Apocynin administration prevents the changes induced by a fructose-rich diet on rat liver metabolism and the antioxidant system

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ABSTRACT

In the present study, we investigated the role of NADPH oxidase in F (fructose)-rich-diet-induced hepatic OS (oxidative stress) and metabolic changes, and their prevention by apocynin co-administration. Wistar rats were fed for 21 days on (i) a control diet, (ii) a control diet plus 10% F in the drinking water, (iii) a control diet with apocynin in the drinking water (CA) and (iv) F plus apocynin in the drinking water (FA). Glycaemia, triglyceridaemia, NEFAs (non-esterified fatty acids) and insulinaemia were determined. In the liver, we measured (i) NADPH oxidase activity, and gene and protein expression; (ii) protein carbonyl groups, GSH and TBARs (thiobarbituric acid-reactive substances); (iii) catalase, CuZn-SOD (superoxide dismutase) and Mn-SOD expression; (iv) liver glycogen and lipid content; (v) GK (glucokinase), G6Pase (glucose-6-phosphatase) and G6PDH (glucose-6-phosphate dehydrogenase) activities; (vi) FAS (fatty acid synthase), GPAT (glycerol-3-phosphate acyltransferase), G6Pase and G6PDH, IL-1β (interleukin-1β), PAI-1 (plasminogen-activator inhibitor-1) and TNFα (tumour necrosis factor α) gene expression; and (vii) IκBα (inhibitor of nuclear factor κB α) protein expression. F-fed animals had high serum TAG (triacylglycerol), NEFA and insulin levels, high liver NADPH oxidase activity/expression, increased OS markers, reduced antioxidant enzyme expression, and increased glycogen, TAG storage and GK, G6Pase and G6PDH activities. They also had high G6Pase, G6PDH, FAS, GPAT, TNFα and IL-1β gene expression and decreased IκBα expression. Co-administration of apocynin to F-fed rats prevented the development of most of these abnormalities. In conclusion, NADPH oxidase plays a key role in F-induced hepatic OS production and probably also in the mechanism of liver steatosis, suggesting its potential usefulness for the prevention/treatment of T2DM (Type 2 diabetes mellitus).

Key words: apocynin, fructose, hepatic metabolic disorder, insulin resistance, NADPH oxidase, pre-diabetes.

Abbreviations: C, control; CA, control + apocynin; DAB, diaminobenzidine; DNPH, dinitrophenylhydrazine; DTT, dithiothreitol; F, fructose; FA, fructose + apocynin; FAS, fatty acid synthase; G6Pase, glucose-6-phosphatase; G6PDH, glucose-6-phosphate dehydrogenase; GK, glucokinase; GPAT, glycerol-3-phosphate acyltransferase; HOMA, homoeostasis model assessment; IGT, impaired glucose tolerance; IκBα, inhibitor of nuclear factor κB α; IL-1β, interleukin-1β; IR, insulin resistance; MDA, malondialdehyde; NASH, non-alcoholic steatohepatitis; NEFA, non-esterified fatty acid; OS, oxidative stress; PAI-1, plasminogen-activator inhibitor 1; qPCR, quantitative PCR; ROS, reactive oxygen species; SOD, superoxide dismutase; T2DM, Type 2 diabetes mellitus; TAG, triacylglycerol; TBARs, thiobarbituric acid-reactive substance; TBS, Tris-buffered saline; TNFα, tumour necrosis factor α.

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INTRODUCTION

The prevalence of T2DM (Type 2 diabetes mellitus) and IGT (impaired glucose tolerance) is growing very fast worldwide, attaining epidemic characteristics [1]. It has been claimed that sedentary habits and the frequent consumption of unhealthy high-calorie diets in modern societies are major causes of such epidemics [2]. Since the increase in total energy consumption is usually accompanied by marked changes in meal nutrient composition [3], it has been suggested that the increased use of F (fructose)-rich syrups and refined carbohydrates has greatly contributed to the epidemics of obesity and T2DM [4–7].

The liver is the primary site of F extraction and metabolism; consequently, many efforts have been devoted to study the impairing effect of its increased availability upon hepatic glucose metabolism [8,9]. This impairment is accompanied by other liver abnormalities, such as increased fat deposits [10] and decreased insulin sensitivity and concentration of its intracellular signalling mediators [11]. We have shown further that normal rats fed on F for 3 weeks undergo significant changes in hepatic carbohydrate and lipid metabolism [12,13].

Experimental evidence has suggested that OS (oxidative stress) could be the underlying mechanism responsible for the detrimental effects of F [14–17]. OS results from an imbalance between ROS (reactive oxygen species) production and antioxidant capacity; F promotes such imbalance by the simultaneous enhancement of ROS production and the down-regulation of the main liver antioxidant enzymes [13]. OS also plays a major role in the pathogenesis of other pathological entities such as endothelial dysfunction, hypertension, increase of inflammatory markers and CVD (cardiovascular disease) [18].

