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Intracellular Localization of Long-Chain Acyl-Coenzyme A Hydrolase and Acyl-L-carnitine Hydrolase in Brown Adipose Tissue from Guinea Pigs

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The activities of long-chain acyl-CoA hydrolase (palmitoyl-CoA hydrolase, EC 3.1.2.2) and long-chain acyl-L-carnitine hydrolase (palmitoyl-L-carnitine hydrolase, EC 3.1.1.28) in brown adipose tissue from cold-exposed and control guinea pigs were studied. Mitochondria from cold-exposed animals hydrolysed 21 nmol of palmitoyl-CoA/min per mg of protein and 1.3 nmol of palmitoyl-L-carnitine/min per mg of protein, and the specific activities were respectively 2 and 5 times as high in cold-exposed as in control animals. The subcellular-localization studies showed that both the long-chain acyl-CoA hydrolase and long-chain acyl-L-carnitine hydrolase were localized in the mitochondria. A location also in the soluble fraction cannot be excluded. The long-chain acyl-CoA hydrolase activity was doubled when the mitochondria were disrupted; this indicates that the enzyme is localized in the matrix compartment.

Brown adipose tissue is an effective thermogenic organ, largely because of the ability of its mitochondria to oxidize fatty acids without being limited by the cellular demand for ATP (Flatmark & Pedersen, 1975; Nicholls, 1976). The tissue has a high capacity for fatty acids and acylglycerol synthesis (Drahota *et al.*, 1970; Joel, 1965), and a high capacity for oxidation of non-esterified fatty acid to acetate (Bernson, 1976). Fatty acids not only serve as oxidizable substrates during the thermogenic activation of the tissue, but also appear to regulate the mitochondrial energy-transducing system (Prusiner, 1970) and proton conductance (Heaton & Nicholls, 1976) in such a way as to permit a major portion of the energy to be dissipated as heat (for review, see Flatmark & Pedersen, 1975).

In isolated mitochondria from brown adipose tissue a carnitine-dependent and less-active carnitine-independent oxidation of fatty acids have been described (Drahota *et al.*, 1970). The mitochondria have an unusually high capacity for net transfer of long-chain fatty acyl groups across the inner mitochondrial membrane (Normann *et al.*, 1978). The long-chain fatty acyl-CoA synthetase is localized exclusively in the outer mitochondrial membrane (Pedersen *et al.*, 1975).

A long-chain acyl-CoA hydrolase activity localized in both the mitochondrial and microsomal fractions, and a long-chain acyl-L-carnitine hydrolase activity localized in the microsomal fraction, have been described in rat liver (Colli *et al.*, 1969; Mahadevan & Sauer, 1969; Berge & Farstad, 1979). The physiological role of these activities remains uncertain at present. Since fatty acid metabolism in brown adipose tissue is very specialized, information on the enzyme activities in this tissue is of considerable interest. The present paper reports data on the intracellular localization of these enzymes in brown adipose tissue from cold-adapted guinea pigs. It is further shown that the specific activities of both palmitoyl-CoA hydrolase and palmitoyl-L-carnitine hydrolase are increased in mitochondria from cold-adapted animals as compared with control animals.

Experimental

Animals and preparation of mitochondria

Weaned guinea pigs were used as control aminals. Cold-adapted animals were obtained by transferring 3-week-old animals from an environment at 25°C to 5°C, where they remained for at least 2 weeks to obtain an optimal increase in the mitochondrial mass (Flatmark *et al.*, 1976). Mitochondria from the animals were isolated in 0.25 M-sucrose/2 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer (pH 7.2)/0.2 mM-EDTA/0.5 mM-ATP as described by Slinde *et al.* (1975).

Preparation of subcellular fractions

The subcellular fractions of cold-adapted animals were prepared essentially as described by de Duve *et al.* (1955), with some modifications of the centrifugal effects (Pedersen *et al.*, 1975), since the sedimentation

coefficient of brown-adipose-tissue mitochondria (approx. 8000S) is markedly lower than that of rat liver mitochondria (approx. 12000S) (Pedersen *et al.*, 1975).

Materials

Palmitoyl-CoA, rotenone and Hepes were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Palmitoyl-L-carnitine chloride was purchased from Supelco (Bellefonte, PA, U.S.A.) and L-carnitine was a product of Koch–Light Laboratories (Colnbrook, Bucks., U.K.). [1-^{14}C]Tyramine hydrochloride was obtained from The Radiochemical Centre, Amersham Bucks., U.K. L-[*Me*-^{3}H]Carnitine was a gift from Professor J. Bremer, Institute for Medical Biochemistry, Oslo, Norway. [1-^{14}C]Palmitoyl-CoA and [1-^{14}C]palmitoyl-L-carnitine were purchased from New England Nuclear, Boston, MA, U.S.A. The scintillation liquid (Scint-Hei 3) was from Koch–Light. All other chemicals were of the highest purity commercially available. Carnitine palmitoyltransferase (EC 2.3.1.21) was prepared as described by Farstad *et al.* (1967).

