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Elevated hepatic mitochondrial and peroxisomal oxidative capacities in fed and starved adult obese (*ob/ob*) mice

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Hepatic mitochondrial and peroxisomal oxidative capacities were studied in young (4–5 weeks old) and adult (6–9 months old) lean and obese *ob/ob* mice that were fed or starved for 24 or 48 h. The adult obese mice showed elevated capacity for mitochondrial oxidation (ng-atoms of O consumed/min per mg of protein) of lipid and non-lipid substrates, with the exception of pyruvate+malate, and elevated activities of citrate synthase and total carnitine palmitoyltransferase. Oxidative rates and enzyme activities were not affected by starvation of lean or obese mice, and both males and females responded similarly. Peroxisomal palmitoyl-CoA oxidation (nmol/min per mg of peroxisomal protein) was also increased in livers of adult obese mice and did not change with starvation. In young mice, hepatic mitochondrial and peroxisomal oxidative capacities in lean and obese mice were comparable. The increased mitochondrial and peroxisomal oxidative capacities appear to develop with maturation in obese *ob/ob* mice.

INTRODUCTION

It has been reported that obese (*ob/ob*) mice may have impaired hepatic fatty acid oxidation (Herberg & Coleman, 1977) and elevated rates of hepatic lipogenesis and associated enzymes (Fried & Antopol, 1966; Chang *et al.*, 1967; Hems *et al.*, 1975; Herberg & Coleman, 1977). This pattern is similar to that in the obese Zucker rat (McCune *et al.*, 1981; Fukuda *et al.*, 1982; Triscari *et al.*, 1982). The regulation of hepatic mitochondrial oxidation of long-chain fatty acids is proposed to be dependent on the reciprocal relationship of fatty acid synthesis and oxidation (McGarry & Foster, 1980). Consistent with this, obese mice and rats may have depressed hepatic β-oxidation of fatty acids, because circulating insulin concentrations are elevated (Dubuc, 1976; Menahan, 1983) and fatty acid-synthesis rates and malonyl-CoA concentrations are high. Increased malonyl-CoA would inhibit CPT-A (also called 'CPT I' or 'outer CPT'; CPT localized on the outer surface of the mitochondrial inner membrane) and thereby decrease hepatic β-oxidation of long-chain fatty acids. However, when we examined hepatic mitochondrial capacity for β-oxidation and keto acid production in Zucker rats, we found no differences between lean and obese rats; concurrently, total CPT activity (sum of inner- and outer-membrane activity) was elevated in obese rats (Brady & Hoppel, 1983*a*).

These data suggested that the regulation of fatty acid oxidation was due not to intrinsic mitochondrial oxidative capacity, but rather to cellular control of substrate or inhibitor concentrations. This lack of change in mitochondrial oxidative capacity has also been noted in states of elevated hepatic ketogenesis induced by diabetes and growth-hormone (somatotropin) administration (DiMarco & Hoppel, 1975).

Fatty acid oxidation has also been shown to occur in peroxisomes (Lazarow, 1978), and one study has shown that hepatic peroxisomal capacity is actually increased in fed obese mice compared with their lean counterparts (Murphy *et al.*, 1979).

Thus the purpose of our studies was to examine fatty acid-oxidative capacity (mitochondrial and peroxisomal) in lean and obese adult mice in the fed state and with 24 and 48 h of starvation, where increased hepatic fatty acid oxidation might be expected to occur. In addition, we also studied young (4–5 weeks old) mice to determine if age affects fatty acid-oxidative capacity, since Fraser & Trayhurn (1983) found that liver mitochondria from adult obese mice exhibited increased capacity for Ca^{2+} uptake compared with lean controls, whereas young obese mice did not share this characteristic.

Our results indicate that hepatic mitochondria and peroxisomes from adult obese mice actually exhibit elevated capacity to oxidize fatty acids and that mitochondria from adult obese mice also exhibit elevated capacity to oxidize non-lipid substrates, with the exception of pyruvate plus malate. Young obese mice (4–5 weeks old) did not exhibit the increased mitochondrial or peroxisomal fatty acid-oxidative capacities observed in adult obese mice.

