Influence of cyclopropene fatty acids (Baobab seed oil) feeding on the in vitro Δ9 desaturation of stearic acid in rat liver microsomes

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Δ9 desaturation of [1-^{14}C] stearic acid to oleic acid was studied in liver microsomes of rats fed either fresh Baobab seed oil, containing cyclopropene fatty acids (malvalic acid and sterculic acids), or heated Baobab oil, containing only minor amounts of these acids. This was compared with rats fed a control oil mixture or a fat-free diet. Up to 50 nmol of substrate, Δ9 desaturation rate was the same in rats fed the three fat diets. From 50 to 150 nmol, the desaturation rates were practically constant in rats fed fresh Baobab seed oil. It increased in heated oil-fed rats at the same rate as in the control group, but more slowly than in the fat-free diet group. Fatty acid composition of liver microsomal lipids did not reflect the in vitro observed desaturation rate because monounsaturated fatty acids were in the same percentages in fresh and heated Baobab seed oil-fed rats. Moreover, the proportion of arachidonic acid was lower, and that of linoleic acid higher for both diets, in comparison with controls, suggesting a decreased Δ6 and/or Δ5 desaturation rate.

Keywords: cyclopropene fatty acids; liver Δ9 desaturation

Introduction

Malvalic acid [7-(2-octyl-1-cyclopropenyl) heptanoic] and sterculic acid [8-(2-octyl-1-cyclopropenyl) octanoic] are cyclopropene fatty acids (CPFA) normally present in seed lipids of different plant families such as Malvaceae, Sterculaceae, or Bombacaceae.[1] CPFA are toxic and held responsible for physiological and metabolic disorders in several species of animals.[2] In particular, sterculic acid (19CE) has been shown to inhibit the enzymic system, which desaturates stearic acid (18:0) to oleic acid (18:1n-9) in hens,[3] in trouts,[4] in rats,[5–8] and in plants.[9]

Seeds of Baobab, a giant tree of the Bombacaceae family, are used to produce an edible oil largely consumed by the Malagasy population after some refining. The fresh oil was demonstrated to exhibit deleterious effects on hens.[10] It has also been reported that heating resulted in a decrease of the oil CPFA content.[11] Accordingly, it was interesting to study the effect of fresh Baobab seed oil feeding on the Δ9 desaturase activity in liver microsomes of rats, which are animals closer to humans than hens, and to observe the effect of oil heating on this desaturation. The fatty acid composition of liver total microsomal lipids was also determined to ascertain how the enzyme activity measured in vitro was regulated in vivo.

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Materials and methods

Chemicals

[1-^{14}C] stearic acid (54 mCi/mmol, 99% radiochemically pure) was obtained from the Radiochemical Centre (Amersham, UK) and used at the specific activity of 10 mCi/mmol after

dilution with unlabeled fatty acid (50 nmol/10 μL of ethanol solution). Coenzymes and unlabeled fatty acid were provided from Sigma Co. Inc. (St Louis, MO, USA). All other chemicals of analytical grade were from Merck (Darmstadt, Germany) and Sigma.

Animals and diets

Weanling male rats of the Wistar strain were randomly divided into four groups of $n=4$ animals. They were fed a different semi-synthetic diet ad libitum for 4 weeks. The experimental diets only differed in the lipid composition. Diet denoted fat free (FF) was composed of a basal diet used to prepare the lipid-containing diets. These contained 10% by weight of the following oils: fresh (BO) and heated (HBO) Baobab seed oils and a mixture of palm oil (95% wt) and sunflower oil (5% wt) used as control (CO), containing the same level of linoleic acid (18:2n-6) as BO. The HBO was heated in a stainless-steel beaker for 8 hours at a controlled temperature of 200° C in the presence of air, as previously described.[11] The composition of the diets, (g/kg diet) was: sucrose, 280; maize starch, 350; casein, 180; agar-agar, 20; cellulose, 20; vitamin mixture, 20; salt mixture, 30; and fat, 100. The fatty acid composition of the diet lipids is shown in *Table 1*. The major fatty acids of the Baobab seed oil were palmitic (16:0), oleic, and linoleic acids. The cyclopropene fatty acids amounted to 12.7% and consisted of 6.2% malvalic acid (18CE) and 6.5% sterculic acid (19CE). Dihydrosterculic acid (19CA), a cyclopropane fatty acid, and a derivate iso 18 were also present in low amounts (1.80 and 0.19%, respectively). Heat treatment of the oil induced a great decomposition of the CPFA because the 18CE and the 19CE content decreased to 0.27 and 0.19%, respectively. The percentage of the other fatty acids increased correlatively.

