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Hepatic Mitochondrial Energetics During Catch-Up Fat With High-Fat Diets Rich in Lard or Safflower Oil

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We have investigated whether altered hepatic mitochondrial energetics could explain the differential effects of high-fat diets with low or high ω6 polyunsaturated fatty acid content (lard vs. safflower oil) on the efficiency of body fat recovery (catch-up fat) during refeeding after caloric restriction. After 2 weeks of caloric restriction, rats were isocalorically refed with a low-fat diet (LF) or high-fat diets made from either lard or safflower oil for 1 week, and energy balance and body composition changes were assessed. Hepatic mitochondrial energetics were determined from measurements of liver mitochondrial mass, respiratory capacities, and proton leak. Compared to rats refed the LF, the groups refed high-fat diets showed lower energy expenditure and increased efficiency of fat gain; these differences were less marked with high-safflower oil than with high-lard diet. The increase in efficiency of catch-up fat by the high-fat diets could not be attributed to differences in liver mitochondrial activity. By contrast, the lower fat gain with high-safflower oil than with high-lard diet is accompanied by higher mitochondrial proton leak and increased proportion of arachidonic acid in mitochondrial membranes. In conclusion, the higher efficiency for catch-up fat on high-lard diet than on LF cannot be explained by altered hepatic mitochondrial energetics. By contrast, the ability of the high-safflower oil diet to produce a less pronounced increase in the efficiency of catch-up fat may partly reside in increased incorporation of arachidonic acid in hepatic mitochondrial membranes, leading to enhanced proton leak and mitochondrial uncoupling.

INTRODUCTION

It is well known that after a period of weight loss or growth retardation, the recovery of body weight is accompanied by a faster rate of body fat gain compared to protein gain (1). The fact that this phenomenon of preferential catch-up fat persists in the absence of hyperphagia, and during refeeding on well-balanced diets (2) suggests that an enhanced efficiency for fat deposition is a fundamental physiological reaction to weight loss or growth retardation. Such an elevated efficiency for recovering the body's fat energy reserves probably had survival advantage in an ancestral lifestyle of intermittent food availability. Nowadays, however, it is a factor that contributes to obesity relapse after therapeutic slimming and to excessive fat accumulation during catch-up growth, particularly during nutritional rehabilitation on energy-dense fatty foods.

Indeed, past studies of refeeding after food restriction in the rat have shown that the high metabolic efficiency that drives catch-up fat on a low-fat diet (LF) is exacerbated by refeeding on a high-fat diet (3,4), albeit to varying degrees depending upon the fatty acid composition of the diet (5). Among the various dietary fat types studied, refeeding with a high-fat diet made from lard (rich in saturated and monounsaturated fatty acids, but low in polyunsaturated fatty acids) resulted in the most pronounced exacerbation in the efficiency of catch-up fat. Conversely, isocaloric refeeding with a high-fat diet made from safflower oil (rich in ω6 polyunsaturated fatty acid, linoleic acid) produced the least exacerbation of the efficiency for catch-up fat (5).

Given our recent findings suggesting that, during refeeding on a LF, the liver contributes importantly to the phenomenon of preferential catch-up fat through enhanced *de novo* lipogenesis supported by an increase in hepatic mitochondrial oxidative capacity (6), we investigated here the extent to which altered hepatic mitochondrial energetics may underlie the differential exacerbation of catch-up fat by diets rich in lard or safflower

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oil. To this end, after evaluating the impact of these fat types on energy balance during high-fat refeeding, we measured state 3 and state 4 respiratory capacities in liver homogenates and isolated mitochondria using nicotinamide adenine dinucleotide and flavin adenine dinucleotide substrates, as well as mitochondrial proton leak. Furthermore, mitochondrial oxidative damage and antioxidant defence were also determined from measurements of lipid peroxidation as well as aconitase, and superoxide dismutase (SOD) specific activity. Finally, we determined the activity of hepatic stearoyl-coenzyme A desaturase 1 (SCD-1), an enzyme that is considered to play an important role in the development of obesity induced by saturated high-fat diets (7).

METHODS AND PROCEDURES

Experimental design

Male Sprague–Dawley rats were caged singly in a temperature-controlled room (23 ± 1 °C) with a 12-h light/dark cycle and adapted to room and cage environments for 1 week. The experiments were conducted after this period of adaptation on rats selected on the basis of body weight being within ±5 g of the mean body weight. The experimental design of semistarvation-refeeding was identical to that previously reported (3–5). Specifically, four groups of 7-week-old rats ($n = 8$) with a body weight of 232 ± 5 g were restricted for 14 days at ~50% of their *ad libitum* food (chow) intake, corresponding to 165 kJ metabolizable energy (ME)/day. Restriction was carried out using pelleted chow diet (Mucedola, Settimo Milanese, Italy) consisting, by energy, of 29.0% protein, 60.4% carbohydrates, and 10.6% fat. Such a reduction in food intake leads to growth arrest, as shown by the fact that at the end of this period, body weight of the rats was 229 ± 4 g (**Figure 1**). After this period, one group was killed for the determination of body energy, lipid, and protein content at the end of food restriction (i.e., prior to refeeding), while the other three groups were refed an amount of energy equal to the energy content of the *ad libitum* food intake of spontaneously growing rats of similar body weight (330 kJ ME/day). Refeeding was carried out for 1 week by using a LF, a high-fat diet made from lard (HF-L), or a high-fat diet made from safflower oil (HF-S), which is rich in unsaturated fatty acids, and specifically in linoleic acid (18:2 ω6). Each refed rat consumed all the food provided on a daily basis (i.e., 330 kJ ME/day) throughout the entire refeeding period. The nutrient composition and fatty acid composition of these three refeeding diets are reported in **Table 1**. At the end of this 1-week period of isocaloric refeeding on low-fat or high-fat diets, the animals were killed, liver removed for biochemical assays, and the rest of the body processed for determination of body energy, fat, and protein. Animals were maintained in accordance with Italian Health Ministry regulations and guidelines for the care and use of laboratory animals.

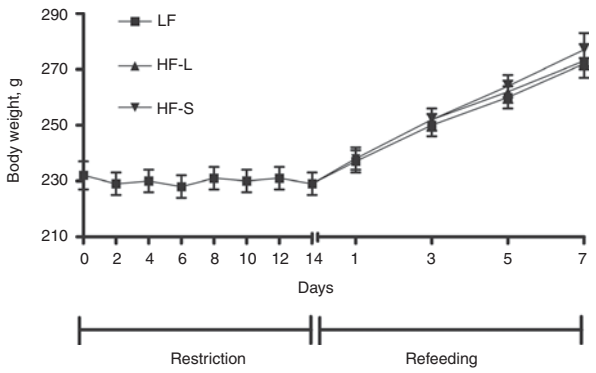

Figure 1 Body weight changes over time during food restriction and refeeding in rats refed with low-fat diet (LF), high-fat diet rich in saturated fatty acids (HF-L) or high-fat diet rich in unsaturated fatty acids (HF-S). Values are the means ± s.e.m. of eight different experiments.

