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Hepatic Fatty Acid-Supported Respiration in Rats Fed an Energy-Dense Diet

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The energy balance and hepatic fatty acid-supported respiration were studied in rats fed a control or an energy-dense diet. In addition, state 3 and 4 respiratory rates as well as ketone body production with palmitoylcarnitine as substrate were determined in isolated mitochondria. Metabolizable energy intake and energy expenditure increased in rats fed an energy-dense diet, but the gain in body weight and lipid content remained unchanged. No variation occurred in the mitochondrial palmitoylcarnitine utilization rate and ketone body production, but a significant increase in the mitochondrial content of ketone bodies and the serum levels was found in rats fed an energy-dense diet. Furthermore, we have shown a significant increase in fatty acid-stimulated respiration in hepatocytes from rats fed an energy-dense diet. The enhanced hepatic fatty acid utilization as an energy substrate found in rats fed an energy-dense diet may contribute to reduce the availability of lipids for storage, thus counteracting the development of obesity.

KEY WORDS—energy-dense diet; energy balance; hepatic oxygen consumption; fatty acid; mitochondria.

INTRODUCTION

It has been suggested that the capacity to avoid diet-induced obesity is related to the ability to increase the oxidation of fat in response to increases in fat intake.[1,2] On the other hand, it has been reported that genetically obese rats have impaired hepatic fatty acid oxidation and ketogenesis.[3–6] We have shown that feeding rats an energy dense diet induces hyperphagia associated with an increase in the resting metabolic rate which was abolished by the administration of propranolol.[7,8] Similar results have been obtained previously in rats fed a cafeteria diet.[9] In addition, in these rats the results of Berry *et al.*[10] and Ma *et al.*[11] suggested that an increase in liver metabolism contributes to the increased oxygen consumption by the body.

In view of these observations and taking into account that the oxidation of fatty acids is a major provider of metabolic energy,[12] it appeared of interest to study hepatic fatty acid-supported respiration in rats fed an energy-dense diet. To this purpose, together with the determination of energy balance, we have measured the oxygen consumption and ketone body (KB) production with palmitoylcarnitine as a substrate in isolated mitochondria, as well as the fatty acid-stimulated respiration in isolated hepatocytes.

MATERIALS AND METHODS

Animals and Experimental Design

Twenty male Wistar rats of about 30 days of age were divided into two groups with the same mean body weight (about 80 g). These were fed for 15 days either a control diet (20 per cent casein, 0·3 per cent methionine, 25 per cent sucrose, 40 per cent cornstarch, 5 per cent alphacel, 5 per cent corn oil, 0·2 per cent choline bitartrate, 3·5 per cent American Institute of Nutrition (AIN) mineral mix, 1 per cent AIN vitamin mix, g/g; the percentage of total metabolizable energy: 19·5 per cent protein, 12 per cent lipid, 68·5 per cent carbohydrate, 16·5 kJ gross energy g^{-1}); or an energy-dense diet which consisted of 13·5 per cent casein, 0·3 per cent methionine, 15 per cent sucrose, 20 per cent cornstarch, 5 per cent alphacel, 5 per

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cent corn oil, 0·2 per cent choline bitartrate, 3·5 per cent AIN mineral mix, 1 per cent AIN vitamin mix, 16·9 per cent lyophilized meat, 18·6 per cent butter, g/g; the percentage of total metabolizable energy; 19·5 per cent protein, 46·1 per cent lipid and 34·4 per cent carbohydrate, 20·2 kJ gross energy g^{-1}. All the rats were maintained singly in grid-bottomed cages at 24°C under an artificial circadian 12-h light/12-h dark cycle. Animal care, housing and killing met the guidelines of the Italian Health Ministry. At the end of the experimental period, five rats of each group were used for the measurement of the energy balance, the isolation of mitochondria and the determination of serum metabolites, while the other five rats were used for the preparation of hepatocytes.

Measurement of Energy Balance

The body weights and intake of food were monitored daily to allow calculations of gain in body weight and gross energy intake. The faeces were also collected daily for measurement of energy content. The collected faeces were dried and ground to a powder before determining their energy content with the bomb calorimeter. The gross energy content of the control and the energy-dense diet was also determined by bomb calorimetry.

