Research Paper

Fructose supplementation worsens the deleterious effects of short-term high-fat feeding on hepatic steatosis and lipid metabolism in adult rats

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New Findings

- **What is the central question of this study?**
  In humans, 'Western-style' diet is characterized by high levels of both saturated fats and fructose. Lipid oversupply to the liver typical of high-fat diets could be exacerbated by the coexistence of high levels of fat and fructose in the diet, thus accelerating the development of metabolic deregulation.
- **What is the main finding and its importance?**
  Short-term consumption of a Western diet, rich in saturated fats and fructose, is more conducive to the development of liver steatosis and deleterious to glucose homeostasis than a high-fat diet. This result points to the harmful effect of adding fructose to the usual Western, high-fat diet.

The purpose of the present study was to examine the short-term effect of high-fat or high-fat–high-fructose feeding on hepatic lipid metabolism and mitochondrial function in adult sedentary rats. Adult male rats were fed a high-fat or high-fat–high-fructose diet for 2 weeks. Body and liver composition, hepatic steatosis, plasma lipid profile and hepatic insulin sensitivity, together with whole-body and hepatic *de novo* lipogenesis, were assessed. Hepatic mitochondrial mass, functionality, oxidative stress and antioxidant defense were also measured. Rats fed the high-fat–high-fructose diet exhibited significantly higher plasma triglycerides, non-esterified fatty acids, insulin and indexes of hepatic insulin resistance compared with rats fed a low-fat or a high-fat diet. Hepatic triglycerides and ceramide, as well as the degree of steatosis and necrosis, were significantly higher, while liver p-Akt was significantly lower, in rats fed high-fat–high-fructose diet than in rats fed high-fat diet. A significant increase in non-protein respiratory quotient and hepatic fatty acid synthase and stearoyl CoA desaturase activity was found in rats fed the high-fat–high-fructose diet compared with those fed the high-fat diet. Significantly lower mitochondrial oxidative capacity but significantly higher oxidative stress was found in rats fed high-fat and high-fat–high-fructose diets compared with rats fed low-fat diet, while mitochondrial mass significantly increased only in rats fed high-fat–high-fructose diet. In conclusion, short-term consumption of a Western diet, rich in saturated fats and fructose, is more conducive to the development of liver steatosis and deleterious to glucose homeostasis than a high-fat diet.

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Experimental Physiology

Introduction

Chronic consumption of high-fat diets contributes to the development of obesity and type 2 diabetes (Lanni *et al.* 2005; Rutledge & Adeli, 2007; Qi *et al.* 2009). In particular, we have previously shown that long-term feeding with diets rich in fat causes the onset of obesity, insulin resistance, liver lipid deposition and mitochondrial derangement in a rat model of adult, sedentary humans (Crescenzo *et al.* 2008). Interestingly, a similar pattern of metabolic deregulation is evident after long-term feeding with a low-fat diet rich in fructose, a popular sugar used industrially to sweeten foods and beverages (Crescenzo *et al.* 2013*b*). Behind these similarities, however, high-fat and high-fructose diets affect whole-body lipid metabolism in different ways. In fact, during high-fat feeding an increased lipid supply to peripheral organs, and particularly to the liver, arises mainly from dietary lipids, while during high-fructose feeding an increased hepatic *de novo* lipogenesis is the main source of circulating lipids (Ren *et al.* 2012).

In humans, consumption of a 'Western-style diet', characterized by high levels of both saturated fats and fructose, could favour the development of metabolic diseases. In fact, it is possible that the deleterious effects of fat or fructose found by us after long-term dietary treatment (Crescenzo *et al.* 2008; Crescenzo *et al.* 2013*b*) could be additive when high-fat–high-fructose diets are consumed. In these condition, lipid oversupply to the liver could be due to both dietary fats and hepatic *de novo* lipogenesis and could, therefore, accelerate the development of metabolic deregulation. In addition, the putative lipid overflow to hepatic cells could influence mitochondrial function.

Taking into account the above considerations, we considered it to be of interest to evaluate hepatic lipid metabolism and mitochondrial dysfunction in rats fed a high-fat or high-fat–high-fructose diet, the latter mimicking a typical unhealthy Western diet. Long-term dietary treatment is not adequate to address this issue, because high fat or high fructose alone each induces metabolic derangement. Assessments were therefore carried out after 2 weeks of dietary treatment, when normal glucose tolerance is maintained in high-fat-fed rats (Iossa *et al.* 2003), so that the possible worsening effect of adding fructose to the high-fat diet could be detected. The results obtained provide evidence of the worsening effect of fructose during short-term high-fat feeding on hepatic insulin sensitivity and lipid metabolism.

Methods

Ethical approval

Treatment, housing and killing of rats met the guidelines set by the Italian Health Ministry. All experimental procedures involving rats were approved by 'Comitato Etico-Scientifico per la Sperimentazione Animale' of the University 'Federico II' of Naples.

Animals

Male Sprague–Dawley rats (Charles River, Lecco, Italy) 90 days of age were caged in a temperature-controlled room (23 ± 1°C) with a 12 h–12 h light–dark cycle (06.30–18.30 h light on) and divided into three groups (each composed of six rats), which were fed the low-fat (LF), high-fat (HF), or high-fat–high-fructose (HF-F) diet for 2 weeks. All the rats were fed with the same amount of calculated metabolizable energy (ME; 250 kJ), corresponding to the amount of energy consumed by the same rats immediately before the start of the dietary regimen. The composition of the three diets is shown in Table 1.

