ROLE OF CARNITINE IN LEUCINE OXIDATION BY MITOCHONDRIA OF RAT MUSCLE

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1. Introduction

Branched-chain amino acids are preferentially oxidized in muscle, in contrast to other amino acids, which are mainly oxidized in liver [1]. In muscular tissue carnitine concentration is considerably higher than in other tissues, except for kidney [2]. It may therefore be physiologically important that carnitine stimulates the oxidation of branched-chain amino acids and their oxo acids in mitochondria and homogenates of rat skeletal muscle [3] and in mitochondria of rat heart [4]. Since the products of branched-chain oxo acid decarboxylation are branched-chain acyl-CoA esters, carnitine might be involved in the transport of their acyl-residues across the mitochondrial membrane. On the other hand, carnitine might stimulate branched-chain amino acid oxidation indirectly by removal of long-chain acyl-CoA esters, which are known to be potent inhibitors of various enzymes [5]. In this study we excluded this latter possibility. It is also shown that carnitine stimulates 2-oxoisocaproate oxidation in intact mitochondria of skeletal muscle and heart, but not in those of liver, kidney cortex and brain, nor in broken mitochondria of skeletal muscle. The stimulation is accompanied by an accumulation of isovaleryl-carnitine. It is proposed that carnitine plays a role in leucine oxidation in muscle by removal of isovaleryl-residues out of the mitochondrion, and that these isovaleryl-residues have to be oxidized in other tissues.

2. Materials and methods

L-carnitine was obtained from Grand Island Biological Company (Grand Island, N.Y.). Isovaleryl-L-carnitine, propionyl-L-carnitine and acetyl-L-carnitine were synthesized according to Solberg and Bremer [6]. Amino acid oxidase, thiamine pyrophosphate and palmitoyl-CoA were purchased from Sigma (St. Louis). [1-^{14}C]leucine and [U-^{14}C]leucine were obtained from the Radiochemical Centre, Amersham. 2-[U-^{14}C]- and 2-[1-^{14}C]oxoisocaproate was synthesized from [U-^{14}C]- and [1-^{14}C]leucine, respectively, by the method of Meister [7].

Male albino Wistar rats (180–240 g) were used, one rat per experiment. The rats were starved for 18–24 h before they were killed. Mitochondria were prepared as earlier described [3]; heparin was omitted during preparation of mitochondria of non-muscular tissue. Oxidation rates were measured in a medium containing 10 mM potassium phosphate, 75 mM Tris–HCl (pH 7.4), 25 mM sucrose, 35 mM KCl, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM 2-oxoglutarate, 1 mM NAD^+, 25 μM cytochrome *c*, 0.1 mM coenzyme A, 5 mM ADP and 2 mM L-carnitine (unless otherwise stated). 0.5 mM L-[1-^{14}C]leucine or 0.5 mM 2-[U-^{14}C]oxoisocaproate was used as substrate (spec. act. 100 μCi/mmol). Incubation occurred in a final volume of 1.0 ml containing 0.10–0.15 mg mitochondrial protein at 37°C for 30 min. $^{14}CO_2$ was trapped in 0.2 ml ethanolamine/ethyleneglycol (1:2, v/v). Reactions were terminated by the addition of 0.2 ml 30% trichloroacetic acid, whereafter the vials were kept on ice for 75 min. Radioactivity of $^{14}CO_2$ was measured in a liquid scintillation counter with 0.4% Omnifluor in toluene/methanol (2:1, v/v). [^{14}C]carnitine-esters were obtained in the supernatant of the incubation mixture and purified from trichloroacetic acid and the main part of 2-oxoisocaproate by two extractions with diethyl ether [6]. After drying the residue was dissolved in ethanol/

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water (1:1, v/v) and the carnitine-esters were separated by thin-layer chromatography on Silicagel G. The chromatograms were developed in diethylether/formic acid (9:1, v/v) and after drying in methanol/chloroform/water/conc. ammonia/formic acid (55:50:10:7.5:2.5, by vol.) [6]. Carnitine-esters were identified by cochromatography of unlabeled carnitine-esters. This twofold thin-layer chromatography appeared to be adequate to separate the carnitine-esters from 2-oxoisocaproate, 2-oxoglutarate and citrate.

Palmitoyl-CoA concentration was determined according to Allred and Guy [8]. Protein concentration was determined by the method of Lowry et al. [9] with bovine serum albumin as standard.

