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ORIGINAL CONTRIBUTION

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Abstract
Purpose To study the effect of isoenergetic administration to adult rats of high-fat or high-fat–high-fructose diet for 2 weeks on skeletal muscle mitochondrial energetic.
Methods Body and skeletal muscle composition, energy balance, plasma lipid profile and glucose tolerance were measured, together with mitochondrial functionality, oxidative stress and antioxidant defense.
Results Rats fed high-fat–high-fructose diet exhibited significantly higher plasma triglycerides and non-esterified fatty acids, together with significantly higher plasma glucose and insulin response to glucose load. Skeletal muscle triglycerides and ceramide were significantly higher in rats fed high-fat–high-fructose diet. Skeletal muscle mitochondrial energetic efficiency and uncoupling protein 3 content were significantly higher, while adenine nucleotide translocase content was significantly lower, in rats fed high-fat or high-fat–high-fructose diet.
Conclusions The results suggest that a high-fat–high-fructose diet even without hyperphagia is able to increase lipid flow to skeletal muscle and mitochondrial energetic efficiency, with two detrimental effects: (a) energy sparing that contributes to the early onset of obesity and (b) reduced oxidation of fatty acids and lipid accumulation in skeletal muscle, which could generate insulin resistance.

Keywords Insulin resistance · Mitochondrial coupling · Obesity · Ceramide

Introduction

The link between mitochondrial dysfunction and insulin resistance is a matter of debate, especially in skeletal muscle, the main tissue involved in glucose homoeostasis. In fact, there are reports in favour or against the existence of such link, in both humans [1, 2] and animal models [3–5]. One of the possible reasons explaining the discrepancy between different studies in humans is the level of physical activity, which is the most powerful stimulus to mitochondrial biogenesis in skeletal muscle [6], since obese and diabetic subjects tend to be more sedentary than control ones. In this respect, animal models are very useful tools, since rats kept in laboratory display a sedentary behaviour, due to standard stabulation conditions [7], and therefore, it is possible to perform studies aiming at the elucidation of the link between insulin resistance and mitochondrial functioning, without the confounding effect of changes in physical activity. Another possible reason of the apparent discrepancy among the various results published on the above issue is the choice of the parameter to be studied in evaluating mitochondrial function. In fact, if the hypothesis is that reduced mitochondrial oxidation of fatty acids causes ectopic fat deposition, that in turn elicits insulin resistance, all the factors contributing to mitochondrial lipid burning must be taken into account. The mitochondrial oxidation of metabolic fuels depends on (1) organelle number, (2) organelle activity and (3) energetic efficiency of the mitochondrial machinery in synthesising adenosine triphosphate (ATP) from the oxidation of fuels. Changes in each of these three factors could theoretically affect lipid

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oxidation and should be monitored to confirm or reject the hypothesis.

Recently, we have found that, in adult rats, long-term feeding a high-fructose–low-fat diet elicits the development of obesity and insulin resistance [8], as well as an increase in mitochondrial energetic efficiency, both in liver [9] and skeletal muscle [10]. In addition, we have previously found that long-term high-fat feeding is associated with the development of insulin resistance [11, 12], while short-term high-fat feeding is associated with normal glucose homoeostasis [13]. Since dietary lifestyle in modern societies is characterised by intake of foods that are rich in fructose and fat, we considered of interest to evaluate the possible alterations in skeletal muscle mitochondrial function at an early phase of high-fat feeding, when insulin sensitivity is maintained, and to verify the worsening effect of fructose supplementation on insulin sensitivity and skeletal muscle mitochondrial function. To this end, a high-fat–high-fructose diet was administered for 2 weeks to adult rats and mitochondrial mass, respiratory activity and energetic efficiency, together with indexes of oxidative stress and antioxidant defence of these organelles, were assessed in skeletal muscle. Whole-body insulin sensitivity was assessed by glucose load, and the experimental model here used was metabolically characterised by determining the main parameters of energy balance, as well as the lipid composition of plasma and skeletal muscle.

Materials and methods

Male Sprague–Dawley rats (Charles River, Italy) of 90 days of age were caged singly in a temperature-controlled room (23 ± 1 °C) with a 12-h light/dark cycle (06.30–18.30) and divided in three groups, which were fed a low-fat, high-fat or high-fat–high-fructose diet for two weeks. The daily amount of estimated metabolisable energy given to the three groups of rats (250 kJ) corresponded to the amount of energy introduced by the same rats immediately before the start of the feeding protocol. The composition of the three diets is shown in Online Resource 1, 2 and 3. 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-Sientifico per la Sperimentazione Animale" of the University "Federico II" of Naples (2012/24688).

During the whole experimental period, faeces and urine were collected daily and the respective energy content assessed with a bomb calorimeter.

At the end of the experimental period, the rats were killed by decapitation, the hind leg skeletal muscles (gastrocnemius, soleus, tibialis anterior and quadriceps) were harvested and the carcasses used for body composition determination.

Glucose tolerance test, insulin tolerance test, plasma TNF-α and lipid profile

Glucose tolerance test was carried out on rats that were feed deprived for 6 h from 08.00. Basal post-absorptive samples obtained from venous blood from a small tail clip were collected in EDTA-coated tubes, and then glucose (2 g/kg body weight) was injected intraperitoneally. Small blood samples were collected after 20, 40, 60, 90, 120 and 150 min.