During the whole experimental period, faeces and urine were collected daily and the respective energy contents assessed with a bomb calorimeter. Daily food consumption was monitored, gross energy density of the diet was measured using the bomb calorimeter, and gross energy intake was calculated. The ME intake was determined by subtracting the energy measured in faeces and urine from the gross energy intake. At the end of the experimental period, the animals were anaesthesized with zolazepam plus tiletamine (1:1) (30 mg kg^{-1} I.P.) and killed by decapitation, the liver harvested, and the carcasses used for determination of body composition.

Non-protein respiratory quotient (NPRQ)

The 24 h oxygen uptake, carbon dioxide output and respiratory quotient of the rats were recorded with an indirect open-circuit calorimeter (Panlab s.r.l., Cornella, Barcelona, Spain). Urine was collected for the whole period (24 h), and urinary nitrogen levels were measured by an enzymatic colorimetric method (FAR S.r.l., Settimo di Pescantina, Verona, Italy). The 24 h NPRQ was then calculated.

Glucose tolerance test, plasma lipid profile and hepatic transaminase

Rats were feed deprived for 6 h from 08.00 h. Basal postabsorptive samples obtained from venous blood from a small tail clip were collected in EDTA-coated tubes and then glucose (2 g kg^{-1}) was injected intraperitoneally. Given that the magnitude of the rise in plasma glucose and insulin concentrations immediately (0–30 min) following the glucose load is proportional to the magnitude of hepatic insulin resistance (Abdul-Ghani *et al.* 2007), and the rise in plasma glucose and insulin concentrations can

be quantified by the incremental area under the curve (AUC) for plasma glucose and insulin, the product of glucose AUC and insulin AUC during the first 30 min after glucose load was calculated and used as an index of hepatic insulin resistance.

After centrifugation at 1400g_a for 8 min at 4°C, plasma was removed and stored at −20°C until used for determination of substrates and hormones. Plasma glucose concentration was measured by a colorimetric enzymatic method (Pokler Italia, Pontecagnano, Italy). Plasma insulin concentration was measured using an ELISA kit (Mercodia AB, Uppsala, Sweden) in a single assay to avoid interassay variations. Basal postabsorptive values of plasma glucose and insulin were used to calculate the Homeostatic Model Assessment (HOMA) index as [glucose (in milligrams per decilitre)] × [insulin (in milliunits per litre)]/405 (Cacho *et al.* 2008).

Commercial kits adopting colorimetric enzymatic methods were used to measure the plasma concentrations of total cholesterol, triglycerides, alanine aminotransferase (ALT; SGM Italia, Rome, Italy) and non-esterified fatty acids (NEFAs; Randox Laboratories Ltd, Crumlin, Co. Antrim, UK).

Body and liver composition

Body energy, lipid and protein content were measured as previously described (Crescenzo *et al.* 2012). Briefly, the alimentary tract was cleaned of undigested food and the carcasses were then autoclaved. After dilution in distilled water and subsequent homogenization of the carcasses, duplicate samples of the homogenized carcass were analysed for energy content by bomb calorimeter. Total body lipids, as well as hepatic lipids, were measured by the Folch extraction method (Folch *et al.* 1957). The energy as lipid, calculated from body lipids by using the coefficient of 39.2 kJ g^{-1}, was then subtracted from total body energy to obtain the energy as protein. Liver triglycerides and cholesterol were measured by a colorimetric enzymatic method using commercial kits (SGM Italia), and glycogen was assessed by a direct enzymatic procedure (Roehrig & Allred, 1974). Liver ceramide content was evaluated by enzyme-linked immunosorbent assay (ELISA; Crescenzo *et al.* 2013*a*).

Liver histology

Fragments of the liver of each animal with weights of ∼1 g were embedded in Tissue-Tek OCT (Sakura, Tokyo, Japan) and stored at −80°C until sectioning. Frozen sections of 10 μm thickness were obtained in a cryostat (JUNG FRIGOCUT 2800N; Leica, Solms, Germany) and dried at room temperature for 4 h. The sections were stained for 1 h in a saturated solution of Sudan III in 70% ethanol, then washed for 15 min in 50% ethanol and then in distilled water. The sections were mounted in glycerol:PBS (9:1).

Ten digital images for each animal were analysed in a random manner and studied to assess steatosis as described by Catta-Preta *et al.* (2011). Software Image Pro Plus, version 6.0.0.260 for Windows 2000/XP Professional (Media Cybernetics, Rockville, MD, USA) was used for the analyses (Fernandes-Santos *et al.* 2013).

Stearoyl CoA desaturase and fatty acid synthase activity

Stearoyl CoA desaturase activity was measured polarographically in liver homogenates at 37°C in a solution containing 0.1 M K_2HPO_4, pH 7.4, 1 μM myxothiazol, 0.12 mM NADH and 0.06 mM stearoyl-CoA as the 5 mM cyanide-sensitive (Strittmatter *et al.* 1974), myxothiazol-insensitive oxygen consumption. Fatty acid synthase activity was measured in liver homogenates at 37°C by following the oxidation of 5 mg ml^{-1} NADPH in a solution containing 0.1 M KH_2PO_4, pH 6.5, 1 mg ml^{-1} acetyl-CoA and 0.5 mg ml^{-1} malonyl-CoA (Penicaud *et al.* 1991).

