Effect of ingestion of thermally oxidized frying oil on desaturase activities and fluidity in rat-liver microsomes

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Fresh and thermally oxidized olive and sunflower oils were fed to rats at a practical level of concentration. Rats were fed a diet ad libitum for 10 months that contained 12% (wt/wt) of a test oil. Fatty acid composition of liver microsomes; physical state of the membrane lipids; and activities of the Δ^9, Δ^6, and Δ^5 desaturases were determined. The results demonstrate that dietary heated oils produce alterations in the desaturase activities of rat liver microsomes with concomitant changes in fatty acid composition of these membranes. Dietary heated oils accelerate the conversion of palmitic to palmitoleic acid by activating the Δ^9 desaturase activity and slow down the synthesis of arachidonic from linoleic acid by inhibiting the Δ^6 and Δ^5 desaturase activities in the rat liver microsomes. Fluorescence polarization studies, using diphenylhexatriene as a probe, showed lower membrane fluidity in olive oil groups than in sunflower oil groups. Moreover, there were significant differences between physical states of the membrane lipids from each group of the heated and non-heated oils. We concluded that thermally oxidized oils appear to produce alterations at the molecular level in the microsomes of rats, which are dependent on the composition of dietary lipids.

Keywords: oil thermal oxidation; desaturase activities; membrane fluidity; liver; rats

Introduction

The prolonged laboratory heating of fats in the presence of air to simulate the conditions of deep fat frying has been reported to cause pathologic responses such as appetite and growth depression, diarrhea, histologic changes in various tissues, and even death in some cases when fed to experimental animals.[1-3] Under thermal conditions, the reactivity of fatty acids increases with the degree of unsaturation, but the distribution and geometry of double bonds also influence the extent of oxidation. Double bonds become conjugated or lost as they are involved in reactions forming various secondary products, some of which have been identified as fatty acid geometrical isomers,[4] cyclic fatty acid monomers,[5-7] dimers, and polymeric triglycerides,[8-10] as well as some oxidative components.[11,12] This research takes a serious view regarding the potential toxicity of heated fats.

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On the other hand, another group of investigators has shown many times that feeding of used commercial frying fats to experimental animals does not lead to harmful effects and that the used frying fats appear to be nutritionally the equal of fresh fats when fed at high levels.[13-15]

Most toxicologic investigations on the effects of thermally oxidized fats have been quite general with little consideration of events at the molecular level. Therefore, this paper deals with nutritional effects of heated oils in rats, starting with chemically well-defined fats using a new analytical system that permits the determination of objective parameters based on the new compounds originated during the thermoxidation.[16] The membrane fluidity of the microsomal phospholipids was measured to correlate changes in desaturase activities with any alteration of the physical state of the vesicular membranes.[17] The investigation also considered the relationships of the membrane bilayer lipid composition to structure and kinetics of integral enzymes through fatty acid desaturation complex.[18] This approach makes possible the comparison of alteration levels of fats used in different essays and, at the same time, proportionates new possibilities in nutritional studies.

Materials and methods

Chemicals and reagents

All cofactors, NADH, CoA (sodium salt) and bovine serum albumin (essentially free of fatty acid), were obtained from Sigma Chemical Corporation (St. Louis, MO, USA). 1,6-Diphenylhexatriene was from Aldrich Chemical Corporation (Steinheim, Germany). All other chemicals were analytical grade. [1-^{14}C]Palmitic acid (specific activity [sa] 56.0 mCi/mmol), [1-^{14}C]linoleic acid (sa, 50.5 mCi/mmol) and [1-^{14}C]eicosa-8,11,14-trienoic acid (sa, 54.9 mCi/mmol) were purchased from New England Nuclear (Boston, MA, USA).

Sample oil preparation

Fats used were olive and sunflower oils, both non-heated and heated in a stainless steel beaker for 100 hours at a controlled temperature of 200° C in the absence of foodstuff. The samples were stored at −16° C under an atmosphere of nitrogen until needed, and changes in composition were not detected during storage. The chemical characteristics of the oils used have been described previously.[16]

Animals and treatments

Male Wistar rats (Iffa-Credo, Lyon, France), obtained at 4 weeks of age, were housed in individual metabolic cages which separated feces from urine. After 1 week for acclimation, rats weighing around 100 g were randomly divided into four groups of 12 animals.

The semi-synthetic lipid-deprived diet was purchased from Panlab SRL (Barcelona, Spain). The experimental diet also contained the selected amount (12%) of a test oil. The composition of the experimental diet is shown in *Table 1*.

The experimental diet was prepared once a week and stored in a closed container kept at a temperature below 5° C. Food and water were provided ad libitum; food intake and body weight were measured every 3–4 days, and the animals were examined on a regular basis to detect early symptoms of toxicity. The room was maintained at 21° ± 2° C with lights on from 08.00 to 20.00 hr.

