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Antioxidant treatment prevents the development of fructose-induced abdominal adipose tissue dysfunction

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Abstract

In the present study, we tested the effect of OS (oxidative stress) inhibition in rats fed on an FRD [fructose-rich diet; 10% (w/v) in drinking water] for 3 weeks. Normal adult male rats received a standard CD (commercial diet) or an FRD without or with an inhibitor of NADPH oxidase, APO (apocynin; 5 mM in drinking water; CD-APO and FRD-APO). We thereafter measured plasma OS and metabolic-endocrine markers, AAT (abdominal adipose tissue) mass and cell size, FA (fatty acid) composition (content and release), OS status, LEP (leptin) and IRS (insulin receptor substrate)-1/IRS-2 mRNAs, ROS (reactive oxygen species) production, NADPH oxidase activity and LEP release by isolated AAT adipocytes. FRD-fed rats had larger AAT mass without changes in body weight, and higher plasma levels of TAG (triacylglycerol), FAs, TBARS (thiobarbituric acid-reactive substance) and LEP. Although no significant changes in glucose and insulin plasma levels were observed in these animals, their HOMA-IR (homoeostasis model assessment of insulin resistance) values were significantly higher than those of CD. The AAT from FRD-fed rats had larger adipocytes, higher saturated FA content, higher NADPH oxidase activity, greater ROS production, a distorted FA content/release pattern, lower insulin sensitivity together with higher and lower mRNA content of LEP and IRS-1-/2 respectively, and released a larger amount of LEP. The development of all the clinical, OS, metabolic, endocrine and molecular changes induced by the FRD were significantly prevented by APO co-administration. The fact that APO treatment prevented both changes in NADPH oxidase activity and the development of all the FRD-induced AAT dysfunctions in normal rats strongly suggests that OS plays an important role in the FRD-induced MS (metabolic syndrome) phenotype.

Key words: abdominal adipocyte, adipokine, fructose-rich diet, insulin signalling, lipid metabolism, metabolic syndrome

INTRODUCTION

According to some authors, the current epidemics of obesity, Type 2 diabetes and the MS (metabolic syndrome) in the U.S. [1] could be ascribed to the drastic increase in the annual *per capita* fructose consumption [2,3]. It has also been shown that administration of an FRD (fructose-rich diet) to normal rats induces the development of features characteristic of the human MS phenotype [4,5], and in humans it speeds up the transition from IGT (impaired glucose tolerance) to Type 2 diabetes [6,7]. Consequently, the US Dietary Guidelines recommend limiting calorie intake (which includes both added sugar and solid fat) to 13% of energy requirement [8].

Although the precise mechanism whereby an FRD induces the MS remains controversial, it has been postulated that an increased rate of OS (oxidative stress) plays a key role [9–13]. In this regard, we have previously reported increased OS in AAT (abdominal adipose tissue) [14] and impaired adipoinsular

Abbreviations: AAT, abdominal adipose tissue; ACTB, β-actin; APO, apocynin; AU, arbitrary units; bw, body weight; CD, commercial diet; C_T, threshold cycle value; DPI, diphenylene iodonium; FA, fatty acid; FAME, fatty acyl methyl ester; FRD, fructose-rich diet; H&E, haematoxylin and eosin; HOMA, homoeostasis model assessment; H_2DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; GLUT, glucose transporter; IGT, impaired glucose tolerance; IR, insulin resistance; IRS, insulin receptor substrate; KRB, Krebs–Ringer–Bicarbonate; LEP, leptin; MDA, malondialdehyde; MS, metabolic syndrome; MUFA, mono-unsaturated FA; NBT, Nitro Blue Tetrazolium; NEFA, non-esterified FA; NEM, *N*-ethylmaleimide; OS, oxidative stress; PKC, protein kinase C; PUFA, polyunsaturated FA; qRT-PCR, quantitative real-time PCR; ROS, reactive oxygen species; RT, reverse transcriptase; SFA, saturated FA; TBARS, thiobarbituric acid-reactive substance; TAG, triacylglycerol.

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axis function [14,15] in normal rats fed on an FRD for 3 weeks.

Fructose is poorly or not metabolized by pancreatic β cells [16,17]; however, it enhances the insulin secretion stimulated by glucose [18]. Such enhancement could be probably mediated through its effect upon digestive tract [19], liver [20] and adipose tissue [21,22], with the consequent release of modulatory metabolites and hormones/adipokines. In fact, the hypertriglyceridaemia and IR (insulin resistance) induced by the intake of an FRD could account for such effects [14,15]. We further hypothesized that all these alterations could be triggered by an initially increased OS rate induced by excess fructose consumption [14].

In an attempt to provide an experimental support to our assumption, we have studied the effect of the co-administration of an FRD and an OS inhibitor, APO (apocynin) (a well-known inhibitor of NADPH oxidase) [23], upon: (i) serum concentrations of OS, metabolic biomarkers and LEP (leptin); (ii) morphometric characteristics of AAT adipocytes; (iii) fatty acyl composition, OS biomarker levels and NADPH oxidase activity in AAT; (iv) *in vitro* ROS (reactive oxygen species) production and LEP release by isolated AAT adipocytes; and (v) AAT mRNA level of LEP and of intracellular mediators of insulin activity IRS (insulin receptor substrate)-1/IRS-2.

MATERIALS AND METHODS

Animals and experimental design

Normal adult male Wistar rats 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 CD (commercial diet; rat chow; Ganave). When the animals reached 180–200 g of bw (body weight), they were randomly divided into four groups and fed *ad libitum* for 3 weeks with (i) a commercial standard chow and tap water (control diet; CD), (ii) CD plus 10 % fructose (w/v; Cicarelli) in drinking water (FRD); (iii) CD plus APO (Sigma; 5 mM in drinking water) [23] (CD-APO) and (iv) FRD plus APO (FRD-APO). Although we have shown previously that this FRD intake provides a comparable total calorie intake in the study groups [15], we registered the total daily energy intake and bw in all groups.

The study protocol complies with international regulations concerning the ethical use and care of animals (Office of Animal Care and Use, NIH).

Blood measurements

Non-fasting animals were killed between 08:30 and 09:00 h and trunk blood was collected into EDTA-coated tubes. Tubes were rapidly centrifuged (4 °C at 2060 ***g***) and plasma samples were immediately analysed or stored at − 20 °C. Commercial assay kits were used to measure plasma levels of glucose (Bio System), TAG (triacylglycerol) (Wiener Laboratories) and NEFAs [non-esterified FAs (fatty acids); Randox Laboratories]. Plasma insulin concentration was determined by RIA [24] and lipid peroxidation by measuring TBARS (thiobarbituric acid-reactive substance) [14]. The amount of TBARS formed was calculated by the extinction coefficient for the MDA (malondialdehyde)–TBA complex of 1.56×10^5 $(\text{mol/l})^{-1} \cdot \text{cm}^{-1}$, and expressed as pmol of MDA/mg of plasma protein, measured with the Bio-Rad Protein Assay kit. LEP concentration in plasma, in the incubation medium samples as well as in AAT extracts was measured by a validated specific RIA (standard curve 0.04–15 ng/ml) [25]. Intra- and inter-assay CV (coefficients of variation) of both these RIAs were 3–7 % and 5–9 % respectively.

Histological studies

AAT pads were removed and immediately fixed in 4 % (v/v) PFA (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, immersed in 70 % (v/v) ethanol, and thereafter embedded in paraffin. Sections of 4 μm were obtained at different levels of the blocks and stained with H&E (haematoxylin and eosin). Quantitative morphometric analysis was performed with a Jenamed 2 Carl Zeiss light microscope, an RGB CCD Sony camera and OPTIMAS software (Bioscan Incorporated) ($\times$40 objective). For each AAT sample, one section and three levels were selected ($n = 4/5$ animals per group). Systematic random sampling was used to select ten fields for each section and a minimum of 100 cells per group were counted. We measured adipocyte diameter and cell volume with the formula $4/3\pi .r^3$ [26].

