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Changes induced by a fructose-rich diet on hepatic metabolism and the antioxidant system

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ABSTRACT

Aims: The effect of a three-week fructose-rich diet (FRD) upon gene expression, protein and activity levels of liver antioxidant system and carbohydrate metabolism was studied.
Main methods: Serum glucose (fasting and after a glucose load), triglyceride and insulin levels of normal male Wistar rats were measured. In liver, we measured gene/protein expression and enzyme activity of catalase (CAT), copper–zinc–superoxide dismutase (CuZnSOD) and glutathione peroxidase (GSHPx); reduced glutathione (GSH); protein carbonyl content; thiobarbituric acid reactive substances (TBARS) content and microsomal membrane susceptibility to lipid peroxidation; glucokinase (GK), glucose-6-phosphatase (G-6-Pase) and glucose-6-phosphate dehydrogenase (G-6-PDH) activity; and glycogen, pyruvate, lactate and triglyceride content.
Key findings: Similar body weights and caloric intake were recorded in both groups. FRD rats had higher serum glucose, insulin and triglyceride levels, molar insulin:glucose ratio, HOMA-IR values and impaired glucose tolerance, whereas CAT, CuZnSOD and GSHPx relative gene expression levels were significantly lower. CAT and CuZnSOD protein expression, CAT activity and GSH content were also lower, while protein carbonyl content was higher. No differences were recorded in CuZnSOD, MnSOD and GSHPx activity, TBARS content and membrane susceptibility to lipid peroxidation. Glycogen, lactate and triglyceride content and GK, G-6-Pase and G-6-PDH activity were significantly higher in FRD rats.
Significance: In the presence of oxidative stress, the liver exhibits changes in the carbohydrate and lipid metabolic pathways that would decrease reactive oxygen species production and their deleterious effect, thus inducing little impact on specific antioxidant mechanisms. This knowledge could facilitate the design and implementation of strategies to prevent oxidative stress-induced liver damage.

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Introduction

The prevalence of diabetes, particularly type 2 diabetes (T2DM), increases exponentially worldwide and it is estimated that by 2025 it will affect 300 million people (King et al. 1998). T2DM is preceded by impaired glucose tolerance (IGT), a state where the metabolic and endocrine changes described in overt diabetes (American Diabetes Association 2008; Butler et al. 2003) are already present, albeit to a lesser extent. Progression of IGT towards T2DM can be effectively prevented or even delayed through lifestyle changes or drug treatment (Tuomilehto et al. 2001; Diabetes Prevention Program Research Group 2002; DREAM et al. 2006).

Sedentary habits and consumption of unhealthy high calorie diets are common characteristics of modern societies (Guthrie and Morton 2000). The increase in total energy consumption has been accompanied by marked changes in the composition of meal nutrients (Putnam and Allshouse 1999): the annual *per capita* consumption of fructose in the US has increased from ~0.2 kg in 1970 to ~28 kg in 1997 (Wei et al. 2006). Some authors have suggested that the increased use of fructose-rich syrups and refined carbohydrates has contributed to the current epidemic of obesity and T2DM (Bray et al. 2004; Elliott et al. 2002; Gross et al. 2004).

The liver is the primary site for fructose extraction and metabolism; consequently, it has been postulated that an increase in fructose flow and availability will impair the hepatic metabolism of glucose (Bizeau and Pagliassotti 2005; Pagliassotti and Horton 2004). Such impairment includes the hepatic release of glucose and lipids (VLDL). Supporting this concept, we have already shown that rats fed with a diet rich in fructose (FRD) for 3 weeks have an increase in liver glucokinase (GK)

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activity, mainly due to a marked increase in 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFK2) (Francini et al. 2009). Liver PFK2 regulates GK activity in normal intact animals, as part of the adaptive response to the increased liver fructose flow to maintain glucose/triglyceride homeostasis (Francini et al. 2009). Other associated changes include increased liver fat deposits (Spolarics and Meyenhofer 2000) as well as changes in the hepatocyte sensitivity to insulin and in the concentration of insulin intracellular signalling mediators (Bezerra et al. 2000).