METHODS

Animals, diet and experimental design

Male and female lean (*ob*/+ and +/+) and obese (*ob/ob*) mice (6–9 months and 4–5 weeks of age) were from the breeding colony of C57 BL/6J-*ob*/+ mice established at Michigan State University [originally derived from heterozygote breeding pairs (*ob*/+ × *ob*/+) purchased from Jackson Laboratories (Bar Harbor, ME, U.S.A.]. Mice were housed in groups in solid-bottom cages with wood-chip bedding with access *ad libitum* to

Abbreviation used: CPT, carnitine palmitoyltransferase (EC 2.3.1.21).
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water and stock diet (Purina Lab Chow). Animal rooms were maintained at 25 °C and lights were on or off for 12 h each. Mice were killed between 07:30 and 08:30 h. In starvation experiments, food was removed either 24 or 48 h before they were killed. The design was a 2 (lean/obese) × 2 (male/female) × 3 (days starved) factorial arrangement, and the data were analysed by analysis of variance as described by Federer & Zelen (1966). Individual treatment means were compared by Tukey's omega procedure (Steel & Torrie, 1960).

Mitochondrial and peroxisomal isolation

Mitochondria and peroxisomes were isolated from the same homogenates by the methods of Hoppel *et al.* (1979) and of Brady & Hoppel (1983*b*). Two mice of each genotype were killed at each time point. A total of three replicates at each time point were obtained. Livers were weighed, then pooled, minced, and homogenized (10%, w/v) in 5 mM-Mops/70 mM-sucrose/220 mM-mannitol/2 mM-EDTA, pH 7.4. Nuclei and cell debris were removed by centrifugation at 700 *g* for 10 min, and mitochondria were isolated by centrifugation at 7000 *g* for 10 min. After two washes and final suspension, mitochondrial protein was measured by the biuret reaction (Gornall *et al.*, 1949).

Peroxisomes were isolated from the 7000 *g* supernatant. This supernatant was centrifuged at 32000 *g* and the peroxisome-enriched pellet resuspended. The subsequent procedures were essentially as described by Neat *et al.* (1981) and Brady & Hoppel (1983*b*). Isolation was effected by centrifugation of the resuspended pellet at 79000 $g_{av.}$ at 5 °C for 30 min [1850–1950 ($\times 10^7$) rad^2/s] on a self-forming 50% (v/v) Percoll gradient containing 250 mM-sucrose, 2 mM-Mops, 5 mM-EDTA and 0.1% ethanol, pH 7.2. Separation was accomplished by ultracentrifugation (Beckman L5) in a vertical rotor (Beckman type VTi 50). The resultant gradient was fractionated into ten 3 ml fractions. The peroxisomal peak was identified on the basis of catalase activity (Baudhuin *et al.*, 1964).

Mitochondrial and peroxisomal oxidation

Mitochondrial oxygen consumption was measured with a Clark-type electrode (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.), maintained in a chamber at 30 °C. The incubation media contained 80 mM-KCl, 50 mM-Mops, 1 mM-EGTA, 5 mM-phosphate and 1 mg of dialysed defatted bovine serum albumin/ml, pH 7.0. State-3 respiration was defined as ADP-stimulated respiration and State-4 respiration as ADP-limited respiration (Chance & Williams, 1955). Respiratory control ratio (RCR) and ADP/O ratios were calculated as described by Estabrook (1967).

Palmitoyl-CoA-dependent NAD^+ reduction was determined in the major peroxisomal peak fraction by using a dual-wavelength spectrophotometer (Aminco DW2a) at 340 nm with a 400 nm reference (Neat & Osmundsen, 1979). Protein was determined by the method of Bradford (1976) with a commercially available kit (Bio-Rad, Richmond, CA, U.S.A.).