Biological procedures

At completion of the study (after 10 months of treatment with the experimental diets), the rats were killed by exsanguination. The livers were immediately excised, trimmed of connective tissues, weighed, and washed in an ice-cold homogenization medium containing 0.25 M sucrose, 10 mM Hepes (hydroxyethylpiperazine-ethanesulfonic acid) (pH 7.4), 20 mM EGTA and 5 mM DTT (dithiothreitol). All subsequent operations were carried out at 4° C. Livers were homogenized in a Potter-Elvehjem homogenizer with ice-cold homogenization medium. Each homogenate was centrifuged for 20 min at 15,000*g*. The supernatant was collected and centrifugation (15,000*g*) was repeated. The 15,000*g* supernatant was centrifuged at 105,000*g* for 60 min in a Sorvall ultracentrifuge, model OTD 50B. The resulting microsomal pellets were immediately frozen in liquid N_2 and stored at −70° C until the assays.

Lipids were extracted from the liver microsomal fractions by the method of Folch et al.[19] For determination of fatty acyl composition, the fatty acids were analyzed as their methyl esters by gas chromatography. The samples were saponified by heating for 5 min with 5 mL of 0.2 M sodium methylate and heated again at 80° C for 5 min with 6% (wt/vol) H_2SO_4 in anhydrous methanol. The fatty acid methyl esters thus formed were eluted with hexane and analyzed with a gas chromatograph

Table 1 Composition of experimental diet

Basal diet (g/100 g)	
Milk casein[a]	19.8
Cornstarch	18.6
Glucose	37.0
Cellulose powder	5.3
Vitamins[b]	1.0
Minerals[c]	6.3
Test oil (g/100 g)	12.0

[a] Lipid deprived.
[b] Vitamins (in 1 kg diet): retinyl acetate, 19,800 IU; cholecalciferol, 6000 IU; thiamine HCl, 20 mg; riboflavin, 15 mg; niacin, 70 mg; pyridoxine HCl, 10 mg; inositol, 150 mg; cyanocobalamin, 50 μg; ascorbic acid, 170 mg; dl-α-tocopherol acetate, 40 mg; phylloquinone, 40 mg; Ca-panthotenate, 100 mg; choline-Cl, 1.36 g; folic acid, 5 mg; p-aminobenzoic acid, 50 mg; biotin, 0.3 mg.
[c] Minerals (in 1 kg diet): P, 7.75 g; Ca, 10.0 g; K, 6.0 g; Na, 4.0 g; Mg, 1.0 g; Mn, 80 mg; Fe, 0.3 g; Cu, 12.5 g; Zn, 45 mg; Co, 90 μg; I, 0.49 mg.

(Hewlett-Packard, model 5710 A, Palo Alto, CA, USA) equipped with a flame-ionization detector. SP-2310 (3%) and SP-2300 (2%) on a 100/120 chromosorb WAW were used in a 200 cm glass column, and N_2 (20 mL/min) served as the carrier gas. The temperature was programmed to rise from 190°–220° C at a rate of 2° C/min. Fatty acid methyl esters were identified by comparison of their retention times against those of standards.

Steady-state fluorescence polarization measurements were made by using a 1 cm light path and excitation-emission wavelengths of 360–440 nm (3–5 nm resolution) respectively, in a Perkin-Elmer 650-40 spectrofluorometer adapted for fluorescence polarization, as previously described,[20] with light modifications. Corrections for light scattering were also routinely made as described by Brasitus et al.[21] The hydrophobic, fluorescence probe, diphenylhexatriene (DPH), was used to label microsome membranes. DPH was stored at −30° C in tetrahydrofuran at a concentration of 0.1% (wt/vol). Native membranes, in an amount corresponding to about 200 μg of lipid, were incubated at 25° C for 1 hr with 1.55 μL DPH dispersed in 3 mL buffered saline (100 mM KCl, 20% (wt/vol) sucrose, 50 mM Tris-HCl, pH 7.4). The static component of membrane fluidity was also assessed by an order parameter, S, where $S = (r\infty/ro)^{1/2}$, as described.[22]