Insulin tolerance test was carried out on rats that were feed deprived for 6 h from 08.00. Basal post-absorptive samples obtained from venous blood from a small tail clip were collected in EDTA-coated tubes, and then insulin (1.5 U/kg body weight) was injected intraperitoneally. Small blood samples were collected after 20, 40, 60, 90 and 120 min.

After centrifugation at $1400 \times g_{av}$ 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 colorimetric enzymatic method (Pokler Italia, Italy). Plasma insulin concentration was measured using an ELISA kit (Mercodia AB, Sweden) in a single assay to remove inter-assay variations. Basal post-absorptive values of plasma glucose and insulin were used to calculate Homoeostatic Model Assessment (HOMA) index as (Glucose (mg/dL) × Insulin (mU/L))/405 [14]. Plasma tumour necrosis factor alpha (TNF-α) concentrations were determined using a rat-specific enzyme-linked immunosorbent assay (R&D Systems, MN, USA) according to manufacturer's instruction. Briefly, the wells of a microtitre plate were coated with 100 µl of mouse anti-rat TNF-α (4 µg/mL) in PBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L Na_2HPO_4, 1.5 mmol/L KH_2PO_4, pH 7.4) and incubated overnight at room temperature. The antibody excess was then removed by washing with Wash Buffer (containing 0.05 % (v/v) Tween 20 in PBS, pH 7.4), and the remaining sites on the plate were blocked with reagent diluent (PBS containing 1 % BSA) (1 h, room temperature). After extensive washing, 100 µL of samples (1:2–1:10 dilution in reagent diluent) was added to the wells and incubated for 2 h at room temperature. After further washing, the wells were incubated with biotinylated goat anti-rat TNF-α (225 ng/mL in reagent diluent) followed by treatment with Streptavidin-HRP (1:200 dilution; 1 h, room temperature). Peroxidase-catalysed colour development from tetramethylbenzidine was measured at 450 nm. Plasma concentrations of triglycerides and non-esterified fatty acids (NEFA) were measured by colorimetric enzymatic method using

commercial kits (SGM Italia, Italy and Randox Laboratories ltd., United Kingdom).

Body composition, skeletal muscle composition and energy balance

Guts were cleaned of undigested food, and the carcasses were then autoclaved. After dilution in distilled water and subsequent homogenisation of the carcasses with a Polytron homogeniser (Kinematica, Switzerland), duplicate samples of the homogenised carcass were analysed for energy content by bomb calorimeter. To take into account the energy content of skeletal muscle, tissue samples were dried and the energy content was then measured with the bomb calorimeter. Total body water content was determined by drying carcass samples in an oven at 70 °C for 48 h. Total body and skeletal muscle lipids were measured by the Folch extraction method [15]. The energy as lipid was calculated from body lipids by using the coefficient of 39.2 kJ/g and was then subtracted from total body energy to obtain the energy as protein [16]. Skeletal muscle triglycerides and cholesterol were measured by colorimetric enzymatic method using commercial kits (SGM Italia, Italy), phospholipids were obtained by subtracting triglycerides and cholesterol content from total lipid content and glycogen was assessed by direct enzymatic procedure [17]. Skeletal muscle ceramide content was evaluated by enzyme-linked immunosorbent assay (ELISA) [10] using 96-well Polysorp plates (Nunc, NY, USA).

Energy balance measurements were conducted by the comparative carcass technique over the experimental period, as detailed previously [18, 19]. Briefly, daily food consumption was monitored, gross energy density of the diet was measured by a bomb calorimeter and gross energy intake was calculated. Metabolisable energy (ME) intake was determined by subtracting the energy measured in faeces and urine from the gross energy intake. Body energy and lipid gain were calculated as the difference between the final and the initial content of body energy and lipid. Initial body energy and lipid content was assessed on a group of rats that were killed at the beginning of the dietary treatment. Energetic efficiency was calculated as the percentage of body energy retained per ME intake, and energy expenditure was determined as the difference between ME intake and energy gain. Net energy expenditure was obtained from energy expenditure by subtracting the cost of lipid and protein storage. The cost of storage was determined taking into account that the energy loss in storing 1 kJ protein is 1.25 kJ, while the energy cost for lipid deposition, in animals consuming a diet with an high percentage of carbohydrate, such as the low-fat diet, is 0.36 kJ/kJ lipid deposited since almost all of the body lipids will be synthesised de novo from glucose [20]. When the level of the fat in the diet is increased, as with the high-fat and the high-fat–high-fructose diet, body lipids are derived directly from dietary lipid, with a much lower energy cost (0.16 kJ/kJ lipid deposited) [21].