Body composition

Guts were cleaned of undigested food and the carcasses were then autoclaved. After dilution (1:2 distilled water) and subsequent homogenization of the carcasses with a Polytron homogenizer (Kinematica, Luzern, Switzerland), the resulting homogenates were frozen at −20 °C until the day of measurements. Duplicate samples of the homogenized carcass were analyzed for energy content by bomb calorimetry. To take into account the energy content of the liver, tissue samples were dried and the energy content was then measured with the bomb calorimeter. Total body fat content was measured by the Folch extraction method (8). Total body water content was determined by drying carcass samples in an oven at 70 °C for 48 h. Total body protein content was determined using

Table 1 Composition, energy content, and fatty acid composition (g/100 g fatty acids) of experimental diets

Component, g	LF	HF-L	HF-S
Standard chow	479	479	479
Casein	94.5	94.0	94.0
Methionine	1	1	1
Choline	0.7	0.7	0.7
AIN vitamin mix	3.7	3.7	3.7
AIN mineral mix	13.4	13.4	13.4
Sunflower oil	11.7	11.7	11.7
Sucrose	191	—	—
Glucose	205	—	—
Lard	—	175	—
Safflower oil	—	—	175
Total weight	1000.0	778.5	778.5
Metabolizable energy, kJ	13980	13934	13934
Protein, % energy[a]	22.05	22.06	22.06
Lipid, % energy[a]	7.02	54.35	54.35
Carbohydrate, % energy[a]	70.93	23.59	23.59
Fatty acid			
4:0–10:0	—	0.22	—
12:0	—	0.22	—
14:0	—	1.27	—
16:0	11.92	24.45	7.55
18:0	3.83	16.30	2.48
20:0	0.25	—	—
14:1ω5	—	0.48	—
16:1ω7	0.58	2.52	0.07
18:1ω9	27.99	39.51	14.31
20:1ω9	1.10	1.11	0.14
22:1ω9	0.79	0.11	0.10
18:2ω6	51.14	12.59	73.26
18:3ω3	2.41	1.20	2.09

HF-L, high-fat diet rich in saturated fatty acids; HF-S, high-fat diet rich in unsaturated fatty acids; LF, low-fat diet.
[a]Estimated by computation using values (kJ/g) for energy content as follows: protein 16.736, lipid 37.656, and carbohydrate 16.736.

a formula relating total energy value of the carcass, energy derived from fat, and energy derived from protein (3); the caloric values for body fat and protein were taken as 39.2 and 23.5 kJ/g, respectively (9).

Plasma FFAs and tumor necrosis factor-α levels, liver lipid content and FAS activity

Plasma was obtained from blood samples and free fatty acid (FFA) levels were measured by colorimetric enzymatic method (Roche Diagnostics, Mannheim, Germany), while tumor necrosis factor-α levels were measured by ELISA kit (R&D Systems, Minneapolis, MN).

Samples of liver tissue were homogenized with distilled water (final volumes equal to twice the sample weight) and analyzed for lipid content by the method of Folch *et al.* (8). Fatty acid synthase (FAS) activity was measured in liver homogenates according to the protocol described by Pénicaud *et al.* (10).

Energy balance

Energy balance measurements were conducted by the comparative carcass technique over 1 week of refeeding, as detailed previously (11). Briefly, during the experimental period, ME intake was determined by subtracting the energy measured in feces and urine from the gross energy intake, determined from daily food consumption and gross energy density of the diet. Body energy, protein, and fat gain were calculated as the difference between the final and initial content of body energy, protein, and fat; the initial values being obtained from the group killed at the end of the food restriction period. Gross efficiency was calculated as the percentage of body energy retained per ME intake. Finally, energy expenditure was determined as the difference between ME intake and energy gain.

Liver mitochondrial respiration, proton leak, lipid peroxidation, and SCD-1, aconitase, and SOD activity

Liver homogenates and isolated mitochondria were prepared as previously reported (12). Control experiments of enzymatic and electron microscopy characterization have shown that our isolation procedure (centrifugation at 3,000g_{av} for 10 min) results in a cellular fraction essentially constituted by mitochondria. In fact, the activity of marker enzymes of the main cellular compartments, namely 5′-AMP phosphatase for plasma membrane, uricase for peroxisomes, acid phosphatase for lysosomes and glucose-6-phosphatase for microsomes, was undetectable when measured in our mitochondrial preparations. Oxygen consumption was measured polarographically with a Clark-type electrode (Yellow Springs Instruments, Yellow Springs, OH) at a temperature of 30 °C. Homogenates or isolated mitochondria were incubated in a medium containing 80 mmol/l KCl, 50 mmol/l HEPES, 5 mmol/l K_2HPO_4, 1 mmol/l EGTA, 0.1% (w/v) fatty acid-free bovine serum albumin, pH 7.0 and were allowed to oxidize their endogenous substrates for 3 min. Either 10 mmol/l glutamate + 2.5 mmol/l malate, or 40 μmol/l palmitoyl-coenzyme A + 2 mmol/l carnitine + 2.5 mmol/l malate, or succinate 10 mmol/l + rotenone 3.75 μmol/l were added as substrate. State 3 oxygen consumption was measured in the presence of 0.3 mmol/l ADP. State 4 respiration was obtained in the presence of oligomycin (4 μg/ml) in the homogenates or in the absence of ADP in isolated mitochondria. Respiratory control ratio was calculated according to Estabrook (13). SCD-1 activity was measured polarographically in liver homogenates at 37 °C in a solution containing 0.1 mol/l K_2HPO_4, pH 7.4, 1 μmol/l myxothiazol, 0.12 mmol/l NADH and 0.06 mmol/l stearoyl-CoA as cyanide (5 mmol/l)-sensitive (14), myxothiazol insensitive oxygen consumption.

Mitochondrial proton leak was assessed by titration of steady state respiration rate as a function of mitochondrial membrane potential in liver mitochondria. In fact, if the activity of the respiratory chain is titrated with inhibitors in the presence of oligomycin to prevent ATP synthesis, the resulting titration curve of mitochondrial membrane potential against respiration rate represents the kinetic response of the proton leak to changes in membrane potential and is an indirect measurement of proton leak, since steady state oxygen consumption rate (i.e., proton efflux rate) in nonphosphorylating mitochondria is equivalent to proton influx rate due to proton leak. Proton leak kinetics were obtained in isolated mitochondria as previously reported (15). Briefly, oxygen consumption was measured polarographically with a Clark-type electrode (Yellow Springs Instruments) maintained at 30 °C, in a medium containing 80 mmol/l LiCl, 50 mmol/l HEPES, 1 mmol/l EGTA, 50 mmol/l Tris-PO_4, pH 7.0, 0.1% (w/v) fatty acid-free bovine serum albumin. Titration of state 4 respiration was carried out by sequential additions of malonate in the presence of 0.5 mg mitochondrial protein, succinate (10 mmol/l), rotenone (3.75 μmol/l), oligomycin (2 μg/ml), safranin O (83.3 nmol/mg), nigericin (80 ng/ml). Mitochondrial membrane potential recordings were performed in parallel with safranin O using a JASCO dual-wavelength spectrophotometer (511–533 nm). The absorbance readings were transformed into mV membrane potential using the Nernst equation: $\Delta\psi = 61\ mV \cdot \log([K^+]_{in}/[K^+]_{out})$. Calibration curves made for each preparation were obtained from traces in which the extramitochondrial K^+ level ($[K^+]_{out}$) was altered in the 0.1–20 mmol/l range. The change in absorbance caused by the addition of 3 μmol/l valinomycin was plotted against $[K^+]_{out}$. Then, $[K^+]_{in}$ was estimated by extrapolation of the line to the zero uptake point. Basal proton leak was measured in the absence of palmitate through titrations carried out with malonate up to 5 mmol/l. Palmitate-induced proton leak was measured in the presence of 85 μmol/l palmitate through titrations carried out with malonate up to 600 mmol/l.

