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Original article

In vivo oxidation of carboxyl-labelled cyclic fatty acids formed from linoleic and linolenic acids in the rat

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Abstract – Cyclic fatty acid monomers (CFAM), which occur from the intramolecular cyclisation of linoleic and linolenic acids, are subsequently present in some edible oils and are suspected to induce metabolic disorders. One may suggest that the presence of a ring would alter the ability of the organism to oxidise these molecules. In order to test this hypothesis, we assessed the oxidative metabolism of CFAM in rats. For this purpose, rats were force-fed from 1.5 to 2.6 MBq of [1-^{14}C]-linoleic acid, [1-^{14}C]-linolenic acid, [1-^{14}C]-CFAM-18:2 or [1-^{14}C]-CFAM-18:3, and $^{14}CO_2$ production was monitored for 24h. The animals were then sacrificed and the radioactivity was determined in different tissues. No significant differences in $^{14}CO_2$ production were found 24h after the administration of CFAM and their respective precursors. Our data clearly demonstrate that, at least for the first β-oxidation cycle, CFAM are oxidised in a similar way as both essential fatty acids.

heating oil / essential fatty acids / oxidation / metabolism

1. INTRODUCTION

Heating oils and fats results in alterations of the structure of fatty acids and in the formation of new compounds [1]. Among them, cyclic fatty acid monomers (CFAM) have been demonstrated to be the most toxic compounds formed in heated oils. CFAM are mainly formed from the C18 polyunsaturated fatty acids of the edible oils (linoleic acid, 18:2n-6 and linolenic acid, 18:3n-3) [2]. Cyclisation of linoleic acid gives rise to thirteen identified monoenoic CFAM containing mostly a C5-membered ring (CFAM-18:2, Fig. 1A) while sixteen dienoic CFAM with a mixture of C5- and C6-membered ring structures have been identified from linolenic acid (CFAM-18:3, Fig. 1B). Studies carried out on animals have shown that CFAM-18:3 cross the gastrointestinal border [3, 4], are incorporated in some tissues [5, 6] and may induce adverse effects including high mortality of rat neonates [6]. In a recent work, we found that CFAM

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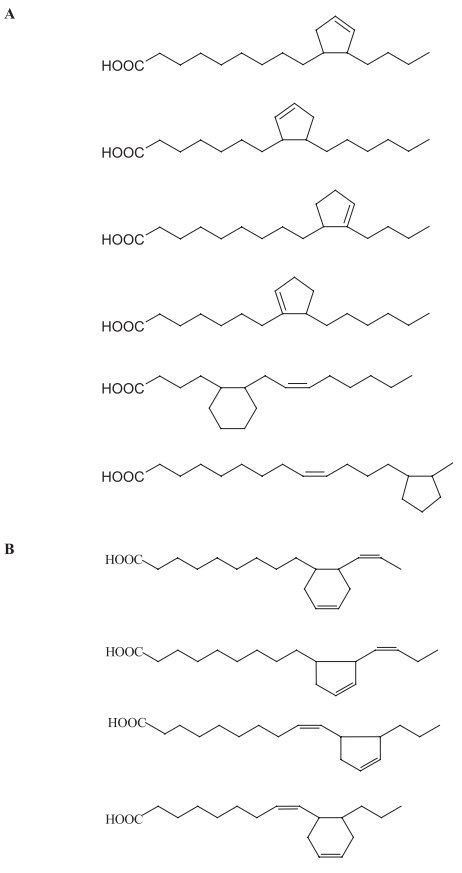

Figure 1. Chemical structures of the cyclic fatty acids formed from linoleic acid (**A**, from [14]) and linolenic acid (**B**, from [15]).

(CFAM-18:2 and CFAM-18:3) are poor substrates for in vitro mitochondrial oxidation enzymes in the rat liver [7], suggesting that the organism may be unable to oxidise these molecules.

In order to determine whether CFAM-18:3 and CFAM-18:2 can be metabolised in vivo via the β-oxidation process, [1-^{14}C] radiolabelled CFAM-18:2 and CFAM-18:3 were orally administered to rats and their oxidation was compared to that of 18:2n-6 and 18:3n-3. $^{14}CO_2$ expiration was monitored for 24 h and the distribution of the radioactivity in the different organs was also determined.

2. MATERIALS AND METHODS

This study was conducted following the *Guidelines for the Care and Use of Experimental Animals* and in accordance with the French official regulations on animal experiments (law 87-848, institutional authorization nr A21100).