Enzyme assays and other analytical methods

Palmitoyl-CoA synthetase (EC 6.2.1.3) was measured as described by Farstad *et al.* (1967), with some modifications (Pedersen *et al.*, 1975). The palmitoyl-CoA hydrolase (EC 3.1.2.2) activity was determined by measuring the release of [1-^{14}C]palmitate from [1-^{14}C]palmitoyl-CoA. The assay mixture contained 0.25M-sucrose, 35μM-[1-^{14}C]palmitoyl-CoA (0.01–0.02μCi), 10mM-Hepes buffer (pH7.5), and protein as indicated. The final volume, 0.3ml, was incubated at 35°C for 0.5 to 3min. The reaction was stopped by adding 2.0ml of propan-2-ol/heptane/1M-H_2SO_4 (20:5:1, by vol.) (Dole, 1956), and unesterified [^{14}C]palmitate was extracted by the method of Bar-Tana *et al.* (1971). A 0.5ml portion of the heptane phase containing the unesterified [^{14}C]palmitate was mixed with 10ml of Scint-Hei 3, and the radioactivity determined by liquid-scintillation counting. The recovery of added [1-^{14}C]palmitate was above 93%. Palmitoyl-L-carnitine hydrolase (EC 3.1.1.28) was measured under identical conditions as described above for palmitoyl-CoA hydrolase but with 35μM-[1-^{14}C]palmitoyl-L-carnitine (0.03μCi) as the substrate. The assays of amine oxidase (EC 1.4.3.4) (Aas, 1971), carnitine palmitoyltransferase (EC 2.3.1.21) (Vollset & Farstad, 1979), malate dehydrogenase (EC 1.1.1.37) (Bergmeyer & Bernt, 1974), rotenone-insensitive NADPH–cytochrome *c* reductase (EC 1.6.2.4) (Sottocasa *et al.*, 1967), succinate–phenazine methosulphate oxidoreductase (EC 1.3.99.1) (Arrigoni & Singer, 1962), acid phosphatase (EC 3.1.3.2) (Bergmeyer, 1970), lactate dehydrogenase (EC 1.1.1.27) (Scandinavian Committee on Enzymes, 1974) and glutamate dehydrogenase (EC 1.4.1.3) (Bergmeyer, 1974) were carried out as described in the references. All spectrophotometric measurements were performed with a Shimadzu recording spectrophotometer MPS 5000. Radioactivity was counted in a Packard Tri-Carb liquid-scintillation spectrometer (model 3385). Protein was determined by using the Folin–Ciocalteu reagent (Lowry *et al.*, 1951), with bovine serum albumin as standard.

Results

Assay conditions for palmitoyl-CoA hydrolase and palmitoyl-L-carnitine hydrolase in homogenates of brown adipose tissue of cold-adapted animals

The reaction kinetics of both palmitoyl-CoA hydrolase and palmitoyl-L-carnitine hydrolase are dependent on substrate and protein concentrations. Fig. 1 shows that the rate of hydrolysis of palmitoyl-CoA at a substrate concentration of 35μM was constant for 8min when 0.15mg of protein/ml was used and for 3min when 0.34mg of protein/ml was used. With 35μM-palmitoyl-L-carnitine and 0.34mg of protein/ml the rate of hydrolysis was constant for at least 10min, and proportional to the amount of protein from 0.15 to 0.50 mg/ml when incubation time was

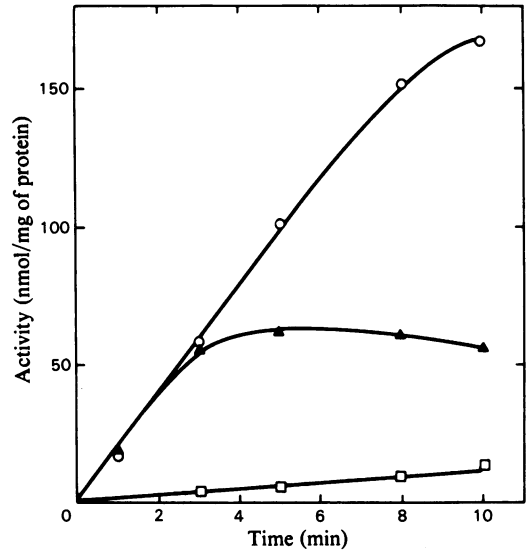

Fig. 1. *Hydrolysis of palmitoyl-CoA* (35μM) *and palmitoyl-L-carnitine* (35μM) *by brown-adipose-tissue homogenates of cold-adapted animals as a function of time*