Antioxidant system and TBARS in AAT

Pre-weighed ATT pads were sonicated in buffer containing 50 mM Tris/HCl (pH 7.4), 1.15 % (w/v) KCl, 1 mM NEM (*N*-ethylmaleimide) and 0.1 % BHT (butylated hydroxytoluene), and then centrifuged at 4 °C (600 ***g***). Supernatants were stored at − 70 °C under argon atmosphere. Contents of GSSG were determined by HPLC [14], and GSH was measured as described previously [14]. Samples for GSSG analyses were obtained in the presence of NEM, and deproteinized using TCA (trichloroacetic acid; 15 % final concentration) [14]. The concentrations of α-tocopherol, lycopene, retinol and β-carotene were determined by HPLC [14]. In addition, pre-weighed AAT pads were immediately frozen in liquid N_2. Tissues were homogenized in a phosphate-buffered solution (pH 7.4) containing 10 % (w/v) KCl; homogenates were stored at − 70 °C until processing for measurement of lipid peroxidation as TBARS [14].

AAT pads and isolated adipocyte incubations

Small pieces (8–10 per tube, 100–200 mg) of AAT pads from animals of each group were incubated in 1 ml of KRB (Krebs–Ringer–Bicarbonate) buffer (118 mM NaCl, 25.96 mM $NaHCO_3$, 4.74 mM KCl, 2.24 mM $CaCl_2$, 1.19 mM $MgSO_4$ and 0.91 mM KH_2PO_4, pH 7.4), with 1 % essentially FA-free BSA (Sigma), for 120 min at 37 °C in a 5 % CO_2/95 % O_2 atmosphere. The spontaneous FA release into the media was then measured as described previously [14].

Isolated adipocytes were obtained from AAT by a minor modification of the Rodbell procedure and processed as previously reported by us [27]. Briefly, isolated adipocytes were diluted with incubation medium to obtain a concentration of approx.. 200 000 adipocytes/0.9 ml. Then this cell volume was distributed in 15 ml conical plastic 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) [27]. At the end of the incubation period, aliquots of medium were carefully separated and kept frozen ($-20\,^{\circ}C$) to measure basal NEFAs as well as basal and insulin-stimulated LEP concentrations. For this analysis, we used samples taken from five different experiments (six replicates per experiment).

AAT NADPH oxidase activity and intracellular ROS production

Superoxide (O_2^-) generation was detected by NBT (Nitro Blue Tetrazolium) assay [28], as described by Oliveira et al. [29]. Pre-weighed AAT pads were quickly rinsed in KRB buffer and transferred into plastic tubes containing 500 μl of KRB buffer with 1% BSA, and incubated for 2 h with 0.2% NBT (37 °C, 95% O_2/ 5% CO_2) in the presence of 3.3 mM glucose with or without 10 μmol/l DPI (diphenylene iodonium), an NADPH oxidase inhibitor. At the end of the incubation period, AAT pads were sonicated in 100 μl of 50% (v/v) acetic acid to dissolve the NBT-reduced formazan; thereafter, the absorbance of each sample was determined at 620 nm. The production of O_2^- ascribed to NADPH oxidase activity was expressed as the difference between the values measured in the absence and the presence of DPI in the reaction tube.

Intracellular oxidants were detected using H_2DCF-DA (2′,7′-dichlorodihydrofluorescein diacetate) [30]. Briefly, adipocytes seeded in 12-well plates (at a density of 100 000 cells/well) were incubated for 1 h at 37 °C in a 95% O_2/5% CO_2 atmosphere, in the absence or presence of the PKC (protein kinase C) activator PMA (10 μM), followed by a 30 min incubation with H_2DCF-DA (20 mM). After these treatments, cells were gently scraped by a lifter and suspended in the same media. For detection of intracellular fluorescence, cells were excited at 385 nm using a Beckman Coulter fluorimeter. The dichlorofluorescein emission was recorded at 535 nm and expressed in AU (arbitrary units) as net (PMA-induced − spontaneous production) of the total ROS production.

Lipid composition analysis

Aliquots of AAT incubation medium or homogenates were processed by GLC. Total lipids were extracted as previously described [14]. GLC of FAME (fatty acyl methyl esters) was performed as previously reported [14], except that in this case we used a capillary column (Supelco) mounted on a Hewlett Packard HP 6890 Series GC System Plus, equipped with a terminal computer integrator system. FAMEs were identified by comparison of their relative retention times with authentic standards, and mass distribution was calculated electronically by quantification of the peak areas. A mixture of pure authentic standards (Avanti Polar Lipids) was routinely run to confirm the identity of the analytical peaks. As already reported [14], the FAs determined were the SFAs (saturated FAs) myristic ($C_{14:0}$), palmitic ($C_{16:0}$) and stearic ($C_{18:0}$) acids, the MUFAs (mono-unsaturated FAs) palmitoleic ($C_{16:1}$) and oleic ($C_{18:1}$) acids, and the PUFAs (polyunsaturated FAs) linoleic ($C_{18:2\,n-6}$), eicosatrienoic ($C_{20:3\,n-6}$), arachidonic ($C_{20:4\,n-6}$) and γ-linolenic ($C_{18:3\,n-3}$) acids. When appropriate, data were expressed as the relative composition of FAs (in μmol), calculated as the percentage of the sum (Σ) of both SFA (ΣSFA) and UFA (unsaturated FAs) (ΣUFA) from the sum of all FAs measured in either AAT pads (content) or the incubation medium (release). Additionally, relationships among different lipidic species were also estimated (e.g. $C_{18:2\,n-6}/C_{20:4\,n-6}$).

AAT RNA isolation and qRT-PCR (quantitative real-time PCR)

Total RNA was isolated from AAT pads of all experimental groups by a modification of the single-step, acid guanidinium isothiocyanate–phenol–chloroform extraction method (TRIzol®; Invitrogen) [31]. The yield and quality of extracted RNA was assessed by 260/280 nm absorbance ratio and electrophoresis in denaturing conditions on 2% (w/v) agarose gel. A total of 1 μg of the total RNA was reverse-transcribed using random primers (250 ng) and Superscript III RNase H-RT (reverse transcriptase; 200 units/μl: Invitrogen). For qRT-PCR the following primers were applied: ACTB (β-actin) (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 bp) (GenBank® accession number: NM_013076); IRS-1 (R): 5′-ACGGTTTCAGAGCAGAGGAA-3′, (F): 5′-TGTGCCAAGCAACAAGAAAG-3′ (176 bp) (GenBank® accession number: NM_012969); IRS-2 (R): 5′-CCAGGGATGAAGCAGGACTA-3′, (F): 5′-CTACCCACTGAGCCCAAGAG-3′ (151 bp) (GenBank® accession number: AF087674); and GLUT (glucose transporter)-4 (R): 5′-TGGACGCTCTCTTTCCAACT-3′, (F): 5′-GCTTCTGTTGCCCTTCTGTC-3′ (166 bp) (GenBank® accession number: NM_012751). A portion (2 μl) of the RT mix was amplified by the QuantiTect Syber Green PCR kit (Qiagen), 0.5 mM of each specific primer and the Light Cycler Detection System (MJ Mini Opticon; Bio-Rad). PCR efficiency was approx. 1. C_T (threshold cycle value) was 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 analysed at the end of amplification. Differences in C_T were calculated in every sample for each gene of interest as follows: C_T gene of interest $- C_T$ reporter gene. ACTB, whose mRNA levels did not differ between control and test groups, was used as a reporter gene. Relative changes in the expression level of one specific gene ($\Delta\Delta C_T$) were calculated as ΔC_T of the test group minus ΔC_T of the control group, and then expressed as $2^{-\Delta\Delta C_T}$.

