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Kinetic parameters of hepatic oxidation of cyclic fatty acid monomers formed from linoleic and linolenic acids

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Abstract

Cyclic fatty acid monomers (CFAM) occur from linoleic (CFAM-18:2) or linolenic (CFAM-18:3) acids present in some edible oils as a result of domestic frying or industrial refining. They present adverse effects in pups and weaning rats. In the present work, we studied the importance of hepatic oxidation in the metabolism of CFAM. For this purpose, kinetic parameters of Carnitine Palmitoyl Transferase I (key enzyme of the channeling of the fatty acids into the mitochondrial β-oxidation pathway) and Acyl CoA Oxidase (key enzyme of the peroxisomal oxidation pathway) towards CFAM-18:2 and CFAM-18:3 were calculated on hepatic sub-cellular fractions of rats. For mitochondrial oxidation of CFAM, we observed a lower oxygen consumption and a lower activity of Carnitine Palmitoyl Transferase compared to 18:2w6 and 16:0. For peroxisomal oxidation, CFAM-18:2 showed the same kinetic parameters (Vm and $K_{0.5}$) as 18:2w6 and 16:0, used for oxidative controls, whereas CFAM-18:3 presented a lower Vm (-50%). This difference should induce a lower catabolism of CFAM-18:3 in liver. This could contribute to their accumulation and probably to their toxic effect.

Keywords: Cyclic fatty acid monomers; Mitochondrial oxidation; Peroxisomal oxidation

1. Introduction

Among all the reactions which take place during domestic or industrial frying, cyclisation of linoleic and linolenic acids leads to the formation of cyclic fatty acid monomers (CFAM) [1,2]. Cyclic monoenoic acids occurring from linoleic acid (CFAM-18:2) contain mainly C5-membered-ring whereas a mixture of dienoic acids with C5- and C6-membered-ring results from the cyclisation of linolenic acid (CFAM-18:3) [3–5].

CFAM-18:3 can be efficiently absorbed [6,7] and incorporated into tissue lipids [8,9]. *In vivo* studies on rats ascribed to CFAM-18:3 some harmful effects on new-born rats [9,10]. Based on a preferential accumulation of the C5- over the C6-membered ring CFAM-18:3, whether in the lipids of cell culture [11,12], of lymph [7] or of rat pups and mother [9], the C5- and C6-membered ring were suspected to be differently metabolized. Moreover, a recent study on male rats showed that CFAM-18:3 induced a phenotypic peroxisome proliferator response, such as an increase of the peroxisomal-associated oxidation [13].

In contrast, no information have so far been published concerning the effects of CFAM-18:2.

The objective of this study was to evaluate the oxidative pathway of the CFAM-18:2 and CFAM-18:3 in liver subcellular fractions. For this purpose, we investigated the importance of mitochondrial and peroxisomal oxidation pathways using CFAM as substrates. This aspect may be of great importance to appraise the real impact of such molecules on health.

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Abbreviations: CFAM, cyclic fatty acid monomers; ACO, acyl-CoA oxidase; CPT, carnitine palmitoyl transferase; ACS, acyl-CoA synthetase; RCI, respiratory control index, ADP:O, adenosine diphosphate over atomic oxygen ratio.

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2. Materials and methods

2.1. Chemicals

Solvents provided from SDS (Peypin, France) were distilled before use. [1-^{14}C]-linoleic acid and [1-^{14}C]-linolenic acid were purchased from NEN (Les Ulis, France). CFAM-18:3 and CFAM-18:2 were prepared from heated linseed and sunflower oils and purified as previously described [14]. The purity of the CFAM was checked by thin layer chromatography (TLC) and by gas liquid chromatography using a HP5 capillary column (8 m long and 0.32 mm i.d.) fitted with a flame ionization detector and run on programmation mode (oven injection temperature set at 60°C, then increased at 30°C/min to 240°C then at 15°C/min to 350°C, held for 40 min). An aliquot was radiolabelled ([1-^{14}C]-CFAM-18:2, 2.1 GBq/mmol, and [1-^{14}C]-CFAM-18:3, 2.09 GBq/mmol) by the Commissariat à l'Energie Atomique (CEA-Saclay, France) using the bromo-decarboxylation reaction [15]. CFAM-18:3, CFAM-18:2, 18:2w6 and 16:0 were chemically condensed in acyl-CoA using free CoASH, and purified by high performance liquid chromatography, as described by Kawaguchi [16].

All other chemicals were purchased from Sigma-Aldrich (Sigma Co., L'Isle-D'Abeau-Chêne, France).

Male Wistar rats, weighing 200–220 g were provided by Charles River (France). They were fed a standard chow diet up to the sacrifice (9.00 am).