Preparation of liver microsomes

At the end of the 4-week period, rats were killed by exsanguination. Each liver was immediately removed, weighed, and homogenized in a Potter-Elvehjem tube with 6 volumes of a solution containing 0.25 M sucrose and 0.05 M phosphate buffer, pH 7.4. The homogenate was centrifuged at 13,000*g* for 20 min. The pellet was discarded and the supernatant was recentrifuged at 105,000*g* for 60 min. The resulting pellet was resuspended in supernatant (1V) and homogenizing solution (2V). All operations were carried out at 4° C. The protein content of the microsomal fractions was estimated by the biuret method.[12]

Table 1 Fatty acid composition of dietary lipids

Dietary group	CO	BO	HBO
14:0	0.81	4.60	4.80
15:0	0.12	2.40	2.80
16:0	39.10	37.30	43.30
16:1n-7	0.10	0.25	0.29
17:0	0.11	0.22	0.25
Iso18	—	0.19	0.14
18CE	—	6.20	0.27
18:0	5.10	4.20	4.60
18:1n-9 + 18:1n-7	40.00	21.30	24.70
18:2n-6	13.30	13.50	14.40
19CE	—	6.50	0.90
18:3n-3	0.48	0.10	0.11
19CA	—	1.80	2.12
20:0	0.44	0.74	0.83
20:1n-9	0.21	0.12	0.12
22:0	0.18	0.15	0.23
Nonidentified	—	—	0.40

Results are expressed as moles% of total fatty acids.
CO, Control mixture of palm and sunflower seed oil.
BO, Baobab seed oil.
HBO, Heated Baobab seed oil.
18CE, Malvalic acid; 19CE: sterculic acid; 19CA: dihydrosterculic acid; Iso 18: derivative of 19CA.

In vitro desaturase assays

The conditions of desaturation assays were essentially those previously described,[13] except that a wide range of substrate levels (0–150 nmol) was used at each assay. All incubations were carried out at 37° C for 15 min in the presence of 5 mg of microsomal protein. The other conditions (incubation medium, total volume) and the separation of the substrate and product ^{14}C fatty acids by reversed phase high performance liquid chromatography in the conditions used in the laboratory were reported previously.[14]

Fatty acid analysis

Lipids from an aliquot of liver microsomes were extracted using the method of Delsal.[15] Fatty acid methyl esters were prepared by transmethylation with sodium methoxide[16] and analyzed by gas liquid chromatography using a glass capillary column.[13]

Statistics

Desaturation rates were expressed as nmol of desaturation product formed/15 min/5 mg microsomal protein and fatty acid composition as moles (%). Both results were means ± SD of four determinations (one per rat). After analysis of variance using a Duncan multiple range test, means were compared in the four groups of rats (FF, BO, HBO, and CO) according to the least significant difference. Values assigned a different superscript letter were significantly different at $P < 0.01$.

Results

Body weight, liver weight, and liver microsomal protein content of the rats maintained on the four different diets are reported in *Table 2*. After 4 weeks of diet, the body weight of animals fed the BO diet was only half that observed in the CO, while the liver weight and microsomal protein content were approximately the same. Results found in rats fed HBO were similar to those in the CO group. No difference was observed with the rats fed the FF diet.