Mitochondrial enzyme assays

Citrate synthase (Srere, 1969) and CPT (Hoppel & Tomec, 1972) were measured in mitochondria that had been stored at −70 °C for 1 month. The CPT assay measures the sum of both CPT-A and CPT-B activities, since it was done on frozen sonicated mitochondria. CPT-A is located on the outer side of the mitochondrial inner membrane and CPT-B on the inner side of this membrane. The assay measures CoA-dependent release of L-[*Me*-^{14}C]carnitine from palmitoyl-L-[*Me*-^{14}C]carnitine at 37 °C. Palmitoylcarnitine hydrolase activity was subtracted by using a blank containing no added CoA. CPT-A activity was measured in the direction of palmitoylcarnitine formation with L-[*Me*-^{14}C]carnitine as substrate. This substrate was prepared by demethylation of L-carnitine (Sigma Tau, Rome, Italy), followed by remethylation with [^{14}C]methyl iodide (New England Nuclear, Boston, MA, U.S.A.) by the method of Ingalls *et al.* (1982).

The CPT assay, at pH 7.0, contained, in a final volume of 0.25 ml: 80 mM-KCl, 59 mM-Mops, 1 mM-EGTA, 1 mg of dialysed defatted bovine serum albumin/ml, 4 mg of sodium tetrathionate/ml, 40 μM-palmitoyl-CoA, 0.5 mM total L-carnitine (unlabelled plus ^{14}C-labelled). The reaction was initiated by the addition of 50 μg of mitochondrial protein, and the reaction allowed to proceed for 2 min at 30 °C. The assay was linear with time for up to 4 min with 100 μg of protein under these conditions.

Materials

Percoll was obtained from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A. L-Carnitine chloride was a gift from Otsuka Pharmaceutical, Osaka, Japan. Bovine serum albumin (Sigma Fraction V) was defatted (Chen, 1967) and dialysed (Hanson & Ballard, 1968). Palmitoyl-L-carnitine was prepared (Ziegler *et al.*, 1967) and its concentration determined with 50% (v/v) methanol present and as carnitine after alkaline hydrolysis (Hestrin, 1949). Palmitoyl-CoA was synthesized (Seubert, 1960). Octanoyl-CoA was obtained from P-L Biochemicals, Milwaukee, WI, U.S.A. ADP, octanoic acid and malic acid were purchased from Sigma Chemical Co., St Louis, MO, U.S.A. All other chemicals were laboratory-reagent grade.

RESULTS

Mitochondrial fatty acid-oxidative capacity

Table 1 presents the data for oxidative phosphorylation in hepatic mitochondria from male mice when palmitoylcarnitine + malate or palmitoyl-CoA + carnitine + malate were the substrates. Results for females were similar to those for the male mice (not shown). With palmitoylcarnitine + malate, mitochondria from obese mice exhibited significantly higher oxidation rates than did mitochondria from lean mice; neither lean nor obese oxidative rates were affected by up to 48 h of starvation. State-4 rates were also slightly, but significantly, elevated in mitochondria from obese mice, with no effect of starvation. Respiratory control ratios (RCR) were higher in mitochondria from obese mice, but values for both preparations agreed well with previous data from this laboratory (Brady & Hoppel, 1983*a*; Hoppel *et al.*, 1979). ADP/O ratios were not significantly affected by genotype or starvation. Both the RCR and ADP/O ratios indicate that high-quality mitochondrial preparations were obtained. The data obtained when palmitoyl-CoA + carnitine + malate were substrates had the same pattern as for palmitoylcarnitine + malate, except that no significant State-4 difference occurred. Again, although data from

Table 1. Hepatic mitochondrial oxidative phosphorylation in *ob/ob* mice

Values are given as the means for three male replicates per time point (6–9 months old). Mitochondria were isolated as described in the Methods section, and incubated at 30 °C. Measurements of State-3 and State-4 respiration, respiratory control ratio (RCR) and ADP/O are described in the Methods section. Values are given in ng-atoms of O consumed/min per mg of mitochondrial protein. Palmitoylcarnitine concentration was 40 μM; malate, 2.5 mM; palmitoyl-CoA, 40 μM; L-carnitine 2 mM. Abbreviations: S.D., standard deviation which is derived from the mean-square error of a 2×3 factorial analysis of variance; G, significant effect of genotype; F, ratio of treatment mean square to error mean square; significance is indicated at the 0.05 level (NS, not significant).