The underlying mechanism responsible for the detrimental effects of a FRD is not quite clear, but there is evidence that such diet promotes gluco-oxidative stress (Busserolles et al. 2002b; Girard et al. 2006; Lange et al. 1980; Thirunavukkarasu et al. 2004). In fact, short-term FRD administration to normal rats induces significant changes in many metabolic, endocrine and oxidative stress markers (Alzamendi et al. 2009).

In the presence of a state of oxidative stress, not all body tissues react in the same manner: while markers of such state undergo no significant changes in the liver (Busserolles et al. 2002a; Kelley et al. 2004; Spolarics and Meyenhofer 2000), they do in tissues such as insulin-producing cells, characterized by their extremely low antioxidant defense (Gurgul et al. 2004; Robertson et al. 2004; Tiedge et al. 1998). However, it is not clear whether such differences depend only on the uneven antioxidant defense of these tissues.

In an attempt to answer this question, we currently studied the effect of a fructose overload upon gene expression, protein and activity levels of the liver antioxidant defense system as well as on the hepatic carbohydrate and lipid metabolic pathways. Such knowledge would help both to better understand the adaptation mechanisms involved in fructose-induced oxidative stress and to develop appropriate strategies for the prevention and treatment of obesity and T2DM triggered by unhealthy diets.

Materials and methods

Chemicals and drugs

Reagents of the purest available grade were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The polyclonal anti-bovine liver catalase (CAT) antibody raised in rabbit was purchased from Rockland (Gilbertsville, Pennsylvania, USA). Anti-rat glutathione peroxidase (GSHPx) and copper–zinc–superoxide dismutase (CuZnSOD) rabbit sera were kindly provided by Prof. Sigurd Lenzen (Medizinische Hochschule Hannover, Hannover, Germany). The secondary antibody (peroxidase-conjugated AffiniPure donkey anti-rabbit IgG) was purchased from Dianova (Hamburg, Germany).

Animals

Normal male Wistar rats weighing 150–180 g were used. They were maintained in a temperature-controlled room (23 °C) with a fixed 12-h light–dark cycle (06:00–18:00 h). Animal experiments and handling were performed according to the "Ethical principles and guidelines for experimental animals" (3rd Edition 2005) of the Swiss Academy of Medical Sciences (mail@samw.ch).

One group of 15 animals (5 different experiments run in triplicate) had free access to a standard commercial diet and tap water (control, C), while another 15 rats had access to the same diet with 10% fructose in the drinking water (FRD). The fiber content of the commercial diet was 7%. Water intake was measured daily, while individual body weight was recorded once a week. Twenty-one days after this treatment, blood samples from 4-h-fasted animals were drawn from the retroorbital plexus under light halothane anesthesia and collected into heparinized tubes to measure plasma glucose, triglyceride and immunoreactive insulin levels. Afterwards, the animals were killed by decapitation and the same portion of the liver (median lobe) was consistently removed to perform all the assays.

Plasma measurements

Glucose was measured by the glucose–oxidase GOD-PAP method (Roche Diagnostics, Mannheim, Germany). Triglycerides were assayed using a commercial immunoenzymatic assay kit implemented in an automated clinical analyzer, and immunoreactive insulin was determined by radioimmunoassay (RIA) (Herbert et al. 1965) using an antibody against rat insulin, rat insulin standard (Linco Research Inc., IN, USA) and highly-purified porcine insulin labeled with ^{125}I (Linde et al. 1980). Serum insulin and fasting serum glucose values were then employed to estimate insulin resistance by HOMA-IR, using the following formula: [Serum Insulin (μU/ml × fasting blood glucose (mM))]/22.5 (Matthews et al. 1985).

Glucose tolerance

The day before sacrifice, glucose tolerance was measured in 12 h-fasted rats after i.p. glucose injection (1 g/kg in saline). Blood samples were obtained from the orbital plexus at 0, 15, 30, 60 and 120 min under light phentobarbital anesthesia (48 mg/kg). Results were expressed as the area under the glucose curve (AUC) in mM/min.

Determination of liver glycogen

Each piece of fresh liver (400 mg) was placed in a tube with 1 ml of 33% KOH and incubated for 20 min at 100 °C. Then, 1.25 ml of alcohol was added to each tube and the mixture incubated for 24 h at 4 °C. After that, the mixture was centrifuged at 2400 rpm for 20 min. The pellets obtained were resuspended in 1 ml of distilled water plus 3 ml of Antrona solution (100 mg diluted in 100 ml of SO_4H_2 84%v/v) and incubated for 20 min at 100 °C. Finally, the optical density (OD) was measured photometrically at 620 nm. Results are expressed as μmol glycogen/mg tissue (Chun and Yin 1998).

