 was significantly reduced. Our study demonstrates that administration of a FRD significantly affects insulin sensitivity and several AAT endocrine/metabolic functions. These alterations could be part of a network of interacting abnormalities triggered by FRD-induced oxidative stress at the AAT level. In view of the impaired glucose tolerance observed in FRD rats, these alterations could play a key role in both the development of metabolic syndrome (MS) and β-cell failure.

Keywords Adipokines · Metabolic syndrome · Insulin signalling · Lipids · Dexamethasone

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Introduction

Fructose syrup has become a popular sweetener used in soft drinks and many other products of massive daily consumption; in countries like the USA, the annual per capita fructose consumption rose from 0.2 kg in 1970 to 28 kg in 1997 [1, 2]. It has been postulated that such increased consumption would contribute to the current obesity, type 2 diabetes and metabolic syndrome (MS) epidemics [3]. Several studies demonstrated that administration of a fructose-rich diet (FRD) to normal rats induces several features of the MS [4, 5]. However, the mechanisms whereby FRD induces the MS are still not fully understood. It has been suggested that the main mechanism triggered by the FRD is an increased production of reactive oxygen species (ROS) that cause oxidative stress [6]. Recent studies from one of our laboratories [7] demonstrated that FRD administration to normal rats for 3 weeks induces simultaneously: (a) an increase of serum triglyceride and free fatty acids (FFAs) levels; (b) a state of insulin resistance together with an impairment in glucose tolerance; (c) an increase in the percentage of saturated fatty acid composition in the abdominal adipose tissue (AAT); (d) larger basal in vitro release of FFAs by AAT pads together with a local pro-oxidative state.

Because adipose tissue is widely recognized to be an active endocrine organ [8] rather than a simple deposit of energy-rich substrates and, on the basis of our previous [7]

and other [9] studies, we currently investigated whether FRD-induced AAT oxidative stress could promote a local (AAT) endocrine dysfunction. For this purpose we studied in normal rats fed a FRD for 3 weeks: (1) peripheral levels of lipids and several adipokines; (2) the AAT mRNA concentration of leptin [10] and of some intracellular insulin mediators (Insulin Receptor Substrate (IRS)1 and IRS2) [11]; (3) morphometric characteristics of AAT adipocytes; and (4) the in vitro endocrine function of adipocytes isolated from AAT.

Results

Effect of FRD on peripheral biomarkers

As expected [7], FRD rats had significantly ($P < 0.05$) higher plasma concentrations of glucose (8.27 ± 0.23 vs. 7.16 ± 0.27 mM), triglyceride (1.28 ± 0.07 vs. 0.76 ± 0.08 g/l), and FFA (9.43 ± 0.41 vs. 6.28 ± 0.30 mM); however, similar concentrations of total cholesterol were found in the two groups (CD and FRD: 0.37 ± 0.03 and 0.39 ± 0.02 g/l). Circulating leptin, adiponectin, and plasminogen activator inhibitor factor-1 (PAI-1) levels were higher ($P < 0.05$) in FRD than in CD rats (Fig. 1: upper, middle, and lower panels, respectively). Conversely, both groups (CD and FRD) had similar levels of C-reactive protein (0.65 ± 0.11 and 0.54 ± 0.04 mg/ml) and corticosterone (9.01 ± 2.62 and 8.22 ± 1.45 μg/dl).

AAT mass and adipocyte characteristics

FRD rats showed a slight but significant increase in AAT mass (2.46 ± 0.29 vs. 1.87 ± 0.21 g; $n = 6/7$ rats per group; $P < 0.05$). AAT pads from FRD rats digested with collagenase yielded a significantly ($P < 0.05$ vs. CD) lower number of adipocytes ($1.74 \pm 0.16 \times 10^6$ vs. $2.69 \pm 0.21 \times 10^6$ cells per gram of AAT; $n = 6/7$ rats per group). These data fully agree with the morphological characteristics of these cells. In fact, FRD AAT pads displayed a decrease in cell number and an increase in both adipocyte diameter and volume (Table 1).