Male Wistar rats (Janvier, Le Genest Saint Isle, France) weighing 260 ± 7 g (mean ± SEM) were housed in individual cages under controlled conditions of temperature (22 ± 1 °C) and relative humidity (55–60%). A 12 h light-dark cycle (lights on from 7:00 am–7:00 pm) was maintained. The animals were fed ad libitum with sterilised commercial pellets (Extralabo, Provins, France) and had free access to tap water. The day before the experiment, they had access to only 10 g of commercial pellets, in order to be partially fasted. All the experiments started at the same time of the day (oral load of the radioactive fatty acid at 9:00 am) in order to avoid diurnal variations.

CFAM-18:3 and CFAM-18:2 were purified from linseed and sunflower oils, respectively, as described by Sébédio et al. [2]. [1-^{14}C]-CFAM-18:2 (2.2 GBq·mmol^{-1}) and [1-^{14}C]-CFAM-18:3 (2.1 GBq·mmol^{-1}) were synthesised from these fatty acids using a bromo-decarboxylation procedure as described by Barton [8]. [1-^{14}C]-18:2n-6 (2.2 GBq·mmol^{-1}) was given by the CEA (Gif-sur-Yvette, France) and [1-^{14}C]-18:3n-3 (2.1 GBq·mmol^{-1}) was purchased from NEN (Les Ulis, France). The radiopurity of the fatty acids was more than 96% (data not shown).

The fatty acids were dissolved in triolein (Sigma Chemicals, L'Isle-d'Abeau, France) and then administered to rats by gastric tubing (280 µg, approximately 1.8 MBq per rat). After tubing, the rats were immediately installed in an airtight Plexiglas metabolic chamber, as previously described [9]. The $^{14}CO_2$ expired was trapped in a bottle containing 500 mL of a CO_2 trapping agent (Carbosorb, Packard, Groningen, the Netherlands). The rats were fasted during the

following 24 h. Without interrupting the bubbling of the expired air in the trapping agent, about 1 mL was removed every 30 min during the first 6 h of the experiment, every single hour during the 10 following hours and hourly again 20 to 24 h after tubing. As the density of the trapping agent increased along the experiment, the weights of each sample and of the bottle at each time of sampling were measured in order to determine the exact radioactivity expired, as described previously [10]. Nine milliliters of a scintillation cocktail (Permafluor E, Packard) were added to each sample and the radioactivity was determined using a TriCarb 2000 CA liquid scintillation counter (Packard).

At the end of the 24 h experimental period, the animals were anaesthetised and blood was withdrawn into a heparinised syringe. Then the tissues (brain, heart, liver, *Gastrocnemius* muscle, lung, kidneys, spleen, adrenals, testis, sus-epidydimal adipose tissue) were removed from the carcass, blotted on filter paper and weighed. The lower portion of the gastrointestinal tract (from the colon to the rectum including the faeces) was analysed separately. The carcass of each animal was weighed before homogenisation.

The radioactivity in 3 aliquots of each tissue (70–80 mg each), in 5 aliquots of carcass and of the lower portion of the gastrointestinal tract (110–130 mg), in blood and urine was determined by liquid scintillation counting after digestion in Soluene (Packard) as described elsewhere [9].

3. STATISTICAL ANALYSIS

Data are presented as means ± SD of 3 independent determinations. The results were analysed by an ANOVA procedure using the SAS software (SAS Institute, Cary, USA). *P* values of less than 0.05 or 0.01 were considered as significant.

4. RESULTS

The weights of the rats and tissues were not significantly different in the four groups (data not shown, $P > 0.05$).

4.1. Oxidation of 18:3n-3 and CFAM-18:3

The distribution of the radioactivity 24 h after oral administration of the [1-^{14}C]-18:3n-3 and [1-^{14}C]-CFAM-18:3 fatty acids is presented in Table I. Almost all the radioactivity administered was recovered into CO_2 and in the different organs (recovery > 98%, data not shown).

Total production of $^{14}CO_2$ at 24 h after administration of CFAM-18:3 and of 18:3n-3 was similar (73.8% and 79.8% of the radioactivity administered, respectively). The kinetics of the oxidation presented similar asymptotic profiles (Fig. 2). For each time of the experiment, no significant differences were observed.