GK activity

Liver pieces were removed and immediately homogenized in a hand-held homogenizer (20 times) suspended in ice cold phosphate saline buffer containing 0.1 mM PMSF, 0.1 mM benzamidin, 2 mM DTT, 4 μg/ml aprotinin and 0.3 M sucrose, pH 7.5. The homogenate was then passed through a 23-gauge needle syringe (5 times) to ensure appropriate sample mixing. Aliquots of these homogenates were centrifuged at 600 × *g* to separate and discard the nuclear fraction. The supernatant was centrifuged twice at 8000 × *g* and 100,000 × *g* at 4 °C, and the resultant supernatant was collected and identified as the cytosolic fraction (CF). Phosphorylation in the 100,000 × *g* soluble CF was measured at 37 °C, pH 7.4, by recording the increase in absorbance at 340 nm in a well-established enzyme-coupled photometric assay containing glucose-6-phosphate dehydrogenase (G-6-P DH), ATP and NADP (Lenzen et al. 1988a,b; Massa et al. 2004). For each assay, five different experiments were done in triplicate. GK activity was obtained by subtracting the activity measured at 1 mM glucose (hexokinase) from that measured at 100 mM glucose. These concentrations were selected after fitting different curves obtained using a wide range of glucose concentrations (3.1, 6.25, 12.5, 25, 50, 100 and 200 mM). Enzyme activity was expressed as mU per milligram of protein. One unit of enzyme activity was defined as 1 μmol glucose-6-phosphate (G-6-P) formed from glucose and ATP/min at 37 °C.

G-6-Pase activity

Homogenization of liver samples and isolation of microsomes were carried out as described by Nordlie and Arion (1966). Homogenization

medium was 0.25 M sucrose/5 mM Tris-acetate/0.5 mM Na-EDTA, pH 7.4 (3 ml/g tissue). Microsomes were washed once with 0.25 M sucrose/5 mM Tris-acetate, pH 7.4, and centrifuged at 100,000×*g*. Untreated microsomes were diluted to the desired final concentration with the sucrose buffered solution and assayed without further treatment.

Fully disrupted microsomes were prepared at 0 °C by adding 0.1 ml of 0.75% (w/v) Triton X-100 to 0.9 ml of untreated microsomes (about 10 mg of protein) and allowed to stand on ice for 20 min. The reaction was stopped by adding 250 µl of 10% TCA, and 2 ml of $MoNH_4$ (diluted in H_2SO_4 1 N) plus 320 µl of $FeSO_4$ (diluted in H_2SO_4 0.15 N) were added to 200 µl of each sample. The OD at 660 nm was determined photometrically and results were expressed as "latency". Latency was calculated according to the following formula: 100×(activity in disrupted microsomes − activity measured in untreated microsomes)/activity measured in disrupted microsomes (Lange et al. 1980).

G-6-P DH activity

Pieces of liver were homogenized in buffer Tris/HCl 0.1 M, 1 mM EDTA, pH 7.6 (10 ml/g tissue). The homogenate was centrifuged 15 min at 10,000×*g* and enzyme activity was measured in the supernatants. The increase in absorbance of the NADPH produced was measured photometrically at 340 nm. G-6-P DH activity was measured according to the method of Beutler (1975).

Liver pyruvate content

Pieces of liver were homogenized in perchloric acid 1 M (10 ml/g tissue) and the homogenates were centrifuged for 5 min at 2500 rpm. Each supernatant obtained was neutralized by addition of 2 M K_2CO_3. The pyruvate content in the sample was determined in an enzyme system that uses lactate dehydrogenase and NADH as electron donors; absorbance was measured at 340 nm. Under these conditions, the decrease in absorbance due to the disappearance of NADH was proportional to the amount of pyruvate present in the sample. Results are expressed as µmol pyruvate/g tissue (Lamprecht and Heinz 1986).