The Δ9 desaturation rates measured in liver microsomes with four levels of substrate are illustrated in *Figure 1*. In the three groups CO, HBO, and FF, the desaturation rate increased linearly with the substrate concentration (except perhaps in the 0–50 nmol range). For each concentration of substrate, values were identical in the HBO and CO groups, whereas those in the FF group were twice as high. Between 0 and 50 nmol of ^{14}C 18:0, values observed in the BO group were those of the HBO and CO groups, but above that value, they reached a plateau. With 150 nmol of substrate, the desaturation rate for the group fed BO was

Table 2 Body weight, liver weight, and liver microsomal protein content of rats fed the different diets

Dietary group	Body weight (g)	Liver weight (g)	Microsomal protein (mg/g of liver)
FF	260 ± 16[a]	11.2 ± 0.7[a]	17.2 ± 1.3[ab]
BO	134 ± 7[c]	9.9 ± 1.0[a]	14.2 ± 1.2[b]
HBO	250 ± 4[b]	10.6 ± 0.6[a]	18.5 ± 1.1[a]
CO	276 ± 12[c]	11.3 ± 0.4[a]	17.9 ± 1.1[a]

FF, Fat-free diet.
CO, Control mixture of palm and sunflower seed oil.
BO, Baobab seed oil.
HBO, Heated Baobab seed oil.
Values are means ± SD for four rats fed each diet. After analysis of variance (Duncan's multiple range test), means were compared according to least significant differences. In each column, means assigned different superscript letters were significantly different ($P < 0.01$).

less than one-half that measured in the CO group. The relative capacity of liver to desaturate 18:0 was calculated by taking into account the microsomal protein amount isolated from each liver. In the four groups of animals, at 150 nmol of substrate, the following data were found: 6070±280, 3110±85, 2980±25, and 960±110 nmol 18:0 converted to 18:1n-9 by the whole liver in 15 min for the FF, HBO, CO, and BO groups, respectively. Therefore, when expressed on the basis of whole organ, the Δ9 desaturation capacity of liver in the HBO group was 3.5 times higher than in the BO group, whereas on the basis of tissue protein, the rate was only 2.2 times higher.

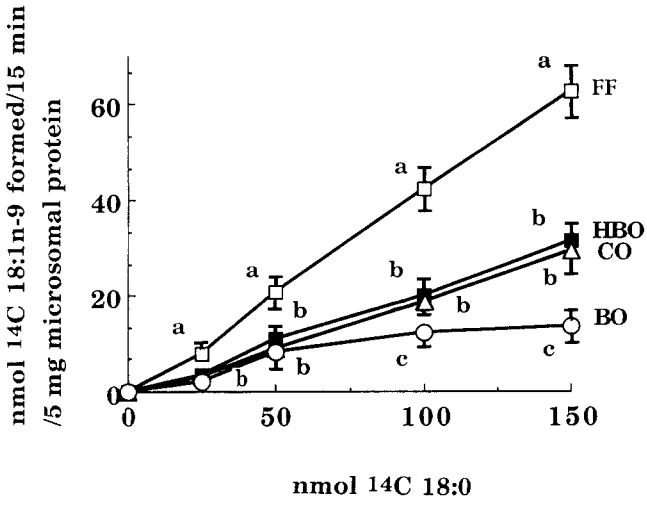

Figure 1 Activity of [1-^{14}C] stearic acid Δ9 desaturation at four concentrations in liver microsomes of rats fed four different diets. Results are means ± SD for four animals per group. Means were compared in the four groups for each concentration of substrate. Means with a different manuscript letter (a, b, c in decreasing order) were significantly different at $P < 0.01$. FF, Fat free; BO, Fresh Baobab seed oil; HBO, Heated Baobab seed oil; and CO, Control oil mixture.

The fatty acid composition of liver microsomal total lipids (principally phospholipids) is reported in Table 3. In the BO group compared with the control group (CO), the percentage of 16:1n-7 was reduced, while 16:0 remained unchanged. The percentage of 18:0 was increased by 24%. This increase was compensated for by a decrease in monounsaturated fatty acids 18:1n-9 and 18:1n-7, the elongation product of 16:1n-7. Con-