2.2. Enzyme assays

All the enzyme assays were performed with appropriate liver subcellular fractions obtained by conventional ultracentrifugation techniques.

The CPT activity and ACO activity were determined on mitochondrial-peroxisomal fractions, previously stored at −80°C, according to Bieber [17] and Lazarrow [18], respectively. We determined the kinetic parameters i.e. maximal velocity (or Vmax), the substrate concentration allowing half of the Vmax (or $K_{0.5}$) and the metabolic efficiency ($Vmax/K_{0.5}$). The different acyl-CoA assayed were CFAM-18:2-CoA, CFAM-18:3-CoA and both 16:0-CoA and 18:2-CoA as reference molecules. The concentrations assayed for the kinetic parameter determination were 5, 10, 20, 40 and 80 μM.

The kinetic activity of acyl-CoA synthetase was studied according to Bar Tana [19] using [1-^{14}C]-CFAM-18:2, [1-^{14}C]-CFAM-18:3 and their respective chemical precursors ([1-^{14}C] linoleic, 2.1 GBq/mmol and [1-^{14}C]-linolenic acid, 2 GBq/mmol). This measurement was carried out on freshly prepared microsomes.

The overall capacity of the mitochondria to oxidize CFAM-CoA substrates was determined by the measurement of mitochondrial oxygen consumption on mitochondrial/peroxisomal fractions freshly prepared. The different acyl-CoA assayed were CFAM-18:2-CoA, CFAM-18:3-CoA and both 16:0-CoA and 18:2-CoA as reference molecules. The parameters of oxidative phosphorylation, including the rates of oxygen consumption in state III (i.e. ADP-stimulated oxygen consumption), state IV (oxygen consumption after total ADP phosphorylation), respiratory control index (RCI) and ADP/O ratio, were determined following the third addition of ADP according to Estabrook [20] and Chance [21].

Table 1
Liver ACS apparent kinetic constants calculated from the different fatty acids.* This measurement was carried out on freshly prepared microsomal fractions.

ACS			
Substrate	V_{app}max (nmol/min/mg)	K_{app}0.5 (μM)	$V_{app}max/K_{app}0.5$
18:2 n-6	49.0 ± 6.94[a]	27.1 ± 6.67[a]	2.0 ± 0.19[a]
CFAM-18:2	17.1 ± 3.39[b]	25.7 ± 3.45[a]	0.7 ± 0.06[b]
18:3 n-3	26.9 ± 2.96[b]	15.5 ± 1.26[a,b]	1.7 ± 0.18[a]
CFAM-18:3	3.9 ± 0.57[c]	10.5 ± 1.97[b]	0.4 ± 0.03[b]

* Kinetic constants are calculated from a conventional Lineweaver & Burk model.

[a,b,c] Numbers in the same column not sharing a common superscript are significantly different ($P < 0.05$) (expressed as mean ± SEM, n = 4 to 5 animals).

V_{app}max: apparent maximal velocity; K_{app}0.5: apparent substrate concentration allowing half of the V_{app}max; $V_{app}max/K_{app}0.5$: index for metabolic efficiency.

2.3. Statistical analysis

The kinetic parameters for CPT-I, ACO and ACS were calculated according to a conventional Lineweaver & Burk model. The data are presented as mean ± SEM (n = 4 to 5 animals). The results were analyzed by a one-way ANOVA and a Student-Newman-Keuls test. The level of significance was set at $P \leq 0.05$.

3. Results

3.1. Acyl-CoA synthetase

The Vmax of ACS using CFAM-18:2 substrate was approximately 3-fold lower than with 18:2 n-6 and the Vmax with CFAM-18:3 was approximately 7-fold lower than with 18:3 n-3 (Table 1, $P < 0.05$). Moreover, the Vmax did not differ significantly between CFAM-18:2 and 18:3 n-3 ($P > 0.05$). The $K_{0.5}$ values were statistically identical among CFAM-18:2 and 18:2 n-6 in one side, and among CFAM-18:3 and 18:3 n-3 on the other side. The $K_{0.5}$ tended to be higher for 18:2 n-6 and CFAM-18:2 than for the 18:3 n-3 and CFAM-18:3 substrates, but the difference reached a statistical threshold for CFAM-18:3 only. The metabolic efficiency of the ACS was always higher for the non-cyclic precursor than for their cyclic counterpart.

Table 2
Liver CPT-1 apparent kinetic constants calculated from the different acyl-CoA.* This measurement was carried out on mitochondrial/peroxisomal fractions previously stored at −80°C.