Mice ...	Lean			Obese				
Period of starvations (days)...	0	1	2	0	1	2	S.D.	F
Palmitoyl-carnitine + malate								
State 3	100.9	91.7	77.3	127.0	129.3	135.4	12.0	G
State 4	16.8	17.3	16.2	19.6	19.7	18.9	1.7	G
RCR	6.1	5.3	4.8	6.5	6.6	7.3	1.0	G
ADP/O	2.5	2.4	2.6	2.7	2.4	2.6	0.4	NS
Palmitoyl-CoA + carnitine + malate								
State 3	77.4	80.0	75.4	106.7	102.5	115.2	9.6	G
State 4	19.2	17.2	14.9	18.2	17.1	16.3	3.2	NS
RCR	4.1	4.7	5.1	6.3	6.0	7.0	0.9	G
ADP/O	2.7	2.5	2.5	2.8	2.4	2.6	0.3	NS

Table 2. State-3 oxidative rates in hepatic mitochondria of *ob/ob* mice

Means ± S.D. are given for three male pairs at each time point (6–9 months old) and are expressed as ng-atoms of O consumed/min per mg. Mitochondria were isolated and incubated as described in the Methods section. Substrate concentrations were: octanoylcarnitine, 200 μM; L-malate, 2.5 mM; octanoyl-CoA, 200 μM; L-carnitine, 2 mM; octanoate, 200 μM; glutamate, 10 mM; pyruvate, 10 mM; succinate, 10 mM; rotenone, 3.75 μM. For abbreviations, see Table 1.

Mice ...	Lean			Obese				
Period of starvation (days)...	0	1	2	0	1	2	S.D.	F
Lipid substrates								
Octanoylcarnitine + malate	109.8	105.7	92.9	147.7	148.9	158.2	9.6	G
Octanoyl-CoA + carnitine + malate	67.3	76.2	77.8	94.5	92.6	114.2	10.9	G
Octanoate + malate	62.8	60.8	50.6	75.8	78.9	76.5	7.0	G
Non-lipid substrates								
Glutamate	74.2	70.4	61.1	90.0	89.2	88.8	11.2	G
Pyruvate + malate	40.8	34.1	32.9	42.9	42.0	42.1	9.8	NS
Succinate + rotenone	185.3	166.9	164.2	221.5	221.1	246.8	23.1	G

female mice are not presented, they were similar to data obtained from male mice.

We also used octanoyl-CoA, octanoylcarnitine and octanoate as lipid substrates, and glutamate, pyruvate + malate and succinate as non-lipid substrates (Table 2). The purpose of using various lipid and non-lipid substrates was to examine different modes of transportation into the mitochondria, different dehydrogenases, and different sites of entry of reducing equivalents into the mitochondrial electron-transport chain. The results were similar to those presented for palmitoylcarnitine; the mitochondrial State-3 capacity to oxidize substrates was elevated in the obese mice in every case, except for pyruvate + malate, where there were no differences attributable to sex, genotype or starvation.

Since CPT-A has been proposed as the rate-limiting step in hepatic mitochondrial β-oxidation of long-chain fatty acids, we measured CPT-activity to see if it was correlated with increased β-oxidation in adult obese mice. Total CPT specific activity is presented in Table 3 and was significantly elevated in obese mice compared with lean, and not affected by starvation. CPT-A activity was not elevated in mitochondria of young obese mice (lean, 4.92 and obese, 5.30 nmol/min per mg), but was elevated in mitochondria from older mice (lean 3.19, and obese, 9.14 nmol/min per mg). CPT-A specific activity increased with 24 h starvation in lean mice (10.09 nmol/min per mg), but not in obese mice (11.22 nmol/min per mg). Thus CPT-A followed the pattern of total CPT activity, except that activity of lean fed mice was depressed compared with activity during starvation. We also measured citrate synthase specific activity as an example of an enzyme not directly involved in the β-oxidative pathway. Citrate synthase specific activity was elevated in mitochondria from obese mice and not affected by starvation.