Preparation of skeletal muscle isolated mitochondria and measurements of mitochondrial oxidative capacities and degree of coupling

Hind leg muscles were freed of excess connective tissue, finely minced, washed in a medium containing 100 mmol/L KCl, 50 mmol/L TRIS, pH 7.5, 5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 5 mmol/L EGTA, 0.1 % (w/v) fatty acid-free BSA and treated with protease (3.6 U/g tissue) for 4 min. Tissue fragments were then homogenised with the above medium (1:8 w/v) at 500 rpm (4 strokes/min). Homogenate was centrifuged at $3{,}000\times g_{av}$ for 10 min, the resulting supernatant was rapidly discarded and the pellet was resuspended and centrifuged at $500\times g_{av}$ for 10 min. The supernatant was then centrifuged at $3{,}000\times g_{av}$ for 10 min, and the pellet was washed once and resuspended in suspension medium (250 mmol/L Sucrose, 50 mmol/L Tris, pH 7.5, 0.1 % fatty acid-free BSA). In control experiments, we assured the quality of our mitochondrial preparation by checking that contamination of mitochondria by other ATPase-containing membranes was lower than 10 %, and addition of cytochrome c (3 nmol/mg protein) only enhanced state 3 respiration by approximately 10 %.

Oxygen consumption was measured polarographically with a Clark-type electrode (Yellow Springs Instruments, OH, USA) in a 3-mL glass cell, at a temperature of 30 °C. Skeletal muscle mitochondria were incubated in a medium containing 30 mmol/L KCl, 6 mmol/L $MgCl_2$, 75 mmol/L sucrose, 1 mmol/L EDTA, 20 mmol/L KH_2PO_4 pH 7.0 and 0.1 % (w/v) fatty acid-free BSA, pH 7.0. The substrates used were 10 mmol/L succinate + 3.8 μmol/L rotenone, 10 mmol/L glutamate + 2.5 mmol/L malate, 40 μmol/L palmitoyl-carnitine + 2.5 mmol/L malate or 10 mmol/L pyruvate + 2.5 mmol/L malate. After the addition of 0.3 mmol/L ADP, maximal ADP-stimulated oxygen consumption was measured and taken as state 3, while state 4 was obtained from oxygen consumption measurements at the end of state 3, when ADP becomes limiting. Respiratory control ratio was calculated as state 3/state 4 ratio.

The degree of coupling was determined in skeletal muscle mitochondria as previously reported [22] by applying equation 11 by Cairns et al. [23]: degree of coupling $= \sqrt{1-(J_o)_{sh}/(J_o)_{unc}}$, where $(J_o)_{sh}$ represents the oxygen consumption rate in the presence of oligomycin that inhibits ATP synthase, and $(J_o)_{unc}$ is the uncoupled rate of oxygen consumption induced by FCCP, which dissipates the transmitochondrial proton gradient. $(J_o)_{sh}$ and $(J_o)_{unc}$ were measured as above using succinate (10 mmol/

L) + rotenone (3.75 μmol/L) in the presence of oligomycin (2 μg/mL) or FCCP (1 μmol/L), respectively, both in the absence and in the presence of palmitate at a concentration of 50 μmol/L.

Mitochondrial lipid peroxidation, aconitase and superoxide dismutase-specific activity

Lipid peroxidation was determined according to Fernandes et al. [24]. Aconitase-specific activity was measured spectrophotometrically by the method of Gardner [25], while superoxide dismutase (SOD)-specific activity was measured according to Flohè and Otting [26].

Western blot quantification of mitochondrial cytochrome c and p-Akt in skeletal muscle tissue and uncoupling protein 3 and adenine nucleotide translocase in isolated mitochondria

To evaluate changes in mitochondrial mass in skeletal muscle, 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 mmol/L Tris pH 6.8, 10 % sucrose, 2 % SDS, 4 % β-mercaptoethanol) and loaded onto a 12 % SDS–polyacrylamide gel. After the run in electrode buffer (50 mmol/L Tris, pH 8.3, 384 mmol/L glycine, 0.1 % SDS), the gels were transferred onto PVDF membranes (Millipore, MA, USA) at 0.8 mA/cm^2 for 90 min. The membranes were preblocked in blocking buffer (PBS, 5 % milk powder, 0.5 % Tween 20) for 1 h and then incubated overnight at 4 °C with monoclonal antibody for cytochrome c (Biomol International, PA, USA, diluted 1:100 in blocking buffer). Membranes were washed 3 times 12 min in PBS/0.5 % Tween 20 and 3 times 12 min in PBS and then incubated 1 h at room temperature with a anti-mouse, alkaline phosphatase-conjugated secondary antibody (Promega, WI, USA). The membranes were washed as above described, rinsed in distilled water and incubated at room temperature with a chemiluminescent substrate, CDP-Star (Sigma-Aldrich, MO, USA). Data detection was carried out by exposing autoradiography films (Eastman Kodak Company, NY, USA) to the membranes. Quantification of signals was carried out by Un-Scan-It gel software (Silk Scientific, UT, USA). To normalise the specific signal of cytochrome c in each lane, actin was detected as above using a rabbit polyclonal antibody (Sigma-Aldrich, MO, USA) and a anti-rabbit, alkaline phosphatase-conjugated secondary antibody (Promega, WI, USA).