Table 3 Main fatty acids of liver microsome total lipids

Dietary group	FF	CO	BO	HBO
16:0	21.10 ± 0.40[a]	21.50 ± 0.60[ab]	22.40 ± 0.50[b]	22.40 ± 0.50[b]
16:1n-7	4.90 ± 0.60[a]	2.80 ± 0.20[b]	0.40 ± 0.10[b]	0.73 ± 0.06[c]
18CE			0.40 ± 0.10[a]	0.13 ± 0.02[b]
18:0	14.20 ± 0.60[a]	16.50 ± 0.40[b]	21.50 ± 0.50[c]	20.50 ± 0.30[d]
18:1n-9	16.70 ± 0.50[a]	10.50 ± 0.30[b]	8.50 ± 0.50[c]	8.60 ± 0.20[c]
18:1n-7	5.30 ± 0.30[a]	3.80 ± 0.50[b]	1.30 ± 0.10[c]	1.80 ± 0.20[d]
18:2n-6	5.90 ± 0.20[a]	11.10 ± 0.20[b]	17.80 ± 0.50[c]	14.80 ± 0.40[d]
19CE			1.13 ± 0.20[a]	0.47 ± 0.07[b]
20:3n-9	7.60 ± 0.50[a]	1.06 ± 0.14[b]	0.84 ± 0.06[c]	1.26 ± 0.11[b]
20:4n-6	13.80 ± 1.00[a]	23.40 ± 0.80[b]	14.40 ± 0.70[a]	18.90 ± 0.20[b]
22:6n-3	2.90 ± 0.10[a]	3.80 ± 0.10[b]	3.60 ± 0.10[b]	3.20 ± 0.10[c]
Total MUFA	26.90 ± 0.50[a]	21.10 ± 0.50[a]	10.20 ± 0.40[b]	11.10 ± 0.20[c]
Total SFA	35.30 ± 0.60[a]	38.00 ± 0.50[b]	43.90 ± 0.50[c]	42.90 ± 0.50[c]
MUFA/SFA	0.76 ± 0.11[a]	0.71 ± 0.12[a]	0.23 ± 0.06[b]	0.26 ± 0.07[b]
20:4n-6/18:2n-6	2.33 ± 0.15[a]	2.11 ± 0.13[a]	0.81 ± 0.10[b]	1.27 ± 0.09[c]

Results are means ± SD for $n = 4$ determinations and are expressed as moles% of fatty acids from total liver microsomes. After analysis of variance (Duncan's multiple range test), means were compared in each row according to the least significant difference. Means assigned different superscript letters were significantly different ($P < 0.01$).
MUFA, Monounsaturated fatty acids.
SFA, Saturated fatty acids.
CO, Control mixture of palm and sunflower seed oil.
BO, Baobab seed oil.
HBO, Heated Baobab seed oil.
FF, Fat-free diet.

sequently, the monounsaturated/saturated fatty acids ratio was three times lower. With regard to the polyunsaturated fatty acids, the percentage of 18:2n-6 was higher, whereas the percentage of 20:4n-6 was lower, the ratio 20:4 /18:2 being 2.6 times lower. Additionally, malvalic (18CE) and sterculic (19CE) acids were present in the BO microsomal lipids, but in much lower concentrations than in the diet lipids *(Table 1)*. If we compare HBO and BO, we can observe that the percentages of 18:1n-9 and 18:0 were practically the same. The percentage of 20:4n-6 was increased and that of 18:2n-6 decreased in the HBO group so that the ratio 20:4/18:2 was definitely higher, however remaining much lower when compared with the control group. The presence of minor levels of cyclopropenoic acids could still be observed in the HBO group, despite their very low concentration in the heated oil *(Table 1)*. In the FF group, the absence of dietary lipids was accompanied by a decrease in 18:2 and 20:4n-6 when compared with the control, but the ratio 20:4/18:2 was not significantly different, as compared with the control group.

Discussion

Growth rate of rats fed fresh Baobab seed oil was considerably lower than in controls. Andrianaivo et al.[17] have shown that this resulted from the very low food intake (271 g for 4 weeks compared with 504 g for the CO group and 468 g for the HBO group). Additionally, the food efficiency (weight gain/food intake) was three times lower (0.11 instead of 0.35). These combined effects can by themselves explain the low weight gain. In rats eating the HBO, the final body weight was not significantly different from the control. CPFA are probably poorly absorbed in the intestine and they interact with absorption of the other nutrients, causing low food efficiency. Such an effect on rat growth was also observed with seed oil from *sterculia foetida*[18] and *maesta*,[19] which also contain cyclopropenoic acids.