Δ^9, Δ^6, and Δ^5 desaturase activities were assayed according to methods previously described.[23,24] The assay was initiated by adding 2–2.5 mg of microsomal protein to the incubation mixture, which contained 1.25 mM NADH, 1.5 mM reduced glutathione, 5 mM ATP, 5 mM $MgCl_2$, 0.3 mM coenzyme A, and 75 μM ^{14}C-palmitic or 75 μM ^{14}C-linoleic or 75 μM ^{14}C-eicosa-8,11,14-trienoic acid in a final volume of 1 mL. Fatty acids were used as sodium salt-albumin-complex (1 μg free fatty acid / 11.5 μg bovine serum albumin). Incubations were carried out at 37° C for 20 min in a metabolic shaker. Enzyme assays were terminated by addition of 1 mL of 10% (wt/vol) KOH in ethanol followed by saponification at 100° C for 30 min and addition of 1 mL of 4 N H_2SO_4. Lipids were extracted twice with 5 mL chloroform/ methanol (2:1, vol/vol). Both organic phases were combined and evaporated under nitrogen. Methylation of fatty acids was carried out with 14% boron trifluoride in methanol.[25] Fatty acid methyl esters were separated on silica gel G-60 thin layer chromatography (TLC) plates containing 5% (wt/vol) $AgNO_3$. Plates were developed three times at 4° C in benzene and radioactivity was analyzed in a Berthold Automatic TLC-

linear Analyzer with the aid of a computer program. The percentage of conversion of substrate to product was calculated.

Microsomal protein was determined by the method of Lowry et al.[26] with bovine serum albumin as the standard.

Statistical assay

Comparisons between the olive and sunflower oil groups were tested by two-factor analysis of variance. Differences between the heated and non-heated oils were established by the unpaired student's *t* test.

Results and discussion

In this work we used the same original and heated samples, refined olive and sunflower oils, chemically evaluated in our laboratory and recently described in a report.[16] The diet of these animals was nutritionally adequate. The recommended level of fat in the diet of the rat is about 5%,[27] but 12% fat was used since most heated fat studies have employed this level of fat, thus enabling comparisons among studies.

In spite of the high level of altered fat consumed by two of the groups of rats, results indicative of acute toxicity were not found.

The data listed in *Table 2* show the fatty acid profile of the liver microsomal lipids from rats fed the test oils. The most striking feature is the low and high content of oleic acid in microsomes of the heated olive and sunflower groups, respectively, as compared with the non-heated oil groups. Furthermore, the relative amount of arachidonic acid, which is considered the major product in the synthesis of (n-6) polyunsaturated fatty acids in microsomal lipids, decreased significantly ($P < 0.05$), both in animals fed the heat-treated olive or sunflower oil.

Table 2 Fatty acid composition of liver microsomal lipid[a]

	Fresh (10)		Heat-treated (11)	
	Olive oil	Sunflower oil	Olive oil	Sunflower oil
14:0	1.4 ± 0.1	—	0.6 ± 0.1	2.3 ± 0.2[c]
16:0	19.2 ± 1.9	19.8 ± 1.8	21.5 ± 2.0	20.6 ± 1.1
16:1 (n-7)	1.2 ± 0.1	1.7 ± 0.1	3.6 ± 0.5[b]	2.6 ± 0.2[b]
18:0	17.4 ± 1.6	18.7 ± 2.1	18.8 ± 1.2	11.6 ± 0.9[b,c]
18:1 (n-6), (n-9)	25.5 ± 2.1	12.2 ± 0.7	19.1 ± 1.5[b]	20.1 ± 1.3[b]
18:2 (n-6)	6.2 ± 0.4	17.1 ± 1.1	9.1 ± 0.6[b]	18.4 ± 1.6[c]
20:0	–	0.9 ± 0.1	1.2 ± 0.1	—
20:4 (n-6)	21.1 ± 1.2	22.5 ± 1.6	16.7 ± 1.1[b]	17.9 ± 1.2[b]
22:5 (n-3)	4.0 ± 0.2	3.0 ± 0.3	3.2 ± 0.1	3.1 ± 0.2
22:6 (n-3)	4.1 ± 0.1	4.2 ± 0.3	4.2 ± 0.2	3.7 ± 0.1
Other	—	1.5 ± 0.1	—	—
SAT[d]	38.0	39.4	42.1	34.5
UNSAT[e]	62.0	59.1	57.9	65.5
UNSAT/SAT	1.63	1.50	1.37	1.90

[a] Results are expressed as mean percentage ± standard deviation of fatty acid methyl esters prepared from total liver microsomal lipids. The numbers in parentheses are the number of animals in each group.
[b] Significantly different from non-heated group, $P < 0.5$.
[c] Significantly different from heated olive oil group, $P < 0.005$.
[d] SAT, total saturated fatty acids.
[e] UNSAT, total unsaturated fatty acids.