For p-Akt detection, additional six rats for each group were feed deprived for 6 h from 08.00, then were administered insulin (10 U/Kg) and were killed 15 min after insulin injection for Western blot analysis. Skeletal muscle tissue samples were treated as above and incubated with polyclonal antibody for p-Akt (Cell Signalling, MA, USA, diluted 1:1,000 in blocking buffer). Akt was detected with polyclonal antibody (Cell Signalling, MA, USA, diluted 1:1,000 in blocking buffer) and used to normalise the p-Akt signal.

For uncoupling protein 3 (UCP3) and adenine nucleotide translocase (ANT) detection, mitochondrial samples were processed as above and incubated with rabbit polyclonal antibody (UCP3 = Millipore, MA, USA, diluted 1:3,000 in blocking buffer, ANT = Biogenesis Ltd., UK, diluted 1:50 in blocking buffer). Equal loading was verified by Ponceau S staining.

Statistical analysis

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

Materials

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

Results

Body composition measurements carried out after 2 weeks of isoenergetic feeding show that body energy and lipids were significantly higher in rats fed high-fat or high-fat–high-fructose diet compared to rats fed low-fat diet (Table 1). Energy balance determination indicates that body energy gain, lipid gain and energetic efficiency were significantly higher in rats fed high-fat or high-fat–high-fructose diet compared to rats fed low-fat diet, while energy expenditure and net energy expenditure were significantly lower in rats fed high-fat or high-fat–high-fructose diet compared to rats fed low-fat diet (Table 1).

HOMA index was found to be significantly higher in rats fed high-fat–high-fructose diet compared to the other two groups (low-fat = 8.2 ± 0.2, high-fat = 8.1 ± 0.3, high-fat–high-fructose = 19.6 ± 0.4, $P < 0.05$, one-way ANOVA followed by Tukey's post-test). The results of the glucose tolerance test carried out in rats fed low-fat, high-fat or high-fat–high-fructose diet at the end of the diet treatment indicate that the glucose (Fig. 1a) and insulin (Fig. 1b) response were significantly higher in rats fed high-fat–high-fructose diet compared to rats fed low-fat or

Table 1 Body composition and energy balance in rats fed low-fat, high-fat or high-fat–high-fructose diet for 2 weeks

	Point 0	Low-fat	High-fat	High-fat–high-fructose
Body weight (g)	485 ± 15	498 ± 12[a]	501 ± 12[a]	506 ± 12[a]
Body energy (kJ)	3,650 ± 45	4,333 ± 33[a]	4,659 ± 28[b]	4,807 ± 33[b]
Body lipids (kJ)	1,835 ± 22	2,485 ± 18[a]	2,837 ± 15[b]	2,946 ± 20[b]
Body proteins (kJ)	1,828 ± 20	1,848 ± 12[a]	1,822 ± 10[a]	1,861 ± 15[a]
Metabolisable energy intake (kJ)		3,568 ± 253[a]	3,494 ± 271[a]	3,645 ± 319[a]
Faecal energy loss (kJ)		695 ± 44	777 ± 33	676 ± 41
Urinary energy loss (kJ)		149 ± 10	146 ± 11	152 ± 9
Energy gain (kJ)		610 ± 45[a]	1,010 ± 65[b]	1,193 ± 68[b]
Lipid gain (kJ)		607 ± 45[a]	1,008 ± 45[b]	1,153 ± 58[b]
Energy expenditure (kJ)		2,958 ± 178[a]	2,484 ± 114[b]	2,452 ± 101[b]
Net energy expenditure (kJ)		2,724 ± 100[a]	2,323 ± 111[b]	2,268 ± 110[b]
Energetic efficiency (%)		18 ± 2[a]	29 ± 2[b]	30 ± 2[b]

Values are the mean ± SEM of 6 different rats. Means without a common letter are significantly different, $P < 0.05$ (one-way ANOVA followed by Tukey's post-test)

high-fat diet. We also calculated an index of skeletal muscle insulin sensitivity [27], i.e. (d*G*/d*t*)/mean plasma insulin concentration (I), where d*G*/d*t* is the rate of decline in plasma glucose concentration and is calculated as the slope of the least square fit to the decline in plasma glucose concentration from peak to nadir, and I represents the mean plasma insulin concentration during the glucose tolerance test. The obtained results show a significant decrease in skeletal muscle insulin sensitivity index (Fig. 1c) in rats fed high-fat–high-fructose diet. The results of the insulin tolerance test carried out in rats fed low-fat, high-fat or high-fat–high-fructose diet at the end of the diet treatment indicate that the degree of glucose decrease (Fig. 1d) was significantly lower in rats fed high-fat–high-fructose diet compared to rats fed low-fat or high-fat diet. Finally, skeletal muscle p-Akt levels after a bolus of insulin were found to be significantly lower in rats fed high-fat–high-fructose diet compared to rats fed low-fat or high-fat diet (Fig. 1e, f).

Plasma lipid profile indicates a significant increase in NEFA in rats fed high-fat diet compared to rats fed low-fat diet (Table 2) and a further significant increase in NEFA in rats fed high-fat–high-fructose diet, compared to rats fed high-fat diet (Table 2). In addition, rats fed high-fat–high-fructose diet exhibited significantly higher triglycerides compared to rats fed low-fat or high-fat diet (Table 2). No variation was found in plasma TNF-α between the three groups of rats (Table 2), thus suggesting that this proinflammatory cytokine is not involved in the early phase of development of obesity.