Leptin, IRS1, and IRS2 gene expression in AAT

In agreement with the higher circulating leptin levels and the size of the adipocytes, leptin mRNA abundance in AAT pads was significantly ($P < 0.05$) higher in FRD-fed than in CD rats (Fig. 2).

Conversely, the expression of IRS1 and IRS2 mRNAs in this tissue was significantly lower ($P < 0.05$) in FRD than in CD rats (Fig. 2).

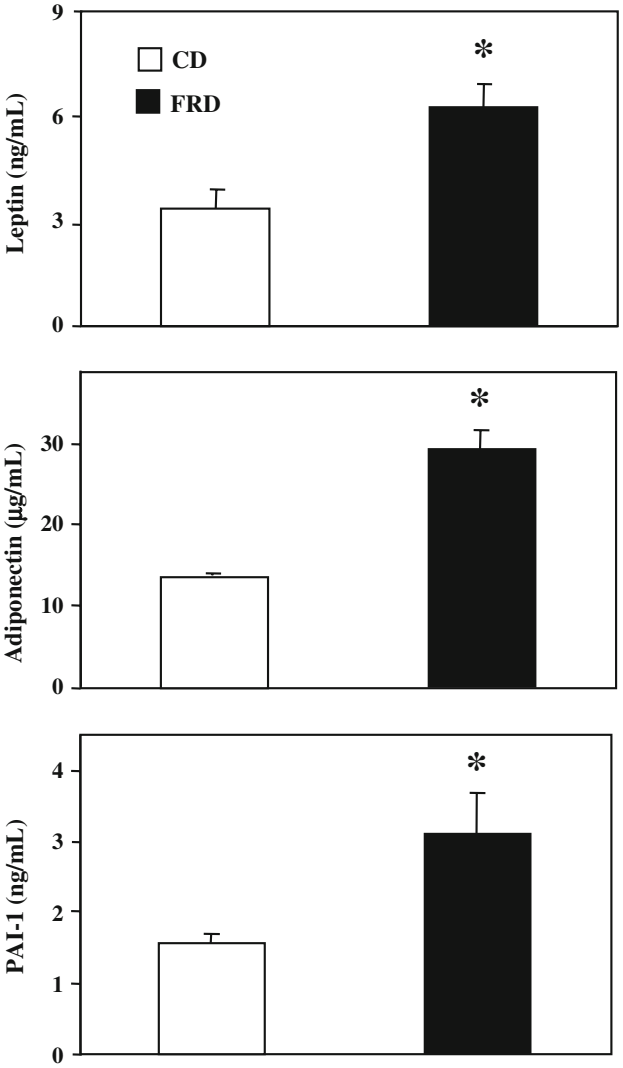

Fig. 1 Plasma concentrations of leptin (upper panel), adiponectin (middle panel), and PAI-1 (lower panel) in CD and FRD rats. Values represent means ± SEM, $n = 6/7$ animals per group. *$P < 0.05$ vs. CD values

Table 1 Abdominal adipose tissue adipocyte number, diameter, and volume (100 cells per section, $n = 3$ animals per group) were measured in CD and FRD rats (values are means ± SEM)

	CD	FRD
Adipocyte number (cells per mm^2)	1.81 ± 0.14	1.22 ± 0.11*
Adipocyte diameter (μm)	48.2 ± 2.6	54.1 ± 2.6*
Adipocyte volume ($\mu m^3 \times 10^3$)	89.57 ± 33.69	119.52 ± 12.83*

* $P < 0.05$ or less vs. CD values

Leptin secretion by isolated AAT adipocytes

Figure 3 shows (left panel) the release of leptin by adipocytes isolated from AAT and incubated in absence (basal)