Despite the deleterious effect on growth, BO did not induce a higher liver weight nor a higher microsome protein content than CO. However, when compared with body weight, liver weight was 1.8 times higher (7.38% versus 4.09%.). This is in agreement with data from others who observed an enlargement of liver and other organs in rats fed cyclopropenoic acids.[17,20] Although poorly absorbed, their concentrations in blood were probably sufficient to stimulate the detoxification processes in liver endoplasmic reticulum, explaining the relative hepatomegaly.

When the Baobab seed oil was heated, as in industrial refining processes or in familial cooking, most of the cyclopropenoic acids were degraded because less than 0.5% moles was still present. In rats fed HBO, body weight, liver weight, and microsomal protein content were those found in the control rats. Thus, according to these biological parameters, heating seems to induce a quality of the oil comparable to the control oil.

Results dealing with the in vitro Δ9 desaturation rates in liver microsomes could support this conclusion because no difference could be observed between HBO and CO groups. However, the fatty acid profiles of the microsomal lipids, which reflect the fatty acid profiles of liver total phospholipids, were different in rats fed these two diets and close to what was observed in rats fed the BO. In particular, the decreased percentage of 18:1n-9 in rats fed HBO as compared with rats fed CO suggests a decreased in vivo transformation of 18:0 by Δ9 desaturation, contrary to what was shown in vitro. In the whole animal, cyclopropenoic acids, absorbed and present in low concentration in blood and hepatocytes, may compete with 18:0 at one or several steps of Δ9 desaturation, or with 18:1n-9 at the step of esterification. This eventual competition cannot occur in vitro, giving an explanation to the discrepancies observed in vitro and in vivo.

This raises the question of the mechanism by which the cyclopropenoic acids inhibit Δ9 desaturation. We can probably discard the hypothesis of an inhibiting effect of the acids incorporated in the lipids of the liver microsomal membrane, inside which the desaturase and its associated proteins are anchored, because the acids were only present in very low concentration in rats fed the fresh oil and they are still present, although at lower concentrations, in rats fed the heated oil. Because in the BO group the Δ9 desaturation system was practically saturated at 50 nmol of substrate in our experimental conditions (and not yet saturated at 150 nmol in the HBO and CO groups), we can hypothesize that the amount of active enzyme was lower in the BO group because a decreased amount of enzymic protein or because part of the active site of the protein was not available to the substrate. It has been suggested that inhibition resulted from the formation of a carbon-sulfur bond between a carbon atom of the cyclopropene ring and a thiol group at the desaturating site of the enzyme.[3,7,9,21] The cyclopropenoic acids exert their inhibiting effect on the desaturating system itself and not at the activation step of the substrate because these acids also inhibit the desaturation of the CoA derivatives of fatty acids.[7]

The problem could be different in vivo because a higher rate of in vitro Δ9 desaturation in HBO group, when compared with the BO group, did not result in higher concentration of 18:1n-9 or 18:1n-7 in microsomal lipids. Minor amounts of free CPFA in liver cytosol either inhibited in vivo Δ9 desaturation or elongation or inhibited esterification of the desaturation product in microsomal lipids. A metabolite derived from CPFA, such as a cyclopropanoic acid, may also be a causative agent for the inhibition of the desaturation or elongation in vivo.

Another point deserves attention in our results. In rats fed BO, the decreased 20:4n-6 content with a concomitant increased 18:2n-6 content of liver microsomal lipids, as compared with the control group, may suggest an impaired synthesis of 20:4n-6 from 18:2n-6 by successive Δ6 and Δ5 desaturations. This possible inhibition could also be apparent in the HBO group,

despite the low concentration of CPFA in the heated oil. An inhibiting effect of these acids on the biosynthesis of essential polyunsaturated fatty acids would much better explain the severe pathological disorders observed in animals fed CPFA-containing oil[20] than inhibition of Δ9 desaturation. In vitro experiments of Δ6 desaturation of 18:2n-6 and Δ5 desaturation of 20:3n-6 (dihomo-γ-linolenic acid) are presently performed in the laboratory to assess this hypothesis.

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