CPT-1			
Acyl-CoA	V_{app}max (nmol/min/mg)	K_{app}0.5 (μM)	V_{app}max/K_{app}0.5
18:2-CoA	25.3 ± 2.26[a]	7.8 ± 0.43[a]	3.3 ± 0.42[a]
16:0-CoA	16.2 ± 3.47[b]	8.4 ± 1.58[a]	2.3 ± 0.70[a,b]
CFAM-18:2-CoA	7.1 ± 1.42[c]	6.9 ± 1.13[a]	1.5 ± 0.75[b]
CFAM-18:3-CoA	6.3 ± 1.04[c]	6.3 ± 0.57[a]	1.1 ± 0.25[b]

* Kinetic constants are calculated from a conventional Lineweaver & Burk model.

[a,b,c] Numbers in the same column not sharing a common superscript are significantly different ($P < 0.05$ for V_{app}max and $P < 0.08$ for V_{app}max/K_{app}0.5) (expressed as mean ± SEM, n = 4 to 5 animals).

V_{app}max: apparent maximal velocity; K_{app}0.5: apparent substrate concentration allowing half of the V_{app}max; V_{app}max/K_{app}0.5: index for metabolic efficiency.

3.2. Carnitine palmitoyl transferase

The kinetic parameters of CPT in rat liver mitochondria membrane were assayed with different acyl-CoA substrates (Table 2). The Vmax for 18:2-CoA was significantly higher than that for 16:0-CoA ($P < 0.05$). The Vmax of CPT was identical among the CFAM-18:2-CoA and CFAM-18:3-CoA substrates (*P = 0.98).* The Vmax values obtained with the CFAM were 3.8- and 2.4-fold less than with 18:2-CoA and 16:0-CoA, respectively. Conversely, the CPT shared a similar $K_{0.5}$ for all acyl-CoAs. As a result, the metabolic efficiency of CPT was in the decreasing order 18:2-CoA > 16:0-CoA > CFAM-18:2-CoA > CFAM-18:3-CoA.

3.3. Mitochondrial oxygen consumption

The rate of mitochondrial oxygen consumption after ADP stimulation (state III) was always lower with CFAM-CoA substrates than with their non-cyclic homologous (Table 3). A similar trend was observed when all ADP was converted to ATP (state IV), although the effect reached statistical significance with CFAM-18:3-CoA merely ($P \leq 0.05$). The other index of the mitochondrial oxidative integrity remained unchanged upon utilization of any of the acyl-CoA substrates (e.g. RCI and ADP:O ratio, Table 3).

3.4. Peroxisomal Acyl-CoA oxidase

The Vmax of the peroxisomal ACO was in the decreasing order 16:0-CoA > 18:2-CoA and CFAM-18:2-CoA > CFAM-18:3-CoA (Table 4), but only the Vmax of CFAM-18:3-CoA was significantly lower than the others. The Vmax of the enzyme was twice lower with CFAM-18:3-CoA than with 18:2-CoA or CFAM-18:2-CoA. The apparent $K_{0.5}$ of the peroxisomal ACO was similar for all substrates assayed. The metabolic efficiency of CFAM-18:3-CoA was 2.6- and 4-fold lower than that of 18:2-CoA (and CFAM-18:2-CoA) and 16:0-CoA, respectively.

Table 3
Mitochondrial oxidative parameters calculated from the different acyl-CoA. This measurement was carried out on freshly prepared mitochondrial fractions.

Mitochondrial oxidative parameters				
Acyl-CoA	State III*	State IV*	RCI	ADP:O
18:2-CoA	32.6 ± 2.70[a]	6.1 ± 0.21[a]	5.4 ± 0.55	2.7 ± 0.08
16:0-CoA	37.7 ± 1.25[a]	7.0 ± 0.36[a]	5.2 ± 0.34	2.6 ± 0.10
CFAM-18:2-CoA	24.5 ± 2.64[b]	6.2 ± 0.79[a,b]	4.0 ± 0.21	2.7 ± 0.09
CFAM-18:3-CoA	22.1 ± 2.09[b]	4.4 ± 0.59[b]	5.6 ± 0.95	3.0 ± 0.14

* Rate of O_2 consumption (in ng O_2.mL^{-1}).

[a,b] Numbers in the same column not sharing a common superscript are significantly different ($P < 0.05$) (expressed as mean ± SEM, n = 5 to 6 animals).

RCI: respiratory control index; ADP:O: adenosine diphosphate over atomic oxygen ratio.

