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Oxidative metabolism of long-chain fatty acids in mitochondria from sheep and rat liver

Evidence that sheep conserve linoleate by limiting its oxidation

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1. Mitochondria isolated from the livers of sheep and rats were shown to oxidize palmitate, oleate and linoleate in a tightly coupled manner, by monitoring the oxygen consumption associated with the degradation of these acids in the presence of 2mM-L-malate. 2. Rat liver mitochondria oxidized linoleate and oleate at a rate 1.2–1.8 times that of palmitate. 3. Sheep liver mitochondria had a specific activity for the oxidation of palmitate that was 50–80% of that of rats and a specific activity for the oxidation of oleate and linoleate that was 30–40% that of rats. This would indicate that sheep conserved linoleate by limiting its oxidation. 4. Carnitine acyltransferase I (CAT I) actively esterified palmitoyl-CoA and linoleate to carnitine in both rat and sheep liver mitochondria, and in both cases the rate for linoleate was faster than for palmitate. 5. The CAT I reaction in both rat and sheep liver was inhibited by micromolar amounts of malonyl-CoA. With 90 μM-palmitoyl-CoA as substrate, CAT I was inhibited by 50% with 2.5 μM-malonyl-CoA in rats, and in sheep, 50% inhibition was found with all malonyl-CoA concentrations tested (1–5 μM). With 90 μM-linoleate as substrate for CAT I, a much larger difference in response to malonyl-CoA was seen, the rat enzyme being 50% inhibited at 22 μM-malonyl-CoA, whereas sheep liver CAT I was 91% and 98% inhibited at 1 μM- and 5 μM-malonyl-CoA respectively. 6. We propose that malonyl-CoA may act as an important regulator of β-oxidation in sheep, discriminating against the use of linoleate as an energy-yielding substrate.

Non-ruminant animals require 1–2% of their dietary energy as linoleate to avoid signs of essential fatty acid deficiency (Holman, 1968), but ruminants, because of the biohydrogenation process in the rumen (Hawke & Silcock, 1970; Dawson & Kemp, 1970), have only 0.3–0.5% of the dietary energy entering the duodenum present as essential fatty acids such as linoleate (Leat & Harrison, 1972), yet they show no signs of essential fatty acid deficiency, indicating that ruminants utilize the available linoleate efficiently even though there is no evidence that enzymes acylating glycerol 3-phosphate and other substrates differ in ruminants from those in non-ruminants (Daae, 1973).

The β-oxidation of essential fatty acids in ruminants may be prevented or be much decreased compared with animals such as the rat, as the production of CO_2 from infused linoleate in the sheep is much less than from infused stearate (Lindsay & Leat, 1977). This could be due to the properties of CAT I, which catalyses the first obligatory step leading to β-oxidation of long-chain fatty acids. CAT I may play an important part in regulating β-oxidation of fatty acids because of its position outside the permeability barrier (Brosnan *et al.*, 1973) and because its activity is modulated by malonyl-CoA (McGarry & Foster, 1980), a potential physiological regulator of CAT I activity and the oxidation of long-chain fatty acids (Ontko & Johns, 1980; Robinson & Zammit, 1982; Saggerson *et al.*, 1982; Zammit, 1983*a*,*b*; Mills *et al.*, 1983).

In this paper we compared the oxidation of palmitate, oleate and linoleate by rat and sheep liver mitochondria and studied the possibility that the properties of CAT I in sheep liver mitochondria might prevent the β-oxidation of linoleate.

Abbreviation used: CAT I, carnitine acyltransferase I.