3. Results

In contrast to pyruvate [3] and 2-oxoisovalerate, we observed that 2-oxoglutarate does not inhibit 2-oxoisocaproate oxidation. Since 2-oxoglutarate was used in experiments with leucine for transamination, it was for a good comparison also added in experiments with 2-oxoisocaproate. In intact mitochondria oxidative decarboxylation of 2-oxoisocaproate is independent of addition of thiamine pyrophosphate and is linear for 40 min (fig.1). In freezed-thawed mitochondria the oxidation rate is reduced and can only be partially restored by the addition of thiamine pyrophosphate and dithiothreitol. Restoration by thiamine pyrophosphate was equal from 0.05–1.00 mM. Oxidation rate in broken mitochondria was linear for 10–30 min.

Oxidation of 2-oxoisocaproate by intact mitochondria of skeletal muscle and heart was stimulated 2.6 and 1.7 times, respectively, by addition of L-carnitine (table 1). No or only a slight stimulation by carnitine was observed in liver, kidney and brain mitochondria. Carnitine did not increase 2-oxoisocaproate oxidation in skeletal muscle mitochondria, which were broken by freezing-thawing (data not shown). Addition of carnitine to skeletal muscle, heart and kidney mitochondria oxidizing 2-[U-^{14}C]-oxoisocaproate caused an accumulation of [^{14}C]-isovaleryl-carnitine (table 2). The amount of [^{14}C]-isovalerylcarnitine was in all experiments equal or larger than the increase in $^{14}CO_2$ production. No

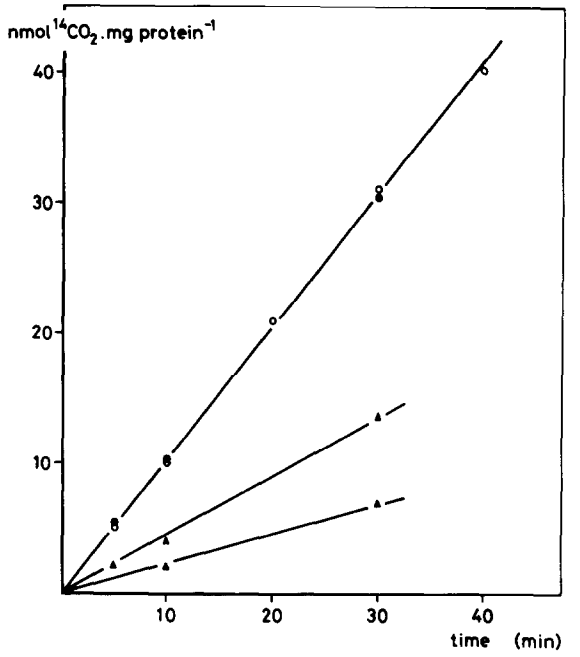

Fig.1. Effect of thiamine pyrophosphate on the oxidative decarboxylation rate of 2-oxoisocaproate by intact and broken mitochondria of rat skeletal muscle. $^{14}CO_2$ production from 0.5 mM 2-[1-^{14}C]oxoisocaproate was determined as described in section 2. Mitochondria were broken by three times freezing at −60°C and subsequent thawing at a concentration of 0.8 mg mitochondrial protein per ml. Thiamine pyrophosphate (0.15 mM) and dithiothreitol (1 mM) were added when indicated. ○–○, Intact mitochondria; ●–●, intact mitochondria + thiamine pyrophosphate and dithiothreitol; △–△, broken mitochondria; ▲–▲, broken mitochondria + thiamine pyrophosphate and dithiothreitol.

formation of [^{14}C]acetyl-carnitine or [^{14}C]propionyl-carnitine was observed with skeletal muscle mitochondria. In liver and brain mitochondria the accumulation of [^{14}C]isovaleryl-carnitine was much less. A slight accumulation of [^{14}C]acetyl-carnitine occurred in liver mitochondria.