Hydrolysis of palmitoyl-CoA: 0.15mg of protein/ml (○) and 0.34mg of protein/ml (▲). Hydrolysis of palmitoyl-L-carnitine: 0.34mg of protein/ml (□). Reproducibility of replicate measurements was better than ±8% for both enzymes.

10min. The rate was about 6% of the palmitoyl-CoA hydrolysis at identical concentrations. In the present study all assays were performed within the linear range in terms of both substrate and enzyme concentrations.

Subcellular-localization studies of palmitoyl-CoA hydrolase and palmitoyl-L-carnitine hydrolase of cold-adapted animals

Table 1 shows the distributions of palmitoyl-CoA hydrolase and palmitoyl-L-carnitine hydrolase compared with marker enzymes in fractions isolated from homogenates of brown adipose tissue. The recovery of both enzymes and protein was in the range 92–124%. About one-third of the activity of the mitochondrial inner-membrane markers carnitine palmitoyltransferase and succinate–phenazine methosulphate oxidoreductase were found in the mitochondrial fraction. The outer mitochondrial-membrane marker enzymes amine oxidase and palmitoyl-CoA synthetase were found in the mitochondrial and microsomal fractions in amounts comparable with those reported by Pedersen *et al.* (1975). The marker enzymes for lysosomes (acid phosphatase), microsomal fraction (NADPH–cytochrome *c* reductase) and the soluble fraction (lactate dehydrogenase) were distributed as expected (Pedersen *et al.*, 1975). Malate dehydrogenase and glutamate dehydrogenase were found in both the mitochondria and the soluble fraction. All of these values were within the variation limits reported by Pedersen *et al.* (1975). A comparison of palmitoyl-CoA hydrolase and palmitoyl-L-carnitine hydrolase shows that their main localization is in the mitochondria. As with malate dehydrogenase and glutamate dehydrogenase, however, a significant amount was also found in the particle-free supernatant. In rat liver, the glutamate hydrogenase is mainly localized in the mitochondria (Hoppel & Tomec, 1972). The activity of both hydrolases in the particle-free supernatant could therefore be due to mitochondrial damage.

Activity of the mitochondrial enzymes in brown adipose tissue of cold-adapted and control guinea pigs

The nutritional and environmental state of an animal influences the synthesis and the oxidation of its fat. The increased yield of mitochondria and change in the fat metabolism on cold-adaptation (for review, see Shapiro, 1977) indicate that changes in the concentrations of enzymes participating in fat metabolism may occur. Table 2 shows that cold-adaptation of guinea pigs caused the activities of palmitoyl-CoA synthetase and carnitine palmitoyltransferase to increase 1.3- and 2.8-fold respectively, and the activities of plamitoyl-CoA hydrolase and palmitoyl-L-carnitine hydrolase increased 2.1- and 5-fold respectively. All mitochondrial preparations were frozen and thawed twice before incubation to ensure complete destruction of the mitochondrial structure. On the other hand, the activity of the tricarboxylic acid-cycle-associated enzyme malate dehydrogenase was decreased by 50%. The inner-membrane marker enzyme succinate–phenazine methosulphate oxidoreductase and outer-membrane marker enzyme

Table 1. *Distribution of palmitoyl-CoA hydrolase and palmitoyl-L-carnitine hydrolase and some marker enzymes in brown-adipose-tissue homogenates of cold-adapted guinea pigs*

The absolute values for enzyme activities in total homogenate are expressed in μmol/min; protein is given in mg. The enzyme activities and the protein contents in the fractions are expressed as per cent of the total in whole homogenate (i.e. cytoplasmic extract + nuclear fraction). The reproducibility of replicate analyses of the same sample was better than ±8%. The differences in the percentage values in samples from two animals were from ±3 to ±12%, in agreement with Pedersen *et al.* (1975).