As for skeletal muscle composition (Table 2), tissue total lipids, triglycerides and ceramide were significantly higher in rats fed high-fat diet compared to rats fed low-fat diet and a further, significant increase was found in rats fed high-fat–high-fructose diet compared to rats fed high-fat diet.

Mitochondrial respiratory capacities were assessed in isolated skeletal muscle mitochondria by using nicotinamide adenine dinucleotide (NAD), flavin adenine dinucleotide (FAD) and lipid substrates. The obtained values for state 3, state 4 and RCR are similar to those by us previously found [10, 28], and state 4 values with succinate are in agreement with published values [29]. In addition, the values of RCR here found indicate the good quality of the mitochondrial preparations. Finally, no variation was found in state 3, state 4 or RCR between the three groups of rats (Table 3). Similarly, no variation was found in whole tissue cytochrome c/actin ratio, taken as an index of skeletal muscle mitochondrial mass (Table 3).

Oligomycin state 4 respiration was significantly lower, both in the absence and in the presence of palmitate, in rats fed high-fat or high-fat–high-fructose diet compared to rats fed low-fat diet (Fig. 2a), while no variation was found in maximal FCCP-stimulated respiration (Fig. 2b). As a consequence, skeletal muscle mitochondrial energetic efficiency, assessed as degree of coupling, was significantly higher in rats fed high-fat or high-fat–high-fructose diet compared to rats fed low-fat diet (Fig. 2c). Mitochondrial UCP3 protein content was significantly higher, while ANT content was significantly lower, in rats fed high-fat or high-fat–high-fructose diet compared to rats fed low-fat diet (Fig. 2d).

Since aconitase activity is very sensitive to superoxide exposure, this enzyme can be used to estimate indirectly the superoxide concentration. We, therefore, measured mitochondrial aconitase activity in isolated mitochondria as an in vivo indicator of ROS damage. To avoid mistakes due to a different amount in total aconitase activity, we measured active aconitase and total aconitase, obtained after reactivation of the inactive fraction of aconitase, and the results are expressed as the active/total aconitase activity ratio. In addition, lipid peroxidation was measured and taken as an index of lipid oxidative damage, while SOD activity was taken as an index of mitochondrial