Since long-chain fatty acids might be associated to the skeletal muscle mitochondria and coenzyme A is present in the incubation system, a low concentration of long-chain acyl-CoA esters might evolve during leucine oxidation. Therefore the effect of palmitoyl-CoA on the rate of oxidative decarboxylation of leucine was tested to exclude the possibility that carnitine artificially stimulates leucine oxidation by

Table 1
Effect of L-carnitine on 2-[U-^{14}C]oxoisocaproate oxidation by mitochondria of rat tissues

Tissue		nmol $^{14}CO_2$.min^{-1}.mg protein^{-1}		+ L-carnitine/ − L-carnitine
		− L-carnitine	+ L-carnitine	
Muscle	(8)	0.36 ± 0.02	0.94 ± 0.05	2.65 ± 0.18[b]
Liver	(6)	0.90 ± 0.13	1.00 ± 0.11	1.15 ± 0.07
Heart	(3)	1.57 ± 0 01	2.71 ± 0.06	1.72 ± 0.03[a]
Kidney cortex	(3)	2.69 ± 0.18	2.93 ± 0.11	1.10 ± 0.04
Brain	(3)	0.62 ± 0.02	0.72 ± 0.04	1.16 ± 0.02

[a] $P < 0.01$
[b] $P < 0.001$

$^{14}CO_2$ production from 0.5 mM 2-[U-^{14}C]oxoisocaproate was determined as described in Materials and methods. The ratios between $^{14}CO_2$ production in the presence of 2.0 mM L-carnitine and without carnitine were separately determined in each experiment. Values are the means ± SEM of the number of experiments indicated between parentheses. Significance was calculated by paired *t* test

Table 2
Increase in $^{14}CO_2$ production and [^{14}C]isovaleryl-carnitine production by carnitine during oxidation of 2-[U-^{14}C]oxoisocaproate by mitochondria of rat tissues

Tissue		ΔCO_2	Isovaleryl-carnitine
Muscle	(6)	0.51 ± 0.04	0.64 ± 0.03
Liver	(6)	0.10 ± 0.06	0.19 ± 0.03
Heart	(3)	0.81 ± 0.07	1.32 ± 0.03
Kidney cortex	(3)	0.24 ± 0.13	0.99 ± 0.07
Brain	(3)	0.10 ± 0.01	0.24 ± 0.03

Production of $^{14}CO_2$ and [^{14}C]isovaleryl-carnitine from 0.5 mM 2-[U-^{14}C]oxoisocaproate was determined with and without addition of carnitine as described in section 2. Without addition of carnitine no isovaleryl-carnitine was detected. Values of increase (in nmol ^{14}C-labeled product.min^{-1}.mg protein^{-1}) represent means ± SEM of the number of experiments indicated within parentheses

skeletal muscle mitochondria. No effect was observed after addition of 30 μM palmitoyl-CoA (fig.2). Addition of 40 μM palmitoyl-CoA or higher concentrations strongly decreased leucine decarboxylation. In intact mitochondria 2-oxoisocaproate oxidation was decreased to a similar extent at palmitoyl-CoA

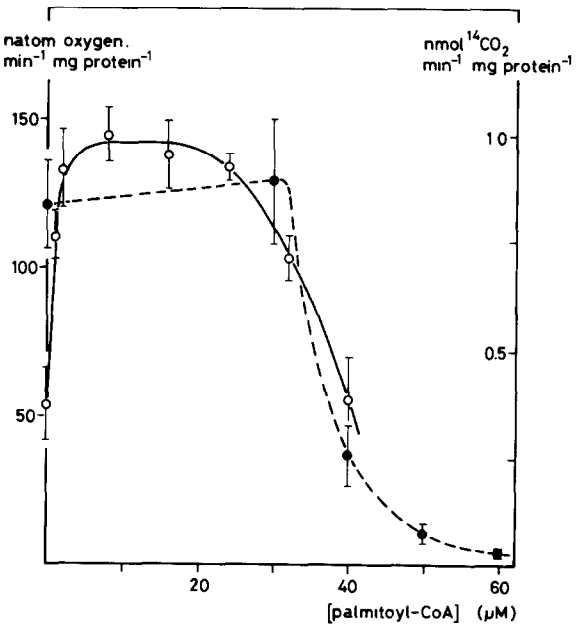

Fig.2 Effect of palmitoyl-CoA concentration on oxygen consumption and leucine decarboxylation by rat skeletal muscle mitochondria. Decarboxylation of 0.5 mM [1-^{14}C]-leucine was determined in the presence of 2.0 mM L-carnitine as described in section 2. Oxygen consumption during oxidation of palmitoyl-CoA was determined polarographically in a similar medium as leucine oxidation, but 0.5 mM L-malate was substituted for 2-oxoglutarate. ○—○, Oxygen consumption; ●—●, leucine decarboxylation. Values represent the means ± SEM of 4 and 6 experiments, respectively.