Mitochondrial respiration

Isolation of liver mitochondria and measurement of state 3 respiration were carried out as previously reported (Crescenzo *et al.* 2012). Briefly, liver tissue fragments were gently homogenized with a medium containing 220 mM mannitol, 70 mM sucrose, 20 mM Hepes, 1 mM EDTA and 0.1% (w/v) fatty acid-free bovine serum albumin, pH 7.4, in a Potter Elvehjem homogenizer set at 500 r.p.m. (4 strokes min^{-1}). The homogenate was then centrifuged at 1000g_a for 10 min, and the resulting supernatant was again centrifuged at 3000g_a for 10 min. The mitochondrial pellet was washed twice and finally resuspended in a medium containing 80 mM KCl, 50 mM Hepes, 5 mM Tris-PO_4, 1 mM EGTA and 0.1% (w/v) fatty acid-free bovine serum albumin, pH 7.0. The oxygen consumption rate was measured polarographically with a Clark-type electrode (Yellow Springs Instruments, Yellow Springs, OH, USA) in a 3 ml glass cell, at a temperature of 30°C in a medium containing 80 mM KCl, 50 mM Hepes, 5 mM K_2HPO_4, 1 mM EGTA and 0.1% (w/v) fatty acid-free bovine serum albumin, pH 7.0. All samples were allowed to oxidize their endogenous substrates for 3 min. Substrates added were as follows: 10 mM glutamate plus 2.5 mM malate; or 10 mM pyruvate plus 2.5 mM malate; or 40 μM palmitoyl-coenzyme A plus 2 mM carnitine plus 2.5 mM malate; or 10 mM succinate plus 3.75 μM rotenone. State 3 oxygen consumption was measured in the presence of 0.3 mM ADP, while state 4 was obtained from oxygen consumption measurements at the end of state 3, when

ADP becomes limiting. The respiratory control ratio (RCR) was calculated as the state 3/state 4 ratio.

Control experiments of enzymatic and electron microscopy characterization have shown that our isolation procedure (centrifugation at 3000g_a for 10 min) results in a cellular fraction that consists essentially of mitochondria.

Mitochondrial lipid peroxidation, aconitase and superoxide dismutase (SOD) specific activity

Lipid peroxidation was determined according to Fernandes *et al.* (2006), by measuring thiobarbituric acid reactive substances (TBARS), using the thiobarbituric acid assay. Aliquots of mitochondrial suspensions were added to 0.5 ml of ice-cold 40% trichloroacetic acid. Then, 2 ml of 0.67% aqueous thiobarbituric acid containing 0.01% of 2,6-di-tert-butyl-*p*-cresol was added. The mixtures were heated at 90°C for 15 min, then cooled in ice for 10 min, and centrifuged at 850*g* for 10 min. The supernatant fractions were collected, and lipid peroxidation was estimated spectrophotometrically at 530 nm. The amount of TBARS formed was calculated using a molar extinction coefficient of 1.56×10^5 mol^{-1} cm^{-1} and expressed as nanomoles of TBARS per milligram of protein.

Active aconitase specific activity was measured spectrophotometrically by following the formation of NADPH (340 nm) at 25°C in a mixture containing 0.2 mM $NADP^+$, 5 mM sodium citrate, 0.6 mM $MnCl_2$, 2 U ml^{-1} final concentration of isocitric dehydrogenase, 50 mM Tris–HCl, pH 7.4, and 30 ml of mitochondrial extract (Gardner, 2002). Aconitase inhibited by reactive oxygen species *in vivo* was reactivated so that total activity could be measured by incubating mitochondrial extracts in a medium containing 50 mM dithiothreitol, 0.2 mM Na_2S and 0.2 mM ferrous ammonium sulphate. The specific activity of SOD was measured in a medium containing 0.1 mM EDTA, 2 mM KCN, 50 mM KH_2PO_4, pH 7.8, 20 mM cytochrome *c*, 0.1 mM xanthine and 0.01 U/ml of xanthine oxidase. Determinations were carried out spectrophotometrically (550 nm) at 25°C, by monitoring the decrease in the reduction rate of cytochrome *c* by superoxide radicals, generated by the xanthine–xanthine oxidase system. One unit of SOD activity is defined as the concentration of enzyme that inhibits cythocrome *c* reduction by 50% in the presence of xanthine plus xanthine oxidase (Flohè & Otting, 1974).

Western blot quantification of mitochondrial cytochrome *c* and hepatic Akt and p-Akt

In order to evaluate changes in liver mitochondrial mass, the amount of a mitochondrial marker protein, cytochrome *c*, was detected by Western blot. To this end, tissue samples were denatured in a buffer (60.0 mM Tris, pH 6.8, 10% sucrose, 2% SDS and 4% β-mercaptoethanol) and loaded onto a 12% SDS-polyacrylamide gel. After the run in electrode buffer (50 mM Tris, pH 8.3, 384 mM glycine, 0.1% SDS), the gels were transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA) at 0.8 mA cm^{-2} for 90 min. The membranes were preblocked in blocking buffer (PBS, 5% milk powder and 0.5% Tween 20) for 1 h and then incubated overnight at 4°C with monoclonal antibody for cytochrome *c* (ENZO Life Sciences, Farmingale, NY, USA; diluted 1:100 in blocking buffer). Membranes were washed three times for 12 min each time in PBS containing 0.5% Tween 20 and three times for 12 min in PBS; and then incubated for 1 h at room temperature with an antimouse, alkaline phosphatase-conjugated secondary antibody (Promega, Madison, WI, USA). The membranes were washed as described above, rinsed in distilled water and incubated at room temperature with a chemiluminescent substrate, CDP-Star (Sigma-Aldrich, St Louis, MO, USA). Data detection was carried out by exposing autoradiography films (Eastman Kodak Company, Rochester, NY, USA) to the membranes. Quantification of signals was carried out by Un-Scan-It gel software (Silk Scientific, Orem, UT, USA). To normalize the specific signal of cytochrome *c* in each lane, actin was detected as above using a rabbit polyclonal antibody (Sigma-Aldrich) and an antirabbit, alkaline phosphatase-conjugated secondary antibody (Promega).