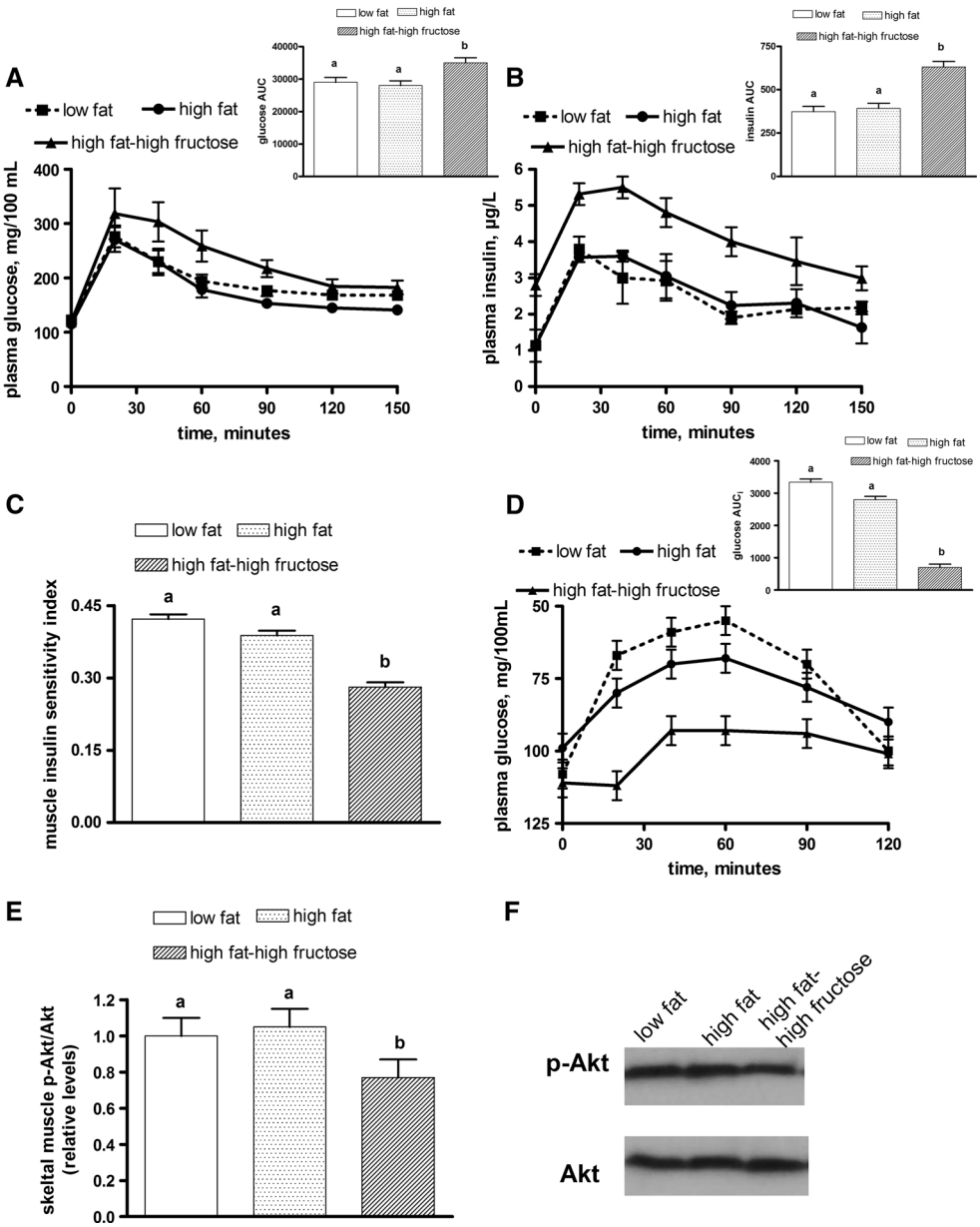

Fig. 1 Plasma glucose (**a**) and insulin (**b**) levels after a glucose load, muscle insulin sensitivity index (**c**), plasma glucose levels after insulin load (**d**) and skeletal muscle pAkt/Akt ratio (calculated with respect to rats fed a low-fat diet) (**e**) with representative Western blots (**f**) in rats fed a low-fat, high-fat or high-fat–high-fructose diet for 2 weeks. Values are the means, with their standard errors represented by *vertical bars*, of six rats per group. Area under the curve (AUC) for glucose load and inverse AUC for insulin load (AUCi) were calculated using the trapezoid method. Means without a *common letter* are significantly different, $P < 0.05$ (one-way ANOVA followed by Tukey's post-test)

antioxidant defence (Table 4). No variation was found in any of the above parameters.

Discussion

In the present paper, we show that short-term isoenergetic consumption of diets rich in fat or fat/fructose is able to induce changes in energy balance so that energy expenditure decreases and lipid gain and content increase, leading to the early phase of obesity development in a rat model of adult sedentary humans. The increased body lipids are partly due to the lower cost of lipid deposition during high-fat feeding [30]. However, the decrease in net energy expenditure (which can be representative of the cost of body energy maintenance) found in rats fed high-fat or high-fat–high-fructose diet suggests that other energy sparing mechanisms take place, with the skeletal muscle as a possible candidate site as it accounts for about 30 % of whole-body energy requirements in rats [31].

A number of studies have suggested that mitochondrial impairment could be at the basis of the onset of diet-induced insulin resistance in skeletal muscle, probably because of an imbalance between the flow of lipid substrates to the tissue and the mitochondrial capacity to burn them [1, 32, 33], with a following ectopic deposition of triglycerides and other lipid intermediates (diacylglycerol, ceramide) [34]. However, this hypothesis has been

Table 2 Plasma lipids and skeletal muscle composition of rats fed a low-fat, high-fat or high-fat–high-fructose diet for 2 weeks

	Low-fat	High-fat	High-fat–high-fructose
Plasma NEFA (mM)	0.27 ± 0.02[a]	0.43 ± 0.03[b]	0.66 ± 0.05[c]
Plasma triglycerides (mg/100 ml)	126 ± 4[a]	125 ± 5[a]	144 ± 5[b]
Plasma TNF-α (pg/ml)	56.4 ± 7.3[a]	55.5 ± 6.8[a]	56.1 ± 8.1[a]
Total lipids (mg/g)	24.1 ± 1.0[a]	28.0 ± 1.0[b]	32.3 ± 1.0[c]
Triglycerides (mg/g)	3.5 ± 0.2[a]	4.6 ± 0.3[b]	5.8 ± 0.3[c]
Cholesterol (mg/g)	0.9 ± 0.1[a]	1.0 ± 0.1[a]	0.9 ± 0.1[a]
Phospholipids (mg/g)	23.1 ± 1.0[a]	21.5 ± 1.0[a]	25.1 ± 1.0[a]
Glycogen (mg/g)	1.0 ± 0.1[a]	1.0 ± 0.1[a]	1.0 ± 0.1[a]
Ceramide (AU/g)	204 ± 20[a]	343 ± 25[b]	496 ± 28b[c]

Values are the mean ± SEM of 6 different rats. Means without a common letter are significantly different, $P < 0.05$ (one-way ANOVA followed by Tukey's post-test)

AU absorbance unit, *NEFA* non-esterified fatty acids, *TNF* tumour necrosis factor

Table 3 Skeletal muscle mitochondrial respiratory capacities and cytochrome *c* content in rats fed a low-fat, high-fat or high-fat–high-fructose diet for 2 weeks