NADPH oxidase is an important source of ROS production in several tissues, including the liver, which has active isoforms of the enzyme [19–24]; thus its inhibition represents an attractive and common treatment target for many diseases [25]. Accordingly, the aim of the present study was to investigate the role of NADPH oxidase in OS induced by F feeding to normal rats, and the possible preventive effect of apocynin co-administration, an NADPH oxidase inhibitor. The results obtained show that the enzyme is up-regulated by F overload, and that its inhibition prevented the changes induced by such a diet. These findings help to better understand the adaptive mechanisms involved in F-induced OS and in the development of 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 and mouse monoclonal anti-β-actin antibody were obtained from Sigma. The polyclonal anti-bovine liver catalase antibody raised in rabbit was purchased from Rockland. Anti-rat-SOD1 [CuZn (copper/zinc)-SOD (superoxide dismutase)] rabbit sera were provided by Professor Sigurd Lenz (Medizinische Hochschule Hannover, Hannover, Germany). Anti-rat-p22phox and anti-rat-SOD2 [Mn (manganese)-SOD] were purchased from Santa Cruz Biotechnology. Secondary antibodies horseradish-peroxidase-conjugated AffiniPure donkey anti-(rabbit IgG) and horseradish-peroxidase-conjugated anti-(mouse IgG) were purchased from Santa Cruz Biotechnology and Abcam respectively.

Animals

Normal male Wistar rats (150–180 g of body weight) were maintained in a temperature-controlled room (23 ℃) with a fixed 12 h light/12 h dark (06.00–18.00 h) cycle. Animals had free access to a standard commercial diet and tap water [C (control)], the same diet plus 10 % F in the drinking water (F), control diet supplemented with 5 mM apocynin in the drinking water (CA), and the F diet supplemented with 5 mM apocynin in the drinking water (FA respectively). Water intake was measured daily, whereas individual body weight and food intake were recorded once a week. The amount of apocynin ingested by each rat was calculated based on the apocynin concentration in the water and the daily water intake. A total of 21 animals were used in each condition.

After 21 days of treatment, blood samples were drawn from all animals from the retroorbital plexus under light halothane anaesthesia after a 4 h fasting period and collected into heparinized tubes to measure plasma glucose, TAG (triacylglycerol) and immunoreactive insulin levels. Afterwards, the animals were killed by decapitation and a portion of the liver was systematically removed to perform all of the assays.

Animal experiments and handling were performed according to the ‘Ethical principles and guidelines for experimental animals’ of the Swiss Academy of Medical Sciences [26].

Plasma measurements

Glucose was measured with the glucose-oxidase GOD-PAP method (Roche Diagnostics). TAG levels were assayed using a commercial immunoenzymatic assay kit (TG colour GPO/PAP AA; Wiener Lab) implemented in an automated clinical analyser; NEFAs (non-esterified fatty acids) were measured using a colorimetric assay with a commercial kit (Randox Laboratories); immunoreactive insulin levels were measured by RIA [27] using an antibody against rat insulin, a rat insulin standard (Linco Research) and highly purified porcine insulin labelled with 125I [28]. Serum insulin and fasting blood glucose values were used to estimate IR (insulin resistance) by HOMA (homoeostasis model assessment)–IR, using.
the equation: [serum insulin (µ-units/ml) × fasting blood glucose (mM)]/22.5 [29].

**NADPH oxidase activity**

Liver pieces were rinsed in Krebs buffer to avoid superoxide production from blood cells; thereafter, 100 mg of liver tissue were homogenized in 1 ml of ice-cold Krebs buffer. The homogenate was centrifuged at 3655 g for 10 min at 4 °C. For assay purposes, this crude homogenate was diluted 1:2 in the same buffer. The protein concentration in the sample was determined using the Bradford method [30]. Aliquots of the homogenates (100 µg of protein) were incubated for 5 min at 37 °C in Krebs–Hepes buffer [118.3 mmol/l NaCl, 4.7 mmol/l KCl, 1.8 mmol/l CaCl₂, 1.2 mmol/l MgSO₄, 1.0 mmol/l KH₂PO₄, 25 mmol/l NaHCO₃, 11 mmol/l glucose and 20 mmol/l Hepes, pH 7.4, at 37 °C]. Lucigenin-enhanced chemiluminescence was used to measure O₂ production with 5 µmol/l lucigenin. The chemiluminescence measured in arbitrary units was recorded with a luminometer (Chameleon; C). For assay purposes, negative controls were prepared adding 4 µl of apocynin to the mixture. NADPH oxidase activity was measured in the presence of 100 µmol/l NADPH and expressed as c.p.m./mg of liver protein.