Materials and methods

Palmitic acid, oleic acid, linoleic acid, palmitoyl-CoA, malonyl-CoA, L-carnitine, D-mannitol, dithiothreitol, ATP, ADP, bovine serum albumin (Cohn fraction V), Tris and EGTA were from Sigma Chemical Co., St. Louis, MO, U.S.A. DL-[*methyl*-^{14}C]Carnitine (50 μCi, 51.4 Ci/mol) was obtained from Amersham International, Amersham, Bucks., U.K., and from this a solution containing 4.32 mM-L-carnitine (0.23 Ci/mol) was prepared. Other reagents were analytical grade, and solutions were prepared in distilled deionized water. All glassware was kept free of detergents by soaking in alkali and then in acid, and finally rinsing in deionized water. Micellar solutions of fatty acids were prepared by titrating the potassium salt to pH 7–8 with HCl. Palmitate solutions were prepared at 70°C and unsaturated fatty acids under N_2. Fatty acid-free albumin was prepared by the method of Chen (1967); the freeze-dried solution was then dialysed as described by Hanson & Ballard (1968), and the final product contained 0.56 μmol of unesterified fatty acid/mol of albumin.

Treatment of animals

Female Sprague–Dawley rats (150–200 g) were kept at 25°C with light from 06:00 to 18:00 h, with access to a commercially available diet and fresh water at all times, except when they were deprived of food for 18–20 h before slaughter, which was by decapitation between 09:00 and 10:00 h.

Preparation of rat and sheep liver mitochondria

Rat livers (15 g) were removed rapidly and rinsed in buffer (225 mM-D-mannitol, 75 mM-sucrose, 50 μM-EGTA, pH 7.4). The liver was chopped into 2–3 mm cubes, rinsed twice with buffer, and homogenized in a Potter–Elvehjem homogenizer (0.25 mm clearance) with 60 ml of buffer, three passes being made by hand. The homogenate was centrifuged (800 *g* for 5 min), and the supernatant was centrifuged (4500 *g* for 20 min) to sediment the mitochondria. The mitochondrial supernatant was discarded, and any fat removed by wiping with a paper tissue. The mitochondria were resuspended, by stirring with a test tube filled with ice, in 30 ml of buffer and resedimented (4500 *g* for 20 min). The supernatant was discarded along with broken mitochondria, which appeared as an opaque fluffy layer. This resuspension procedure was done twice, and the final pellet (6–8 mg of mitochondrial protein/g of liver) was used in subsequent experiments.

Sheep liver was obtained from the freezing-works slaughterhouse and kept on ice for 30 min before preparation of mitochondria was started with the removal of the parenchyma and passage of 12–15 g of liver tissue through a tissue press (1.5 mm-diameter holes). This material was washed, homogenized and centrifuged as described above for rats.

β-Oxidation assay conditions

The rate of β-oxidation of long-chain fatty acid substrates was determined at 25°C by measuring the ADP-stimulated O_2 uptake with a Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH, U.S.A.). The standard reaction mixture contained 50 mM-sucrose, 15 mM-KCl, 2 mM-EGTA, 50 mM-Tris/HCl (pH 7.4), 25 mM-KH_2PO_4 (pH 7.4), 0.6 mM-carnitine, 25 μM-CoA in rats, or 5 μM-CoA in sheep, 1 mM-dithiothreitol, 1.5 mM-ATP, unesterified fatty acids and albumin in a 7:1 molar ratio (fatty acid:albumin) and 2 mM-malate. The reaction mixture was saturated with air, and 3–5 mg of mitochondrial protein was added, followed by 10 μl of 60 mM-ADP to give a total volume of 3 ml. The difference between O_2 uptake before and after the addition of ADP was taken as the rate of O_2 consumption.