Table 3 In vitro desaturase activities and order parameter (S) of liver microsomes of rats fed fresh or heat-treated oil diets[a]

	Fresh (9)		Heat-treated (11)	
	Olive oil	Sunflower oil	Olive oil	Sunflower oil
Δ^9	0.94 ± 0.08	0.93 ± 0.08	1.68 ± 0.09[b]	2.14 ± 0.11[b]
Δ^6	2.75 ± 0.12	3.06 ± 0.10	1.39 ± 0.06[b]	1.57 ± 0.08[b]
Δ^5	3.82 ± 0.14	4.72 ± 0.18	2.55 ± 0.11[b]	2.93 ± 0.12[b]
S	0.761 ± 0.003	0.723 ± 0.002	0.777 ± 0.003[b]	0.730 ± 0.002[b,c]

[a] Δ^9, Δ^6, and Δ^5 desaturase activities are expressed as pKat per mg protein. All values are means ± standard deviation. The numbers in parentheses are the number of animals in each group.
[b] Significantly different from non-heated group, $P < 0.05$.
[c] Significantly different from heated olive oil group, $P < 0.05$.

The results from the in vitro measurements of Δ^9, Δ^6, and Δ^5 desaturase activities in liver microsomes from rats fed the different experimental diets are presented in *Table 3*. The rats in the present study were subjected to controlled lighting conditions and were killed at a fixed time to reduce the influence of possible diurnal changes on the measured enzyme activities.[28,29] The Δ^9 desaturase activity in the microsomes of the heated oil groups was significantly ($P < 0.05$) increased. These findings can be attributed to high ratios of palmitoleic/palmitic acid and oleic/stearic acid observed for the heated olive and sunflower groups, respectively. In fact, such changes result from the metabolic effects due to any stimulation of Δ^9 desaturase,[17,30]

The Δ^6 desaturase activity in the microsomes of the heated olive and sunflower groups was significantly ($P < 0.05$) lower than in the non-heated groups. The accumulation of 18:2(n-6), the substrate of Δ^6 desaturase, and the decreased level of 20:4(n-6) observed in the microsomal lipids of the heated oil groups compared with the non-heated oil groups are consistent with the tendency toward decreased microsomal Δ^6 desaturase activity obtained in vitro (*Table 3*).

The Δ^5 desaturase activity was also significantly ($P < 0.05$) lower in the microsomes of the heated olive and sunflower groups than in the non-heated groups, and significant differences were observed between groups (*Table 3*). These results agree with the studies using liver microsomes from animals, deficient and sufficient in EFA, that were fed diets containing positional isomers of fatty acids produced by hydrogenation during the processing of cooking oils.[23,31] These unnatural fatty acids inhibit liver microsomal desaturation of 18:2(n-6) and 20:3(n-6), just the substrates for Δ^6 and Δ^5 desaturases. The Δ^5 desaturase activities obtained in vitro were higher than those of Δ^6 desaturase, as shown in *Table 3*.

The whole desaturation system in the lipid bilayer of the microsomal membrane limits the kinetic reactions to a bidimensional system and the physicochemical properties of the lipids and, therefore, the lipid composition may play a role in the desaturase activity. In order to determine whether changes in de-

saturase activity were related to alterations in the physical state of the microsomal membrane caused by changes in the microsomal phospholipid fatty acids between dietary treatments, steady-state fluorescence polarization studies were carried out. Although order parameter S_{DPH} is related to the spatial lipid organization rather than to the motion rate of the molecules,[32] it is considered to be the reciprocal of the membrane fluidity. The values of the order parameters S_{DPH} were significantly higher in the olive oil groups than in the sunflower oil groups (*Table 3*), which agrees well with the fluidizing effect of dietary sunflower oil,[33] essentially due to the high incorporation of 18:2(n-6) in the lipid microsomal membrane from rats fed the sunflower oil diet. On the other hand, the order parameters S_{DPH} were significantly ($P < 0.05$) higher in the heated olive and sunflower oil groups than in the non-heated olive and sunflower oil groups. It represents a higher membrane lipid matrix packing order[34] and may be considered to indicate a lower microsomal membrane fluidity caused by heated oil treatments.

If a decrease of the unsaturated/saturated acid ratio in microsomal membrane bilayer really decreases the fluidity of the membrane, as is considered in olive oil groups, an increase or decrease of membrane fluidity that would decrease or increase the biosynthesis of double bonds, therefore modifying the packing of the membrane, could trigger a self-regulatory mechanism. However, this mechanism may not be used to explain the decrease of membrane fluidity in the heated sunflower group because the unsaturated/saturated acid ratio in microsomal membrane bilayer is increased in the non-heated sunflower group. These observations are consistent with the previous discussion since the kinetic properties of desaturases have been shown not to be exclusively dependent on the physical state of the membrane.[35,36]

In conclusion, thermally oxidized oils appear to produce alterations at the molecular level in the microsomes of rats fed diets for a long time, but further studies will be needed to determine the reasons for the observed changes in desaturase activity and membrane fluidity of these animals.

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