For p-Akt detection, an additional six rats for each group were feed deprived for 6 h from 08.00 h, then administered insulin (10 U kg^{-1}) and killed 15 min after insulin injection for Western blot analysis. Liver tissue samples were treated as above and incubated with polyclonal antibody for p-Akt (Cell Signaling, Danvers, MA, USA; diluted 1:1000 in blocking buffer). Akt was detected with polyclonal antibody (Cell Signaling; diluted 1:1000 in blocking buffer) and used to normalize the p-Akt signal.

Statistical analysis

Data are given as means ± SEM. Statistical analyses were performed by one-way ANOVA followed by Tukey's *post hoc* test. Probability values <0.05 were considered to indicate a significant difference. All analyses were performed using GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA).

Materials

All chemicals used were of analytical grade and were purchased from Sigma (St Louis, MO, USA).

Table 1. Composition of diets

Component	Low-fat diet	High-fat diet	High-fat–high-fructose diet
Total weight of the daily portion (g)	18.78	16.18	16.18
Of which:			
Standard chow (g)*	4.21	4.21	4.21
Sunflower oil (g)	0.62	0.62	0.62
Casein (g)	2.34	2.34	2.34
Butter (g)	—	2.07	2.07
Cornstarch (g)	8.41	3.74	—
Fructose (g)	—	—	3.74
AIN mineral mix (g)	2.00	2.00	2.00
AIN vitamin mix (g)	1.00	1.00	1.00
Choline (g)	0.10	0.10	0.10
Methionine (g)	0.10	0.10	0.10
Metabolizable energy content of the daily portion (kJ)†	250	250	250
Protein [J (100 J)$^{-1}$]	20.85	20.87	20.87
Lipids [J (100 J)$^{-1}$]	11.29	42.53	42.53
Carbohydrates [J (100 J)$^{-1}$]	67.86	36.60	36.60
Of which:			
Fructose	—	—	25.00
Starch	67.86	36.60	11.60
SFA (%)	13.4	52.9	52.9
UFA (%)	86.6	47.1	47.1
MUFA (%)	33.4	30.1	30.1
PUFA (%)	53.2	17.0	17.0
UFA/SFA	6.5	0.9	0.9

*Mucedola 4RF21 (Settimo Milanese, Milan, Italy) containing (in g kg^{-1}): protein, 185; lipid, 30; and fibre, 60; metabolizable energy 11.20 kJ g^{-1}. †Estimated by computation using values (in kJ g^{-1}) for energy content as follows: protein, 16.736; lipid, 37.656; and carbohydrate, 16.736. Abbreviations: AIN, American Institute of Nutrition; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; UFA, unsaturated fatty acids.

Results

Body and liver composition, plasma parameters and *de novo* lipogenesis

The HF and HF-F rats exhibited a significant increase in body energy, body lipids and epididymal fat in comparison to LF rats (Table 2), although the three groups of rats exhibited similar ME intake (LF, 3412 ± 211 kJ; HF, 3392 ± 189 kJ; and HF+F, 3453 ± 223 kJ). In addition, in HF-F rats, epididymal fat increased significantly compared with HF rats.

Regarding liver composition, HF rats exhibited a significant decrease in liver glycogen compared with LF rats, while in HF-F rats the hepatic glycogen content was similar to that found in LF rats and significantly higher compared with HF rats (Table 2). A significant increase in hepatic lipids, triglycerides and cholesterol was found in HF and HF-F rats compared with LF rats. A further increase was found in hepatic lipids and triglycerides in HF-F rats compared with HF rats. Higher hepatic ceramide content was found in HF-F rats compared with LF and HF rats (Table 2). The degree of hepatic steatosis was significantly higher in HF and HF-F rats compared with LF rats (+152 and +577%, respectively), and a significant increase was also evident when HF-F rats were compared with HF rats (+168%) (Fig. 1). In addition, plasma levels of ALT, considered to provide a reliable index of hepatocellular necrosis (Ozera *et al.* 2008), were significantly higher in HF-F rats compared with LF and HF rats (Table 2).

The plasma lipid profile (Table 2) indicates a significant increase in total cholesterol and NEFA in HF and HF-F rats compared with LF rats. The HF-F rats exhibited significantly higher triglycerides compared with LF and HF rats, and NEFA exhibited a further significant increase compared with HF rats. The NPRQ, an index of whole-body *de novo* lipogenesis, was significantly lower in HF rats than in LF rats, while in HF-F rats it was significantly lower than in LF rats, but significantly higher than in HF rats (Table 2). These data suggest an inhibition of whole-body *de novo* lipogenesis induced by high-fat diet, which is partly removed by fructose supplementation.

This picture is substantially confirmed by the evaluation of hepatic lipogenic capacity, carried out by the assessment of two rate-limiting enzymes, namely fatty acid synthase and stearoyl CoA desaturase (Table 2). The results show

Table 2. Whole-body and liver composition, plasma lipid profile and *de novo* lipogenesis in rats fed a low-fat, a high-fat or a high-fat–high-fructose diet for 2 weeks