Digestible energy intake (taking into account the spillage of food) was obtained by subtracting the energy measured in the faeces from the total energy intake as measured from the daily food consumption. Metabolizable energy (ME) intake was expressed as digestible energy intake $\times 0{\cdot}96$.[13] The gain in energy was obtained by subtracting the energy content of an initial group (five rats killed for the energy content at the beginning of the study) from that of each of the two experimental groups. At the end of the experiment the animals were anesthetized by the intraperitoneal injection of chloral hydrate (40 mg $(100\,g)^{-1}$ body weight), blood was collected from the abdominal aorta and the liver was removed. Then, after removal of the gut content, the carcasses were autoclaved, chopped into small pieces, mixed thoroughly, and homogenized in water (volumes equal to twice the carcass weight) with a Polytron. Samples of homogenates were dessicated into a dry powder from which small pellets (about 200 mg) were made. The energy content was measured with a Parr adiabatic calorimeter calibrated with a dry benzoic acid standard. The lipid content of the carcasses was also determined according to the method of Folch *et al.*[14] Corrections were made for the energy content of the liver. Energy expenditure was calculated from the difference between ME intake and energy gain.

Circulating Metabolite Concentrations

Serum samples were stored at −20°C until the time of analysis. For determining the serum ketone body (KB) concentrations, serum samples were deproteinized with 1 M $HClO_4$. After removal of the precipitated proteins, the supernatant solution was neutralized with 1 M NaOH and stored at −20°C until it was analysed. Samples of the neutralized extracts were used for the assays of β-hydroxybutyrate (Sigma kit) and acetoacetate, measured enzymatically by standard spectrophotometric procedures coupled to the appearance or disappearance of NADH.[15] Triglycerides were measured by the lipase/glycerol kinase method, and free fatty acids (FFA) by the acyl-CoA synthetase/acyl-CoA oxidase method. The measurements were made with the enzymic kits obtained from Boehringer-Mannheim Biochemia, Milano, Italia.

The preparation and Incubation of Isolated Mitochondria

The livers were minced finely and washed with a medium containing 220 mM mannitol, 70 mM sucrose, 20 mM Tris, at pH 7·4, 1 mM EDTA, and 1 per cent (w/v) fatty-acid-free bovine serum albumin. Tissue fragments were gently homogenized with the same medium (1 : 10, w/v) in a Potter Elvehjem homogenizer set at 500 r.p.m. (4 strokes min^{-1}). The homogenate was filtered through sterile gauze and freed of debris and nuclei by centrifugating at $1000 \times g_{av}$ for 10 min; the resulting supernatant was centrifuged at $3000 \times g_{av}$ for 10 min, the mitochondrial pellet was washed twice and finally resuspended in a medium containing 80 mM KCl, 50 mM Hepes at pH 7·0 and 5 mM KH_2PO_4. The protein content of the mitochondrial suspension was determined by the method of Hartree[16] with bovine serum albumin as the protein standard.

Mitochondrial Respiration

Mitochondrial oxygen consumption was measured polarographically with a Clark-type electrode (Yellow Springs Instruments, Yellow Springs, OH, U.S.A.), maintained in a chamber at 30°C, with the above suspension medium supplemented with

0·1 per cent (w/v) bovine serum albumin. Measurements were made within 2 h following the isolation of the mitochondria. The mitochondria were allowed to oxidize their endogenous substrates for a few minutes. Palmitoylcarnitine 40 μM + malate 2·5 mM were then added to determine the state 4 oxygen consumption rate. Six minutes later, ADP (at a final concentration of 0·3 mM) was added and the state 3 rate was measured. State 3 respiration was also measured, omitting malate, in the presence of palmitoylcarnitine 40 μM, either alone or in combination with rotenone 3·75 μM or rotenone 3·75 μM + vitamin K_3 180 μM. The ratio between state 3 and 4 (RCR) and the ADP/O ratios were calculated according to Estabrook.[17]

Ketone Body (KB) Content and Production

KB production was measured in isolated mitochondria incubated at 30°C for 15 min in the suspension medium supplemented with 0·1 per cent (w/v) fatty-acid-free bovine serum albumin, 40 μM palmitoylcarnitine and 10 mM ADP. The incubation was stopped by adding 0·1 ml of 20 per cent (v/v) $HClO_4$; after the precipitated proteins were removed, the supernatant solution was neutralized with 1 M NaOH. Samples of the neutralized extracts were used for the assays of β-hydroxybutyrate and acetoacetate, measured enzymatically by standard spectrophotometric procedures[15] coupled to the appearance or disappearance of NADH. Initial concentrations of KB before the addition of palmitoylcarnitine were also measured.