**Protein carbonyl groups, GSH and TBARSs (thiobarbituric acid-reactive substances)**

Protein carbonyl content was assayed by the procedure described by Levine et al. [31]. Liver homogenates were centrifuged and 0.5 ml of the supernatant was mixed with 10 mM 2,4'-DNPH (dinitrophenylhydrazine) in 2 M HCl and incubated for 1 h at room temperature (23 °C); thereafter, the mixture was precipitated with 20 % trichloroacetic acid. The pelleted protein was washed three times by resuspension in ethanol/ethyl acetate (1:1, v/v). Proteins were then solubilized in 6 M guanidine hydrochloride and centrifuged at 16000 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. Results are expressed as nmol of carbonyl residues/mg of protein based on the molar absorption coefficient (ε) of 21 000 M⁻¹·cm⁻¹. GSH in liver was determined using the method of Ellman, as described in [32]. Briefly, liver homogenates were centrifuged at 1000 g and 1 ml of the supernatant were mixed with 1 ml of trichloroacetic acid (10%) and incubated at 4 °C for 1 h. The mixture was then centrifuged for 20 min at 3500 g and 100 µl of the supernatant was mixed with 900 µl of trichloroacetic acid (5%), 2 µl of 0.01 M Tris/HCl buffer (pH 8.9) and 50 µl of DTNB [5,5'-dithiobis-(2-nitrobenzoic acid); 0.4 % in methanol] and the GSH content was measured spectrophotometrically at 414 nm. Results are expressed as µmol of GSH/g of tissue.

TBARSs were measured in the liver by the method described by Pomppella et al. [33]. Liver homogenates were mixed with 10 % trichloroacetic acid and the samples were cooled on ice and then centrifuged at 3000 g for 10 min. The clear supernatant was mixed with 0.7 % thiobarbituric acid and heated at 100 °C for 15 min. Samples were cooled at room temperature, and the corresponding absorbance was measured at 535 nm against a blank (reaction mixture without liver homogenate but subjected to the same procedure). TBARS levels are expressed in nmol of MDA (malondialdehyde)/g of tissue using a molar absorption coefficient (ε) of 1.56 × 10⁵ M⁻¹·cm⁻¹.

**Isolation of total RNA**

Total liver RNA was isolated from C-, F-, CA- and FA-fed rats using TRIzol® Reagent (Gibco) [34]. The integrity of the 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). RT–PCR (reverse transcription–PCR) was performed using the SuperScript III (Gibco) and total RNA from C-, F-, CA- and FA-fed rat livers was used as a template.

**Gene expression by real-time PCR [qPCR (quantitative PCR)]**

qPCR was performed with a Mini Opticon Real-Time PCR Detector Separate MJR (Bio-Rad Laboratories), using SYBR Green I as a fluorescent dye which binds only double-stranded DNA. Then 10 ng of cDNA was amplified in 25 µl of the reaction mixture containing 0.6 µM of each primer, 3 mM MgCl₂, 0.2 mM dNTPs and 0.15 µl of platinum Taq DNA polymerase (6 units/µl; Invitrogen). Samples were first denatured at 95 °C for 3 min followed by 40 PCR cycles. Each cycle comprised a melting step at 95 °C for 30 s, an annealing step at 62 °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 PCRs were empirically defined. Each PCR amplification was performed in triplicate. The oligonucleotide primers (forward and reverse) (Invitrogen) used are listed in Table 1. All amplicons were designed in a size range of 90–288 bp. β-Actin was used as a 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% agarose gel electrophoresis and ethidium bromide staining. Results are expressed as relative gene expression after normalization to the \( \beta\)-actin housekeeping gene using the Qgene96 and LineRegPCR software [35].

**Western blot analysis**

Immunodetection of SOD1, SOD2, catalase, \( p22^{phox} \) and \( \text{I} \kappa \text{B} \) [inhibitor of NF-\( \kappa \text{B} \) (nuclear factor \( \kappa \text{B} \))] was performed in liver homogenates. The protein concentration was quantified by the Bio-Rad Laboratories protein assay [30]. Thereafter DTT (dithiothreitol) and Bromophenol Blue were added to a final concentration of 100 mM and 0.1% respectively. Aliquots of homogenates containing 100 \( \mu \)g of whole protein were separated under reducing conditions by SDS/PAGE (12% gel) and electroblotted on to PVDF membranes to quantify SOD1, SOD2 and \( p22^{phox} \), and 50 \( \mu \)g of whole protein were separated under reducing conditions by SDS/PAGE (10% gel) for catalase and \( \text{I} \kappa \text{B} \) quantification.