Parameter	Low-fat diet	High-fat diet	High-fat–high-fructose diet
Final body weight (g)	492 ± 14	506 ± 17	507 ± 18
Body lipids [g (100 g bwt)$^{-1}$]	12.6 ± 0.4	14.3 ± 0.4*	14.7 ± 0.5*
Epididymal fat weight [g (100 g bwt)$^{-1}$]	1.00 ± 0.10	1.28 ± 0.11*	1.69 ± 0.12**†
Epididymal fat weight [g (100 g lipids)$^{-1}$]	7.9 ± 0.2	8.9 ± 0.2	11.5 ± 0.1****††††
Body proteins [g (100 g bwt)$^{-1}$]	16.1 ± 0.6	15.9 ± 1.3	15.9 ± 0.8
Body energy (kJ g^{-1})	8.7 ± 0.1	9.3 ± 0.1*	9.5 ± 0.2**
Liver lipids (mg g^{-1})	51.2 ± 3.0	63.7 ± 3.0*	78.3 ± 3.0****††
Liver triglycerides (mg g^{-1})	20.4 ± 2.0	38.6 ± 2.0****	52.5 ± 2.0****†††
Liver cholesterol (mg g^{-1})	3.7 ± 0.3	5.7 ± 0.3*	5.6 ± 0.7*
Liver ceramide (AU g^{-1})	367 ± 21	350 ± 23	446 ± 20*†
Liver glycogen (mg g^{-1})	28.5 ± 2	22.5 ± 1.2*	26.6 ± 1.6†
Liver ALT (U l^{-1})	13.3 ± 1.0	12.4 ± 1.0	20.0 ± 1.1***†††
Plasma triglycerides [mg (100 ml)$^{-1}$]	126 ± 4	125 ± 5	144 ± 5*†
Plasma cholesterol [mg (100 ml)$^{-1}$]	51 ± 2	65 ± 2***	72 ± 2****
Plasma NEFA (mM)	0.27 ± 0.02	0.43 ± 0.03*	0.66 ± 0.09***†
Non-protein respiratory quotient	1.10 ± 0.03	0.86 ± 0.03***	0.98 ± 0.03*†
Hepatic fatty acid synthase specific activity [μU min^{-1} (mg protein)$^{-1}$]	0.23 ± 0.03	0.14 ± 0.01*	0.29 ± 0.02†††
Hepatic stearoyl-CoA desaturase specific activity [ng atoms oxygen min^{-1} (g tissue)$^{-1}$]	703 ± 25	293 ± 18****	393 ± 22****†

Values are the means ± SEM of six rats. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ compared with low-fat diet; †$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$, ††††$P < 0.0001$ compared with high-fat diet (one-way ANOVA followed by Tukey's *post hoc* test). Abbreviations: ALT, alanine aminotransferase; AU, absorbance unit; NEFA, non-esterified fatty acids; and U, unit.

a significant decrease in fatty acid synthase activity in HF rats compared with LF rats, and a significant increase in fatty acid synthase activity in HF-F rats compared with HF rats. Stearoyl CoA desaturase activity was significantly lower in HF and HF-F rats compared with LF rats, but significantly higher in HF-F rats compared with HF rats (Table 2).

Glucose homeostasis

Significantly higher plasma insulin and HOMA index values were found in HF-F rats, in comparison to LF and HF rats (Fig. 2*A*). In addition, hepatic insulin resistance index, calculated during the early phase of the glucose tolerance test, was found to be significantly higher in HF-F rats compared with LF and HF rats (Fig. 2*B*). Hepatic insulin sensitivity was also assessed by determining the degree of phosphorylation of the kinase Akt, a distal effector of insulin signalling, which was found to be significantly lower in HF-F compared with LF and HF rats (Fig. 2*C*).

Mitochondrial compartment

State 3 and state 4 oxidative capacity assessed in isolated liver mitochondria was found to be significantly decreased in HF and HF-F rats compared with LF rats (Fig. 3*A* and *B*), while no variation was evident in RCR values (Fig. 3*C*). Whole-tissue cytochrome *c*/actin ratio, an index of hepatic mitochondrial mass, was found to be significantly increased in HF-F rats compared with LF and HF rats (Fig. 3*D* and *E*). Lipid peroxidation and aconitase specific activity were taken as an index of lipid and protein oxidative damage, respectively (Table 3). A significant increase in lipid peroxidation and a significant decrease in active/total aconitase ratio were found in HF and HF-F rats compared with LF rats. No significant variation was found in SOD activity, taken as an index of mitochondrial antioxidant defence (Table 3).

Discussion

Here we show that short-term combined effects of dietary fat and fructose, in quantities that mimic a Western diet, are similar to those induced by high-fat diet alone, i.e. increased body energy and lipid content. From these results, it appears that the supplementation with fructose does not worsen the deleterious effects of high-fat feeding on whole-body composition, at least after short-term dietary treatment. In contrast, high-fat–high-fructose diet worsens plasma lipid profile, by increasing circulating

Table 3. Hepatic mitochondrial oxidative status in rats fed a low-fat, a high-fat or high-fat–high-fructose diet for 2 weeks

Parameter	Low-fat diet	High-fat diet	High-fat–high-fructose diet
Active aconitase [mU (mg protein)$^{-1}$]	7.6 ± 0.5	5.8 ± 0.5*	4.9 ± 0.3**
Total aconitase [mU (mg protein)$^{-1}$]	15.7 ± 0.8	17.6 ± 0.8	13.8 ± 1.0
Active aconitase/total aconitase	0.49 ± 0.02	0.33 ± 0.02***	0.36 ± 0.02***
Lipid peroxidation [nmol (mg protein)$^{-1}$]	1.36 ± 0.10	1.78 ± 0.10*	1.77 ± 0.10*
Superoxide dismutase specific activity [U (mg protein)$^{-1}$]	172 ± 10	163 ± 14	176 ± 15

Values are the means ± SEM of six rats. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with low-fat diet (one-way ANOVA followed by Tukey's *post hoc* test).

lipids, in the form of triglycerides and NEFA. The increased triglyceride plasma levels of HF-F rats are very likely to reflect the increased lipid synthesis in the liver dictated by high fructose intake. In agreement, we show an inhibition of *de novo* lipogenesis at the whole-body and liver level in HF rats, while *de novo* lipogenesis is stimulated at the whole-body and liver level in HF-F rats. This increased lipid synthesis could be due to the hyperinsulinaemic condition of the HF-F rats, because insulin is able to stimulate the pathway of *de novo* lipogenesis, even in conditions of hepatic insulin resistance (Choi & Ginsberg, 2011).