Liver lactate content

The samples were processed using the same procedure as that described for pyruvate determination. The content of lactate in the sample was determined in an assay system that uses lactate dehydrogenase and glutamic–pyruvic transaminase with NAD^+ as electron acceptor; absorbance was measured at 340 nm. The increase in absorbance due to the appearance of NADH was proportional to the amount of lactate in the sample. Results are expressed as µmol lactate/g tissue (Noll 1986).

Liver triglyceride content

Liver triglycerides were extracted following the protocol described by Schwartz and Wollins (2007), and the enzymatic assay of triglyceride levels was carried out with a commercial kit, as described for serum measurements.

Determination of antioxidant enzyme activity

Liver pieces removed from each animal were immediately homogenized 20 times with a hand-held homogenizer in the same homogenization buffer as that used for GK activity. The homogenate was then passed through a 23-gauge needle syringe (5 times) to ensure that the sample was well-mixed. Aliquots of these homogenates were stored at −80 °C to measure CAT, CuZnSOD, manganese–superoxide dismutase (MnSOD) and GSHPx activities. Liver microsomes were prepared by standard differential centrifugation technique as described by Slater and Sawyer (1971). Aliquots of microsomal suspensions in 30 mM phosphate buffer pH 7.4, containing 8.8 g/L KCl were stored at −80 °C until measurements.

Aliquots of post-mitochondria supernatants were used to determine the enzymatic activities of the antioxidant defense system. The selenium-dependent glutathione peroxidase (Se-GSHPx) activity was assayed according to the method of Lawrence and Burk (1976). CuZnSOD and MnSOD activities were measured using the procedure described by Misra and Fridovich (1977) and CAT activity was determined by the method of Aebi (1984).

Isolation of total RNA

Total liver RNA was isolated from C and FRD rats using TRIzol Reagent (Gibco-BRL, Rockville, MD, USA) (Chomczynski and Sacchi 1987). The integrity of isolated RNA was checked by 1% agarose-formaldehyde gel electrophoresis. Possible contamination with protein or phenol was tested by measuring the 260/280 nm absorbance ratio, while DNA contamination was avoided using DNAase I digestion (Gibco-BRL). RT-PCR was performed using the SuperScript III (Gibco-BRL) and total RNA (50 ng) from C and FRD liver as a template.

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

qPCR was performed with a Mini Opticon Real-Time PCR Detector Separate MJR (Bio-Rad Laboratories, Inc, Hercules, California, USA), using SYBR Green I as a fluorescent dye that binds only double-stranded DNA. 10 ng of cDNA were amplified in a 25 µl qPCR reaction containing 0.6 µM of each primer, 3 mM $MgCl_2$, 0.3 mM dNTPs, and 0.2 µl Platinum Taq DNA polymerase 6U/µl (Invitrogen, Carlsbad, California, USA). Samples were first denatured at 94 °C for 3 min, followed by 40 PCR cycles. Each cycle comprised a melting step at 94 °C for 30 s, an annealing step at 63 °C for 45 s, and an extension step at 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. The optimal parameters for the PCR reactions were empirically defined. Each PCR amplification was performed in triplicate. The following oligonucleotide primers (Invitrogen) were used: β-actin gene (GenBank® accession number NM_019130), 5′-AGAGG-GAAATCGTGCGTGAC-3′ and 5′CGATAGTGATGACCTGACCGT-3′; CAT gene (GenBank® accession number NM_012520), 5′-CCTCAGAAACCC-GATGTCCTG-3′ and 5′-GTCAAAGTGTGCCATCTCGTCG-3′; CuZnSOD gene (GenBank® accession number NM_017050), 5′-GTGCAGGGCGT-CATTCACTTC-3′ and 5′-GCCTCTCTTCATCCGCTGGA-3′ and GSHPx gene (GenBank® accession number NM_030826), 5′-TGAGAAGGCT-CACCCGCTCT-3′ and 5′GCACTGGAACACCGTCTGGA-3′All amplicons were designed in a size range of 90 to 250 bp. β-actin was used as housekeeping gene. SYBR Green fluorescence emission was determined after each cycle. The purity and specificity of the amplified PCR products was verified by melting curves generated at the end of each PCR. Product length and PCR specificity were further checked by 2% (w/v) agarose gel electrophoresis and ethidium bromide staining. Data are expressed as relative gene expression after normalization to the β-actin housekeeping gene using the *Qgene96* and LineRegPCR software (Muller et al. 2002).