The Preparation and Incubation of Liver Cells

Rat liver cells were prepared as described by Seglen,[18] except that the rats were anesthesized by the intraperitoneal injection of chloral hydrate (40 mg $(100\,g)^{-1}$ body weight). The hepatocytes were washed and suspended in a medium containing 120 mM NaCl, 5 mM KCl, 50 mM Hepes, 1 mM KH_2PO_4, 2 mM $CaCl_2$, 1·2 mM $MgSO_4$ and 2 per cent (w/v) fatty-acid-free bovine serum albumin. The final cell suspension was stained with Trypan blue and counted in a Burker chamber in order to assess the viability (routinely >90 per cent).

The hepatocyte oxygen consumption was measured polarographically with a Clark-type electrode maintained in a chamber at 37°C. Aliquots corresponding to about 10^6 viable cells were incubated in the suspension buffer with 4 mM hexanoate.

Statistical Analysis

The results are given as the means ± SEM. Statistical significance between the means was examined by the two-tailed Student's *t*-test. Probability values of less than 0·05 were considered to indicate a significant difference.

Materials

ADP, palmitoylcarnitine, malate, β-hydroxybutyrate, acetoacetate, β-hydroxybutyrate dehydrogenase, NAD, NADH, hydrazine hydrate, rotenone, vitamin K_3, hexanoate were purchased from Sigma Chemical Co., St. Louis, MO. All other reagents were of the highest purity commercially available.

RESULTS

Table 1 shows the results of measurement of the energy balance in rats fed a control or an energy-dense diet for 15 days. The mean initial and final body weights were not significantly different between the two groups, yielding a daily body weight gain of about 7 g in both groups. The metabolizable energy intake and energy expenditure of the rats fed an energy-dense diet during the whole period of treatment were 28 per cent ($P < 0·05$) and 50 per cent ($P < 0·05$) higher, respectively, than in the control rats. At the end of the experimental period, the carcass lipid content was about 12 per cent both in rats fed a control and an energy-dense diet.

Figure 1 gives the results of serum metabolite concentrations in rats fed a control or an energy-dense diet. Serum concentrations of triglycerides and FFA were significantly higher (+50 per cent and +38 per cent, respectively; $P < 0·05$) in rats fed an energy-dense diet compared to the controls.

Table 1. Energy balance of rats fed a control or an energy-dense diet.

	Control diet	Energy-dense diet
Initial body weight (g)	80 ± 3	82 ± 3
Final body weight (g)	195 ± 10	196 ± 12
Carcass lipid content (%)	11·6 ± 0·5	12·0 ± 0·7
ME intake (kJ)	2552 ± 32	3275 ± 200*
Energy expenditure (kJ)	1540 ± 97	2315 ± 99*

The results are the means ± SEM of five different rats.
* $P < 0·05$ compared to the control diet.

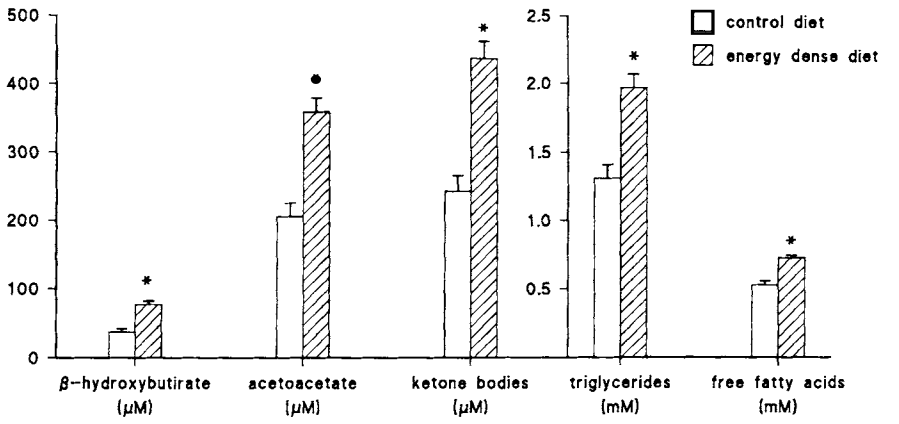

Figure 1. Serum metabolite concentrations in rats fed a control or an energy-dense diet. The values are the means ± SEM of five different rats. * $P < 0.05$ compared to control diet.