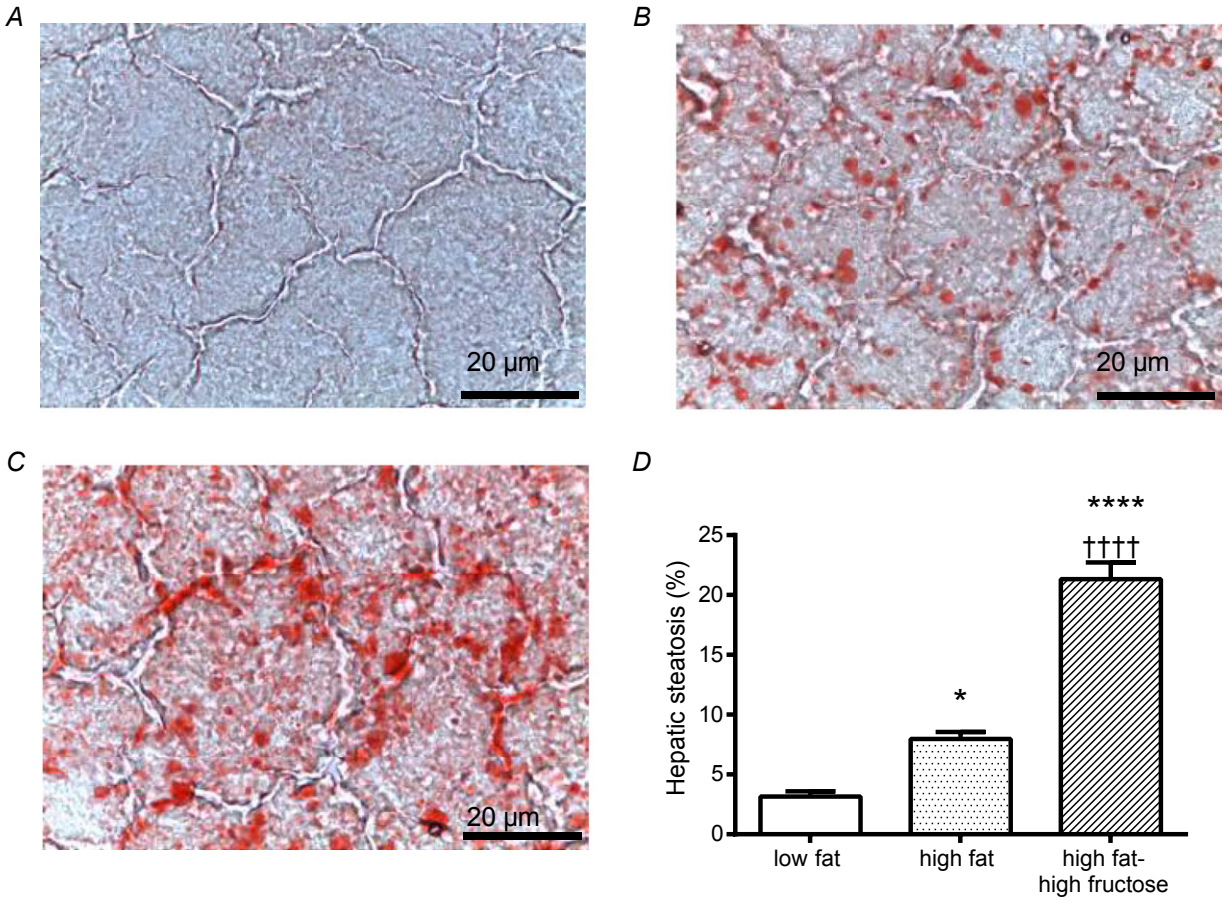

Figure 1. Liver sections from rats fed a low-fat (*A*), a high-fat (*B*) or high-fat–high-fructose diet (*C*) for 2 weeks were stained with Sudan III, and 10 digital images per animal were analysed in a random manner for quantitative assessment (means ± SEM) of steatosis (*D*)
*$P < 0.05$, ****$P < 0.0001$ compared with low fat, ††††$P < 0.0001$ compared with high fat (one-way ANOVA followed by Tukey's *post hoc* test).

It should be noted that here short-term high-fat feeding is associated with an increase in circulating NEFA in the absence of signs of hepatic insulin resistance, while in previous studies we have found that after long-term high-fat feeding, higher plasma NEFAs are associated with insulin resistance (Lionetti *et al.* 2007; Crescenzo *et al.* 2008). It can be hypothesized, therefore, that increased plasma NEFAs contribute to the development of hepatic insulin resistance during high-fat feeding, while the consumption of a high-fat–high-fructose diet accelerates the development of metabolic disturbances elicited by high-fat feeding, as indicated by a further increase in plasma NEFAs. In addition, in HF-F rats, we found an increased HOMA index and hepatic insulin resistance index, together with a decreased p-Akt/Akt ratio and increased glycogen content. These variations are indicative of hepatic insulin resistance in HF-F rats. In fact, indexes derived from measurements of fasting plasma glucose and insulin concentrations, such as the HOMA index, primarily reflect hepatic insulin resistance (Abdul-Ghani *et al.* 2007), because in the postabsorptive state ∼80–85% of glucose originates in the liver, so that the higher the rate of glucose production and the plasma insulin concentration, the greater the severity of hepatic insulin resistance (Abdul-Ghani *et al.* 2007). In addition, during the first 30 min after oral glucose load, the rise in plasma glucose and insulin concentrations is proportional to the magnitude of hepatic insulin resistance (Abdul-Ghani *et al.* 2007). In the liver, insulin resistance usually refers to the impaired ability of insulin to suppress hepatic glucose production (Farese *et al.* 2012), so that hepatic glycogen content can be considered an indirect index of insulin failure. Finally, the insulin-stimulated signal transduction pathway that suppresses hepatic glucose production includes Akt, which is phosphorylated upon stimulation by insulin, so that the relative amount of p-Akt is a further indication of the insulin sensitivity of the tissue.