Lipid peroxidation was determined in isolated mitochondria according to Fernandes *et al.* (16). The specific activities of active aconitase, total aconitase and SOD were measured in isolated mitochondria as previously described (17).

Mitochondrial mass

The activity of the mitochondrial marker enzyme citrate synthase (CS) was measured in liver homogenates and isolated mitochondria according to Srere (18). Mitochondrial mass, expressed as mg/g wet liver, was calculated from the ratio between CS activity in the homogenate and CS activity in isolated mitochondria.

Fatty acid composition of mitochondrial phospholipids

Lipids were extracted from isolated mitochondria as described (8). The extracts were subjected to thin-layer chromatography on silica gel plates with petroleum ether-diethyl ether-acetic acid 50:50:1 (v/v/v). Phospholipids, remaining at the origin, were excised and incubated in 1% (w/v) sulfuric acid in methanol at 50 °C for 16 h to produce fatty acid methyl esters. The latter were extracted with hexane and were separated in a Hewlett Packard 5890 Series II gas chromatograph (Hewlett Packard, Waldbronn, Germany) equipped with a 30-m long AT-WAX capillary column from Alltech (Deerfield, IL) and a flame ionization detector. The column temperature was programmed from 160 to 250 °C at 5 °C/min. The carrier gas was helium at a flow rate of 1 ml/min (at 160 °C). Methyl esters were identified in the chromatograms with the aid of the HP 3365 ChemStation software from Hewlett Packard by comparing their retention times to those of pure methyl esters and were quantified by considering the integrated area under each peak as proportional to mass. The molar amount of each methyl ester (and hence, fatty acid) was then used to calculate its percentage relative to the sum of fatty acids.

Statistical analysis

Data are given as means ± s.e.m. Statistical analyses were performed by one-way ANOVA followed by Tukey 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).

Chemicals

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

Table 2 Body composition, energy balance, and plasma FFA levels in rats refed with low- or high-fat diets

	LF	HF-L	HF-S
Body energy, kJ/g	8.8 ± 0.1	9.7 ± 0.1*	9.4 ± 0.2*
Body fat, %	12.7 ± 0.6	17.3 ± 0.4*	15.1 ± 0.3*,**
Body protein, %	16.3 ± 0.5	14.2 ± 0.5*	15.3 ± 0.3
Body water, %	64.3 ± 0.7	61.5 ± 0.5	63.0 ± 0.3
Hepatic lipids, mg/g	41.7 ± 1.1	58.8 ± 5.0*	61.9 ± 3.7*
Plasma FFA, mmol/l	0.369 ± 0.020	0.544 ± 0.010*	0.393 ± 0.021**
ME intake, kJ	2319 ± 24	2296 ± 16	2290 ± 33
Energy gain, kJ	1099 ± 34	1490 ± 83*	1315 ± 57*
Fat gain, kJ	910 ± 70	1397 ± 30*	1200 ± 47*,**
Protein gain, kJ	197 ± 15	93 ± 6*	124 ± 8*,**
Energy expenditure, kJ	1220 ± 46	806 ± 98*	975 ± 61*,**
Gross efficiency, %	45 ± 2	65 ± 2*	57 ± 2*,**

Values are means ± s.e.m. of eight different experiments. Values of ME intake, energy expenditure, gross efficiency and protein, lipid and energy gain refer to the whole experimental period. Energy expenditure was calculated from the difference between ME intake and energy gain.
FFA, free fatty acid; HF-L, high-fat diet rich in saturated fatty acids; HF-S, high-fat diet rich in unsaturated fatty acids; LF, low-fat diet.
*$P < 0.05$ compared to LF; **$P < 0.05$ compared to HF-L (one-way ANOVA followed by Tukey test).

RESULTS

Table 2 shows the data on body composition of rats refed for 1 week with diets differing in lipid content (high-fat vs. low-fat), as well as in fatty acid composition (HF-L vs. HF-S). The three groups of rats displayed similar body weight at the end of refeeding (Figure 1), but the group refed the HF-L diet displayed significantly higher body energy and fat, as well as lower body protein, compared to the rats refed with the LF. These differential effects between HF-L vs. LF groups were however less marked in rats refed with the HF-S diet. Thus, while body energy and fat were higher in the HF-S rats than in LF rats, they were nonetheless lower than those found in HF-L rats. Similarly, although body protein tended to be lower in the HF-S rats than in LF rats, it was higher than in the HF-L animals. Finally, both groups of animals refed on high-fat diets (HF-L and HF-S) displayed significantly higher values for hepatic lipids compared to those refed the LF, while plasma FFA were significantly higher only in HF-L rats. The data on energy balance measurements show that, despite no differences in ME intake across the three groups of rats, HF-L and HF-S had significantly higher energy and fat gain, while protein gain was significantly lower compared to LF rats. Comparison between the two groups refed high-fat diets shows that the energy and fat gain were significantly lower, while protein gain was significantly higher in HF-S than in HF-L group. Both groups refed high-fat diets (HF-L and HF-S) showed significantly lower values of energy expenditure and significantly higher values of gross efficiency, compared to LF rats, but energy expenditure was significantly higher, and gross efficiency significantly lower, in HF-S rats than in HF-L rats.

Liver mitochondrial state 3 and 4 respiratory capacities were first measured in homogenates to take into account changes in protein mass and oxidative capacity of mitochondria of the whole tissue (Figure 2). Using glutamate + malate or palmitoylCoA + carnitine + malate, significantly higher values for state 3 respiratory capacities were found in HF-L rats compared to LF and HF-S rats, while no significant difference was found when using succinate. In addition, a significant decrease in hepatic FAS activity was found in HF-L and HF-S rats compared to LF rats, while SCD-1 activity was significantly lower only in HF-L rats compared to LF and HF-S rats.

Respiratory capacities were also measured in isolated liver mitochondria to take into account changes in specific oxidative capacity (Figure 3). Using glutamate + malate or palmitoyl-CoA + carnitine + malate, significantly higher values for state 3 respiratory capacities were found in HF-L rats compared to LF and HF-S rats, while no significant difference was found when using succinate.

Figure 4 shows the titrations of respiration rate as a function of mitochondrial membrane potential, that are an indirect measure of mitochondrial proton leak. Nonlinear regression analysis showed that experimental data fitted a sigmoidal dose–response (variable slope) equation. Comparison of nonlinear regression curve fits revealed that basal (Figure 4a) and palmitate-induced (Figure 4b) proton leak were significantly higher in liver mitochondria from HF-S rats compared to LF and HF-L rats. To assess oxidative damage and antioxidant defence in liver mitochondria, lipid peroxidation, aconitase specific activity, and SOD specific activity were tested (Figure 4c). The results show no significant changes in oxidative damage, while a significant decrease in SOD activity and hence in antioxidant defences was found only in HF-S rats compared to LF and HF-L rats. In addition, we found no differences in plasma tumor necrosis factor-α levels, biomarkers of systemic inflammation.