Statistical analysis

Data were analysed by ANOVA (two-factor: diet and treatment), followed by post-hoc comparisons with a Fisher's test [32]. When appropriate, the non-parametric Mann–Whitney test was used to analyse data from adipose tissue mRNA expression [32]. Results are expressed as means $\pm$ S.E.M., and differences were considered significant when P values were <0.05.

RESULTS

Rat bw and energy intake

All experimental animals (10/12 rats per group) had comparable bw at the beginning of the experimental design (CD group, 193.14 $\pm$ 3.01; CD-APO group, 197.11 $\pm$ 8.15; FRD

Table 1 Circulating levels of several markers of the adipoinsular axis function, TBARS and HOMA index
Values are means ± S.E.M. ($n = 8/10$ rats per group). *$P < 0.05$ compared with CD; $^{+}P < 0.05$ compared with FRD. FFA, non-esterified 'free' fatty acid.

	Diet			
Parameter	**CD**	**CD-APO**	**FRD**	**FRD-APO**
Glucose (mM)	6.34 ± 0.14	5.96 ± 0.15	6.17 ± 0.21	6.07 ± 0.22
Insulin (ng/ml)	0.42 ± 0.03	0.45 ± 0.05	0.57 ± 0.07	0.41 ± 0.04
TAG (mM/100 g of bw)	0.42 ± 0.03	0.33 ± 0.05	0.62 ± 0.05*	0.32 ± 0.06^{+}
FFA (mM/100 g of bw)	0.19 ± 0.01	0.21 ± 0.03	0.27 ± 0.02*	0.16 ± 0.03^{+}
LEP (ng/ml per 100 g of bw)	1.64 ± 0.11	1.52 ± 0.18	2.17 ± 0.16*	1.47 ± 0.24^{+}
TBARS (pmol/mg per ml)	58.72 ± 4.45	68.97 ± 4.45	73.71 ± 5.51*	67.10 ± 5.51^{+}
HOMA-IR	2.981 ± 0.001	2.842 ± 0.009	5.271 ± 0.011*	2.673 ± 0.009^{+}

group, 191.89 ± 4.22; FRD-APO group, 190.89 ± 5.49 g). At 3 weeks after the diet/treatment, rats from the CD and FRD groups also showed comparable bw values (292.83 ± 9.14 and 302.33 ± 5.19 g respectively), whereas those co-treated with APO had lower but not significantly different values (274.67 ± 8.04 and 279.17 ± 9.59 g in the CD-APO and FRD-APO groups respectively). A comparable individual daily energy intake was registered over the 3-week experimental period in all animals, regardless of treatment and diet (21-day average): CD group, 108.76 ± 8.65 kJ/day per 100 g of bw; CD-APO group, 107.68 ± 12.04 kJ/day per 100 g of bw; FRD group, 121.79 ± 14.25 kJ/day per 100 g of bw; FRD-APO group, 118.79 ± 12.46 kJ/day per 100 g of bw.

Circulating biomarker levels and HOMA (homoeostasis model assessment) score

Lipids and OS biomarker

Rats fed on the FRD had significantly higher plasma concentrations of TAG, NEFAs and TBARS compared with CD-fed rats (Table 1; $P < 0.05$). APO administration to FRD-fed rats effectively prevented all these increments (Table 1).

Adipoinsular axis function and HOMA score

Although FRD administration did not significantly modify the circulating levels of glucose and insulin, these levels showed an increasing trend (Table 1). Conversely, significantly higher HOMA values were measured in FRD-fed rats ($P < 0.05$), together with a significant increase in the circulating levels of LEP ($P < 0.05$ compared with CD-fed rats; Table 1). Co-administration of APO to these rats significantly prevented both the higher HOMA values and the increased plasma LEP levels ($P < 0.05$; Table 1).

AAT mass and adipocyte characteristics

AAT mass was significantly larger in FRD- than in CD-fed rats (2.48 ± 0.17 compared with 1.87 ± 0.21 g; $n = 8/10$ rats per group; $P < 0.05$). Such a difference was no longer observed in APO-treated rats (1.83 ± 0.21 and 1.97 ± 0.23 g in CD-APO and FRD-APO groups respectively; $n = 8/10$ rats per group).

AAT adipocytes from FRD-fed rats were significantly larger (in diameter and volume) than adipocytes from CD-fed rats ($P < 0.05$; Figures 1E and 1F respectively). In FRD-APO-fed rats, adipocyte size and volume were similar to those observed in CD-fed rats, whereas no changes were observed in cells from CD-APO-fed rats (Figures 1E and 1F).

AAT non-enzymatic antioxidant defence system and TBARS content

Although the total GSH content of the AAT homogenate was not modified by the FRD, this diet significantly increased and decreased (compared with CD values) GSSG and GSH respectively (Table 2). Consequently, the GSH/GSSG ratio decreased significantly in FRD-fed animals. Similarly, the lipid-soluble antioxidants measured as the content of α-tocopherol (the amount of the γ-isomer was negligible), β-carotene, retinol and lycopene were also significantly lower in FRD- than in CD-fed rats (Table 2).

Concomitantly, the TBARS concentration was significantly higher in FRD- than in CD-fed rats (Table 2). Taken together, these changes demonstrate that the FRD induced a significant decrease of the non-enzymatic antioxidant system in AAT. Although APO co-administration did not introduce significant changes in CD-fed rats, it significantly improved the antioxidant capacity of AAT in FRD-fed rats (Table 2).

Analysis of AAT fatty acyl composition and release into the incubation medium

The FA composition of extracted lipids from AAT pads is shown in Table 3. FRD administration did not modify the proportion of the saturated myristic acid; however, it significantly increased the proportion of other SFAs (palmitic and stearic acids). Moreover, this diet induced an overall enhancement of SFA (ΣSFA) ($P < 0.05$ compared with CD values). Although longer chain PUFA ($>C_{20}$ species) were minimally detected in CD pads, they became undetectable in pads from FRD-fed animals. The amount of summed MUFAs (ΣMUFA) and grouped PUFAs (ΣPUFA) was significantly reduced in pads from FRD-fed rats ($P < 0.05$ compared with CD values). Both the $C_{18:2\ n-6}/C_{20:4\ n-6}$ and $C_{20:3\ n-6}/C_{20:4\ n-6}$ ratios were not modified by the FRD. A detrimental metabolic effect of such a diet was also observed after analysing the ratios of both summed SFAs to MUFAs and PUFAs (ΣSFA/ΣMUFA and ΣSFA/ΣPUFA respectively). Indeed, such ratios were drastically increased by this diet ($P < 0.05$ compared with CD values; Table 3). It should also be noted that co-administration of APO to FRD-fed rats resulted in the full prevention of the deleterious FRD effect on AAT fatty acyl composition (Table 3).

The qualitative profile of FAs released by incubating AAT pads was quite similar to that found in tissue contents (Table 4). Although myristic acid was released only in traces (or even at

Table 2 Total glutathione, GSH, GSSG, non-enzymatic antioxidants and TBARS in AAT pads from the different experimental groups

Values are means ± S.E.M. ($n = 5$ pads per group). *$P < 0.05$ compared with CD; $^{+}P < 0.05$ compared with FRD.