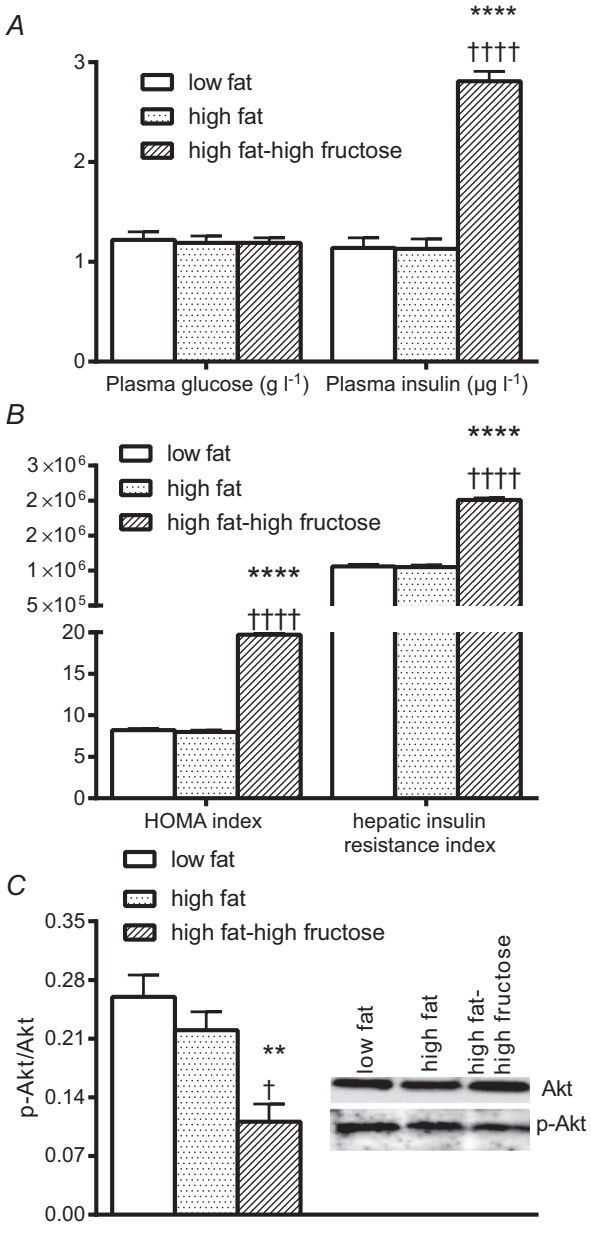

Figure 2. Plasma glucose and insulin (*A*), homeostasis model assessment (HOMA) index and hepatic insulin resistance index (*B*) and p-Akt/Akt ratio (*C*) in rats fed a low-fat, a high-fat or high-fat–high-fructose diet for 2 weeks
Values are the means ± SEM of six rats. $^{**}P < 0.01$, $^{****}P < 0.0001$ compared with low fat; †$P < 0.05$, ††††$P < 0.0001$ compared with high fat (one-way ANOVA followed by Tukey's *post hoc* test).

It can be suggested that the higher plasma NEFAs found in HF-F rats impair hepatic glucose homeostasis, thus accelerating the effects we observed previously after long-term high-fat feeding (Lionetti *et al.* 2007; Crescenzo *et al.* 2008). In fact, the increased availability of fatty acids in the form of plasma NEFA, found in HF and HF-F rats, implies higher lipid flow to tissues, such as liver and skeletal muscle. If this increased fatty acid availability is in excess compared with the cellular oxidation capacity, it could give rise to ectopic lipid deposition, one of the main factors in the onset of insulin resistance (Carobbio *et al.* 2011). To address this issue, we evaluated hepatic deposition of lipids and found that the triglyceride content in the liver paralleled the NEFA plasma levels. In fact, increased hepatic triglycerides were found in HF rats, and a further increase was evident in HF-F rats, coupled with a significant increase in liver ceramide. Thus, we can hypothesize that the onset of hepatic insulin resistance in HF-F rats arises from increased hepatic triglyceride and ceramide content.

The issue of mitochondrial adaptations to changes in fuel supply is crucial in understanding the events leading to cellular insulin resistance. In addition, it is a matter of

debate whether the impairment of mitochondrial function precedes, and could thus be a cause of, the onset of insulin resistance. We were therefore interested in assessing the possible alterations in hepatic mitochondrial function at an early stage of high-fat feeding, when insulin sensitivity is maintained, and to verify the impact of fructose on mitochondrial compartment. From our results, it appears that hepatic mitochondrial impairment is an early event induced by increased lipid content of the diet, because it is already evident after 2 weeks of dietary treatment, and that the presence of fructose does not have a further impact on mitochondrial function. In addition, the impairment occurs both in HF rats, with normal hepatic insulin sensitivity, and in HF-F rats, with altered hepatic insulin sensitivity, and it can therefore be suggested that mitochondrial alterations precede, and could potentially contribute to, hepatic insulin resistance.