Table 3. Hepatic carnitine palmitoyltransferase and citrate synthase in adult *ob/ob* mice

Data are expressed as means ± S.D. for three replicates per time point. CPT activity was measured as described in the Methods section. Citrate synthase activity was measured at 412 nm at 37 °C in 0.1 M-Tris/HCl, pH 8.1, containing 0.1 mM-5,5′-dithiobis-(2-nitrobenzoic acid), 0.3 mM-acetyl-CoA and 0.5 mM-oxalacetate. The reaction was corrected for acetyl-CoA deacylase; then the oxaloacetate was added and the reaction rate measured over 3 min. For abbreviations, see Table 1.

	Activity (nmol/min per mg of protein)							
Mice ...	Lean			Obese				
Period of starvation (days)...	0	1	2	0	1	2	S.D.	F
CPT	333	392	325	610	628	703	110	G
Citrate synthase	234	305	267	334	452	442	89	G

Table 4. Hepatic mitochondrial oxidative phosphorylation in 4–5-week-old mice

Values are expressed as means ± S.D., for six experiments in the fed state using four mice per experiment. Mitochondria were isolated and incubated as described in the Methods section. Substrate concentrations were: malate, 2.5 mM; palmitoylcarnitine, 40 μM; palmitoyl-CoA, 40 μM; L-carnitine, 2 mM. For abbreviations, see Table 1.

	State-3 oxidative rates (ng-atoms/min per mg)			
Mice ...	Lean	Obese		
Substrate			S.D.	F
Palmitoylcarnitine + malate	86.4	88.4	11.6	NS
Palmitoyl-CoA + carnitine + malate	73.3	74.4	9.7	NS

Table 5. Peroxisomal palmitoyl-CoA-oxidative capacity

This was determined as palmitoyl-CoA-dependent NAD^+ reduction (nmol/min per mg of peroxisomal protein) at 35 °C by using an Aminco DW2a dual-wavelength spectrophotometer at 340 nm with a 400 nm reference. Values are expressed as means ± S.D. (see Table 1) for six adult mice (three males + three females) and for six young mice (four male + two female). A statistically significant effect ($P < 0.01$) of obesity was observed for adult, but not for young, mice.

	Period of starvation (days) or otherwise		Oxidation	
		Mice ...	Lean	Obese
Adult	0		15.2 ± 2.2	23.4 ± 2.9
	1		8.6 ± 2.8	34.4 ± 8.9
	2		12.8 ± 3.6	33.4 ± 4.0
Young	Fed		16.4 ± 1.6	17.2 ± 4.6

We also measured the oxidation of palmitoylcarnitine + malate and palmitoyl-CoA + carnitine + malate in hepatic mitochondria from young (4–5-weeks-old) mice. There was no significant difference in oxidative rates in the fed state (Table 4) in young mice.

Peroxisomal oxidative capacity for palmitoyl-CoA in adults and young mice

Peroxisomal palmitoyl-CoA oxidation is presented in Table 5. Expressed as nmol/min per mg of peroxisomal protein, there was a significantly elevated rate in the obese-mouse liver; this did not increase with starvation for up to 48 h. In addition, catalase activity was significantly elevated in peroxisomes from obese mice (results not shown). Catalase activity was about 40% higher among the obese mice and in agreement with the values reported by Murphy *et al.* (1979).

The capacity of isolated hepatic peroxisomes from young obese mice to oxidize palmitoyl-CoA was not significantly increased (Table 5). Thus, as with mitochondrial oxidative capacity, genotype differences are not apparent in young mice.

DISCUSSION

Our basic conclusions from this study are that: (1) adult obese (*ob/ob*) mice have elevated hepatic mitochondrial and peroxisomal capacity to oxidize lipid and non-lipid substrates compared with lean controls, and (2) these elevated capacities are not present in young obese mice and appear to be a consequence of the developmental pattern of obese mice.