Western blot analysis

Protein concentration was quantified by a Bio-Rad protein assay (Bradford 1976). Thereafter, dithiothreitol and bromophenol blue were added to a final concentration of 100 mM and 0.1%, respectively. Aliquots of liver homogenates (100 µg whole protein) were placed in reducing 10% SDS-PAGE and electroblotted to polyvinylidene difluoride membranes. The amount of protein loaded was measured by the Bradford method (1976), and the uniformity of protein loading in each lane was assessed by staining the blot with Ponceau-S (Fig. 1D).

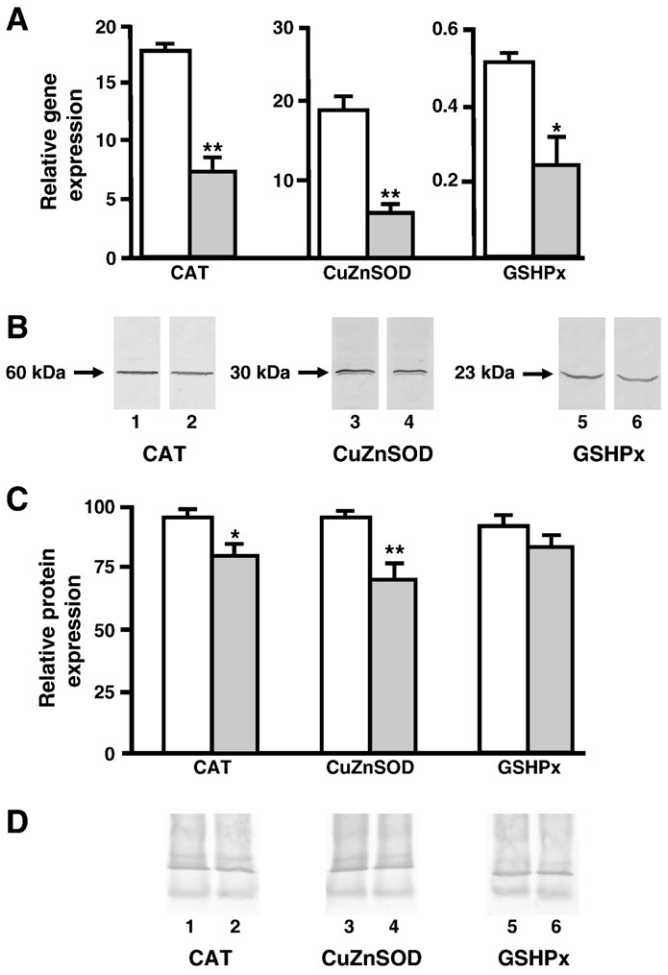

Fig. 1. CAT, CuZnSOD and GSHPx relative gene (A) and protein (B–C) expression. (B) Representative blots show the bands corresponding to CAT, CuZnSOD and GSHPx protein in C (lines 1, 3 and 5) and FRD (lines 2, 4 and 6), respectively. (C) Band intensities were measured in C (white bars) and FRD (gray bars) animals. Results are means ± S.E.M. of 5 different experiments run in triplicate. *$P<0.05$, **$P<0.001$. (D) Ponceau-S staining as loading control corresponding to CAT, CuZnSOD and GSHPx Western blots for C (lines 1, 3 and 5) and FRD (lines 2, 4 and 6).

Nonspecific binding sites of the membranes were blocked by overnight incubation with non-fat dry milk at 4 °C. Enzyme identification and quantification were performed using specific primary antibodies against CAT (final dilution of 1:5000), CuZnSOD (final dilution of 1:3000) and GSHPx (final dilution of 1:7500). After 1-h incubation, the membranes were rinsed in TBS and further incubated for 1 h with the corresponding second antibody, peroxidase-conjugated AffiniPure donkey anti-rabbit IgG (final dilution of 1:10,000). Diaminobenzidine (DAB, Sigma Co., St Louis, MO) was used for color development. Briefly, the membranes were placed in 20 ml TBS plus 10 mg DAB, 10 µl $NiCl_2$ (10%v/v) and 15 µl H_2O_2 (30%v/v). After color development, the reaction was stopped and the membrane was rinsed in distilled water. Finally, the bands were quantified by densitometry using the Gel-Pro Analyzer software.