4. Discussion

Although there are several isomers per CFAM family (those formed from linoleic and linolenic acids), apparent kinetic parameters were ascribed to CFAM-18:2 or CFAM-18:3 family for different reasons. Firstly, cyclisation of linoleic or linolenic acids present in edible oils always generated a mixture of isomers. Secondly, the composition of each mixture of CFAM used in this study (CFAM-18:2 or CFAM-18:3) was similar to CFAM occurring in heating oils [3]. Thirdly, harmful effects described in newborn rats were ascribed to a mixture of CFAM and not to a specific isomer.

Activation of fatty acid. In the liver microsomes, the long-chain ACS activity is essential since it catalyzed the initial

Table 4
Liver ACO apparent kinetic constants calculated from the different acyl-CoA.* This measurement was carried out on mitochondrial/peroxisomal fractions previously stored at −80°C.

ACO			
Acyl-CoA	V_{app}max (nmol/min/mg)	K_{app}0.5 (μM)	V_{app}max/K_{app}0.5
18:2-CoA	8.9 ± 0.68[a]	13.2 ± 1.66[a]	0.7 ± 0.13[a,b]
16:0-CoA	11.0 ± 1.85[a]	11.0 ± 1.23[a]	1.1 ± 0.32[a]
CFAM-18:2-CoA	8.1 ± 1.38[a]	12.4 ± 1.85[a]	0.8 ± 0.19[a,b]
CFAM-18:3-CoA	3.7 ± 0.80[b]	14.8 ± 2.25[a]	0.3 ± 0.11[b]

* Kinetic constants are calculated from a conventional Lineweaver & Burk model.

[a,b,c] Numbers in the same column not sharing a common superscript are significantly different ($P < 0.05$ for V_{app}max and $P < 0.08$ for V_{app}max/K_{app}0.5) (expressed as mean ± SEM, n = 4 to 5 animals).

V_{app}max: apparent maximal velocity; K_{app}0.5: apparent substrate concentration allowing half of the V_{app}max; V_{app}max/K_{app}0.5: index for metabolic efficiency.

step of the fatty acid activation prior to any further metabolism [22–24]. In agreement with other studies [25], we found that the essential fatty acids, linoleic and linolenic acids, featured a high metabolic efficiency. In addition, the ACS metabolic efficiency was far less for the CFAM-18:2 and CFAM-18:3 compared to their non-cyclic parent fatty acids. Anyhow, CFAM-18:2 and CFAM-18:3 were poorer substrates for ACS than linoleic and linolenic acids. This could potentially considerably limit their CoA-dependent cellular metabolism (oxidation or esterification).

Mitochondrial and peroxisomal oxidation. One detoxification pathway required one or two steps of oxidation that increased the hydrophilic status of the compounds and also enabled their excretion. Mitochondria oxidized the major part of the medium- and long-chain fatty acids. CFAM are also 18-carbon fatty acids and we could speculate that the preferential pathway for their oxidation was the mitochondrial β-oxidation. Nevertheless, our results suggested that CFAM were poor substrates for the mitochondrial oxidation enzymes, as suggested by a poor metabolic efficiency of CPT I and a poor O_2 consumption. These results were in agreement with those of Yamada [26] who found that the introduction of a phenyl group to the ω-position of acyl-chains decreased both the activity and the affinity of the carnitine acyltransferase, and thereby their mitochondrial oxidation.

The other organites responsible for cellular oxidation are the peroxisomes. They are involved in the degradation of a wide variety of lipophilic compounds that may be eliminated [27]. In fact, peroxisomal oxidation is unusual in that it does not go to completion but rather catalyzes a limited number of β–oxidation cycles and hence acts as a chain-shortening system. We found that the rate limiting enzyme for peroxisomal oxidation, namely ACO, displayed the same kinetic constants with CFAM-18:2 than with 18:2 n-6 or 16:0. In contrast, we found a lower rate of ACO activity for CFAM-18:3 than for the other fatty acids. This could be due to the size of the ring, since CFAM-18:2 are mainly C5-membered ring compounds, whereas CFAM-18:3 are both C5- and C6-membered ring compounds.

In conclusion we found that activation of CFAM-18:2 and CFAM-18:3 into their CoA derivatives should be a limiting step in their metabolism. Hence, the cell bioavailability of CFAM may be modulated via an other pathway. The CoA activation step is not required for ω- or (ω-1)-hydroxylation by CYP450 (phase I enzymes) and the subsequent processing by the phase II detoxifying enzymes (conjugation). The efficiency of these detoxifying pathways to process both the C5- and the C6-CFAM would be worth for further investigations. Moreover, our data on hepatic oxidation were obtained on liver sub-cellular fractions. To complete our results, it will be interesting to study *in vivo* the oxidation of radiolabelled CFAM-18:2 and CFAM-18:3.

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