Both β-hydroxybutyrate and acetoacetate serum concentrations were significantly higher (+108 per cent and +74 per cent, respectively; $P < 0.05$) in the rats fed an energy-dense diet.

Table 2 shows the state 3 and 4 respiratory rates, the RCR and ADP/O values determined in isolated mitochondria with palmitoylcarnitine + malate as substrate. A significant decrease in state 3 and state 4 respiration measured in isolated mitochondria was found in rats fed an energy-dense diet (−22 per cent and −37 per cent, respectively; $P < 0.05$). We have also found that no significant variation occurred in rats fed an energy-dense diet compared to control rats when malate was omitted and state 3 respiration was measured with palmitoylcarnitine as a substrate, either alone or in the presence of rotenone or rotenone + vitamin K_3 (Table 3).

We also measured fatty acid-supported mitochondrial respiration in isolated hepatocytes: hexanoate was chosen as the substrate to eliminate any peroxisomal contribution to the cellular respiration. The results show that liver cells from rats fed an energy-dense diet exhibited a significant increase (+50 per cent; $P < 0.05$) in hexanoate-supported respiration (Table 2).

Table 4 shows the KB content and production in isolated mitochondria from rats fed a control or an energy-dense diet. The KB content increased significantly in rats fed an energy-dense diet, due to an increase in the β-hydroxybutyrate content, so that the β-hydroxybutyrate/acetoacetate ratio (B/A

Table 2. Fatty acid oxidation in hepatic mitochondria and hepatocytes from rats fed a control or an energy-dense diet.

	Control diet	Energy-dense diet
Fatty acid-supported respiration in hepatocytes (nmol O_2 min^{-1} × 10^6 cells)	22 ± 1	33 ± 1*
Respiratory rates of isolated mitochondria (nmol O_2 min^{-1} × mg protein)		
State 3	97 ± 4	76 ± 6*
State 4	18·4 ± 1·5	11·6 ± 0·9*
RCR	5·4	6·6
ADP/O	2·3 ± 0·1	2·2 ± 0·1

State 4 oxygen consumption was measured in the presence of palmitoylcarnitine 40 μM + malate 2·5 mM; state 3 was achieved by adding ADP at a final concentration of 0·3 mM.
The results are the means ± SEM of five different rats.
* $P < 0.05$ compared to control diet.

Table 3. State 3 oxygen consumption with palmitoylcarnitine as the substrate in hepatic mitochondria from rats fed a control or an energy-dense diet.

	Control diet	Energy-dense diet
Addition		
None	65 ± 4	72 ± 3
Rotenone	14 ± 1	13 ± 1
Rotenone + vitamin K_3	43 ± 5	41 ± 8

State 3 oxygen consumption was measured in the presence of palmitoylcarnitine 40 μM and ADP 0·3 mM; when added, rotenone and vitamin K_3 concentrations were 3·75 μM and 180 μM, respectively.
The results are the means ± SEM of five different rats and are expressed as nmol O_2 min^{-1} × mg protein.

Table 4. KB content and production in hepatic mitochondria from rats fed a control or an energy-dense diet.

	Control diet	Energy-dense diet
Content (nmol mg^{-1} protein)		
Acetoacetate	2·7 ± 0·3	2·6 ± 0·5
β-hydroxybutyrate	6·7 ± 1·0	10·8 ± 1·0*
Total KB	9·4 ± 1·2	13·6 ± 1·0*
B/A ratio	2·9 ± 0·1	4·2 ± 0·2*
Production (nmol $(15\,min)^{-1}$ × mg protein)		
Acetoacetate	13·9 ± 1·2	12·7 ± 1·3
β-hydroxybutyrate	3·7 ± 0·2	3·1 ± 1·1
Total KB	17·6 ± 1·7	15·8 ± 1·6

The results are the means ± SEM of five different rats.
B/A ratio, ratio of β-hydroxybutyrate to acetoacetate.
* $P < 0·05$ compared to the control diet.

ratio) was also increased. No significant variation was found in β-hydroxybutyrate and acetoacetate production in rats fed an energy-dense diet compared to the control rats.