Assessment of protein carbonyl groups, reduced glutathione (GSH) and lipid peroxidation

Protein carbonyl content was assayed by the procedure of Levine et al. (1990). Liver homogenates were centrifuged and 0.5 ml of the supernatant were mixed with 10 mM 2,4-Dinitrophenylhydrazine (DNPH) in 2 M hydrochloric acid and incubated for 1 h at room temperature; thereafter, the mixture was precipitated with 20% trichloroacetic acid (TCA). The pelleted protein was washed thrice by resuspension in ethanol/ethyl acetate (1:1). Proteins were then solubilized in 6 M guanidine hydrochloride, and centrifuged at 16 000 × *g* for 5 min to remove any trace of insoluble material. The carbonyl content was measured spectrophotometrically at 366 nm. A tissue blank incubated with 2 M HCl without DNPH was included for each sample. The results were expressed as nmol of carbonyl residues/mg protein based on the molar extinction coefficient of 21,000 M^{-1} cm^{-1}. GSH in liver was determined by the method of Ellman (Sedlak and Lindsay 1968). Thiobarbituric acid reactive substances (TBARS) were determined by a spectroscopic technique. The absorbance at 535 nm was measured and TBARS were expressed in nmol MDA/g tissue using an extinction coefficient of 1.56 × 105 M^{-1} cm^{-1} (Schinella et al. 2000).

Susceptibility of liver microsomal membrane to lipid peroxidation

The assay for microsomal lipid peroxidation was measured with enzymatic and non-enzymatic systems able to generate free radical species (Schinella et al. 2000).

Lipid peroxidation induced by the Fe2+/ascorbate system

Reaction mixtures contained 2 mg of microsomal protein/ml in 0.1 M Tris–HCl buffer, pH 7.4. Lipid peroxidation was induced by addition of 5 mM $FeSO_4$ and 500 mM ascorbate. The samples (triplicates) were incubated 20 min at 37 °C in the presence of different concentrations of the extracts. Products of lipid peroxidation were determined by the TBARS method, measuring the absorbance at 535 nm.

Lipid peroxidation induced by the Cl_4C/NADPH system

Reaction mixtures contained 1.5 mg of microsomal protein and a NADPH-generating system (0.2 mM NADPH, 4 mM G-6-Pase, 0.6 units G-6-P DH) in the same buffer as above. Peroxidation was started by addition of 0.02 M Cl_4C. After 15 min of incubation at 37 °C, TBARS were determined as above.

Statistical analysis

The experimental data were analyzed using the Student's *t*-test. Differences were considered significant when $P<0.05$.

Results

Body weight and water intake

Similar body weights were recorded in FRD and C animals after the 3-week treatment period (294.2 ± 5.4 vs. 289.4 ± 14.6 g). FRD animals drank a larger volume of water than C (41.6 ± 2.5 vs. 31.8 ± 3.1 ml/day; $P<0.02$). Conversely, the amount of solid food ingested was significantly larger in C (C vs. FRD, 21.2 ± 1.4 vs. 15. 5 ± 1.8 g/animal/day; $P<0.001$). This fact resulted in a different percent of daily intake of nutrients by C vs. FRD rats (carbohydrates:proteins:lipids, 45:43:12 vs. 60:31:9, respectively) and a comparable caloric intake (55.6 ± 4.5 vs. 50.8 ± 3.4 Kcal/day, respectively).

Serum glucose, insulin and triglyceride levels

FRD rats evinced significantly higher serum glucose (8.3 ± 0.2 vs. 7.2 ± 0.3 mM; $P<0.005$), insulin (4.7 ± 0.6 vs. 2.7 ± 0.5 ng/ml; $P<0.02$) and triglyceride (1.3 ± 0.1 vs. 0.8 ± 0.1 mM; $P<0.001$) levels than C rats. The higher molar insulin:glucose ratio measured in FRD rats (0.57 ± 0.04 vs. 0.37 ± 0.02; $P<0.001$) and the HOMA-IR values (43 ± 3 vs. 26 ± 2; $P<0.001$) demonstrate the existence of an insulin resistance state in these rats.

Glucose tolerance

Plasma glucose AUC values following i.p. glucose administration were significantly higher in FRD than in C rats (4.31 ± 0.5 vs. 1.57 ± 0.63 mM/min; $P<0.01$).