	Group			
Parameter	**CD**	**CD-APO**	**FRD**	**FRD-APO**
Total GSH (nmol/g)	35.6 ± 0.75	34.2 ± 0.61	36.8 ± 0.45	38.61 ± 0.69
GSH (nmol/g)	31.15 ± 0.55	30.11 ± 0.49	27.78 ± 0.35*	31.94 ± 0.62^{+}
GSSG (nmol/g)	4.45 ± 0.15	4.09 ± 0.11	9.02 ± 0.25*	6.66 ± 0.22^{+}
GSH/GSSG	7.01 ± 0.11	7.28 ± 0.15	3.09 ± 0.05*	5.82 ± 0.09^{+}
Retinol (μg/g)	0.56 ± 0.02	0.58 ± 0.06	0.22 ± 0.02*	0.41 ± 0.08^{+}
β-Carotenoids (μg/g)	1.15 ± 0.06	0.99 ± 0.05	0.78 ± 0.03*	1.19 ± 0.04^{+}
α-Tocopherol (μg/g)	202.5 ± 5.15	222.1 ± 4.80	174.4 ± 2.95*	197.3 ± 3.15^{+}
Lycopenes (μg/g)	0.31 ± 0.04	0.29 ± 0.06	0.21 ± 0.03*	0.33 ± 0.04^{+}
TBARS (nmol MDA/mg of AAT protein)	22.13 ± 3.51	20.32 ± 5.81	50.51 ± 7.99*	15.35 ± 4.33^{+}

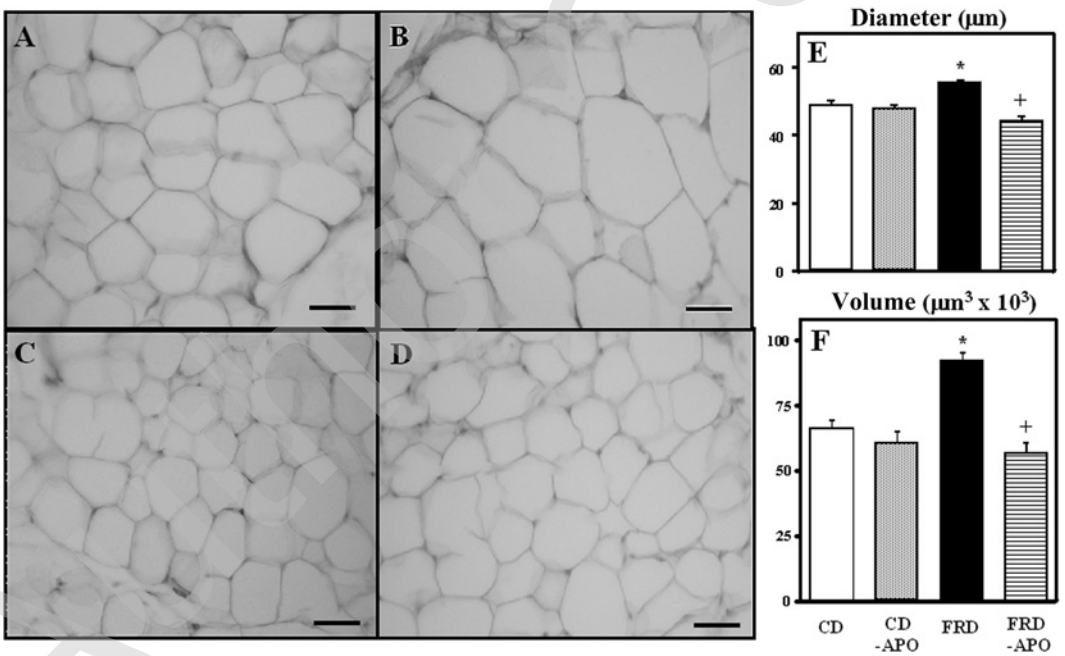

Figure 1 FRD-induced adipocyte hypertrophy

Representative fields of AAT from CD- (**A**), FRD- (**B**), CD-APO- (**C**) and FRD-APO- (**D**) fed rats stained with H&E. Scale bar represents 50 μm; magnification ×400). Adipocyte diameter (**E**) and volume (**F**) in AAT pads from the different groups are also shown. Results are means ± S.E.M. ($n = 4/5$ rats per group). *$P < 0.01$ compared with CD; $^{+}P < 0.01$ compared with FRD.

undetectable levels) by AAT pads from different origins, palmitic and stearic acid release was higher in FRD-fed than in CD-fed rats ($P < 0.05$); the same profile was recorded when SFAs were grouped (ΣSFA). On the other hand, the AAT pad release of oleic acid and other unsaturated FAs (linoleic, γ-linolenic, eicosatrienoic and arachidonic acids, and longer FAs) by AAT pads from FRD-fed rats was significantly reduced ($P < 0.05$ compared with CD values). Concomitantly, the amount of grouped MUFAs (ΣMUFA) and PUFAs (ΣPUFA) was also decreased. Similar to the changes recorded in the FA composition of pads, the analytical ratios $C_{18:2\,n-6}/C_{20:4\,n-6}$ and $C_{20:3\,n-6}/C_{20:4\,n-6}$ were significantly higher in the incubation medium of FRD pads ($P < 0.05$ compared with CD values). These ratios indirectly reflect the conversion rate of the precursor ($C_{18:2\,n-6}$) into the other members of the $n-6$ essential FA family accomplished by the Δ^6 and Δ^5 desaturases. In agreement with these data, a significant increase in the ΣSFA/ΣPUFA ratio was observed after the analysis of AAT pads from FRD-fed rats ($P < 0.05$ compared with CD values). The beneficial effect of APO co-administration to FRD-fed rats was also evident at the level of the incubation medium of their AAT pads. Thus these findings clearly indicate that APO could prevent the FRD-induced changes of fatty acyl chains in complex lipids of the AAT (Tables 3 and 4).

Effectiveness of APO treatment on AAT NADPH oxidase activity

NADPH-oxidase-dependent O_2^- production was significantly higher in AAT pads from FRD- than from CD-fed rats ($P < 0.05$;

Table 3 FA content of AAT pads from the different experimental groups
Various sums (Σ) and ratios are also shown. Values are means ± S.E.M. ($n = 5$ pads per group from three or four experiments). $^{*}P < 0.05$ compared with CD values; $^{+}P < 0.05$ compared with FRD values.

	Group			
FA content (μmol/mg of tissue)	**CD**	**CD-APO**	**FRD**	**FRD-APO**
$C_{14:0}$	2.2 ± 0.1	2.0 ± 0.1	2.6 ± 0.2	2.0 ± 0.1^{+}
$C_{16:0}$	30.3 ± 1.5	29.6 ± 1.1	35.3 ± 1.4*	31.6 ± 1.2
$C_{16:1}$	1.8 ± 0.1	1.5 ± 0.1	1.5 ± 0.06*	2.0 ± 0.1^{+}
$C_{18:0}$	22.2 ± 1.0	23.3 ± 1.1	26.3 ± 0.8*	23.5 ± 0.6^{+}
$C_{18:1}$	12.0 ± 0.7	11.9 ± 0.5	10.2 ± 0.5	12.3 ± 0.2^{+}
$C_{18:2\ n-6}$	18.0 ± 0.7	17.3 ± 0.8	16.6 ± 0.8	17.8 ± 0.4
$C_{18:3\ n-3}$	0.9 ± 0.03	0.8 ± 0.03	0.5 ± 0.05*	0.8 ± 0.1^{+}
$C_{20:3\ n-6}$	0.8 ± 0.01	0.7 ± 0.02	0.8 ± 0.03	0.9 ± 0.05
$C_{20:4\ n-6}$	23.5 ± 1.1	24.3 ± 1.0	17.3 ± 0.9*	24.8 ± 0.8^{+}
$>C_{20}$ PUFAs	0.1 ± 0.01	<0.10	<0.10	0.1 ± 0.03
ΣSFAs	54.7 ± 2.3	54.9 ± 2.0	64.6 ± 1.9*	57.1 ± 1.9^{+}
ΣMUFAs	13.8 ± 0.8	13.4 ± 0.8	11.7 ± 0.6	14.3 ± 0.9
ΣSFAs/ΣMUFAs	4.0 ± 0.1	4.1 ± 0.05	5.5 ± 0.1*	4.0 ± 0.01^{+}
$C_{18:2\ n-6}/C_{20:4\ n-6}$	0.8 ± 0.05	0.7 ± 0.05	1.0 ± 0.1	0.7 ± 0.03
$C_{20:3\ n-6}/C_{20:4\ n-6}$	0.03 ± 0.01	0.03 ± 0.01	0.05 ± 0.02	0.04 ± 0.01
ΣPUFAs	25.2 ± 0.6	25.8 ± 0.8	18.6 ± 0.4*	26.5 ± 0.9^{+}
ΣSFAs/ΣPUFAs	2.2 ± 0.1	2.1 ± 0.05	3.5 ± 0.1*	2.2 ± 0.1^{+}

Table 4 FA release by incubated AAT pads from different groups
Various sums (Σ) and ratios are also shown. Values are means ± S.E.M. ($n = 5$ pads per group per experiment from three or four experiments). $^{*}P < 0.05$ compared with CD values; $^{+}P < 0.05$ compared with FRD values.