Carnitine acyltransferase assay conditions

CAT I activity was assayed in intact mitochondria (0.2–0.5 mg of mitochondrial protein). The standard reaction conditions were 225 mM-D-mannitol, 70 mM-sucrose, 1 mM-EGTA, 10 mM-phosphate buffer (pH 7.4), 5 mM-$MgCl_2$, 1 mM-dithiothreitol, 1.5 mM-ATP, 25 μM-CoA in rats or 5 μM-CoA in sheep, and unesterified fatty acid and albumin in a 7:1 molar ratio (fatty acid:albumin). When palmitoyl-CoA was used as substrate, dithiothreitol, ATP, CoA, unesterified fatty acid and albumin were omitted. The reaction mixture was preincubated at 30°C for 1 min and the reaction was started by the addition of [^{14}C]carnitine (0.6 mM) in a total reaction volume of 0.35 ml. After 3 min the reaction was stopped by adding 2 ml of butanol and 10 ml of 0.1 M-boric acid saturated with butanol. The butanol phase was removed, washed with 0.1 M-boric acid saturated with butanol, and the acyl[^{14}C]carnitine in butanol was counted for radioactivity in 10 ml of scintillation fluid [40 mg of 2,5-diphenyloxazole and 1 mg of 1,4-bis-(5-phenyloxazole-2-yl)benzene in redistilled toluene/Triton X-100 (2:1, v/v)] in a Beckman LS8000 Liquid-Scintillation system, with a quench correction being applied.

Mitochondrial protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Statistical comparisons were by Student's *t* test.

Results

The respiratory control ratio, i.e. the rate of O_2 consumption in the presence of ADP compared with the rate after the ADP had been converted into ATP, for the rat liver mitochondria was 4–7 with 10mM-succinate as substrate, and these mitochondria oxidized succinate at a rate of 4–6 μmol of O_2/h per mg of protein, which is slightly faster than reported in the literature (Johnson & Lardy, 1967). Sheep liver mitochondria oxidized 10mM-succinate at a rate of 2–3 μmol of O_2/h per mg of protein and showed respiratory control ratios of 3–4. Respiratory control ratios varied by less than 10% after three successive additions of ADP in all mitochondria used in the experiments on fatty acid oxidation, indicating that unesterified fatty acids were not uncoupling oxidative phosphorylation at the concentrations used. All mitochondria were assayed in conditions where CoA and carnitine concentrations were optimal. The carnitine concentration, 0.6mM, was the same for sheep and rats, but CoA was optimal at 25 μM for rats and 5 μM for sheep, higher concentrations having an inhibitory effect on sheep liver mitochondria. Increasing the concentration of ATP up to 8mM had no effect on the rate of oxidation of 10 μM-palmitate in rat liver mitochondria.

Rates of O_2 uptake, with various amounts of fatty acids as substrates, with rat and sheep liver mitochondria are shown in Table 1. Ketone bodies were not measured, as the presence of 2mM-malate in the assay mixture effectively channels acetyl-CoA through the tricarboxylic acid cycle (Van den Bergh, 1967). The rates of O_2 uptake with no added fatty acid as substrate were 0.38 and 0.15 μmol of O_2/h per mg of protein for rats and sheep respectively.

The assay of CAT I was performed with mitochondria prepared as described above in a final reaction mixture which had a different osmolality from that in the O_2-uptake experiment, but this did not affect the rate of O_2 uptake in the presence of succinate. The CAT I reactions deliver enough fatty acid to sustain the O_2 uptakes measured in the previous experiments, with a small excess which is probably recycled by the CAT II and carnitine acyltransferase, as described by Houslay & Stanley (1982). The results shown in Table 2 indicate that the ratio activity with palmitate/activity with linoleate in the rats (1.8) are similar to those ratios obtained from the O_2-uptake experiments, but the results from sheep are very different from those in the O_2-uptake experiment.