Carbohydrate metabolic pathways

We recorded a simultaneous increase in the activity of GK, G-6-Pase (increase in the futile glucose cycling) and G-6-P DH, together with an increase in glycogen storage and lactate production, without changes in the levels of pyruvate (Table 1).

Liver triglyceride content

FRD-fed rats had higher liver triglyceride content than C animals (693.2 ± 35.1 vs. 496.8 ± 14.7 μg/100 mg tissue; $P<0.001$).

Antioxidant enzyme gene expression (qPCR)

Significantly lower relative levels of CAT, CuZnSOD and GSHPx gene expression were recorded in FRD than in C animals (18.3 ± 0.6 vs. 7.81 ± 1.1, $P<0.001$; 18.45 ± 1.92 vs. 5.11 ± 0.8, $P<0.01$; 0.5 ± 0.02 vs. 0.25 ± 0.07, $P<0.01$, respectively) (Fig. 1A). In all cases, values were expressed as a function of the housekeeping gene β-actin.

Antioxidant enzyme protein analysis

Western blot performed in samples from C and FRD animals using specific CAT, CuZnSOD and GSHPx antibodies showed a single band of about 60, 30 and 23 kDa, respectively, compatible with the molecular weight of the corresponding enzymes. The intensity of the specific bands increased as a function of the protein concentration used, supporting the reliability and specificity of the immune measurement used (data not shown).

Analysis of liver homogenates showed a lower protein expression for both CAT and CuZnSOD in FRD compared with C animals (78.8 ± 5.8 vs. 96 ± 2.6, $P<0.02$ and 69.8 ± 7.8 vs. 96.8 ± 1.9, OD relative units, $P<0.003$, respectively) (Fig. 1B, C). Conversely, the protein expression of GSHPx showed comparable values in both experimental groups.

Antioxidant enzyme activity

CAT activity was significantly lower in FRD than in C animals (0.93 ± 0.04 vs. 1.14 ± 0.04 k/mg protein; $P<0.05$). A unit of CAT is a rate constant of a first order reaction catalysed by catalase. No significant differences were recorded in CuZnSOD, MnSOD and GSHPx activities between C and FRD rats.

Assessment of protein carbonyl groups, GSH and lipid peroxidation

Protein carbonyl content was significantly higher en FRD than in C rats (1.9 ± 0.07 vs. 1.5 ± 0.08 nmol/mg protein; $P<0.05$). The total content of GSH was significantly lower in FRD than in C rats (3.2 ± 0.26 vs. 4.2 ± 0.28 μmol/g tissue; $P<0.05$, respectively). Conversely, no significant changes were recorded between groups either in TBARS content or in membrane susceptibility to lipid peroxidation under both enzymatic and non-enzymatic assay methods.

Table 1
Liver metabolite and enzyme activity levels.

	C	FRD
Glycogen (μg/mg tissue)	6.5 ± 0.7	17.7 ± 3.3$^{\#}$
Glucokinase (mU/mg protein)	11.3 ± 0.3	18.3 ± 0.4*
G-6-Pase (% latency)	4.7 ± 0.6	9.8 ± 1.3$^{\#}$
G-6-P DH (mU/mg protein)	60 ± 10	150 ± 20$^{\#}$
Lactate (μmol/g tissue)	1.3 ± 0.3	2.7 ± 0.3*
Pyruvate (μmol/g tissue)	0.27 ± 0.04	0.30 ± 0.05

Values are means ± S.E.M. of 5 individual experiments run in triplicate for C and FRD animals. $^{\#}P<0.005$ and $^{*}P<0.05$.

Discussion

We have previously shown that normal Wistar rats fed with a FRD for 3 weeks had significant changes in glucose (IGT) and lipid metabolism (high serum free fatty acid and triglyceride levels), endocrine dysfunction (increased serum levels of insulin, leptin, adiponectin and plasminogen activator inhibitor-1) and insulin resistance (increased HOMA-IR index) (Alzamendi et al. 2009). In this study, FRD rats reproduced the changes in serum glucose and triglyceride levels, insulin resistance and IGT. These altered markers suggest a multiple organ function compromise, such as the adipose tissue (increased FFA levels) and the liver (high blood glucose and triglyceride levels). The high level of insulin together with IGT indicates that β-cells also have some degree of functional compromise, since they could not cope with the increased demand of insulin due to insulin resistance.