	Group			
FA release (μmol/l of medium)	**CD**	**CD-APO**	**FRD**	**FRD-APO**
$C_{14:0}$	<0.10	0.10 ± 0.005	<0.10	0.10 ± 0.015
$C_{16:0}$	4.10 ± 0.05	3.20 ± 0.10	5.50 ± 0.05*	3.90 ± 0.05
$C_{16:1}$	<0.10	<0.10	<0.10	0.10 ± 0.01
$C_{18:0}$	6.20 ± 0.05	5.80 ± 0.10	8.30 ± 0.10*	6.20 ± 0.15
$C_{18:1}$	<0.10	0.20 ± 0.01	<0.10	0.1 ± 0.015
$C_{18:2\ n-6}$	1.50 ± 0.10	1.30 ± 0.10	0.70 ± 0.05*	1.30 ± 0.05
$C_{18:3\ n-3}$	0.10 ± 0.0015	0.10 ± 0.005	<0.10	0.10 ± 0.02
$C_{20:3\ n-6}$	<0.10	0.10 ± 0.015	<0.10	<0.10
$C_{20:4\ n-6}$	0.20 ± 0.05	0.30 ± 0.02	<0.10	0.20 ± 0.015
$>C_{20}$ PUFAs	0.20 ± 0.02	0.20 ± 0.025	<0.10	0.10 ± 0.005
ΣSFA	10.30 ± 0.10	9.1 ± 0.21	13.80 ± 0.15*	10.2 ± 0.22^{+}
ΣMUFA	0.18 ± 0.05	0.25 ± 0.03	0.16 ± 0.04	0.20 ± 0.03
ΣSFA/ΣMUFA	57.78 ± 3.31	36.4 ± 1.90	86.75 ± 4.47*	51.00 ± 3.45^{+}
$C_{18:2\ n-6}/C_{20:4\ n-6}$	7.50 ± 0.31	4.33 ± 0.22	18.75 ± 1.11*	6.50 ± 0.31^{+}
$C_{20:3\ n-6}/C_{20:4\ n-6}$	0.40 ± 0.11	0.33 ± 0.05	2.50 ± 0.26*	0.40 ± 0.03^{+}
ΣPUFA	2.00 ± 0.17	2.2 ± 0.18	0.70 ± 0.05*	1.9 ± 0.12^{+}
ΣSFA/ΣPUFA	5.15 ± 0.59	4.14 ± 1.17	19.70 ± 3.00*	5.37 ± 1.83^{+}

Table 5). In AAT from APO co-treated rats the NADPH-oxidase-dependent O_2^- production was drastically reduced regardless of the diet administered. In fact, anion production by AAT pads from CD-APO-fed rats was significantly lower than in APO-untreated CD-fed rats ($P < 0.05$). Moreover, APO co-treatment fully prevented the FRD-induced O_2^- production (Table 5). It has to be stressed that the profile of total AAT O_2^- production followed a similar pattern to that of NADPH oxidase activity (results not shown).

Isolated adipocyte ROS production and LEP secretion: effects of APO treatment

PMA-induced net ROS production *in vitro* was significantly higher in AAT adipocytes isolated from FRD-fed rats than in

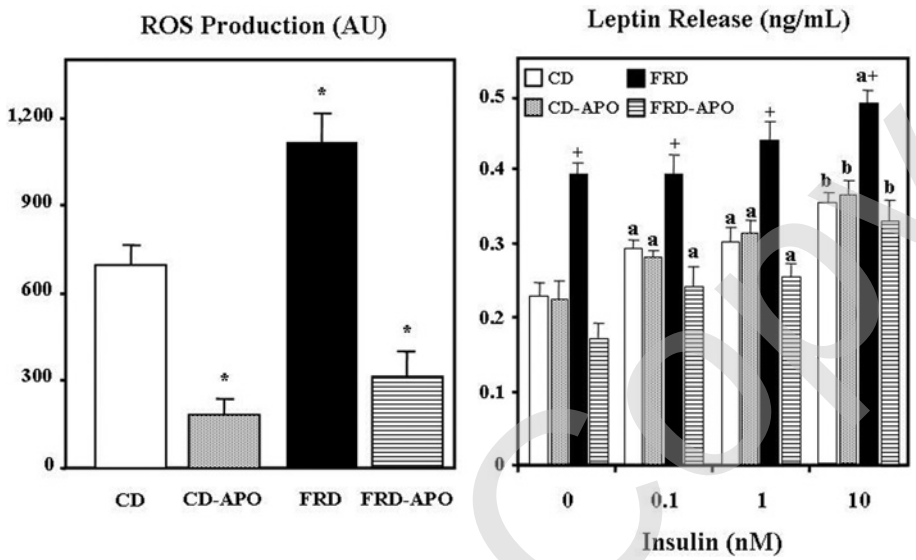

Figure 2 FRD-induced changes in adipocyte OS and adipokine production

PMA-elicited AAT isolated adipocyte ROS production in different experimental groups (left-hand panel). The effect of increasing concentrations of insulin (0–10 nM) on LEP release by isolated adipocytes from AAT pads of the different groups is also shown (right-hand panel). Values are means ± S.E.M. ($n = 4$ different experiments, six replicates per condition). $^{*}P < 0.05$ compared with CD, $^{a}P < 0.05$ compared with 0 nM insulin; $^{b}P < 0.05$ compared with 0.1 nM insulin; $^{+}P < 0.05$ compared with the CD, CD-APO and FRD-APO groups under a similar condition.

Table 5 NADPH oxidase activity (measured as O_2^- production) measured in AAT from CD, CD-APO, FRD and FRD-APO rats

Values are means ± S.E.M. from three different experiments ($n = 5$ pads per group) and represent the difference between the O_2^- value measured in the absence and presence of DPI in the incubation medium. $^{*}P < 0.05$ compared with CD; $^{+}P < 0.05$ compared with FRD.

Group	AAT O_2^- production (AU)
CD	101.09 ± 7.86
CD-APO	69.43 ± 5.68*
FRD	179.04 ± 15.28*
FRD-APO	75.98 ± 6.11*+

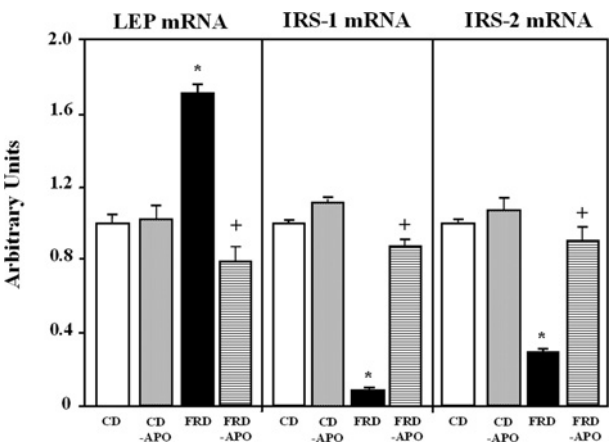

Figure 3 LEP, IRS-1 and IRS-2 mRNA levels in AAT pads from CD- and FRD-fed rats without or with APO treatment

Results (expressed in AU) are normalized to the levels of ACTB and then presented as relative to values obtained in AAT pads from CD-fed rats. Results are means ± S.E.M. ($n = 5$ pads per group). $^{*}P < 0.05$ compared with CD; $^{+}P < 0.05$ compared with FRD.

those from CD-fed rats ($P < 0.05$; Figure 2, left-hand panel). This increased ROS production was markedly reduced in rats co-treated with APO, regardless of the diet received ($P < 0.05$ compared with CD values; Figure 2, left-hand panel).