The amount of protein loaded on to the gel was assessed using the Bradford method [30], and the uniformity of protein loading in each lane by staining the blot with Ponceau S. Non-specific binding sites of the membranes were blocked by previous overnight incubation with non-fat dried skimmed milk powder at 4°C. Enzyme identification and quantification were performed using specific primary antibodies against catalase (final dilution of 1:5000), SOD1 (final dilution of 1:3000), SOD2 (final dilution of 1:1000), \( p22^{phox} \) (final dilution of 1:200), \( \text{I} \kappa \text{B} \) (final dilution of 1:1000) and \( \beta\)-actin (final dilution of 1:10000). After a 2 h incubation for catalase, a 3 h incubation for SOD1, a 1.5 h incubation for SOD2, a 2.5 h incubation for \( p22^{phox} \), an overnight incubation for \( \text{I} \kappa \text{B} \) and a 1 h incubation for \( \beta\)-actin, the membranes were rinsed in TBS (Tris-buffered saline) and further incubated for 1 h with the secondary horseradish-peroxidase-conjugated AffiniPure donkey anti-(rabbit IgG) antibody (final dilution of 1:10000). In the case of \( \beta\)-actin, the secondary horseradish-peroxidase-conjugated anti-(mouse IgG) antibody (final dilution of 1:2000) was used. The specific \( \text{I} \kappa \text{B} \) protein band was visualized by chemiluminescence using the enhanced chemiluminescence detection system (ECL® Western blotting detection reagents; GE Healthcare). For the other proteins, DAB (diaminobenzidine; Sigma) was used for colour development. Briefly, the membranes were placed in 20 ml of TBS plus 10 mg of DAB, 10 \( \mu \)l of NiCl\(_2\) (10%) and 15 \( \mu \)l of H\(_2\)O\(_2\) (30%). After colour development, the reaction was stopped and the membranes were rinsed in distilled water. Finally, the bands were quantified by densitometry using the Gel-Pro Analyser software. \( \beta\)-Actin density was used to normalize the target proteins.

**Liver glycogen content**

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 ethanol was added to each tube and the mixture was incubated for 48 h at 4°C and finally centrifuged at 700 g for 20 min. The pellets obtained were resuspended in 1 ml of distilled water plus 3 ml of Antrone solution (0.1% in 84% H\(_2\)SO\(_4\)) and incubated for 20 min at 100°C. The absorbance was measured photometrically at 620 nm and the results expressed as \( \mu \)mol of glycogen/mg of tissue [36].

**Liver GK (glucokinase) activity**

Livers were removed and immediately homogenized in a hand-held homogenizer (20 times) suspended in ice-cold PBS containing 0.1 mM PMSF, 0.1 mM benzamidine, 2 mM DTT, 4 \( \mu \)g/ml aprotinin and 0.3 M

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**Table 1** Primer sequences

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<th>GenBank accession no.</th>
<th>Sequences</th>
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<td>gp91(^{phox})</td>
<td>NM_023965.1</td>
<td>FW, 5′-CCAGTGCTGGCTGAAACTCTCCT-3′; RV, 5′-ACACCACTCCACGTGTAACA-3′</td>
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<td>β-Actin</td>
<td>NM_031144.2</td>
<td>FW, 5′-AGAGGGAATGCGTGTCAGG-3′; RV, 5′-CGATAGTGATGACCTGACCT-3′</td>
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sucrose, pH 7.5. The homogenate was then passed through a 23-gauge needle syringe (five 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 and 100 000 g at 4 °C, and the resultant supernatant was collected and identified as the cytosolic fraction. Rates of glucose phosphorylation in the 100 000 g soluble cytosolic fraction 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 G6PDH (glucose-6-phosphate dehydrogenase), ATP and NADP [12,37]. 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 is expressed as m-units/mg of protein. One unit of enzyme activity was defined as 1 μmol of glucose 6-phosphate formed from glucose and ATP/min at 37 °C. For each assay, five different experiments were performed in triplicate.

**Liver G6Pase (glucose-6-phosphatase) activity**

Homogenization of liver samples and isolation of microsomes were carried out as described by Nordlie and Arion [38]. Homogenization medium was 0.25 M sucrose/5 mM Tris-acetate/0.5 mM EDTA, pH 7.4 (3 ml/g of 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 % Triton X-100 to 0.9 ml of untreated microsomes (approximately 10 mg of protein) and allowed to stand on ice for 20 min. Then 50 μl of the mixture and 50 μl of G6Pase sodium salt (0.8 M) were added and samples were incubated for 10 min at 30 °C. The reaction was stopped by adding 250 μl of 10 % trichloroacetic acid; then, 2 ml of MoNH4 (diluted in 1 M H2SO4) plus 320 μl of FeSO4 (diluted in 0.15 M H2SO4) were added to 200 μl of each sample. The absorbance 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 [16].

**Liver G6PDH activity**

Pieces of liver were homogenized in 0.1 M Tris/HCl and 1 mM EDTA, pH 7.6 (10 ml/g of 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. G6PDH activity was measured according to the method as described by Beutler [39].

**Liver lipid and TAG content**

Lipid accumulation in the liver of animals from each experimental group was measured (histochemistry) with Sudan Red staining. For that purpose, samples were fixed with 5 % formalin solution and frozen tissue was cut with a freezing microtome. Frozen sections (>5 μm) were stained for 5 min at room temperature with Sudan Red, prepared as a saturated solution of the dye in an ethanol (70 %)/acetone mixture (1:1). Thereafter, the sections were washed for 5 min in ethanol (50 %) in order to remove excess dye. The morphological analysis was performed using a Nikon Eclipse 80i light microscope and photography was carried out with a Nikon digital Sight DS-U1 camera.

Liver TAGs were extracted following the protocol described by Schwartz and Wollins [40], and the enzymatic assay of TAG levels was performed with a commercial kit, as described for serum measurements.