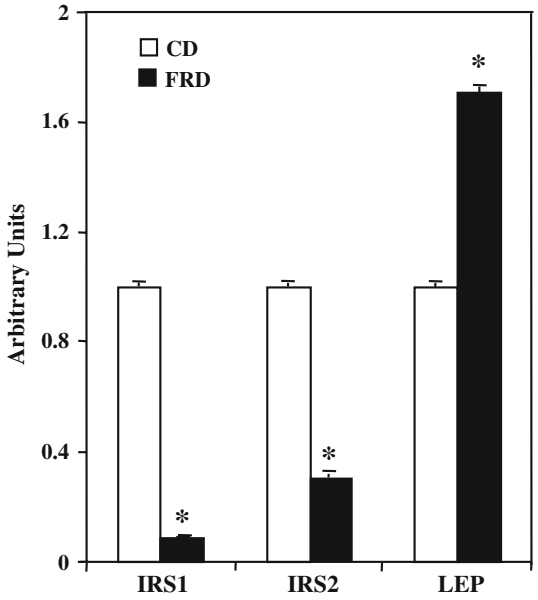

Fig. 2 Leptin (LEP), IRS1 and IRS2 mRNA concentration measured in AAT pads from CD and FRD rats by q-PCR. Data were normalized to the levels of ACTB, and then presented as relative to values obtained in fat pads from CD rats. Values are means ± SEM (n = 3–4 pads per group). *$P < 0.05$ vs. CD values

or in presence of increasing concentrations of insulin (0.1–10 nM). In basal condition, adipocytes from FRD released significantly larger amounts of leptin than those from CD ($P < 0.05$). Insulin increased this release in both groups in a concentration-dependent fashion, but at any concentration tested leptin release was higher in adipocytes from FRD animals. In AAT from FRD, however, the response curve shifted to the right, i.e., only the highest concentration of insulin tested (10 nM) increased leptin secretion significantly. Similar leptin response was elicited by adipocytes incubated with dexamethasone (Dxm; 25–100 mM): it significantly stimulated leptin release in presence of its highest concentration (Fig. 3, right panel).

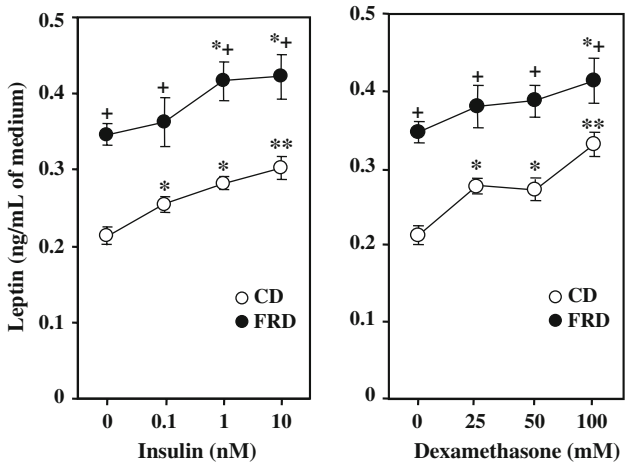

Fig. 3 Effects of increasing concentrations of insulin (left) and dexamethasone (right) on leptin release by adipocytes isolated from AAT of CD and FRD rats. Values represent means ± SEM (n = 4–5 different experiments). *$P < 0.05$ vs. respective 0 nM insulin/dexamethasone values. **$P < 0.05$ vs. insulin 0 and 0.1 nM values. $^{+}P < 0.05$ vs. CD values in similar conditions

Discussion

In the present study we confirmed that, administration of a FRD to normal male rats for 3 weeks increases circulating levels of glucose, triglyceride, and FFAs [7]. These changes were associated with a significant increase of serum leptin, adiponectin, and PAI-1 levels. No changes were recorded in total cholesterol, C-reactive protein, or the corticoadrenal hormone. This metabolic and endocrine profile observed in FRD rats resembles most of the characteristics of the human MS [12] and strongly indicates a switch of a selective set of biomarkers from the homeostatic to the allostatic system.