In agreement with this suggestion, it has been shown that enhancement of hepatic mitochondrial fatty acid oxidation capacity is able to reverse insulin resistance in mice fed a high-fat–high-fructose diet (Monsénégo *et al.* 2012). The events linking mitochondrial impairment to insulin resistance could be a reduced capacity to cope with the increased lipid supply to hepatocytes, thus giving rise to lipotoxicity. In this respect, the increased mitochondrial mass could be a compensatory response to decreased oxidative capacities found in HF-F rats in order to counteract elevated fatty acid substrate availability. However, it is also possible that mitochondrial impairment could contribute to insulin resistance through increased oxidative stress, a factor commonly considered important in this condition (Rains & Jain, 2011), because increased oxidative stress is evident in liver mitochondria from HF and HF-F rats. The present data are also in line with the postulated importance of lipid availability in the onset of insulin resistance (Oakes *et al.* 2013), because, with the same alteration in the mitochondrial oxidative capacity, the higher plasma NEFA levels found in HF-F rats probably worsen lipid overflow to liver cells, increasing ectopic deposition.

In conclusion, we provide evidence that short-term consumption of a typical Western diet, rich in saturated fats and fructose, is more deleterious than a high-fat diet for hepatic lipid metabolism and glucose homeostasis. These results indicate the harmful effect of adding fructose to the usual Western, high-fat diet.

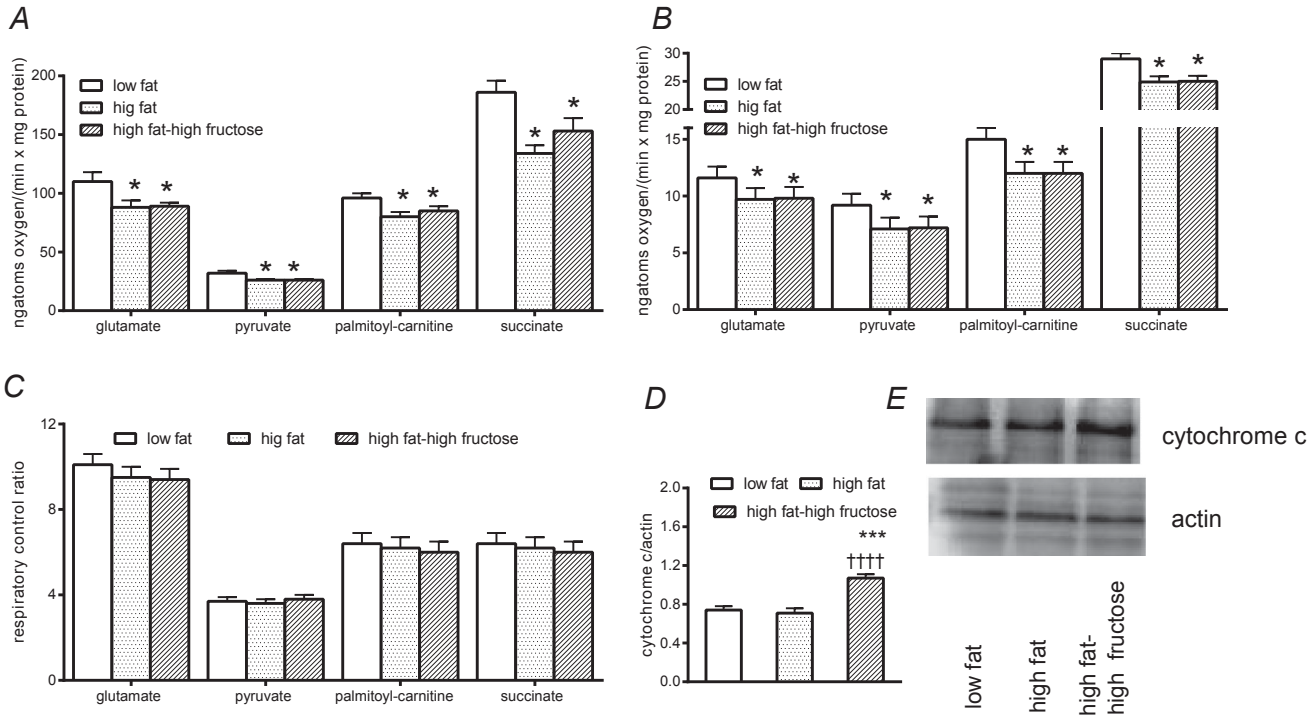

Figure 3. Hepatic mitochondrial state 3 (*A*) and state 4 respiratory capacities (*B*), respiratory control ratios (*C*) and cytochrome *c* content (*D*) with representative Western blots (*E*) in rats fed a low-fat, a high-fat or a high-fat–high-fructose diet for 2 weeks
Values are the means ± SEM of six rats. $^{*}P < 0.05$, $^{***}P < 0.001$ compared with low fat; ††††$P < 0.0001$ compared with high fat (one-way ANOVA followed by Tukey's *post hoc* test).

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Additional Information

Competing interests

None declared.

Author contributions

S.I. designed and supervised the study. S.I. and G.L. obtained funding and provided administrative, technical and material support. Ra.Cr., F.B., P.C., A.M., M.T. and Ro.Ca. performed the experiments. Ra.Cr. and S.I. contributed to the analysis of data and interpretation of the results, Ra.Cr., S.I. and G.L. wrote the draft of the manuscript, and all the authors critically reviewed the manuscript and approved the final version for publication.

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