**Statistical analysis**

Results are expressed as means ± S.E.M. for the indicated number of observations or illustrated by an observation representative of results obtained from several different experiments. Statistical analysis was performed using ANOVA followed by Dunnet’s test for multiple comparisons using the Prism analysis program (GraphPad). Differences were considered significant when P < 0.05.

**RESULTS**

**Body weight and water intake**

Comparable body weights were recorded in all of the experimental groups after the 3-week treatment period (Table 2). F- and FA-fed animals drank a larger volume of water than C- and CA-fed animals. Conversely, the amount of solid food intake was significantly larger in C- and CA-fed animals compared with F- and FA-fed animals (Table 2). Consequently, the groups had a different percentage of daily nutrients, but a comparable calorie intake (Table 2).

The amount of apocynin administered per rat/day was 25.4 ± 1.4 and 33.1 ± 3.4 mg for CA- and FA-fed animals respectively (P < 0.05).

**Plasma measurements**

Although all groups had comparable serum glucose levels (Table 3), F-fed animals had significantly higher serum
Table 2  Body weight and water intake  
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Diet</th>
<th>C</th>
<th>CA</th>
<th>F</th>
<th>FA</th>
<th>ANOVA</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td></td>
<td>294.2 ± 8.3</td>
<td>269.5 ± 4.9</td>
<td>284.3 ± 5.7</td>
<td>285.2 ± 5.9</td>
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<tr>
<td>Water consumption (ml/day)</td>
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<td>27.8 ± 2.7</td>
<td>25.4 ± 1.4</td>
<td>38.6 ± 8.2a</td>
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<td>Food intake (g/day)</td>
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<td>20.9 ± 1.1</td>
<td>19.4 ± 0.5</td>
<td>15.9 ± 0.9a</td>
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<td>Calorie intake (kcal/day)</td>
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<td>55.9 ± 1.5</td>
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<td>61.9 ± 3.7</td>
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Table 3  Serum measurements  
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<th>FA</th>
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<td>Insulin (ng/ml)</td>
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<td>0.90 ± 0.11</td>
<td>0.86 ± 0.13</td>
<td>1.70 ± 0.16a</td>
<td>1.14 ± 0.2b</td>
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<td>Glucose (mg/dl)</td>
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<td>112 ± 3</td>
<td>106 ± 1</td>
<td>113 ± 3</td>
<td>110 ± 3</td>
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<td>TAG (mg/dl)</td>
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<td>114 ± 5</td>
<td>89 ± 7</td>
<td>193 ± 9a</td>
<td>101 ± 9b</td>
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<td>NEFA (mM)</td>
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<td>0.59 ± 0.04</td>
<td>0.58 ± 0.08</td>
<td>0.77 ± 0.05a</td>
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<td>HOMA-IR</td>
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<td>12.8 ± 1.2a</td>
<td>7.6 ± 1.1b</td>
<td>P &lt; 0.05</td>
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</table>

Figure 1  Assessment of protein carbonyl groups (A) and GSH (B)  
C- (white bars), CA- (white dotted bars), F- (grey bars) and FA- (grey dotted bars) fed rats. Results are means ± S.E.M. (n = 15). ANOVA, P < 0.05. aP < 0.05 compared with C-fed animals, and bP < 0.05 compared with F-fed animals.

TAG, NEFA and insulin levels than C rats (Table 3). In FA-fed rats, apocynin co-administration induced a significant reduction in TAG, NEFA and insulin levels (Table 3).

The higher HOMA–IR values measured in F-fed rats (Table 3) demonstrate the existence of an IR state in these rats, which was controlled in FA-fed animals.

Assessment of protein carbonyl groups, GSH and TBARS  
Although the protein carbonyl content was significantly higher and total GSH content was significantly lower in F-fed rats as compared with C-fed rats (Figure 1), these changes were no longer observed in FA-fed animals.

Conversely, no significant changes were recorded among groups in liver TBARS content (C, 6.0 ± 0.8 × 10−3; F, 5.5 ± 0.3 × 10−3; CA, 5.9 ± 0.5 × 10−3; FA, 5.6 ± 0.4 × 10−3 nmol of MDA/g of tissue).

NADPH oxidase activity  
Liver NADPH oxidase activity measured in the presence of 100 μmol/l NADPH was significantly higher in F-fed rats compared with C-fed rats (Figure 2). Apocynin co-administration to F-fed rats reduced the enzyme’s activity to values comparable with those recorded in C-fed rats (Figure 2).
NADPH oxidase subunits gene (qPCR) and protein (Western blot) expression
We recorded significantly higher relative gene expression levels of gp91<sub>phox</sub> and p22<sub>phox</sub> in F-fed animals than in C-fed animals (Figures 3A and 3B). Treatment with apocynin decreased these high values to numbers comparable with those recorded in C-fed rats (Figures 3A and 3B). In all cases, values were expressed as a function of the housekeeping gene β-actin.