	Absolute values, total	Percentage values					
		Nuclear fraction	Mitochondrial fraction	Light mitochondrial fraction	Microsomal fraction	Particle-free supernatant	Recovery
Protein	165	10	9	6	9	66	100
Palmitoyl-CoA hydrolase	0.67	7	47	26	7	36	123
Palmitoyl-L-carnitine hydrolase	0.04	7	46	17	5	37	112
Carnitine palmitoyltransferase	1.9	12	39	16	18	13	98
Palmitoyl-CoA synthetase	2.7	4	35	22	20	25	106
Succinate–phenazine methosulphate oxidoreductase	55	15	32	21	14	15	97
Amine oxidase	0.14	11	34	17	13	18	93
Malate dehydrogenase	94	14	33	11	5	39	102
Glutamate dehydrogenase	13	10	25	10	8	44	97
Acid phosphatase	0.64	13	7	15	21	43	99
NADPH–cytochrome *c* reductase	1.2	8	6	10	42	39	105
Lactate dehydrogenase	11	3	0.3	0.1	0.4	95	99

Table 2. *Mitochondrial enzyme activities in brown adipose tissue from control and cold-adapted guinea pigs*
The mitochondria were frozen and thawed before incubation. The reproducibility of replicate determinations was better than ±8% for all enzymes. The differences between samples from two animals were about 15% for palmitoyl-L-carnitine hydrolase, and about ±8 to ±10% for the other enzymes. Values are given as nmol/min per mg of protein.

Enzyme	Control	Cold-adapted	Increase (fold)
Palmitoyl-CoA synthetase	55	71	1.3
Carnitine palmitoyltransferase	17	48	2.8
Palmitoyl-CoA hydrolase	10	21	2.1
Palmitoyl-L-carnitine hydrolase	0.26	1.3	5.0
Malate dehydrogenase	4100	2100	0.5
Amine oxidase	2.8	2.5	0.9
Succinate–phenazine methosulphate oxidoreductase	12400	11600	0.9

amine oxidase remained almost at a constant activity. The inner membrane of intact mitochondria is impermeable to palmitoyl-CoA (Wojtczak, 1975). Disruption of the mitochondria by freezing and thawing increased the hydrolysis of palmitoyl-CoA from 12.5 to 21 nmol/min per mg of protein, and this suggests that the mitochondrial long-chain acyl-CoA hydrolase in brown adipose tissue is localized in the matrix compartment.

Discussion

Long-chain acyl-CoA hydrolase activity has been found in several organs of different species. In rat liver, the enzyme activity has been localized in the cytosol (Kurooka *et al.*, 1972), the microsomal fraction (Barden & Cleland, 1969; Jezyk & Hughes, 1971), and also in the mitochondrial matrix (Colli *et al.*, 1969; Berge & Farstad, 1979). In aorta and brain of rat, the enzyme has been found in the microsomal fraction (Hashimoto & Dayton, 1975; Brophy & Vance, 1976). In fatty acid-oxidizing tissues, such as heart, long-chain acyl-CoA hydrolase has been found also in the lysosomal fraction (Kako & Patterson, 1975). In rat liver acyl-L-carnitine hydrolase has been found in the microsomal fraction (Mahadevan & Sauer, 1969; Berge & Farstad, 1979). The distribution of palmitoyl-CoA hydrolase and palmitoyl-L-carnitine hydrolase between the different subfractions of brown adipose tissue was found to be very equal. The high activities of the marker enzymes of the outer mitochondrial membrane in the microsomal fraction and particle-free supernatant are due to fragmentation of the mitochondria on homogenization of this tissue (Pedersen *et al.*, 1975). The relatively higher activities of palmitoyl-CoA hydrolase and palmitoyl-L-carnitine hydrolase in the soluble fraction compared with the above-mentioned activities also suggest a possible cytosolic localization. The high activities of malate dehydrogenase and glutamate dehydrogenase in the particle-free supernatant indicate, however, that the apparent cytosolic localization could be due to leakage from mitochondrial matrix.

The physiological role of hydrolysis of acyl-CoA and acyl-L-carnitine in brown adipose tissue is unclear at present. In both control and cold-adapted guinea pigs the activity of palmitoyl-CoA hydrolase was about 50% of the palmitoyl-CoA synthetase activity (Table 2). The activity of palmitoyl-L-carnitine hydrolase was, however, only 2.5–6% of the palmitoyl-CoA hydrolase activity. The increase in activity in the mitochondria during cold-exposure of the guinea pigs (Table 2) indicates that the hydrolase activities may either have a function as a safety device for releasing carnitine, or play a role in thermogenesis as a futile cycle releasing energy as heat. The hydrolases may also be related to the loose coupling of brown-adipose tissue mitochondria by promoting a high concentration of non-esterified fatty acids during thermogenesis (Heaton & Nicholls, 1976; Cannon *et al.*, 1977).

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