Although there have been some reports of altered hepatic fatty acid oxidation in obese mice, extensive data are not available. We examined fatty acid oxidation at the organelle level, as both mitochondria and peroxisomes can oxidize fatty acids. In contrast with what we expected, both mitochondria and peroxisomes from adult obese mice had increased capacity to oxidize palmitoyl-CoA. Oxidation of other lipid and non-lipid substrates was also elevated in mitochondria from adult obese mice, except for pyruvate plus malate. Other capacity studies have been reported in isolated hepatic mitochondria of obese mice which support our present conclusions. Katyare & Howland (1978) found that hepatic mitochondrial oxidation of β-hydroxybutyrate, succinate dehydrogenase activity, and cytochrome *c* were increased in obese mice. Fraser & Trayhurn (1983) found that the capacity of hepatic mitochondria from adult obese mice to take up Ca^{2+} was elevated compared with lean mice, and, similar to results reported here, the young mice did not exhibit differences. Others have found that fatty acid oxidation in perfused livers and isolated hepatocytes of

obese Zucker rats was depressed (McCune *et al.*, 1981; Fukuda *et al.*, 1982; Triscari *et al.*, 1982). However, when we previously examined mitochondrial and peroxisomal oxidative capacity in Zucker rats, we found that mitochondrial oxidative capacity and keto acid production were not depressed in obese-rat liver, nor was the peroxisomal capacity to oxidize palmitoyl-CoA depressed in obese rats. This suggests that it is hepatic cellular regulation rather than the capacity to oxidize fatty acids which limits hepatic fatty acid oxidation in obese Zucker rats.

We have used mitochondrial substrates that have different modes of transport into the mitochondria, different dehydrogenases, and different sites of entry for reducing equivalents into the mitochondrial electron-transport chain. For the fatty acid substrates, the mitochondria from obese mice demonstrated increased oxidative rates irrespective of where in the pathway the substrate enters. Glutamate, an amino acid, is NAD^+-linked, whereas succinate, a dicarboxylic acid, is FAD-linked. Oxidative rates for both substrates were elevated in mitochondria from obese compared with lean mice. This supports the notion that hepatic mitochondria from obese mice have an increased capacity for metabolizing reducing equivalents, whether they are NAD^+- or FAD-linked. The similarity of mitochondrial oxidative rates with pyruvate plus malate in lean and obese mice suggests that either pyruvate transport or the pyruvate dehydrogenase complex is rate-limiting for pyruvate oxidation and this rate-limiting step is identical in mitochondria from obese and lean mice. Furthermore, we have found that starvation for up to 48 h does not alter the elevated oxidative capacity in hepatic mitochondria from obese mice or the similarity in pyruvate oxidation.

Peroxisomal capacity for palmitoyl-CoA oxidation followed the same pattern as in the mitochondria. We have previously observed concomitant increases in fatty acid oxidative capacity in these organelles in the Zucker rat (Brady & Hoppel, 1983*b*). To rule out the possibility of mitochondrial contamination of the peroxisomal preparation, samples were initially run in the presence and absence of KCN. Cyanide did not alter the putative peroxisomal palmitate-oxidation rate. Thus we are confident that peroxisomal rates of oxidation were measured.

The degree of the increase in peroxisomal and mitochondrial fatty acid-oxidative capacity is considerably greater in the obese mice than we had previously observed in the Zucker rat. Murphy *et al.* (1979) have reported elevated rates of hepatic peroxisomal fatty acid oxidation in obese (*ob/ob*) mice. We have now observed that both mitochondrial and peroxisomal capacities for fatty acid oxidation are elevated in the adult obese-mouse liver, but not in liver of young mice. This suggests that the change in hepatic fatty acid-oxidation rates is an effect of the development of obesity rather than its cause. Kaplan & Leveille (1981) have shown that hepatic fatty acid synthesis and associated enzyme activities decline with age in both males and females from a peak at 4–12 weeks. Others have reported that insulin concentrations are elevated from 4 weeks and are still elevated at 20–24 weeks (Mahler *et al.*, 1976). The data suggest that a factor other than circulating insulin may be regulatory at this age. Another possibility is that insulin-sensitivity decreases with age in liver, as has been shown in other tissues (Czech *et al.*, 1977). The sensitivity of hepatic gluconeogenesis and ketogenesis to insulin is depressed in obese mice (Bray & York, 1979; Mobley *et al.*, 1977) and it is not unreasonable to postulate that elevated oxidation rates can be at least partially explained by hepatic insulin resistance.

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