Using specific anti-p22<sub>phox</sub> antibodies a significant increase in the p22<sub>phox</sub> protein level (Western blot) was recorded in F-fed animals (a single band of approximately 22 kDa, corresponding to the molecular mass of the corresponding enzymes). Once again, this increase was prevented by apocynin co-administration (FA) (Figure 3C). The values were normalized using β-actin as a housekeeping protein.

Antioxidant enzyme gene expression (qPCR)
Relative gene expression values of catalase, SOD1 and SOD2 were significantly lower in F-fed animals than in C-fed rats (Figures 4A, 4D and 4G). Catalase and SOD1 expression levels remained within the range of values recorded in C-fed rats when apocynin was co-administered with F (Figures 4D and 4G). In all cases, values were expressed as a function of the housekeeping gene β-actin.

Antioxidant enzyme protein analysis
Western blotting performed in samples of animals from all groups using specific catalase, SOD1 and SOD2 antibodies showed a single band of approximately 60, 30 and 25 kDa respectively, comparable with the molecular mass of the corresponding enzyme. 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 (results not shown).

In liver homogenates from F-fed rats, catalase, SOD1 and SOD2 protein expression levels were significantly lower than in C-fed animals (Figures 4B, 4C, 4E, 4F, 4H and 4I). In FA-fed rats, values were comparable with those measured in C-fed animals (Figures 4C and 4D). In all cases, values were normalized using β-actin as a housekeeping protein.
Table 4  Enzymes and metabolites of carbohydrate metabolism

Values are means ± S.E.M. (n = 15). *P < 0.05 compared with C-fed animals, and †P < 0.05 compared with F-fed animals.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Diet</th>
<th>C</th>
<th>CA</th>
<th>F</th>
<th>FA</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>GK (m-units/mg of protein)</td>
<td></td>
<td>2.1 ± 0.3</td>
<td>1.6 ± 0.15</td>
<td>5.2 ± 0.4*</td>
<td>5.6 ± 0.3</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>G6Pase (% latency)</td>
<td></td>
<td>4 ± 0.7</td>
<td>10 ± 0.4†</td>
<td>12 ± 1.6</td>
<td>12 ± 1.7</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>G6PDH (m-units/mg of protein)</td>
<td></td>
<td>0.05 ± 0.002</td>
<td>0.05 ± 0.005</td>
<td>0.08 ± 0.007†</td>
<td>0.13 ± 0.008b</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Glycogen (μg/mg of tissue)</td>
<td></td>
<td>3.1 ± 0.7</td>
<td>6.1 ± 0.2†</td>
<td>6.2 ± 0.5†</td>
<td>6.8 ± 0.9</td>
<td>P &lt; 0.05</td>
</tr>
</tbody>
</table>

Carbohydrate metabolic pathways

Supporting our previous reports [13,41], F induced a simultaneous and significant increase in the activity of GK, G6Pase (increase in the futile glucose cycling) and G6PDH, together with an increase in glycogen storage (Table 4). These changes were not prevented by apocynin co-administration (FA-fed rats) (Table 4).

Gene expression (qPCR) of enzymes involved in carbohydrate and lipid metabolism

Relative FAS (fatty acid synthase), GPAT (glycerol-3-phosphate acyltransferase), G6Pase and G6PDH gene expression were significantly higher in F-fed animals than in C-fed rats (Figure 5). FAS and GPAT expression levels returned to control values when apocynin was co-administered with F. The changes in G6Pase and G6PDH gene expression, however, were not fully prevented by apocynin.

Liver lipid and TAG content

The histological analysis showed a significant increase of ectopic fat deposition in the liver of F-fed animals compared with C-fed rats (Figures 6A and 6B). This accumulation was prevented by apocynin treatment (Figure 6C). F-fed rats also had higher liver TAG content than C-fed animals (503 ± 13 compared with 383 ± 21 μg/100 mg of tissue; P < 0.001). FA-fed animals had significantly lower TAG content than F-fed rats (393 ± 20 compared with 503 ± 13 μg/100 mg of tissue; P < 0.001). Conversely, apocynin administration did not modify liver TAG content in C-fed animals (420 ± 16 μg/100 mg of tissue).

Expression of inflammatory markers

The relative gene expression of both TNFα (tumour necrosis factor α) and IL-1β (interleukin-1β) was significantly increased in F-fed animals (Figure 7), but apocynin co-administration only prevented the increased expression of the former. Even when PAI-1 (plasminogen-activator inhibitor-1) expression also increased in F-fed animals, this change did not attain statistical significance. Complementarily, the relative protein expression of IκBα decreased significantly in F-fed rats (98.4 ± 2.2) compared with C-fed animals (126.4 ± 7.1; P < 0.05). As this compound is a physiological blocker of NF-κB, the results suggest that its activity could be increased in these animals. Apocynin treatment to FA-fed rats did not restore these values (102.2 ± 9.3) to those measured in C-fed animals.