No differences were found in CS activity measured in the homogenate (LF = 10.0 ± 0.2, HF-L = 9.7 ± 0.4, HF-S = 9.9 ± 0.5 μmol/min × g wet liver), as well as in CS-specific activity measured in isolated mitochondria (LF = 0.28 ± 0.01, HF-L = 0.28 ± 0.01, HF-S = 0.26 ± 0.01 μmol/min × mg protein). Therefore, mitochondrial mass, calculated as the ratio between CS activity in the homogenate and isolated mitochondria, was not significantly different in the three groups of rats (LF = 36.9 ± 1.8, HF-L = 34.7 ± 1.8, HF-S = 36.6 ± 2.1 mg/g wet liver).

Determination of fatty acid composition of mitochondrial phospholipids (Table 3) shows a significant increase in unsaturation index in HF-S rats compared to LF rats, with a decrease in the percentage of total saturated fatty acids (40 vs. 46–47%) and a concomitant increase in the percentage of total unsaturated fatty acids (60 vs. 53–54%). Analysis of the data on the two classes of unsaturated fatty acids indicate that in HF-L and HF-S rats a decrease in monounsaturated fatty acids was

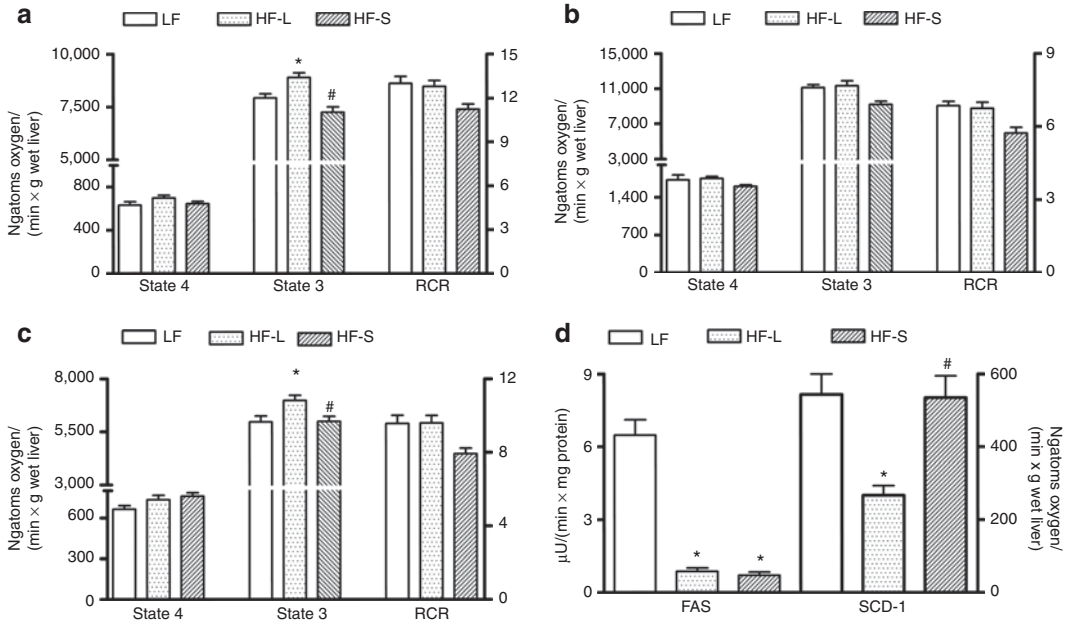

Figure 2 Mitochondrial state 3 respiration, state 4 respiration and RCR values using (**a**) glutamate + malate, (**b**) succinate + rotenone, or (**c**) palmitoylCoA + carnitine + malate as substrate, as well as (**d**) FAS activity and SCD-1 activity in liver homogenates from rats refed with low-fat diet (LF), high-fat diet rich in saturated fatty acids (HF-L) or high-fat diet rich in unsaturated fatty acids (HF-S). Values are means ± s.e.m. of eight different experiments. **P* < 0.05 compared to LF, #*P* < 0.05 compared to HF-L (one-way ANOVA followed by Tukey test). FAS, fatty acid synthase; RCR, respiratory control ratio; SCD-1, stearoyl CoA desaturase 1.

associated with an increase in polyunsaturated fatty acids. In addition, a significant increase in ω6 fatty acids, in particular in arachidonic acid (20:4ω6), was found in HF-S rats compared to HF-L or LF rats. Finally, the bioconcentration of docosahexaenoic acid in mitochondrial phospholipids found by us in the three groups of rats is in line with other reports (19–21) and can be explained taking into account that linolenic acid is a preferential substrate to linoleic acid for Δ6-desaturase (22,23).

DISCUSSION

The most important findings in the present study comparing hepatic mitochondrial energetics in rats refed isocalorically a LF vs. high-fat diets rich in lard or safflower oil can be summarized as follows: (i) the changes in hepatic mitochondrial energetics cannot explain the higher efficiency of body fat recovery (catch-up fat) on the high-lard diet than on the LF; (ii) the less pronounced increase in the efficiency of catch-up fat, and hence lower fat gain, on the high-safflower oil diet (rich in linoleic acid) than on the high-lard diet could result from an increased hepatic proton leak, possibly induced by increased incorporation of arachidonic acid (the main metabolite of linoleic acid) in the inner membrane of liver mitochondria.

Exacerbated efficiency of catch-up fat

The results presented here on energy balance are in line with previous findings suggesting that the elevated energetic efficiency for catch-up fat is exacerbated by high-fat diets (3,4), albeit to a much lesser extent when the fat source derives from safflower oil than from lard (5). In fact, compared to rats that were refed the LF, the increase in efficiency for catch-up fat and the decrease in energy expenditure found here are less pronounced during refeeding in rats refed with the high-lard diet than in rats refed with the high-safflower oil diet.

Fatty liver

Another undesirable consequence of refeeding rats with high-fat diets is the induction of fatty liver, i.e., an increase in hepatic lipid deposition that is known to be one of the two "hits" for the induction of nonalcoholic steatohepatitis (24). This increase occurs even if *de novo* lipogenesis is reduced, as shown by decreased hepatic FAS activity in both groups refed with high-fat diets. This result is different from that previously obtained in rats refed with a LF, in which no increase in hepatic lipid content was found, compared to controls, despite these rats exhibited markedly higher hepatic FAS activity and *de novo* lipogenesis than controls (6). An interesting observation from our present study here and that previously reported (6) is that fatty liver seems to be associated with the lipid content of the diet rather than with the occurrence of *de novo* lipogenesis. In addition, in the use of high-fat diets during nutritional rehabilitation, a high proportion of unsaturated fatty acids can be valuable in limiting fat deposition and favoring protein deposition, but the issue of hepatic steatosis remains unresolved.