Among new altered components identified in the FRD rats we found: (a) a significant increase in AAT mass and its adipocyte size; (b) a significantly decreased expression of IRS1 and IRS2 genes in AAT; (c) higher LEP gene expression in AAT; (d) increased basal and stimulated release of leptin from adipocytes isolated from AAT; and (e) a shift to the left in the in vitro concentration-response curve of leptin release induced by insulin or Dxm.

The lower sensitivity of the AAT adipocytes to the stimulatory effect of insulin on leptin release and the lower expression of IRS1 and IRS2 currently described, demonstrate that this tissue plays a role in the insulin resistant state observed in FRD rats [7, 13, 14].

On the other hand, our data shows that FRD rats have a clear alteration of the AAT endocrine function: there is a significant increase in peripheral concentrations of leptin and adiponectin, and of LEP mRNA in AAT. These adipocytes release a larger amount of leptin in both basal and stimulated (insulin/Dxm) conditions. The changes in the serum concentration and in the in vitro release of leptin by adipocytes observed in FRD rats could be ascribed to the larger adipocyte size measured in these animals and to their increased expression of the LEP mRNA [15]. On due time, the high leptin production could further contribute to impair adipocyte response to insulin by affecting both the hormone binding to its receptor [10] and the expression of IRS1 and IRS2 downstream receptor mediators [16, 17].

FRD rats displayed high circulating levels of both insulin [7] and adiponectin, similar to the hormone pattern previously observed in human beings with defective insulin receptor function [18], and in mice with selective inactivation of insulin receptor gene in their adipocytes [19]. Since adiponectin is a recognized enhancer of insulin

sensitivity [20] and FA oxidation [21], its increase in animals with insulin resistance such as the FRD rats could represent a compensatory autocrine mechanism to counteract the local impairment in insulin sensitivity [22]. Regarding the high plasma PAI-1, its production has been associated with enhanced oxidative stress [22], enlarged adipocyte size, and increased lipid content [23]. Since these latter alterations are present in our FRD rats, they could explain, at least partly, their high circulating PAI-1 levels.

All the FRD-induced endocrine changes currently described plus those previously reported in fatty acid composition and in glycoxidative stress markers [7], could be part of an AAT network of interacting abnormalities namely: the lower insulin antilipolytic effect observed in these rats could be responsible for their high AAT FFA release and the abnormal pattern of saturated/unsaturated ratio [7]. The high circulating levels of saturated FFAs could in turn enhance AAT NADPH oxidase activity and ROS production [24]. The increased local ROS production could lead thereafter to the AAT endocrine dysfunction [24]. Following similar reasoning, Brownlee et al. have proposed [25] a unifying hypothesis to explain the cause–effect relationship between oxidative stress and metabolic/endocrine dysfunctions such as those found in FRD rats. This hypothesis is based on the following: an increased offer of a metabolic substrate will increase ROS mitochondrial production; in turn, ROS sets off a chain reaction that: (a) decreases antioxidant defense, (b) stimulates protein kinase C isoform activity (switching tyrosine to serine-treonine phosphorylation, thus decreasing insulin mediator effectiveness), and (c) increases both PAI-1 production and the nonenzymatic glycosylation rate. Thus, it could be assumed that FRD intake-induced oxidative stress at the AAT level could represent a main and early change responsible for most of the metabolic/endocrine abnormalities observed in these animals.

We previously reported that administration of a FRD to normal rats induces an increase in serum insulin levels together with an impairment of glucose tolerance [7]. This latter alteration suggests that AAT metabolic/endocrine dysfunction could secondarily affect other tissue functions such as β-cell insulin secretion. This effect could be ascribed to the deleterious action of saturated FAs on β-cell function and mass [26], as well as of ROS due to the low islet antioxidant defense capacity [27].