The effect of malonyl-CoA on the activity of CAT I is shown in Fig. 1. The behaviour of sheep and rat liver mitochondria was very different when enzymes were assayed with linoleate as substrate in the presence of malonyl-CoA

Discussion

Mitochondria prepared from rat liver showed maximal rates of fatty acid oxidation, which were comparable with those reported by Lopes-Cardozo & Van den Bergh (1974) and Vaartjes & Van den Bergh (1978), assuming that the palmitate was completely oxidized. This argues against substantial production of ketone bodies or acetate in our

Table 1. *Rates of oxidation of palmitate, oleate and linoleate by rat and sheep liver mitochondria*
The results are the means of two experiments. For further details see the text. Rat mitochondria oxidize fatty acids at faster rates at higher fatty acid concentrations (1.6 μM versus 17 μM, $P = 0.029$; 1.6 μM versus 50 μM, $P = 0.0026$). The rates for sheep mitochondria oxidizing $C_{16:0}$ acid are slower than those for rat mitochondria oxidizing $C_{16:0}$ ($P = 0.0011$), and sheep oxidize $C_{18:1}$ and $C_{18:2}$ acid slower than do rats ($P < 0.0001$ for both substrates).

		Rate of oxidation (μmol of O_2/h per mg of mitochondrial protein)			
	Unesterified fatty acid concn. (μM)	$C_{16:0}$	$C_{18:1}$	$C_{18:2}$	Ratio $\frac{C_{18:2}}{C_{16:0}}$
Rat	1.6	1.42	1.66	2.21	1.7
	8.2	1.61	2.54	2.73	1.9
	17	1.88	2.46	2.95	1.7
	50	2.21	2.55	2.58	1.2
	P value compared with $C_{16:0}$		=0.02	<0.0001	
Sheep	1.6	1.39	1.10	1.03	0.7
	8.2	1.26	0.99	1.04	0.8
	17	1.26	1.04	1.08	0.8
	50	1.29	1.08	1.08	0.8
	P value compared with $C_{16:0}$		<0.0001	<0.0001	

Table 2. *Rates of CAT I activity of rat and sheep liver mitochondria*
Results are means ± S.D. Each point is the mean of two observations performed in duplicate. For further details see the text.

	Concn. of acyl groups (μM)	Rate of production of acylcarnitine (nmol/min per mg of protein) $C_{16:0}$	$C_{18:2}$	Ratio $\frac{C_{18:2}}{C_{16:0}}$
Rat	45	4.0±0.26	7.4±0.46	1.8
	90	4.6±0.16	8.4±0.34	1.8
	135	4.6±0.12	10.0±0.38	2.2
	180	3.0±0.18	11.2±0.80	3.8
	270	3.0±0.16	10.8±0.60	3.7
Sheep	45	2.0±0.08	2.8±0.20	1.2
	90	2.2±0.10	3.0±0.74	1.3
	135	2.4±0.12	3.6±0.30	1.5
	180	1.6±0.14	4.2±0.14	2.5
	270	1.4±0.18	4.2±0.16	2.8