SCD-1 activity

The robust decrease in hepatic SCD-1 activity in rats refed the high-lard diet is in agreement with previous reports obtained after 1 week of high-fat feeding in spontaneously growing control animals (25,26). This enzyme, which catalyzes the

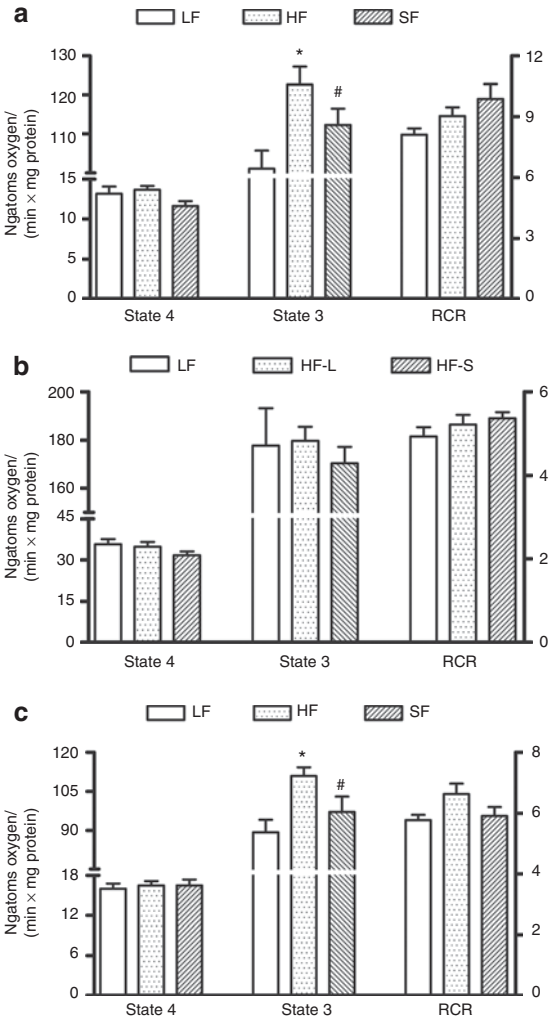

Figure 3 Mitochondrial state 3 respiration, state 4 respiration and RCR values using (**a**) glutamate + malate, (**b**) succinate + rotenone, or (**c**) palmitoylCoA + carnitine + malate as substrate in liver mitochondria from rats refed with low-fat diet (LF), high-fat diet rich in saturated fatty acids (HF-L) or high-fat diet rich in unsaturated fatty acids (HF-S). Values are means ± s.e.m of eight different experiments. *$P < 0.05$ compared to LF, $^{\#}P < 0.05$ compared to HF-L (one-way ANOVA followed by Tukey test). RCR, respiratory control ratio.

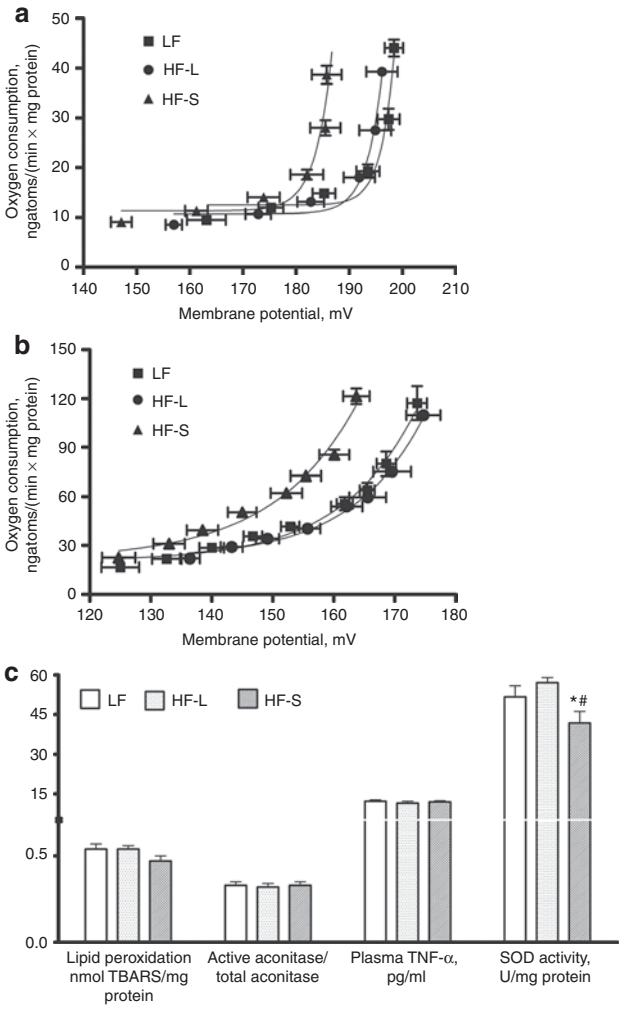

Figure 4 Mitochondrial coupling and oxidative status assessed by (**a**) basal proton leak kinetics, (**b**) palmitate-induced proton leak kinetics and (**c**) markers of oxidative stress and antioxidant defenses in liver mitochondria from rats refed with low-fat diet (LF), high-fat diet rich in saturated fatty acids (HF-L) or high-fat diet rich in unsaturated fatty acids (HF-S). Values are the means ± s.e.m. of eight different experiments. Nonlinear regression curve fit showed that proton leak was significantly ($P < 0.05$) higher in mitochondria from HF-S rats compared to LF and HF-L rats. *$P < 0.05$ compared to LF, $^{\#}P < 0.05$ compared to HF-L (one-way ANOVA followed by Tukey test). SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TNF, tumor necrosis factor.

rate-limiting reaction of monounsaturated fatty acid synthesis, has been implicated in the development of obesity on the basis that mice null for *SCD-1* gene have reduced adiposity and increased energy expenditure (7). However, our present data suggest that physiological decreases in hepatic SCD-1 are not protective against excess fat deposition during catch-up fat. As hepatic SCD-1 is suppressed by leptin (27) but induced by insulin (28), the possibility arises that our findings of the lower hepatic SCD-1 activity during refeeding on the high-lard diet may result from the combination of higher leptinemia and lower insulin sensitivity that characterize rats showing catch-up fat on the high-lard diet compared to those on the LF (4,5). Consistent with this contention are our findings here that refeeding on the high-safflower oil diet, which was previously shown to result in similar insulin sensitivity as refeeding on the LF (5), did not result in depressed SCD-1 activity.