In most tissues (liver, heart, kidneys, brain, lung, spleen, adrenals, testis and blood) the radioactivity recovered after administration of CFAM-18:3 was significantly higher than after feeding 18:3n-3 (from 1.5 to 2.2-fold, Tab. I). On the contrary, in the sus-epidydimal adipose tissue and in the lower portion of the gastrointestinal tract, the radioactivity recovered after administration of CFAM-18:3 accounted for only 30% and 50% of the radioactivity recovered after administration of 18:3n-3, respectively.

4.2. Oxidation of 18:2n-6 and CFAM-18:2

The distribution of the radioactivity 24 h after the oral administration of the [1-^{14}C]-18:2n-6 and [1-^{14}C]-CFAM-18:2 is presented in Table II. The yield of ^{14}C recovery ranged from 87% to 95% (data not shown).

Total $^{14}CO_2$ recovery 24 hours after administration of 18:2n-6 and CFAM-18:2 did not show any significant differences. Nevertheless, these values were lower than those obtained with 18:3n-3 and CFAM-18:3 (60–63% for CFAM-18:2 and 18:2n-6 compared to 73–80% for CFAM-18:3 and 18:3n-3).

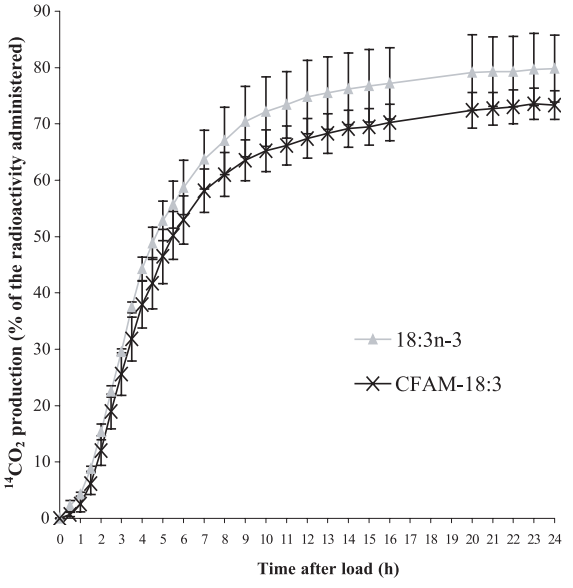

Figure 2. In vivo production of $^{14}CO_2$ observed after administration of [1-^{14}C] radiolabelled 18:3n-3 and CFAM-18:3 to the fasting rat. The results are expressed as means ± SD of the radioactivity administered recovered as $^{14}CO_2$. Data were obtained from three male Wistar rats for each fatty acid.

Table I. ^{14}C distribution observed 24 h after oral administration of [1-^{14}C]-18:3n-3 and [1-^{14}C]-CFAM-18:3 to the fasting rat.

	18:3n-3	CFAM-18:3	%[c]
$^{14}CO_2$ (final value at 24 h)	79.8 (5.92)	73.3 (2.53)	
Adrenals	2.80 (0.25)	4.89 (0.31)[a]	180%
Blood	0.34 (0.03)	0.74 (0.01)[a]	220%
Brain	0.30 (0.04)	0.50 (0.01)[a]	170%
Carcass	0.95 (0.05)	0.97 (0.17)	
Gastrocnemius muscle	0.35 (0.07)	0.41 (0.02)	
Gastrointestinal tract (lower portion)	0.37 (0.07)	0.19 (0.03)[b]	50%
Heart	0.66 (0.07)	1.01 (0.02)[a]	150%
Kidneys	1.34 (0.09)	1.90 (0.23)[b]	140%
Liver	1.86 (0.31)	2.67 (0.20)[b]	140%
Lung	0.92 (0.04)	1.65 (0.23)[a]	180%
Spleen	0.98 (0.09)	1.62 (0.09)[a]	170%
Sus-epidydimal adipose tissue	1.57 (0.16)	0.45 (0.03)[a]	30%
Testis	0.37 (0.04)	0.69 (0.05)[a]	190%
Urine	2.75 (0.26)	2.23 (0.33)	

The results are expressed as means (SD, $n = 3$) of the percentage of the administered radioactivity for the $^{14}CO_2$ and the percentage of the administered radioactivity per gram for tissue blood or urine.
[a] Significantly different from the corresponding value 24 h after administration of 18:3n-3 at $P < 0.05$.
[b] Significantly different from the corresponding value 24 h after administration of 18:3n-3 at $P < 0.01$.
[c] Ratio between the means of recovery after administration of CFAM-18:3 and 18:3n-3.

Table II. ^{14}C distribution observed 24 h after oral administration of [1-^{14}C]-18:2n-6 and [1-^{14}C]-CFAM-18:2 to the fasting rat.