concentrations higher than 30 μM, but it was not affected by palmitoyl-CoA in broken mitochondria (data not shown). The decrease of the oxidative decarboxylation rate of leucine and 2-oxoisocaproate coincides with a decrease of the oxygen consumption at palmitoyl-CoA oxidation (fig.2). Since the latter decrease is known to be caused by inhibition of the adenylate translocator [8,9], the effect of high palmitoyl-CoA concentrations on leucine and 2-oxoisocaproate oxidation might also be caused by the elevated redox state in the mitochondrion. During assay of leucine oxidation palmitoyl-CoA concentration appeared, however, to be less than 2 μM. Therefore it can be excluded that carnitine stimulates leucine oxidation by removal of long-chain acyl-CoA esters.

4. Discussion

Although several authors reported the presence of branched-chain acyl-carnitine esters in several tissues during oxidation of branched-chain amino acids or oxo acids [6,12–14], the role of these esters is unknown [14]. Using [^{3}H]carnitine Spydevold et al. [13] observed accumulation of ^{3}H-labeled isovaleryl-carnitine and acetyl-carnitine during oxidation of 2-oxoisocaproate by rat skeletal muscle. In other tissues propionyl-carnitine was observed too [6,12]. These carnitine esters were also extracted from skeletal muscle, heart liver and testis of rats that had been starved for 18 h [14,15]. With 2-[U-^{14}C]oxoisocaproate we could only demonstrate formation of [^{14}C]isovaleryl-carnitine with skeletal muscle mitochondria. Acetyl-carnitine and propionyl-carnitine will therefore be formed from other substrates in this tissue. The exclusive accumulation of [^{14}C]isovaleryl-carnitine agrees with the observation that skeletal muscle tissue lacks a 3-hydroxy-3-methyl-glutaryl-CoA lyase [16]. [^{14}C]isovaleryl-carnitine also accumulated during oxidation of [U-^{14}C]isovaleryl-carnitine by heart and kidney cortex mitochondria. It is interesting to note that [^{14}C]isovaleryl-carnitine markedly accumulated with mitochondria of those tissues, which physiologically contain a high concentration of carnitine [2], but only slightly in liver and brain mitochondria. Probably the latter tissues have a limited activity of a suited carnitine transferase or translocase. Such a limitation might also explain why 2-oxoisocaproate oxidation is not stimulated by carnitine in brain mitochondria, which are deficient in 3-hydroxy-3-methyl-glutaryl-CoA lyase activity [16], whereas it markedly stimulated in mitochondria of skeletal muscle and heart.

Bieber and Choi [14] proposed that carnitine might carry isovaleryl-residues into the mitochondrion. The suggestion that the branched-chain oxo acid dehydrogenase complex of liver would be situated at the outer surface of the inner mitochondrial membrane [17] would be in favor of this proposal, but was recently questioned [4]. The proposed mechanism would not be advantageous in muscular tissue, since it cannot further degrade the isovaleryl-group [16]. This study presents evidence that carnitine is involved in leucine oxidation in muscular tissue by removal of isovaleryl-residues out of the mitochondrion to liberate intramitochondrial coenzyme A for further oxidation. Formation of isovaleryl-CoA within the mitochondrion was indicated by the observations that (a) oxidation of 2-oxoisocaproate was stimulated by thiamine pyrophosphate only in broken mitochondria, whereas (b) carnitine stimulated this oxidation only in intact mitochondria. Also the parallel effect of palmitoyl-CoA on the adenylate translocator and on oxidation of leucine and 2-oxoisocaproate suggest an inner mitochondrial localization of the branched-chain oxo acid dehydrogenase complex. The fate of isovaleryl-carnitine in the cytoplasm of skeletal muscle is not clear. In kidney and liver isovaleryl-carnitine may act as a reservoir of substrate, which would be further oxidized after depletion of 2-oxoisocaproate. In muscle it appears more likely that the isovaleryl-residues will leave the muscular tissue to become degraded in other tissues. Since transamination and 2-oxoisocaproate decarboxylation are largely performed in muscle [18], leucine oxidation appears to be divided over more than one tissue.

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