Hepatic respiratory capacity

Our results show that refeeding with a diet rich in saturated fat as lard leads to an increase in liver mitochondrial respiratory capacity, but also that no such inductions are observed when the lard is substituted by safflower oil. This increase, due to the high-lard diet, can be detected both in homogenates and in isolated mitochondria and occurs without any change in hepatic mitochondrial mass between the three groups of rat. It can be suggested that refeeding rats with high-fat diets rich in saturated

Table 3 Percentage molar distribution of fatty acids and indices of the fatty acid profile in hepatic mitochondrial phospholipids of rats refed with low- or high-fat diets

Fatty acid, %	LF	HF-L	HF-S
12:0	0.10 ± 0.03	0.17 ± 0.05	0.16 ± 0.04
14:0	1.16 ± 0.15	1.14 ± 0.23	0.86 ± 0.15
16:0	21.56 ± 0.70	16.86 ± 0.53*	15.02 ± 0.47*
16:1ω7	1.79 ± 0.13	1.15 ± 0.42	1.48 ± 0.31
18:0	24.19 ± 0.79	27.61 ± 1.32	24.14 ± 0.68**
18:1ω9	5.42 ± 0.42	4.14 ± 0.22*	3.33 ± 0.23*
18:1ω7	2.50 ± 0.43	1.17 ± 0.12*	1.72 ± 0.11
18:2ω6	8.04 ± 0.78	7.83 ± 1.00	10.20 ± 0.54
18:3ω6	0.15 ± 0.02	0.22 ± 0.03	0.32 ± 0.14
18:3ω3	0.47 ± 0.12	0.62 ± 0.09	0.49 ± 0.12
18:4ω3	2.16 ± 0.42	2.85 ± 0.72	2.02 ± 0.41
20:1ω9	0.16 ± 0.04	0.15 ± 0.04	0.26 ± 0.05
20:3ω6	1.29 ± 0.13	0.36 ± 0.14*	0.42 ± 0.15*
20:4ω6	11.17 ± 1.11	14.28 ± 1.12	19.94 ± 1.42*,**
22:5ω6	0.65 ± 0.20	0.80 ± 0.26	0.94 ± 0.10
22:5ω3	1.23 ± 0.31	1.80 ± 0.28	1.07 ± 0.25
22:6ω3	17.91 ± 2.11	18.86 ± 2.42	17.63 ± 0.90
Total	100.00	100.00	100.00
Saturated, %	47.0 ± 0.8	45.8 ± 0.9	40.2 ± 0.5*,**
Unsaturated, %	53.0 ± 0.8	54.2 ± 0.9	59.8 ± 0.5*,**
Monounsaturated, %	9.9 ± 0.6	6.6 ± 0.5*	6.8 ± 0.5*
Polyunsaturated, %	43.1 ± 1.2	47.6 ± 1.0*	53.0 ± 0.7*,**
ω6, %	21.3 ± 1.8	23.5 ± 2.0	31.8 ± 1.8*,**
ω3, %	21.8 ± 2.8	24.1 ± 2.7	21.2 ± 1.4
Unsaturation index	202 ± 10	221 ± 10	235 ± 3*

Values are the means ± s.e.m. of eight different experiments.
HF-L, high-fat diet rich in saturated fatty acids; HF-S, high-fat diet rich in unsaturated fatty acids; LF, low-fat diet.
*$P < 0.05$ compared to LF, **$P < 0.05$ compared to HF-L (one-way ANOVA followed by Tukey test).

fatty acids is able to upregulate the activity of rate-limiting enzymes for the oxidation of lipid and nicotinamide adenine dinucleotide-linked substrates. In fact, the effect of the high-fat lard diet to induce the increase in mitochondrial respiration capacity is evident when using nicotinamide adenine dinucleotide-linked or lipid substrates, but not with flavin adenine dinucleotide-linked substrate. This result is different from those previously reported in rats fed a high-fat diet *ad libitum* (29), when no differences in hepatic mitochondrial oxidative capacity was found after 2 weeks of dietary treatment. This increase in hepatic mitochondrial respiratory capacity during high-lard refeeding can be explained by the enhanced plasma FFA found in HF-L rats. In fact, increased plasma FFA could give rise to increased intracellular FFA levels, and FFA are natural ligands of peroxisome proliferator-activated receptor (30). Since peroxisome proliferator-activated receptor-α, which is expressed in liver, regulates the transcription of genes coding for enzymes of the mitochondrial fatty acid oxidation pathway (31), it can be speculated that increased plasma FFA here found could stimulate their own oxidation via peroxisome proliferator-activated receptor-α activation in the liver. We suggest that these changes likely represent a homeostatic response to attempt to compensate for elevated availability of lipids in the liver. However, this mechanism fails to dispose excess fatty acids, that are consequently accumulated in liver and adipose tissue, probably since their hepatic oxidation is limited by cellular ATP needs. Overall, our suggestion of a link between increased plasma FFA and increased hepatic mitochondrial oxidative capacity in rats refed the high-lard diet is in line with the finding that in rats refed the high-safflower oil diet, in which no difference is found in plasma FFA levels relative to the LF group, there is also no difference in hepatic mitochondrial respiratory capacity.

Hepatic mitochondrial uncoupling

As mitochondrial proton leak is considered to be an important factor in the efficiency of energy conversion in several tissues (including the liver), we therefore assessed whether the differential effects of diets on whole-body metabolic efficiency and catch-up fat could be attributed to altered hepatic mitochondrial proton leak. Our results however indicate that there are no differences in rats refed with the high-lard diet and the LF in the kinetics of proton leak in liver mitochondria. By contrast, the less pronounced increase in the efficiency of catch-up fat during refeeding on the high-safflower oil diet could arise from decreased efficiency at the level of hepatic mitochondria, since basal and palmitate-induced proton leak in the liver were significantly increased with this diet. The fatty acid composition of mitochondrial phospholipids reveals an increased unsaturated content in rats refed the high-safflower oil diet compared to the other diets. As this increase results mostly from an increase in arachidonic acid (20:4ω6)—the main metabolite of linoleic acid which constitutes virtually all the ω6 polyunsaturated fatty acid content of safflower oil—it follows that the changes in mitochondrial inner membrane permeability may be linked to the composition of the diet. In fact, an increased percentage of unsaturated fatty acids, in particular polyunsaturated fatty acids, in mitochondrial membranes has been correlated with increased proton leak (32). Therefore, the increased incorporation of arachidonic acid found here in liver mitochondrial membrane of rats refed the high-safflower oil diet may play an important role in the induction of proton leak. Accordingly, it has been shown that arachidonic acid content is positively correlated with proton flux (33). Gerson *et al.* (21) found a direct correlation between proton leak and linoleic and arachidonic acid mitochondrial levels, and Stillwell *et al.* (34) have shown that mitochondria from old rats contain more arachidonic acid and have lower mitochondrial coupling.

One of the postulated roles for proton leak is to control the production of reactive oxygen species by the mitochondria, by keeping the membrane potential below a critical threshold (35). Therefore, the increased proton leak found in liver mitochondria from rats refed the high-safflower oil diet could also contribute to avoid oxidative damage. In fact, no variation in aconitase activity and in lipid peroxidation, taken as index of

oxidative damage, was found in these rats, despite the decrease in SOD activity, taken as index of antioxidant defence. The absence of oxidative stress is also indicated by the finding of no significant variation in plasma levels of tumor necrosis factor-α in the three groups of rats.