	18:2n-6	CFAM-18:2	%[c]
$^{14}CO_2$ (final value at 24 h)	60.3 (1.39)	63.1 (4.05)	
Adrenals	4.72 (0.40)	3.98 (0.14)	
Blood	0.32 (0.07)	0.59 (0.03)[a]	180%
Brain	0.33 (0.01)	0.40 (0.03)[b]	120%
Carcass	1.19 (0.09)	0.99 (0.08)	
Gastrocnemius muscle	0.82 (0.20)	0.51 (0.07)	
Gastrointestinal tract (lower portion)	0.25 (0.14)	1.15 (0.26)[a]	460%
Heart	1.88 (0.13)	1.07 (0.08)[a]	60%
Kidneys	1.55 (0.17)	1.62 (0.22)	
Liver	2.27 (0.38)	2.32 (0.08)	
Lung	1.19 (0.10)	1.50 (0.38)	
Spleen	1.66 (0.33)	1.27 (0.07)	
Sus-epididymal adipose tissue	1.73 (0.23)	0.64 (0.06)[a]	40%
Testis	0.88 (0.03)	0.54 (0.09)[a]	60%
Urine	1.78 (0.09)	2.78 (0.18)[a]	160%

The results are expressed as means (SD, $n = 3$) of the percentage of the administered radioactivity for the $^{14}CO_2$ and the percentage of the administered radioactivity per gram for tissue blood or urine.
[a] Significantly different from the corresponding value 24 h after administration of 18:2n-6 at $P < 0.05$.
[b] Significantly different from the corresponding value 24 h after administration of 18:2n-6 at $P < 0.01$.
[c] Ratio between the means of recovery after administration of CFAM-18:2 and 18:2n-6.

$^{14}CO_2$ production from CFAM-18:2 increased rapidly reaching a plateau 12 h after the oral load whereas the $^{14}CO_2$ production from 18:2n-6 increased more slowly during the whole experiment (Fig. 3). Between 2.5 and 11 h, the recovery of $^{14}CO_2$ was significantly lower after oral administration of 18:2n-6 than after the administration of CFAM-18:2.

Some differences in the recovery of the radioactivity in tissues were observed for rats fed the 18:2n-6 or the CFAM-18:2 (Tab. II). After administration of CFAM-18:2, the radioactivity was higher in the brain (+21%), urine (+56%) and blood (+84%) than after administration of 18:2n-6. Similarly, the ^{14}C content of the lower portion of the gastrointestinal tract observed after administration of CFAM-18:2 was about 5-fold the value obtained after tubing linoleic acid. On the contrary, the testis and the sus-epididymal adipose tissue incorporated less radioactivity after administration of CFAM-18:2 than after administration of 18:2n-6 (–39% and –63% in the testis and adipose tissue, respectively).

5. DISCUSSION

Most of the radioactivity administered as essential fatty acids has been recovered into $^{14}CO_2$ (from 60.3% for 18:2n-6 to 79.8% for 18:3n-3), alpha-linolenic acid being more oxidised than linoleic acid 24 h after oral administration. This result was in agreement with previous data reported in 21-day-old rats [11] and in young rats [9, 10]. In a