In summary, our study demonstrates the appearance of indicators of the MS in normal rats shortly (3 weeks) after administration of a FRD. They include a state of decreased insulin sensitivity and several AAT endocrine/metabolic dysfunctions. The alteration in these markers could be part of a network of interacting abnormalities triggered by early FRD-induced AAT oxidative stress [7], and probably plays a key role in both the development of MS and β-cell failure.

Materials and methods

Animals and experimental design

Normal adult male Wistar rats (180–200 g body weight) were kept in a temperature-controlled environment (23°C) with a fixed 12-h light/dark cycle and fed ad libitum for 1 week (stabilization period) with a standard commercial rat chow (Ganave, Argentina). Thereafter, the rats were randomly divided into two groups (30 animals each): rats fed ad libitum during 3 weeks with a commercial standard chow and tap water without (control diet group, CD), and with the addition of 10% fructose (w/v) (FRD). Body weight as well as food and fluid intake were recorded daily. This protocol provides comparable total calorie intake and body weight in both groups [7]. We complied with international regulations concerning the ethical use of animals.

Blood measurements

Nonfasting animals were sacrificed (between 08:30 and 09:00 h) and trunk blood was collected into EDTA coated tubes. Tubes were rapidly centrifuged (4°C at 3000 rpm) and plasma samples immediately analyzed or stored at −20°C. We measured plasma glucose (Bio System, Argentina), triglyceride (Bio System), FFA [7], and total cholesterol (Wiener Laboratories Argentina) levels. Leptin concentration in plasma and in the incubation medium was measured by a previously validated radioimmunoassay (RIA) (standard curve 0.04–15 ng/ml) [28], as well as plasma corticosterone concentrations by RIA (standard curve 0.05–50 μg/dl) [28]. Coefficients of variation (CV) intra and interassay of both RIAs were 3%–7% and 5%–9%, respectively. Circulating levels of total adiponectin (Linco Research, Cat. # EZRADP-62 K; standard curve: 3–100 ng/ml), total PAI-1 (American Diagnostica, Inc., CT, USA, IMUCLONE Cat. # 601; standard curve: 1–20 ng/ml) and C-reactive protein (Life Diagnostics, Inc., PA, USA, Cat. # 2210-2; standard curve: 0.5–30 ng/ml) were also measured. CVs intra and interassay for all these adipokines were 0.5%–3% and 4%–9%, respectively.

Histological studies

AAT pads were removed and immediately fixed in 4% paraformaldehyde (in 0.2 M phosphate buffer), at 4°C for a maximum of 3 days. Tissues were then washed with 0.01 M PBS, and immersed in 70% ethanol for 24 h before being processed and embedded in paraffin. Sections of 4 μm were obtained at different levels of the blocks and stained with hematoxylin–eosin, then examined with a Jenamed 2 Carl Zeiss light microscope; quantitative morphometric analysis was performed using a RGB CCD Sony camera together

with the OPTIMAS software (Bioscan Incorporated, Edmons, WA, USA) (40× objective). For each AAT sample, 1 section and 3 levels were selected ($n = 3$ animals per group). Systematic random sampling was used to select 10 fields for each section and a minimum of 100 cells per group were examined. We then measured the number of adipocytes, their diameter and volume ($4/3\pi r^3$) [29].