In conclusion, our results indicate that the fatty acid composition of the diet during high-fat refeeding affects body fat deposition during weight recovery after caloric restriction. In fact, the exaggerated suppression of thermogenesis that exacerbates catch-up fat on high-fat diets is to some extent counteracted by a diet rich in ω6 fatty acids, in part by a shift in energy partitioning from body fat to protein, and in part via increased thermogenesis which is contributed by increased hepatic mitochondrial proton leak and decreased mitochondrial efficiency. It has been shown that mitochondrial proton leak accounts for about 20% of liver metabolic rate, and since liver contribution to resting metabolism is about 20%, it follows that hepatic mitochondrial proton leak contribution to resting metabolism is about 4% (36). Therefore, an increase in hepatic mitochondrial proton leak can influence whole-body energy expenditure, even if our study does not allow quantitative estimation of the contribution of increased proton leak to total increase in whole-body energy expenditure. Other mechanisms and other organs can also contribute to the increased energy expenditure induced by refeeding the high-fat diet enriched with safflower oil. In particular, diets rich in safflower oil have been shown to enhance sympathetic activity and uncoupling protein 1 in brown adipose tissue, albeit in spontaneously growing rats (37,38).

Our results here in liver, together with those from previous studies in skeletal muscle and adipose tissue using this same rat model of semistarvation-refeeding, suggest that these organs and tissues co-ordinately contribute to catch-up fat via different pathways, as depicted in **Figure 5**. In fact, during refeeding with LF, skeletal muscle, through a reduction in mass and

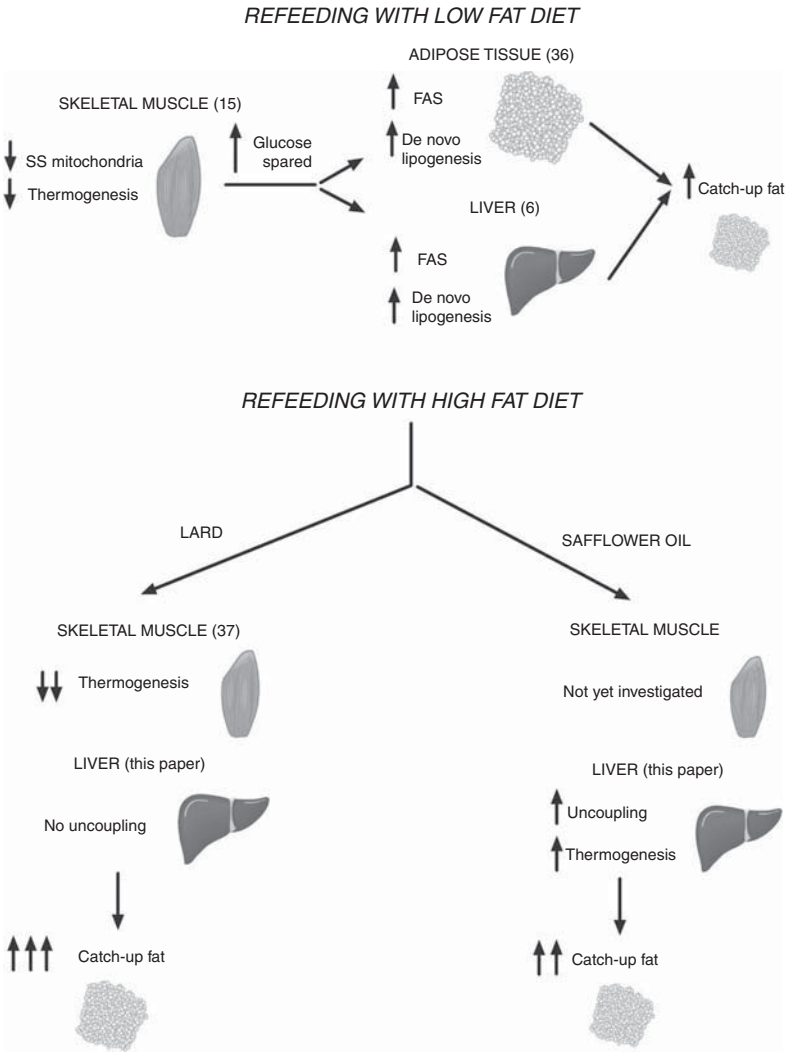

Figure 5 Interaction between different tissues during refeeding with low-fat or high-fat diets. With low-fat diet, skeletal muscle contributes to the suppressed thermogenesis through a reduction in mass and respiratory activity of subsarcolemmal mitochondria (15), while liver and adipose tissue contribute to catch-up fat via an enhancement in *de novo* lipogenesis (6,39). With high-fat diets, both insulin resistance and thermogenesis suppression are exacerbated in skeletal muscle (40), while liver partly counteracts the catch-up fat through diminished mitochondrial efficiency. Numbers in parentheses indicate the references. FAS, fatty acid synthase; SS, subsarcolemmal.

respiratory activity of subsarcolemmal mitochondria, could be contributing directly to the suppressed thermogenesis that favors glucose sparing for catch-up fat (15). The liver and adipose tissue, by contrast, through enhanced FAS activity, might be contributing to catch-up fat via an enhancement in their machinery for *de novo* lipogenesis (6,39). As for catch-up fat with high-fat diets, available data show that, in skeletal muscle, lard-rich diet induces a further decrease in PI3K signaling as well as in AMPK activation, thus exacerbating both insulin resistance and thermogenesis suppression (40). By contrast, it seems that the role of the hepatic tissue in the phenomenon of catch-up fat depends on the composition of the diet. In fact, here we show that, with high-fat diets rich in ω6 fatty acids, the liver partly counteracts the exacerbation in catch-up fat through diminished mitochondrial efficiency. It remains to be investigated whether similar lowering of hepatic mitochondrial efficiency can also account for some of the effects of high-fat diets rich in ω3 fatty acids (α-linolenic acid) in reducing excessive fat storage during refeeding (41). Whether the replacement of some of the ω6 fatty acids by ω-3 fatty acids would also protect against the increase in liver lipids induced by high-fat refeeding warrants further study in the search for optimal nutritional rehabilitation diets that would favor rapid weight regain while limiting the excessive deposition of fat in adipose tissues and liver.

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DISCLOSURE

The authors declared no conflict of interest.