DISCUSSION

The ability of high-fat diets to produce a large increase in body weight and obesity in rats is highly variable both across and within strains.[2-9] In addition, Bray *et al.*[20] have found that it can take considerable time to produce obesity by feeding high-fat diets when starting with young rats. In the same review, they concluded that 'all experimental obesity results from an absolute or relative decrease in the nutrient-stimulated activity of the thermogenic components of the sympathetic nervous system' (SNS). These components may serve as feedback inhibitors of food intake as well as activators of peripheral thermogenic mechanisms.[20]

In the present study, young rats fed an energy-dense diet showed an increase in energy intake; however, the gain in body weight and lipid content remained unchanged (Table 1). In agreement with this finding, we found a compensatory increase in energy expenditure (Table 1) which was useful to balance the increase in energy intake found in rats fed an energy-dense diet. We have previously shown that these rats showed an increase in resting respiratory rates which is mediated by the SNS.[7,8,21] Accordingly, it has been shown that feeding of fat stimulates SNS activity in rats.[22,23] Rats fed an energy-dense diet also showed an increase in serum triglycerides, FFA and KB (Figure 1). The increase in triglycerides could be due to the high-fat content of the energy-dense diet. On the other hand, the increase in FFA could be explained by an increased lipolysis in white and brown adipose tissue, promoted by the enhanced activity of the SNS.[24] The increase in blood KB concentrations (Figure 1) is in agreement with the higher KB content found in isolated mitochondria from rats fed an energy-dense diet (Table 4). High blood β-hydroxybutyrate levels have also been found in rats resistant to obesity when eating a high-fat diet[25] and it has been suggested that β-hydroxybutyrate can be one of the signals responsible for the activation of the SNS.[26]

We have also found that isolated hepatocytes from rats fed an energy-dense diet showed an increase in fatty acid-supported mitochondrial respiration compared to hepatocytes isolated from control rats (Table 2). On the other hand, when we measured respiratory rates in isolated mitochondria with palmitoylcarnitine and malate, we found significantly decreased state 3 and state 4 oxygen consumption in rats fed an energy-dense diet (Table 2). However, no variation was found when malate was omitted and state 3 oxygen consumption was measured in the presence of palmitoylcarnitine, rotenone, and vitamin K_3 (Table 3); this causes a marked oxidation of the mitochondrial pyridine nucleotides.[27] Under these conditions, we only measure oxygen consumption due to $FADH_2$ oxidation.[28] These results can be explained taking into account that we have previously shown a decrease in the capacity of hepatic mitochondria to oxidize NADH in rats fed an energy-dense diet.[21] The decrease in the capacity of NADH oxidation leads to an increase in the NADH/NAD ratio, as also shown by the higher B/A ratio found in the present study (Table 4). It is well known that the mitochondrial NADH/NAD ratio regulates β-oxidation;[29] thus it is possible that, during our measurements of oxygen consumption with palmitoylcarnitine + malate as substrate, the flux through the β-oxidation pathway could have been inhibited by the unusually high NADH/NAD ratio. This possibility is supported by the finding that oxygen consumption was similar in rats fed the control or the energy-dense diet, when NADH was oxidized by vitamin K_3 or when the NADH/NAD ratio was lowered by omitting malate (Table 3). The excess mitochondrial NADH coming from lipid oxidation in rats fed an energy-dense diet, could be oxidized *in vivo* through an energy-wasting cycle. In fact, we have previously found an increase in

the α-glycerophosphate shuttle and a decrease in the malate–aspartate shuttle in rats fed an energy-dense diet;[21] on the basis of these results we have suggested that, in rats fed an energy-dense diet, the high NADH/NAD ratio favours the formation of malate from oxaloacetate and the export of malate to the cytosol. Here malate can be converted to pyruvate or oxaloacetate, with the formation of NADPH or NADH. The reducing equivalents are re-transported into the mitochondria through the α-glycerophosphate shuttle and finally oxidized through the respiratory chain from complex II onwards. This mechanism could lead to a decrease in hepatic metabolic efficiency.[21]

It can be concluded that in rats fed an energy-dense diet the increased hepatic fatty acid-supported respiration together with the increased mitochondrial KB content indicate an increase in liver fatty acid-oxidation which may contribute to reducing the availability of lipids for storage in adipose tissue, thus counteracting the development of obesity. In addition, the enhanced hepatic fatty acid utilization as an energy substrate, if coupled with an energy-wasting cycle,[21] could provide a mechanism by which the liver contributes to the increased energy expenditure of rats fed an energy-dense diet.

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