AAT adipocytes isolated from all the experimental groups and incubated with increasing concentrations of insulin (0–10 nM) released LEP in a concentration-dependent fashion (Figure 2, right-hand panel). At any condition tested, adipocytes from FRD-fed rats released a significantly higher amount of LEP than those from CD-fed rats ($P < 0.05$ compared with CD values). Furthermore, the threshold for insulin-induced LEP release by adipocytes from FRD-fed rats shifted to the right, thus showing decreased insulin sensitivity. In fact, in FRD-fed rats a significant increase in LEP release ($P < 0.05$ compared with baseline) started at a 100 times greater insulin concentration (10 compared with 0.1 nM) than in adipocytes from CD-fed rats. APO co-administration did not affect LEP release by adipocytes from CD-fed rats, but it reduced the high LEP release by adipocytes from FRD-fed rats to values comparable with those of adipocytes from CD-fed rats and re-established the normal threshold of insulin-induced LEP release (Figure 2, right-hand panel).

mRNA content of LEP and intracellular insulin mediators in AAT

The mRNA content of LEP in AAT pads was significantly higher in FRD- than in CD-fed rats ($P < 0.05$; Figure 3, left-hand panel).

Although APO administration to CD-fed rats did not modify such content (compared with CD-fed rats), it abolished the enhancement observed in FRD pads.

IRS-1 and IRS-2 mRNA contents were significantly lower in AAT from FRD- compared with CD-fed rats ($P < 0.05$; Figure 3, middle and right-hand panels respectively). APO co-administration fully prevented the detrimental effect of the FRD on these two intracellular insulin mediators ($P < 0.05$). Additionally, neither treatment- nor diet-dependent changes were found in AAT GLUT-4 mRNA content (results not shown).

DISCUSSION

The results of our present study support the reliability of the FRD-fed rat model to induce several metabolic and endocrine dysfunctions [14,15] that resemble those present in the human MS phenotype [4–6,33]. These are characterized by high values of the HOMA index (indicative of an IR state) and elevated plasma levels of lipids (TAG and NEFAs), adipokines (LEP) and OS biomarkers (specifically TBARS) that indicate increased lipid peroxidation. Thus the model results in a useful tool to study the pathogenesis of the dietary-induced MS and test the effectiveness of different prevention/treatment strategies. The abnormally high levels of these biomarkers confirm those reported in different studies [4–6,14,15,34], as well as their association with several metabolic/endocrine dysfunctions, such as impaired insulin sensitivity, IGT and abnormal lipid metabolism. In the present study, however, we have selectively focused our attention on the metabolic/endocrine AAT dysfunction and the role played by the local and peripheral OS increase. To assess this role, and in order to establish a pathogenic event sequence, we administered an FRD together with APO, an effective inhibitor of NADPH oxidase activity [23].

Brownlee [35] has shown that the excessive provision of a metabolic substrate (fructose in our case) enhances mitochondrial ROS production, which results in a decreased antioxidant defence mechanism, increased PKC isoform activities (mechanism driving to tissue impaired insulin signalling), enhanced adipokine production and promotion of high non-enzymatic glycosylation rate. The high ROS production and TBARS concentration demonstrate the increased OS in the AAT of our FRD-fed rats. This process would simultaneously trigger several dysfunctions, namely, decreased non-enzymatic antioxidant defence ([14], and the present results) and insulin sensitivity, a higher release of FAs, and a significant elevation of the ΣSFA/ΣUFA ratio [14]. The high release of FAs with the consequent increase of their circulating levels would stimulate NADPH oxidase activity [36], establishing a positive-feedback mechanism that would maintain a higher ROS production. In fact, we found that a 3-week FRD intake resulted in a local (AAT) enhancement in NADPH oxidase activity, which tightly paralleled both the increased AAT ROS production and plasma TBARS levels. Moreover, co-administration of FRD and APO significantly prevented all these FRD-induced changes. Interestingly, the NADPH oxidase inhibitor treatment was an effective sequential corrector of the AAT NADPH oxidase activity/ROS production firstly and, thereafter, of most of the metabolic and AAT dysfunctions that occur after feeding the animals with the FRD.

Curtis et al. [37] recently suggested that protein carbonylation (due to the increased OS ratio) plays a major role in mitochondrial dysfunction and may be linked to the development of IR in the adipocyte. Thus this mechanism could contribute to the IR state present in the AAT of FRD-fed rats. The fact that adipose tissue of markedly obese insulin-resistant individuals shows lower AMPK (AMP-activated protein kinase) and higher OS values than insulin-sensitive patients [38] lends further support to the usefulness of the FRD-fed rat model to study the intrinsic mechanisms of AAT dysfunctions and their relationship with Type 2 diabetes development. Complementarily, the high levels of tissue ROS, plasma FAs, NF-κB (nuclear factor κB) and other pro-inflammatory transcription factors [39] may also contribute to the transition from the state of IGT to Type 2 diabetes in rats chronically fed on a high-carbohydrate diet [40].

The link between insulin sensitivity and FA composition was reported early on by Borkman et al. [41], showing that decreased insulin sensitivity was associated with decreased concentrations of PUFAs in skeletal-muscle phospholipids, thus suggesting that changes in FA composition in this tissue modulate the hormone's action. This relationship was also shown in adipose tissue ([14] and the present results).

The biosynthesis of high PUFAs is mainly modulated by the Δ^6 and Δ^5 desaturases through dietary and hormonal stimulated mechanisms, and insulin activates both enzymes [42]. Consequently, the lower insulin sensitivity present in the AAT of FRD-fed rats (demonstrated by the lower IRS-1/IRS-2 expression and the decreased insulin-induced LEP release) could explain their high ΣSFA/ΣUFA ratio. Since enhanced OS could also inhibit Δ^5 and/or Δ^6 desaturase activities/y [43], such an effect would contribute to increase this particular ratio. Furthermore, the decreased β-cell mass present in these rats due to the OS-induced apoptotic rate [44] would actively contribute to the lower insulin effect.

Concurrent with the impaired lipid metabolism, AAT from FRD-fed rats underwent profound changes in its mass (enlarged), cell morphology (increased adipocyte size/volume) and expression of adipokines (enhanced LEP mRNA content) and intracellular insulin mediator (decreased IRS-1/IRS-2 mRNAs) genes. The presence of large adipocytes has been associated with LEP overproduction [45], as shown by data reproduced in our model both under *in vivo* and *in vitro* conditions, as well as with its higher gene expression. It is known that LEP significantly affects insulin binding to its receptor [46] and the expression of IRS-1/IRS-2 downstream of the insulin receptor [47,48]. These LEP effects reinforce the concept that the AAT tissue dysfunction plays a relevant role in the development of the overall IR observed in FRD-fed rats (high HOMA values).