DISCUSSION

We have shown previously that normal Wistar rats fed with F for 3 weeks developed multiple metabolic and endocrine disorders: IR (increased HOMA–IR index), IGT, impaired lipid metabolism (high serum NEFA and TAG levels), haemorheologic alterations and increased serum levels of insulin, leptin, adiponectin and PAI-1 [13,41]. We have also reported that in the liver of these animals, a significant increase of several OS markers was associated with changes in carbohydrate metabolism that would switch liver metabolites away from mitochondrial oxidation, i.e. increased substrate cycle GK/G6Pase and pentose phosphate pathway as well as glycogen storage [12,13].

The significant decrease in GSH content and the increase of carbonyl groups currently observed, together with the decreased gene and protein expression of catalase, SOD1 and SOD2, demonstrate an increased OS,
thus reproducing our previous reports. Taken together, the findings reinforce the concept that the F-induced OS plays an active role in the mechanism by which this diet impairs many liver functions.

In the liver of our F-fed rats, we measured a combined and significant increase in NADPH oxidase activity, in the gene expression of two of its subunits (gp91phox and p22phox), and in the protein expression of p22phox. Comparable dietary-induced disturbances of NADPH oxidase have been reported in other tissues: using a similar F load but administered for a longer period (16 weeks), Liu et al. [42] reported a significant increase in brain NADPH oxidase (p47phox) gene expression. Similarly, consumption of a high-fat/high-sucrose diet resulted in an up-regulation of NADPH oxidase and a down-regulation of several key antioxidant enzymes in the kidney and vascular tissues [18]; consequently, the authors suggested that in these animals the up-regulation of NADPH oxidase could be the link between increased ROS generation, OS and endothelial dysfunction [18]. The administration of this diet for 12 weeks induced similar changes in striated and heart muscle but not in the liver [43]. The fact that (i) animals fed with a cholesterol-rich diet presented an increased protein expression of gp91phox and of biochemical markers of hepatic oxidative burden [44], and (ii) there is an increased enzyme activity in adipose tissue of mice fed with a fat-rich diet that leads to ROS production [45] lend further support to the assumption that NADPH oxidase plays an active role in the development of OS induced by dietary manipulation.

We have demonstrated in the present study that co-administration of apocynin, a specific NADPH oxidase inhibitor, prevents F-induced changes in the activity as well as in the gene and protein expression of the enzyme. Apocynin also prevented the changes induced by F upon liver OS markers, and it would therefore decrease the consecutive hepatic oxidative burden. These results are in agreement with those described by Lu et al. [44], who found that apocynin alleviated oxidative liver injury in rats with hypercholesterolaemia. It has also been demonstrated that treatment with this NADPH oxidase inhibitor effectively ameliorates the development of the metabolic syndrome associated with obesity [45]. The improvement in F-induced IR by apocynin co-administration currently reported provides additional support to the active participation of NADPH oxidase in this process.

Inflammatory markers were also increased in F-fed rats, but apocynin co-administration only prevented the increase of TNFα gene expression. Since it has been demonstrated that TNFα plays a casual role in the onset of F-induced liver damage and IR [46] comprehension of the link between NADPH oxidase and inflammation in the F model merits further research.

The mechanism by which apocynin affects NADPH oxidase activity is not totally known; the NADPH oxidase family of proteins consists of several isoforms that catalyse the one-electron reduction of oxygen using NADH or NADPH as the electron donor, thereby generating superoxide [22]. The NADPH oxidase structure comprises two membrane-bounded elements (gp91phox and p22phox), three cytosolic components (p67phox, p47phox and p40phox) and a low-molecular-mass G-protein (rac1 or 2). Its activation probably occurs when the cytosolic components of the enzyme migrate to the cell membrane and interact with the catalytic transmembrane proteins so that the complete oxidase can be assembled [25]. Therefore the effect of apocynin could be ascribed, at least partially, to

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**Figure 6** Liver lipid accumulation

Photomicrographs of liver sections from C- (A), F- (B) and FA- (C) fed rats. The histological features show mixed microvesicular and macrovesicular fat deposits (white arrows), with zonal distribution (zone two). Sudan Red/200. Scale bar represents 100 μm.

**Figure 7** Inflammation-related gene expression in liver

Relative PAI-1, TNFα and IL-1β gene expressions were measured in C- (white bar), CA- (white dotted bar), F- (grey bar) and FA- (grey dotted bar) fed animals. Results are means ± S.E.M. (n = 21). In all cases, β-actin was used as a housekeeping gene. ANOVA, *P* < 0.05 for TNFα and IL-1β. *aP* < 0.05 compared with C-fed animals, and *bP* < 0.05 compared with F-fed animals.
the inhibition of the different subunit assembly at the membrane level [47–49]. In our case, apocynin prevented not only the increase in NADPH oxidase activity, but also the enhancement of its gene and protein expression. Consequently, and as suggested by others [44,45], the induction of NADPH oxidase gene and protein expression observed in our F-fed animals could be associated with the pro-oxidant environment that will further increase ROS production. Apocynin treatment would interrupt this vicious cycle, driving the enhanced enzyme expressions to values comparable with those recorded in control animals.