REFERENCES

1. Keys A, Brozek J, Henschel A *et al*. *The Biology of Human Starvation*. University of Minnesota Press: Minneapolis, MN, 1950.
2. Dulloo AG, Jacquet J, Montani JP. Pathways from weight fluctuations to metabolic diseases: focus on maladaptive thermogenesis during catch-up fat. *Int J Obes Relat Metab Disord* 2002;26 Suppl 2:S46–S57.
3. Dulloo AG, Girardier L. Influence of dietary composition on energy expenditure during recovery of body weight in the rat: implications for catch-up growth and obesity relapse. *Metabolism* 1992;41:1336–1342.
4. Crescenzo R, Samec S, Antic V *et al*. A role for suppressed thermogenesis favoring catch-up fat in the pathophysiology of catch-up growth. *Diabetes* 2003;52:1090–1097.
5. Dulloo AG, Mensi N, Seydoux J, Girardier L. Differential effects of high-fat diets varying in fatty acid composition on the efficiency of lean and fat tissue deposition during weight recovery after low food intake. *Metabolism* 1995;44:273–279.
6. Crescenzo R, Bianco F, Falcone I *et al*. Hepatic mitochondrial energetics during catch-up fat after caloric restriction. *Metabolism* 2010;59:1221–1230.
7. Sampath H, Miyazaki M, Dobrzyn A, Ntambi JM. Stearoyl-CoA desaturase-1 mediates the pro-lipogenic effects of dietary saturated fat. *J Biol Chem* 2007;282:2483–2493.
8. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 1957;226:497–509.
9. Armsby HP. *The Nutrition of Farm Animals*. The Macmillan Company: New York, 1917.
10. Pénicaud L, Ferré P, Assimacopoulos-Jeannet F *et al*. Increased gene expression of lipogenic enzymes and glucose transporter in white adipose tissue of suckling and weaned obese Zucker rats. *Biochem J* 1991;279 (Pt 1):303–308.
11. Iossa S, Lionetti L, Mollica MP, Barletta A, Liverini G. Energy intake and utilization vary during development in rats. *J Nutr* 1999;129:1593–1596.
12. Iossa S, Lionetti L, Mollica MP *et al*. Effect of high-fat feeding on metabolic efficiency and mitochondrial oxidative capacity in adult rats. *Br J Nutr* 2003;90:953–960.
13. Estabrook RW. Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios. *Methods Enzymol* 1967;10:41–47.
14. Strittmatter P, Spatz L, Corcoran D *et al*. Purification and properties of rat liver microsomal stearyl coenzyme A desaturase. *Proc Natl Acad Sci USA* 1974;71:4565–4569.
15. Crescenzo R, Lionetti L, Mollica MP *et al*. Altered skeletal muscle subsarcolemmal mitochondrial compartment during catch-up fat after caloric restriction. *Diabetes* 2006;55:2286–2293.
16. Fernandes MA, Custódio JB, Santos MS, Moreno AJ, Vicente JA. Tetrandrine concentrations not affecting oxidative phosphorylation protect rat liver mitochondria from oxidative stress. *Mitochondrion* 2006;6:176–185.
17. Lionetti L, Mollica MP, Crescenzo R *et al*. Skeletal muscle subsarcolemmal mitochondrial dysfunction in high-fat fed rats exhibiting impaired glucose homeostasis. *Int J Obes (Lond)* 2007;31:1596–1604.
18. Srere PA. Citrate synthase. *Methods Enzymol* 1969;13:3–5.
19. Khairallah RJ, Sparagna GC, Khanna N *et al*. Dietary supplementation with docosahexaenoic acid, but not eicosapentaenoic acid, dramatically alters cardiac mitochondrial phospholipid fatty acid composition and prevents permeability transition. *Biochim Biophys Acta* 2010;1797:1555–1562.
20. Giudetti AM, Sabetta S, di Summa R *et al*. Differential effects of coconut oil- and fish oil-enriched diets on tricarboxylate carrier in rat liver mitochondria. *J Lipid Res* 2003;44:2135–2141.
21. Gerson AR, Brown JC, Thomas R, Bernards MA, Staples JF. Effects of dietary polyunsaturated fatty acids on mitochondrial metabolism in mammalian hibernation. *J Exp Biol* 2008;211:2689–2699.
22. Maniongui C, Blond JP, Ulmann L *et al*. Age-related changes in delta 6 and delta 5 desaturase activities in rat liver microsomes. *Lipids* 1993;28:291–297.
23. Ivanetich KM, Bradshaw JJ, Ziman MR. Delta 6-desaturase: improved methodology and analysis of the kinetics in a multi-enzyme system. *Biochim Biophys Acta* 1996;1292:120–132.
24. Malaguarnera M, Di Rosa M, Nicoletti F, Malaguarnera L. Molecular mechanisms involved in NAFLD progression. *J Mol Med* 2009;87:679–695.
25. de Fourmestraux V, Neubauer H, Poussin C *et al*. Transcript profiling suggests that differential metabolic adaptation of mice to a high fat diet is associated with changes in liver to muscle lipid fluxes. *J Biol Chem* 2004;279:50743–50753.
26. Scheja L, Toedter K, Mohr R *et al*. Liver TAG transiently decreases while PL n-3 and n-6 fatty acids are persistently elevated in insulin resistant mice. *Lipids* 2008;43:1039–1051.
27. Biddinger SB, Miyazaki M, Boucher J, Ntambi JM, Kahn CR. Leptin suppresses stearoyl-CoA desaturase 1 by mechanisms independent of insulin and sterol regulatory element-binding protein-1c. *Diabetes* 2006;55:2032–2041.
28. Mauvoisin D, Rocque G, Arfa O *et al*. Role of the PI3-kinase/mTor pathway in the regulation of the stearoyl CoA desaturase (SCD1) gene expression by insulin in liver. *J Cell Commun Signal* 2007;1:113–125.
29. Iossa S, Lionetti L, Mollica MP, Barletta A, Liverini G. Fat balance and hepatic mitochondrial function in response to fat feeding in mature rats. *Int J Obes Relat Metab Disord* 1999;23:1122–1128.
30. Desvergne B, Wahli W. Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocr Rev* 1999;20:649–688.
31. Michalik L, Wahli W. Peroxisome proliferator-activated receptors: three isotypes for a multitude of functions. *Curr Opin Biotechnol* 1999;10:564–570.
32. Hulbert AJ, Turner N, Storlien LH, Else PL. Dietary fats and membrane function: implications for metabolism and disease. *Biol Rev Camb Philos Soc* 2005;80:155–169.
33. Brand MD, Couture P, Hulbert AJ. Liposomes from mammalian liver mitochondria are more polyunsaturated and leakier to protons than those from reptiles. *Comp Biochem Physiol Biochem Mol Biol* 1994;108:181–188.
34. Stillwell W, Jenski LJ, Crump FT, Ehringer W. Effect of docosahexaenoic acid on mouse mitochondrial membrane properties. *Lipids* 1997;32:497–506.
35. Korshunov SS, Skulachev VP, Starkov AA. High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. *FEBS Lett* 1997;416:15–18.

36. Rolfe DF, Newman JM, Buckingham JA, Clark MG, Brand MD. Contribution of mitochondrial proton leak to respiration rate in working skeletal muscle and liver and to SMR. *Am J Physiol* 1999;276: C692–C699.
37. Matsuo T, Shimomura Y, Saitoh S *et al*. Sympathetic activity is lower in rats fed a beef tallow diet than in rats fed a safflower oil diet. *Metabolism* 1995;44:934–939.
38. Matsuo T, Komuro M, Suzuki M. Beef tallow diet decreases uncoupling protein content in the brown adipose tissue of rats. *J Nutr Sci Vitaminol* 1996;42:595–601.
39. Summermatter S, Marcelino H, Arsenijevic D *et al*. Adipose tissue plasticity during catch-up fat driven by thrifty metabolism: relevance for muscle-adipose glucose redistribution during catch-up growth. *Diabetes* 2009;58:2228–2237.
40. Summermatter S, Mainieri D, Russell AP *et al*. Thrifty metabolism that favors fat storage after caloric restriction: a role for skeletal muscle phosphatidylinositol-3-kinase activity and AMP-activated protein kinase. *FASEB J* 2008;22:774–785.
41. Yepuri G, Marcelino H, Shahkhalili Y *et al*. Dietary modulation of body composition and insulin sensitivity during catch-up growth in a rat model: effects of oils rich in n6 or n3 polyunsaturated fatty acids. *Br J Nutr* 2011; 105:1750–1753.