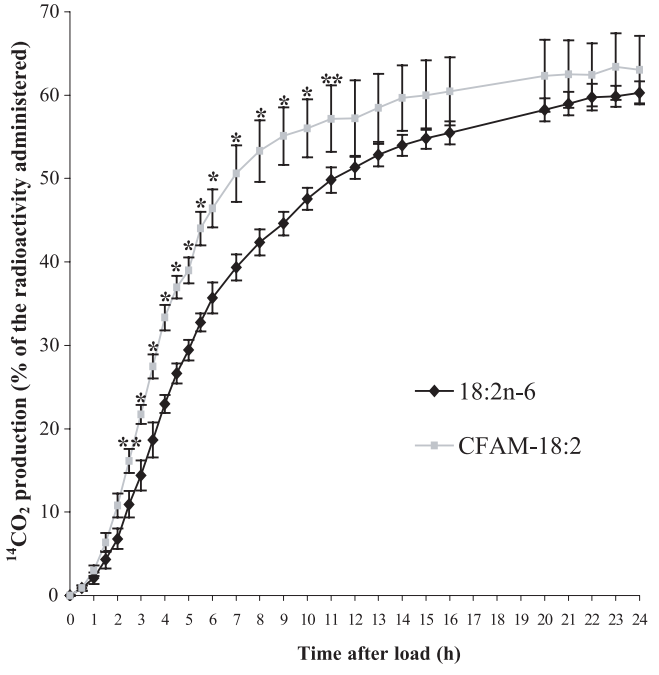

Figure 3. In vivo production of $^{14}CO_2$ observed after administration of [1-^{14}C] radiolabelled 18:2n-6 and CFAM-18:2 to the fasting rat. The results are expressed as means ± SD of the radioactivity administered recovered as $^{14}CO_2$. Data were obtained from three male Wistar rats for each fatty acid. *, ** Significantly different from the corresponding values after administration of 18:2n-6 at $P < 0.05$ and $P < 0.01$, respectively.

fasting status, the animals mainly oxidise fatty acids to produce ketone bodies as glucose-replacing fuel for extrahepatic tissues including the brain. After oral administration of the lipids, β-oxidation and energy production via the tricarboxylic acid cycle were enhanced and that could explain the high rates of $^{14}CO_2$ recovery.

The maximum ^{14}C production was observed between 2 and 4 h after oral administration (data not shown). This time can be related to the time for the molecules to reach and cross the gastrointestinal barrier, to be incorporated in the chylomicrons in the enterocytes and to be delivered to the tissues.

CFAM-18:2 and CFAM-18:3 are atypical fatty acids. Due to the presence of a cycle, one may hypothesise that these fatty acids would be poor substrates for mitochondrial oxidation. Indeed, we have previously shown that in vitro liver mitochondria exhibit lower oxygen consumption and Carnitine Palmitoyl Transferase I activity when incubated with CFAM [7]. However, our present results demonstrate that they are efficiently metabolised by the β-oxidation pathway. To explain this apparent discrepancy, the following hypothesis may be suggested. The production of $^{14}CO_2$ monitored in the present work arose from the first cycle of β-oxidation only from trace amounts of radiolabelled CFAM (about 280 µg). As a consequence, whatever the number of oxidation cycles would occur and even if the intramolecular ring would impair the molecule to be fully oxidised, the remaining

molecule would be formed in very low amounts that may unlikely be toxic. On the contrary, during the in vitro experiment [7], mitochondria were incubated with rather large quantities of CFAM that in the same way as in the present study would be metabolised but in a greater level of magnitude that may lead to greater amounts of molecules with a potential toxicity that remains to be verified.

The ^{14}C distribution in rat tissues remains difficult to interpret because it only accounts for the distribution of the radioactivity 24 h after oral administration of the tracer without any information on the nature of the ^{14}C compounds. Nevertheless, some differences may be underlined. We may suggest that the radioactivity was present in hydrophilic compounds produced by the detoxification of CFAM via the drug-metabolising pathway as previously suggested by Iwaoka and Perkins [12] and Damy Zarambaud and Grandgirard [13]. In our experimental conditions of partial fasting, two different prominent metabolic pathways may be suggested for the fatty acids: oxidation and elimination by detoxification without prior β-oxidation. As suggested by the important rate of $^{14}CO_2$ formation, the four fatty acids are equally prone to enter the β-oxidation pathway, at least for the first steps of activation, transport to the mitochondria (carnitine palmitoyltransferase/translocase shuttle) and first cycle of oxidation. But it must be remarked that despite the presence of a cycle in the molecule, the difference observed between the oxidation of CFAM-18:2 and CFAM-18:3 was of the same order of magnitude as that between linoleic and linolenic acids, the 18:2-fatty acids being less oxidised than the 18:3-fatty acids. This finding was unexpected and remains unexplained. Indeed, no structural similarity can be found between CFAM-18:2 and 18:2n-6 or between CFAM-18:3 and 18:3n-3 which could have explained such findings. On the contrary, the steric hindrance of CFAM-18:2 is closer to CFAM-18:3 than to C18:2n-6. The radioactivity recovered in the lower section of the gastrointestinal tract and in the urine may account for the non-metabolisable part of the fatty acid intake. In this view, our result would suggest that the CFAM-18:2 might be poorly metabolised compared to their corresponding precursor, linoleic acid.

In conclusion, we demonstrated that the presence of an intramolecular ring does not alter the ability of the CFAM to enter and follow the first cycle of oxidation. Further studies with CFAM labelled within or at the vicinity of the ring, are needed to determine whether the CFAM are fully oxidised. In addition, the nature and the side effects, if any, of the chain-shortened CFAM remain largely unknown.

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