The development of all the morphological, metabolic and endocrine dysfunctions previously described was effectively prevented by co-administration of APO with the FRD. APO is a highly effective inhibitor of the NADPH oxidase system by

interfering, at least in part, with the assembly of cytosolic elements of the enzyme at the cell membrane level [49–51]. As a result, catalytic transmembrane proteins cannot reach complete enzyme functionality [52], thus reducing ROS production. Consequently, this preventive effect strongly plays in favour of our departing hypothesis that OS is the first and main cause of the FRD-induced human MS-like phenotype in normal rats. We cannot, however, discard that some beneficial effects of APO can be partly ascribed to its NADPH-oxidase-independent action [53].

In brief, the result of the present study show that administration of an FRD for 3 weeks to normal rats induces multiple morphological, metabolic and endocrine alterations whose development can be effectively prevented by inhibiting the main enhancer of endogenous OS, NADPH oxidase activity, with the highly effective inhibitor APO. These results could help to better understand the pathogenesis of these alterations and also suggest that the use of such an inhibitor could help to prevent, at least in rats, the development of dietary-induced MS.

CLINICAL PERSPECTIVES

- The current epidemics of obesity, Type 2 diabetes and the MS could be ascribed to the drastic increase in fructose consumption. On the other hand, administration of an FRD to normal rats induces the development of the human-like MS phenotype. The increased rate of OS and an associated AAT dysfunction would play a key role in this process.
- In the present study, we have shown that normal rats fed on an FRD for 3 weeks have larger AAT mass and multiple metabolic and endocrine alterations whose development can be effectively prevented by inhibiting the main enhancer of endogenous OS, NADPH oxidase activity, with a highly effective inhibitor (APO).
- These results could help to better understand the pathogenesis of these alterations, and this knowledge would facilitate the development of treatment and preventive strategies for dietary-induced MS.

AUTHOR CONTRIBUTION

Juan Fariña, Ana Alzamendi, Andrés Giovambattista and María García performed experiments and collected and analysed the data. María García revised the paper. Carlos Marra performed experiments and revised the paper. Eduardo Spinedi performed final data analysis, designed illustrations and drafted the paper. Juan Gagliardino conceived and designed the study, performed data analysis and drafted the paper. All authors read and approved the final paper.

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REFERENCES

1 Popkin, B. M. (2010) Patterns of beverage use across the lifecycle. Physiol. Behav. **100**, 4–9
2 Malik, V. S., Popkin, B. M., Bray, G. A., Després, J. P., Willett, W. C. and Hu, F. B. (2010) Sugar-sweetened beverages and risk of metabolic syndrome and type 2 diabetes: a meta-analysis. Diabetes Care **33**, 2477–2483
3 de Koning, L., Malik, V. S., Rimm, E. B., Willett, W. C. and Hu, F. B. (2011) Sugar-sweetened and artificially sweetened beverage consumption and risk of type 2 diabetes in men. Am. J. Clin. Nutr. **93**, 1321–1327
4 Verma, S., Bhanot, S., Yao, L. and McNeill, J. H. (1997) Vascular insulin resistance in fructose-hypertensive rats. Eur. J. Pharmacol. **322**, R1–R2
5 Hwang, I. S., Ho, H., Hoffman, B. B. and Reaven, G. M. (1987) Fructose-induced insulin resistance and hypertension in rats. Hypertension **10**, 512–516
6 Basciano, H., Federico, L. and Adeli, K. (2005) Fructose, insulin resistance, and metabolic dyslipidemia. Nutr. Metab. **2**, 5
7 Hu, F. B. and Malik, V. S. (2010) Sugar-sweetened beverages and risk of obesity and type 2 diabetes: epidemiologic evidence. Physiol. Behav. **100**, 47–54
8 United States Department of Health and Human Services and United States Department of Agriculture Dietary Guidelines Advisory Committee Report (www.health.gov/dietaryguideline/dga2005/report)
9 Delbosc, S., Paizanis, E., Magous, R., Araiz, C., Dimo, T., Cristol, J. P., Cros, G. and Azay, J. (2005) Involvement of oxidative stress and NADPH-oxidase activation in the development of cardiovascular complications in a model of insulin resistance, the fructose-fed rat. Atherosclerosis **179**, 43–49
10 Cavarape, A., Feletto, F., Mercuri, F., Quagliaro, L., Daman, G. and Ceriello, A. (2001) High-fructose diet decreases catalase mRNA levels in rat tissues. J. Endocrinol. Invest. **24**, 838–845
11 Faure, P., Rossini, E., Lafond, J. L., Richard, M. J., Favier, A. and Halimi, S. (1997) Vitamin E improves the free radical defense system potential and insulin sensitivity of rats fed high fructose diets. J. Nutr. **127**, 103–107
12 Faure, P., Rossini, E., Wiernsperger, N., Richard, M. J., Favier, A. and Halimi, S. (1999) An insulin sensitizer improves the free radical defense system potential and insulin sensitivity in high fructose-fed rats. Diabetes **48**, 353–357
13 Wei, Y., Wang, D., Topczewski, F. and Pagliassotti, M. J. (2007) Fructose-mediated stress signaling in the liver: implications for hepatic insulin resistance. J. Nutr. Biochem. **18**, 1–9
14 Rebolledo, O. R., Marra, C. A., Raschia, A., Rodriguez, S. and Gagliardino, J. J. (2008) Abdominal adipose tissue: early metabolic dysfunction associated to insulin resistance and oxidative stress induced by an unbalanced diet. Horm. Metab. Res. **40**, 794–800

15 Alzamendi, A., Giovambattista, A., Raschia, A., Madrid, V., Gaillard, R. C., Rebolledo, O., Gagliardino, J. J. and Spinedi, E. (2009) Fructose-rich diet-induced abdominal adipose tissue endocrine dysfunction in normal male rats. Endocrine **35**, 227–232

16 Ashcroft, S. J., Hedeskov, C. J. and Randle, P. J. (1970) Glucose metabolism in mouse pancreatic islets. Biochem. J. **118**, 143–154

17 Giroix, M. H., Scruel, O., Ladriere, L., Sener, A., Portha, B. and Malaisse, W. J. (1999) Metabolic and secretory interactions between D-glucose and D-fructose in islets from GK rats. Endocrinology **140**, 5556–5565

18 Sener, A. and Malaisse, W. J. (1988) Hexose metabolism in pancreatic islets. Metabolic and secretory responses to D-fructose. Arch. Biochem. Biophys. **261**, 16–26

19 Kyriazis, G. A., Soundarapandian, M. M. and Tyrberg, B. (2012) Sweet taste receptor signaling in beta cells mediates fructose-induced potentiation of glucose-stimulated insulin secretion. Proc. Natl. Acad. Sci. U.S.A. **109**, E524–E532

20 McGuinness, O. P. and Cherrington, A. D. (2003) Effects of fructose on hepatic glucose metabolism. Curr. Opin. Clin. Nutr. Metab. Care **6**, 441–448

21 Hajduch, E., Darakhshan, F. and Hundal, H. S. (1998) Fructose uptake in rat adipocytes: GLUT5 expression and the effects of streptozotocin-induced diabetes. Diabetologia **41**, 821–828

22 Litherland, G. J., Hajduch, E., Gould, G. W. and Hundal, H. S. (2004) Fructose transport and metabolism in adipose tissue of Zucker rats: diminished GLUT5 activity during obesity and insulin resistance. Mol. Cell. Biochem. **261**, 23–33

23 Cui, C., Chen, A. F., Jiang, Z., Wu, Q., Lin, J., Wen, H. and Zeng, J. (2007) Inhibition of NAD(P)H oxidase reduces fibronectin expression in stroke-prone renovascular hypertensive rat brain. Clin. Exp. Pharmacol. Physiol. **34**, 304–309

24 Alzamendi, A., Castrogiovanni, D., Ortega, H. H., Gaillard, R. C., Giovambattista, A. and Spinedi, E. (2010) Parametrial adipose tissue and metabolic dysfunctions induced by fructose-rich diet in normal and neonatal-androgenized adult female rats. Obesity **18**, 441–448