The potential interaction among NADPH oxidase activity, liver carbohydrates and lipid metabolism has not so far been studied [24]. We have demonstrated previously that F-fed rats, together with an increased OS, showed multiple significant changes in liver carbohydrate metabolism, such as an increase in (i) the glycogen storage, (ii) the pentose phosphate pathway, (iii) the activity of both GK and G6Pase (increase in the futile glucose cycling) and (iv) the fat storage and the gene expression of enzymes involved in fat synthesis [13]. In that report, we postulated that 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 pathways, resulting in a lower fuel provision to the mitochondria with the consequent decrease in ROS production. In the present study, apocynin treatment did not affect significantly the changes induced by F on carbohydrate metabolism; its administration to C-fed rats increased the glycogen deposit and G6Pase activity. This uneven effect of apocynin upon liver OS and glucose metabolism rather than contradicting such assumption might only indicate that prevention of the impaired glucose metabolism induced by F takes longer than that induced in OS. The fact that apocynin treatment induced a non-significant reduction in the gene expression of G6Pase and G6PDH could favour this assumption. However, longer treatment periods should be tested to definitely address this point.

On the other hand, it has been postulated that in the absence of an enhanced NADPH oxidase activity (such as in our C-fed rats), apocynin could have a deleterious oxidative effect [50]. Although we have not observed any effect upon OS markers or antioxidant enzymes in CA rats, the changes observed in many carbohydrate metabolic markers are similar to those observed under OS conditions (F-fed animals).

Since NADPH is a cofactor for recycling GSSG to GSH, the observed GSH reduction in F-fed animals could be related to the oxidation in NADPH due to the enhanced activity of NADPH oxidase, as reported in hypercholesterolaemic rats [44]. However, the activity of G6PDH, the rate-limiting enzyme of the pentose phosphate pathway which generates NADPH, is increased in F-fed animals. In the present study, we have detected an even higher activity of the enzyme in FA-fed rats; whether this is an additional protective or compensatory mechanism against GSH reduction due to NADPH content reduction should be further investigated.

In contrast with what happens with glucose metabolism, we demonstrated that apocynin prevented the F-induced increase in TAG and NEFA serum levels and liver lipid content together with a reduction to basal levels in the gene expression of enzymes involved in lipid synthesis (FAS and GPAT). These results would support the concept that ROS production may be a causal factor for the development of NASH (non-alcoholic steatohepatitis) [24]. This fact might be particularly important in the context of the ‘two hits’ theory for NASH pathogenesis [51]. In our F-rich diet model, the increased NADPH oxidase activity with the consequent increase in ROS generation and liver steatosis could represent the first hit. In this context, apocynin administration could be a promising therapeutic tool to alleviate/prevent non-alcoholic fatty liver disease consequent to high-F/sucrose diet intake. Further studies are necessary to prove this assumption.

In conclusion, the results of the present study suggest that NADPH oxidase plays a key role in F-induced hepatic ROS production and probably in the other associated metabolic and endocrine dysfunctions as well. The changes observed in plasma and liver TAG content also suggest that the enzyme could be involved in pathways leading to liver steatosis. The prevention of these alterations by apocynin co-administration suggests that this compound might become a useful tool in the treatment of the metabolic syndrome and T2DM.

**Clinical Perspectives**

- The growing prevalence of T2DM and IGT is attaining epidemic proportions and some evidence suggests that the increased consumption of F-rich syrups has contributed greatly to such growth. Experimental data support the assumption that oxidative stress could be the underlying mechanisms responsible for the detrimental effects of F. As NADPH oxidase is an important source of ROS production, its inhibition would be an attractive treatment target for many diseases.

- In the present paper, we show that NADPH oxidase plays a key role in F-induced hepatic ROS production and probably in the other associated metabolic and endocrine dysfunctions, and its inhibition prevented the changes induced by such a diet.

- Thus our results suggest that inhibiting NADPH oxidase may be potentially useful in the prevention/treatment of the metabolic syndrome and T2DM triggered by the consumption of unhealthy diets.
AUTHOR CONTRIBUTION

María Massa, Flavio Francini and Juan Gagliardino conceived and designed the study, performed the statistical analysis, and drafted the paper. María Castro and María Massa carried out the Western blot analysis and metabolic determinations (liver TAG and glycogen contents, and GK, G6Pase and G6PDH activities). Guillermo Schinella and Flavio Francini determined the protein carbonyl groups, GSH and TBARS. Claudia Caldz and Flavio Francini measured NADPH oxidase activity. María Castro and Flavio Francini performed total RNA isolation and gene expression measurements, handled the experimental animals and carried out the biochemical determinations. Marí­a Zubiría measured NEFA content. All authors read and approved the final paper.

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REFERENCES

16 Lange, A. J., Arion, W. J. and Beaudet, A. L. (1980) Type Ib glycogen storage disease is caused by a defect in the glucose-6-phosphate translocase of the microsomal glucose-6-phosphatase system. J. Biol. Chem. 255, 8381–8384
30 Swiss Academy of Medical Sciences (2005) Ethical Principles and Guidelines for Experimental Animals, 3rd edn, Swiss Academy of Medical Sciences, Basel

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