	Low-fat	High-fat	High-fat–high-fructose
Glutamate			
State 3	867 ± 77[a]	933 ± 28[a]	923 ± 33[a]
State 4	56.5 ± 3.4[a]	62.6 ± 2.9[a]	62.7 ± 3.1[a]
RCR	15.3 ± 0.3[a]	14.9 ± 0.4[a]	14.7 ± 0.3[a]
Pyruvate			
State 3	356 ± 22[a]	434 ± 28[a]	394 ± 25[a]
State 4	45.5 ± 2.5[a]	49.3 ± 3.5[a]	46.1 ± 2.3[a]
RCR	7.8 ± 0.2[a]	8.8 ± 0.2[a]	8.5 ± 0.2[a]
Palmitoylcarnitine			
State 3	304 ± 18[a]	343 ± 37[a]	343 ± 22[a]
State 4	56.4 ± 3.2[a]	54.2 ± 2.2[a]	52.5 ± 2.1[a]
RCR	5.4 ± 0.1[a]	6.3 ± 0.2[a]	6.5 ± 0.2[a]
Succinate			
State 3	909 ± 33[a]	874 ± 48[a]	929 ± 46[a]
State 4	185 ± 9[a]	161 ± 4[a]	163 ± 3[a]
RCR	4.9 ± 0.1[a]	5.4 ± 0.2[a]	5.7 ± 0.2[a]
Cytochrome c/actin	0.49 ± 0.02[a]	0.49 ± 0.02[a]	0.52 ± 0.02[a]

Values are the mean ± SEM of 6 different rats. State 3 and state 4 respiratory capacities are expressed as ngatoms oxygen/(min × mg protein). Means without a common letter are significantly different, $P < 0.05$ (one-way ANOVA followed by Tukey's post-test)

RCR respiratory control ratio

challenged on the basis of another set of results pointing to a dissociation between mitochondrial function and insulin sensitivity [35]. One of the reasons of the above discrepancy could be that the majority of the reported studies have focused their attention on mitochondrial impairment in terms of reduced mass and/or oxidative activity. It is well known that the amount of fuels oxidised by the cell is dictated mainly by ATP turnover rather than by mitochondrial oxidative activity, and therefore, in resting skeletal muscle, changes in organelle number and/or activity could be without consequence for cellular bioenergetics, while modifications in mitochondrial energetic efficiency certainly alter the amount of oxidised fuels, even if ATP turnover does not vary. However, to our knowledge, data on the energetic efficiency in skeletal muscle mitochondria in conditions of obesity and insulin resistance are scarce. In trying to keep further insight into the subject, we measured mitochondrial mass, respiratory activity and energetic efficiency of isolated mitochondria, since each of these factors could affect mitochondrial performance. The present results show no variation in mitochondrial mass and activity and are at variance with our previous findings obtained after 2 weeks of ad libitum (hyperphagic) feeding a high-fat diet [13], thus suggesting that our previous results reflected the effect of hyperphagia rather than high-fat diet itself. In addition, our present results show an increase in mitochondrial energetic efficiency in rats fed high-fat or high-fat–high-fructose diet. In agreement, skeletal muscle mitochondrial content of ANT, which highly contributes to mitochondrial energetic efficiency [36], significantly decreases in rats fed high-fat or high-fat–high-fructose diet. Increased mitochondrial energetic efficiency implies that less substrates need to be burned to obtain the same amount of ATP, while higher plasma NEFA and triglycerides found in rats fed a high-fat–high-fructose diet dictate higher lipid substrate flux to skeletal muscle. As a consequence, it is possible that a condition of imbalance takes place in skeletal muscle cell, with substrate influx exceeding substrate burning and thus favouring ectopic lipid deposition, as shown by higher tissue triglyceride and ceramide content. In addition, the fact that mitochondrial energetic efficiency is higher both in rats with normal insulin sensitivity (high-fat-fed rats) and in those with decreased insulin sensitivity (high-fat–high-fructose-fed rats) let us to hypothesise that this mitochondrial modification is not caused by, but could contribute to the onset of insulin resistance. In agreement with this suggestion, skeletal muscle triglyceride and ceramide content are higher in rats fed high-fat diet but even higher in rats fed high-fat–high-fructose diet and therefore it is possible that in the latter group of rats their concentrations have reached a threshold level able to partly block insulin transduction pathway. The present data, in agreement with others [37], also point to the importance of lipid availability in the onset of skeletal muscle insulin resistance, since with the same alteration in the mitochondrial degree