AAT adipocyte isolation and incubation

Adipocytes were isolated from preweighed AAT pads as previously described [30]. Briefly, fat pads were transferred into sterile plastic tubes containing 4 ml/g fat of Krebs-Ringer-MOPS medium with 1% w/v of BSA, antibiotics (streptomycin–penicillin), and supplemented with 1 mg/ml collagenase type 1 (Sigma) (pH 7.4). Tubes were incubated at 37°C with gentle shaking for 40 min. Fat suspension was then filtered through a nylon cloth and centrifuged (30 s at 400 rpm) at room temperature. Infranatants were aspirated, and adipocytes were washed with 10 ml of fresh sterile Krebs-Ringer-MOPS-BSA medium and centrifuged (3 times) as described above. After washes, cells were diluted with 3–4 ml of sterile Dulbeccos's Modified Eagle's Medium (Sigma) (supplemented with 1% w/v of BSA (Sigma), 1% v/v of FCS and antibiotics (25 mg/l streptomycin and 15 mg/l potassium penicillin G), pH 7.4 (Incubation Medium)) and counted. Cells were then diluted with incubation medium in order to obtain ∼200,000 adipocytes/900 µl of medium, distributed in 15 ml conical tubes and incubated for 45 min at 37°C, in a 95% O_2–5% CO_2 atmosphere without (basal) or with insulin (0.1–10 nM, Novo Nordisk Pharma AG, Switzerland) [31] or dexamethasone (Dxm; Sidus Lab., Argentina, 25–100 mM) [31]. Each condition was run in five replicates in 3–4 different experiments. At the end of the incubations, aliquots of media were carefully aspirated and kept frozen (−20°C) for leptin concentration measurements.

AAT RNA isolation and real-time quantitative PCR

Total RNA was isolated from AAT pads of both experimental groups by a modification of the single step, acid guanidinium isothiocyanate–phenol–chloroform, extraction method (Trizol; Invitrogen, Life Tech., USA; cat. # 15596-026) [32]. The yield and quality of extracted RNA were assessed by 260/280 nm optical density ratio and electrophoresis in denaturing conditions on 2% agarose gel. One microgram of total RNA was reverse-transcripted using random primers (250 ng) and Superscript III Rnase H-Reverse Transcriptase (200 U/µl Invitrogen, Life Tech, USA; cat # 18989-093). For quantitative real-time PCR the following primers were applied: β-actin (ACTB) (R): 5′-ACCCTCATAGATGGGCACAG-3′, (F): 5′-AGCCATGTACGTAGCCATCC-3′ (115 pb) (GenBank accession number: NM_031144); LEP (R): 5′-CTCAGCATTCAGGGCTAAGG-3′; (F): 5′-GAGACCTCCTCCATCTGCTG-3′ (192 pb) (GenBank accession number: NM_013076); IRS1 (R): 5′-ACGGTTTCAGAGCAGAGGAA-3′, (F): 5′-TGTGCCAAGCAACAAGAAAG-3′ (176 bp) (GenBank accession number: NM_012969); and IRS2 (R): 5′-CCAGGGATGAAGCAGGACTA-3′, (F): 5′-CTACCCACTGAGCCCAAGAG-3′ (151 pb) (GenBank accession number: AF087674).

Two microliters of the reverse transcription mix were amplified using the QuantiTect Syber Green PCR kit (Qiagen, cat. # 204143), 0.5 µM of each specific primer and the LightCycler Detection System (MJ Mini Opticon, Biorad). PCR efficiency was ∼1. The threshold cycles (Ct) were measured in separate tubes and in duplicate. The identity and purity of the amplified product were checked by electrophoresis on agarose mini gels, and the melting curve was analyzed at the end of amplification. Differences between the Ct were calculated in every sample for each gene of interest as follows: Ct gene of interest−Ct reporter gene. ACTB, whose mRNA levels do not differ between control and test groups, was used as reporter gene. Relative changes in the expression level of one specific gene (ΔΔCt) were calculated as ΔCt of the test group minus ΔCt of the control group, and then expressed as $2-\Delta\Delta Ct$.

Statistical analysis

Data were analyzed by ANOVA (one or two factor), followed by post hoc comparisons with Fisher's test. Morphometric data were analyzed by the Least Significant Difference test for multiple comparisons [29]. The non-parametric Mann–Whitney test was used to analyze data from adipose tissue mRNA expression [33]. Results are expressed as mean ± SEM, and P values lower than 0.05 were considered statistically significant.

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