In the liver of FRD rats, we recorded a significant 24% and 18% decrease in total GSH content and CAT activity, respectively, and a significant 20% increase in protein carbonyl content, without changes in the activities of CuZnSOD and GSHPx. This single change in antioxidant enzyme activity was accompanied by a significant reduction in CAT, CuZnSOD and GSHPx gene expression, and in CAT and CuZnSOD (not GSHPx) protein expression. We can thus assume that, during the study period, a) FRD lowers the reducing power of the liver, b) changes in gene expression, protein level and enzyme activity do not always run in parallel, and c) in intact animals, enzyme activity does not only depend on its protein content, as shown for CuZnSOD in adrenal and liver tissue (Azhar et al. 1995). On account of the decrease in mRNA and protein expression of the enzymes, we could hypothesize that feeding the rats for longer than 3 weeks would decrease the enzyme's protein content below a threshold level, with the consequent decrease in the activity of all the enzymes tested.

Administration of a FRD generates a general pro-oxidant environment evinced by an increase of TBARS levels in serum (Busserolles et al. 2003; Faure et al. 1997) and adipose tissue (Alzamendi et al. 2009). Under our model conditions, however, the FRD did not affect the lipid peroxidation rate since there were no changes either in TBARS content or membrane susceptibility to lipid peroxidation. Other authors have reported a similar lack of effect of high fructose intake upon lipid peroxidation (Kelley et al. 2004), as well as a different impact of oxidative stress upon liver and other tissue functions (Busserolles et al. 2002a,b; Kelley et al. 2004; Leipnitz et al. 2009; Spolarics and Meyenhofer 2000).

The decrease in GSH and the higher protein carbonyl groups reported in our FRD animals evinced a moderate oxidative stress in the liver. We could therefore assume that the apparent small impact of oxidative stress upon this tissue could be due to its higher content of antioxidant enzymes (Robertson et al. 2004; Tiedge et al. 1998) or to its capacity to put forward additional defense mechanisms against excessive reactive oxygen species (ROS) production.

In this regard, it has been shown that the events associated to the respiratory chain in the mitochondria are the main responsible for the increased production and cell accumulation of ROS (Brownlee 2005). Thus, an overload of nutrients, such as that provided by the FRD, could lead to ROS overproduction/accumulation and to the consequent oxidative stress.

Our FRD rats showed multiple significant changes in liver carbohydrate metabolism: an increase in glycogen storage, in the pentose phosphate pathway (higher G-6-P DH activity) and in lactate production, without changes in the levels of pyruvate. We also recorded a simultaneous increase in the activity of both GK and G-6-Pase (increase

in the futile glucose cycling). The increased activity of G-6-P DH, the first and rate limiting enzyme of the pentose hexose monophosphate shunt, may provide an additional liver mechanism against oxidative stress (Spolarics and Meyenhofer 2000). The simultaneous increase in triglyceride content suggests that these rats have channelled the exceeding substrates towards lipid synthesis. All these changes might represent an adaptive mechanism to the carbohydrate load by which the liver switches its metabolism from the oxidative to the non-oxidative pathway, resulting in a lower fuel provision to the mitochondria with the consequent decrease in ROS production.

In brief, our data show that FRD administration to normal rats for 3 weeks a) increase triglyceride levels, insulin resistance and IGT, b) affects significantly liver CAT, CuZnSOD and GSHPx gene expression and CAT and CuZnSOD protein content, only decreasing significantly CAT activity, c) significantly increases protein carbonyl groups, reduces GSH, without producing changes in TBARS concentration and membrane susceptibility to lipid peroxidation, and d) induce significant changes in carbohydrate metabolism that switch metabolites away from mitochondrial metabolism.

Conclusion

These novel data show that in the presence of oxidative stress, the liver exhibits changes in carbohydrate and lipid metabolic pathways that would decrease ROS production and their deleterious effect, thus inducing little impact on specific antioxidant mechanisms. This knowledge could facilitate the design and implementation of strategies to prevent oxidative stress-induced liver damage.

Conflict of interest statement

There is no conflict of interest.

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