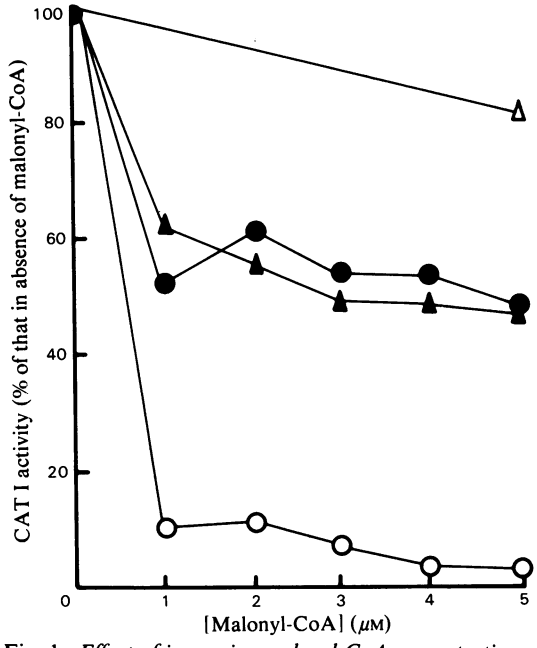

Fig. 1. *Effect of increasing malonyl-CoA concentration on the activity of CAT I from rat and sheep liver mitochondria*
Mitochondria were incubated with either 90 μM-palmitoyl-CoA (▲, rat; ●, sheep) or 90 μM-linoleate in the presence of CoA and ATP (△, rat; ○, sheep). The rates with no malonyl-CoA present were 5.2 and 2.8 nmol of acylcarnitine formed/min per mg of mitochondrial protein in rats and sheep respectively, with 90 μM-palmitoyl-CoA as substrate, and 12.2 and 2.20 nmol of acylcarnitine formed/min per mg in rats and sheep respectively, with 90 μM-linoleate as substrate. Rat mitochondria esterifying linoleate were tested at higher concentrations of malonyl-CoA and had 80%, 68%, 42% and 25% of the activity with no malonyl-CoA at 10 μM-, 15 μM-, 25 μM-, and 50 μM-malonyl-CoA respectively. Each point is the mean of two observations performed in duplicate.

assay system. Sheep liver mitochondria showed a lower specific activity than did those from rats. Koundakjian & Snoswell (1970) were unable to show fatty acid oxidation in their sheep liver mitochondria preparations.

Rat liver mitochondria oxidized both saturated and unsaturated fatty acids without uncoupling and showed a higher specific activity towards linoleate and oleate than with palmitate. Sheep liver mitochondria oxidized palmitate at rates in excess of those for linoleate. Thus sheep can exist on a lower intake of linoleate, because there is discrimination against the use of linoleate as an energy source (Lindsay & Leat, 1977), and a site for this discrimination is the mitochondrial β-oxidation pathway.

The role of CAT I in the discriminating treatment of linoleate was investigated. The ratio of CAT I activity with palmitate to that with linoleate from rats shows that the CAT I rates run in parallel to the rates of oxidation of palmitate and linoleate, but in sheep the rate of the CAT I reaction with linoleate was higher than with palmitate, but the rate of oxidation of linoleate was lower than that of palmitate oxidation. Therefore, discrimination against linoleate oxidation must be at a step or steps other than CAT I in the absence of malonyl-CoA (Tables 1 and 2).

CAT I inhibition by malonyl-CoA was very sensitive in sheep when linoleate was the substrate, there being over 90% inhibition at 1 μM- and 98% at 4 μM-malonyl-CoA. However, with linoleate as substrate, rat liver mitochondria CAT I was 50% inhibited at 22 μM, suggesting that malonyl-CoA would be a very effective inhibitor of linoleate oxidation in sheep but not in rats. The maximum inhibition of CAT I when the substrate was palmitoyl-CoA was 50% over the range tested, 1–5 μM-malonyl-CoA, in sheep liver mitochondria. Thus

the presence of malonyl-CoA in sheep liver would allow the β-oxidation of palmitate but prevent the β-oxidation of linoleate, whereas in rat liver, where the CAT I is 50% inhibited by 2.5 μM-malonyl-CoA with palmitoyl-CoA as substrate, and this is the maximum inhibition observed in these experiments, the presence of malonyl-CoA would allow a greater oxidation of linoleate than of palmitate at malonyl-CoA concentrations in the 0–5 μM range.

Sheep liver contains acetyl-CoA carboxylase (Ballard *et al.*, 1969) even though it is not a major site of fatty acid biosynthesis. Tissues in rats with a low fatty acid-synthesizing capacity are extremely sensitive to inhibition by malonyl-CoA at the CAT I site (Mills *et al.*, 1983; Saggerson & Carpenter, 1981), and possibly malonyl-CoA is synthesized as a regulatory control molecule in these tissues and sheep liver, independent of fatty acid synthesis.

The role of malonyl-CoA in discriminating against linoleate oxidation in sheep is uncertain, as we do not know the intracellular malonyl-CoA concentration. We propose that acetate derived from rumen fermentation is converted into malonyl-CoA in sheep liver and prevents β-oxidation of fatty acids by acting at the CAT I site and that this is an important regulatory molecule in preventing the oxidative degradation of essential fatty acids such as linoleate.

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