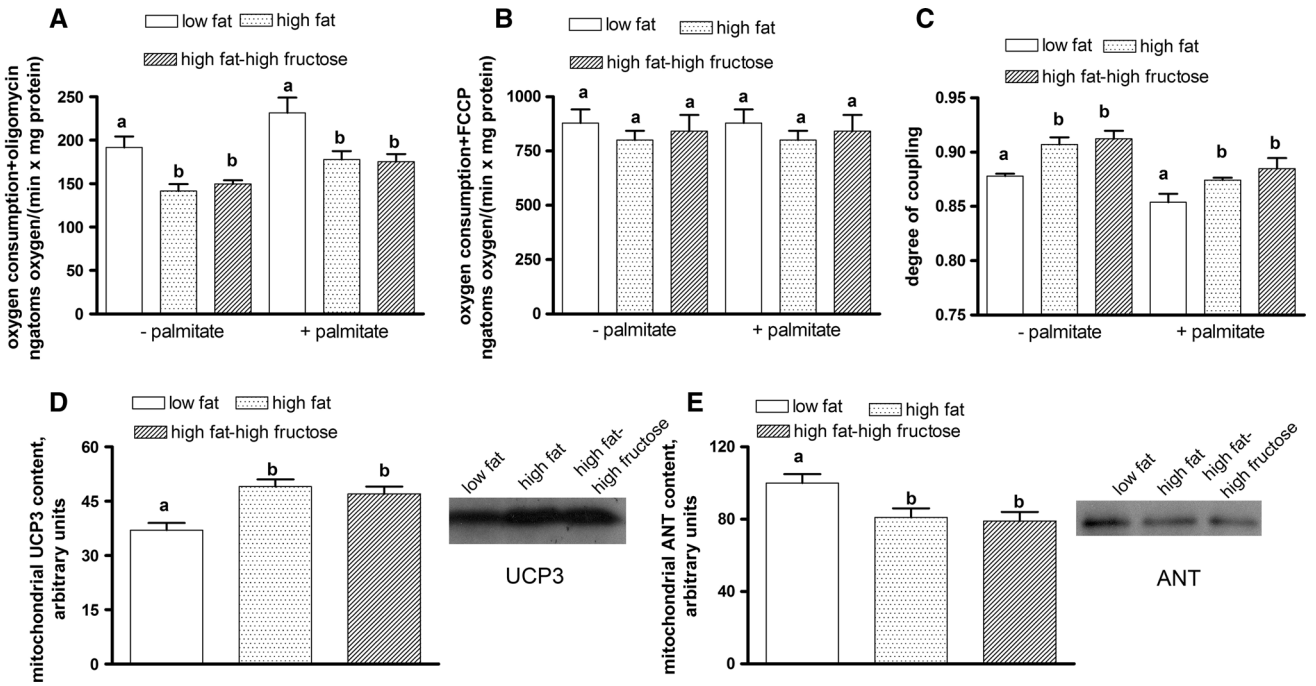

Fig. 2 Oxygen consumption in the presence of oligomycin (**a**) or uncoupled by FCCP (**b**), degree of coupling values calculated from oxygen consumption in the presence of oligomycin and uncoupled by FCCP (**c**), in the absence and in the presence of palmitate, uncoupling protein 3 (UCP3) (**d**) and adenine nucleotide translocase (ANT) content (**e**) (with representative blots) in skeletal muscle mitochondria from rats fed a low-fat, high-fat or high-fat–high-fructose diet for 2 weeks. Values are the means, with their standard errors represented by *vertical bars*, of six rats per group. Means without a *common letter* are significantly different, $P < 0.05$ (one-way ANOVA followed by Tukey's post-test)

Table 4 Oxidative status in skeletal muscle mitochondria in rats fed a low-fat, high-fat or high-fat–high-fructose diet for 2 weeks

	Low-fat	High-fat	High-fat–high-fructose
Active aconitase (mU/mg protein)	106.1 ± 4.9[a]	90.9 ± 6.2[a]	98.5 ± 5.8[a]
Total aconitase (mU/mg protein)	128.9 ± 5.2[a]	115.9 ± 8.3[a]	120.3 ± 13.0[a]
Active aconitase/total aconitase	0.83 ± 0.05[a]	0.80 ± 0.06[a]	0.85 ± 0.06[a]
Lipid peroxidation (nmol of thiobarbituric acid reactive substances/mg protein)	5.26 ± 0.25[a]	5.39 ± 0.48[a]	5.38 ± 0.33[a]
Superoxide dismutase (U/mg protein)	203.4 ± 19.9[a]	206.7 ± 12.6[a]	201.2 ± 15.3[a]

Values are the mean ± SEM of 6 different rats. Means without a common letter are significantly different, $P < 0.05$ (one-way ANOVA followed by Tukey's post-test)

of coupling the higher plasma triglycerides and NEFA levels found in rats fed high-fat–high-fructose diet probably worsen lipid overflow to skeletal muscle cell, increasing ectopic deposition. The increased lipid overflow is a direct consequence of the replacement of starch with fructose, and this results point to the harmful effect of adding fructose to a high-fat diet.

Higher degree of coupling should imply increased reactive oxygen species production [38], but skeletal muscle mitochondria in rats fed high-fat or high-fat–high-fructose diet display no sign of increased oxidative stress, probably because of the significant increase in UCP3 mitochondrial content found in these rats. In fact, it has been proposed that UCP3 plays a role in the defence from oxidative damage [39]. The up-regulation of UCP3 is also in line with the increased plasma NEFA, since it is well known that UCP3 synthesis is stimulated in all the conditions characterised by increased plasma NEFA, such as starvation, exercise, and high-fat diet [40].

In conclusion, the extrapolation of our present findings from small rodents to humans suggests that brief periods of high-fat–high-fructose diet even without hyperphagia are extremely dangerous in sedentary human subjects. In fact, this lifestyle is able to increase lipid flow and mitochondrial energetic efficiency in skeletal muscle, with two detrimental metabolic effects: (a) energy sparing that contributes to the early onset of obesity and (b) reduced oxidation of fatty acids in skeletal muscle and consequently

lipid accumulation, which could generate muscle insulin resistance, an initial step in the progression towards type 2 diabetes.

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Conflict of interest The authors declare no conflicts of interest.

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