25 Giovambattista, A., Chisari, A. N., Gaillard, R. C. and Spinedi, E. (2000) Food intake-induced leptin secretion modulates hypothalamo-pituitary-adrenal axis response and hypothalamic Ob-Rb expression to insulin administration. Neuroendocrinology **72**, 341–349

26 Moreno, G., Perelló, M., Camihort, G., Luna, G., Console, G., Gaillard, R. C. and Spinedi, E. (2006) Impact of transient correction of increased adrenocortical activity in hypothalamo-damaged, hyperadipose female rats. Int. J. Obes. **30**, 73–82

27 Giovambattista, A., Gaillard, R. C. and Spinedi, E. (2008) Ghrelin gene-related peptides modulate rat white adiposity. Vitam. Horm. **77**, 171–205

28 Schrenzel, J., Serrander, L., Bánfi, B., Nüsse, O., Fouyouzi, R., Lew, D. P., Demaurex, N. and Krause, K. H. (1998) Electron currents generated by the human phagocyte NADPH oxidase. Nature **392**, 734–737

29 Oliveira, H. R., Verlengia, R., Carvalho, C. R., Britto, L. R., Curi, R. and Carpinelli, A. R. (2003) Pancreatic β-cells express phagocyte-like NADPH oxidase. Diabetes **52**, 1457–1463

30 Moini, H., Packer, L. and Saris, N. E. (2002) Antioxidant and prooxidant activities of alpha-lipoic acid and dihydrolipoic acid. Toxicol. Appl. Pharmacol. **182**, 84–90

31 Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**, 156–159

32 McElroy, W. D. and Swanson, C. P. (eds) (1974) Biostatistical Analysis, Prentice Hall, Englewood Cliffs, NJ, U.S.A.

33 Grundy, S. M., Hansen, B., Smith, Jr, S. C., Cleeman, J. I. and Kahn, R. A. (2004) Clinical management of metabolic syndrome: report of the American Heart Association/National Heart, Lung and Blood Institute/American Diabetes Association conference on scientific issues related to management. Circulation **109**, 551–556

34 Miatello, R., Vázquez, M., Renna, N., Cruzado, M., Zumino, A. P. and Risler, N. (2005) Chronic administration of resveratrol prevents biochemical cardiovascular changes in fructose-fed rats. Am. J. Hypertens. **18**, 864–870

35 Brownlee, M. (2005) The pathobiology of diabetic complications: a unifying mechanism. Diabetes **54**, 1615–1625

36 Furukawa, S., Fujita, T., Shimabukuro, M., Iwaki, M., Yamada, Y., Nakajima, Y., Nakayama, O., Makishima, M., Matsuda, M. and Shimomura, I. (2004) Increased oxidative stress in obesity and its impact on metabolic syndrome. J. Clin. Invest. **114**, 1752–1761

37 Curtis, J. M., Hahn, W. S., Stone, M. D., Inda, J. J., Droullard, D. J., Kuzmicic, J. P., Donoghue, M. A., Long, E. K., Armien, A. G., Lavandero, S. et al. (2012) Protein carbonylation and adipocyte mitochondria function. J. Biol. Chem. **287**, 32967–32980

38 Xu, X. J., Gauthier, M. S., Hess, D. T., Apovian, C. M., Cacicedo, J. M., Gokce, N., Farb, M., Valentine, R. J. and Ruderman, N. B. (2012) Insulin sensitive and resistant obesity in humans: AMPK activity, oxidative stress, and depot-specific changes in gene expression in adipose. J. Lipid Res. **53**, 792–801

39 Alam, S. E., Singh, R. B., Gupta, S., Dherange, P., De Meester, F., Wilczynska, A., Dharwadkar, S., Wilson, D. and Hungin, P. (2012) Nutritional aspects of epigenetic inheritance. Can. J. Physiol. Pharmacol. **90**, 989–994

40 Gutman, R., Basílico, M. Z., Bernal, C., Chicco, A. and Lombardo, Y. B. (1987) Long-term hypertriglyceridemia and glucose intolerance in rats fed chronically an isocaloric sucrose-rich diet. Metabolism **36**, 1013–1020

41 Borkman, M., Storlien, L. H., Pan, D. A., Jenkins, A. B., Chisholm, D. J. and Campbell, L. V. (1993) The relation between insulin sensitivity and the fatty-acid composition of skeletal-muscle phospholipids. N. Engl. J. Med. **328**, 238–244

42 Brenner, R. R. (2003) Hormonal modulation of delta6 and delta5 desaturases: case of diabetes. Prostaglandins Leukotrienes Essent. Fatty Acids **68**, 151–162

43 Montanaro, M. A., Lombardo, Y. B., González, M. S., Bernasconi, A. M., Chicco, A., Rimoldi, O. J., Basabe, J. C. and Brenner, R. R. (2005) Effect of troglitazone on the desaturases in a rat model of insulin-resistance induced by a sucrose-rich diet. Prostaglandins Leukotrienes Essent. Fatty Acids **72**, 241–250

44 Maiztegui, B., Borelli, M. I., Madrid, V. G., Del Zotto, H., Raschia, M. A., Francini, F., Massa, M. L., Flores, L. E., Rebolledo, O. R. and Gagliardino, J. J. (2011) Sitagliptin prevents the development of metabolic and hormonal disturbances, increased β-cell apoptosis and liver steatosis induced by a fructose-rich diet in normal rats. Clin. Sci. **120**, 73–80

45 Couillard, C., Mauriège, P., Imbeault, P., Prud'homme, D., Nadeau, A., Tremblay, A., Bouchard, C. and Després, J. P. (2000) Hyperleptinemia is more closely associated with adipose cell hypertrophy than with adipose tissue hyperplasia. Int. J. Obes. Relat. Metab. Disord. **24**, 782–788

46 Walder, K., Filippis, A., Clark, S., Zimmet, P. and Collier, G. R. (1997) Leptin inhibits insulin binding in isolated rat adipocytes. J. Endocrinol. **155**, R5–R7

47 Cohen, B., Novick, D. and Rubinstein, M. (1996) Modulation of insulin activities by leptin. Science **274**, 1185–1188

48 Krempler, F., Hell, E., Winkler, C., Breban, D. and Patsch, W. (1998) Plasma leptin levels: interaction of obesity with a common variant of insulin receptor substrate-1. Arterioscler. Thromb. Vasc. Biol. **18**, 1686–1690

49 Johnson, D. K., Schillinger, K. J., Kwait, D. M., Hughes, C. V., McNamara, E. J., Ishmael, F., O'Donnell, R. W., Chang, M. M., Hogg, M. G., Dordick, J. S. et al. (2002) Inhibition of NADPH oxidase activation in endothelial cells by ortho-methoxy-substituted catechols. Endothelium **9**, 191–203

50 Barbieri, S. S., Cavalca, V., Eligini, S., Brambilla, M., Caiani, A., Tremoli, E. and Colli, S. (2004) Apocynin prevents cyclooxygenase 2 expression in human monocytes through NADPH oxidase and glutathione redox-dependent mechanisms. Free Radical Biol. Med. **37**, 156–165

51 Peters, E. A., Hiltermann, J. T. and Stolk, J. (2001) Effect of apocynin on ozone-induced airway hyperresponsiveness to methacholine in asthmatics. Free Radical Biol. Med. **31**, 1442–1447

52 Stefanska, J. and Pawliczak, R. (2008) Apocynin: molecular aptitudes. Mediators Inflamm. **2008**, 106507

53 Heumüller, S., Wind, S., Barbosa-Sicard, E., Schmidt, H. H., Busse, R., Schröder, K. and Brandes, R. P. (2008) Apocynin is not an inhibitor of vascular NADPH oxidases but an antioxidant. Hypertension **51**, 211–217

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