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Comparison of Recovery of Previously Depressed Hepatic Δ6 Desaturase Activity in Adult and Old Rats

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Abstract
The ability to recover hepatic Δ6 desaturase (Δ6D) activity with linoleic acid as substrate was compared in adult and old rats. Male rats fed a diet deficient in α-linolenic acid were used either at 6 or 21 months. From these two ages onward, animals were fed a diet containing 10% fish oil for 3 months to reduce Δ6D activity. After this period, some of the animals were killed. The other animals were returned to the original diet deficient in α-linolenic acid. Fatty acid composition in liver and brain and hepatic Δ6D activity were analysed 3 and 7 days after the change in diet. When rats were fed the diet containing 10% fish oil, Δ6D activity was lower than in those fed the diet deficient in α-linolenic acid. The liver fatty acid composition was altered with disappearance of 22:5 n–6 and a decrease in 18:2 n–6, 20:4 n–6 and 22:4 n–6 accompanied by an increase in 20:5 n–3, 22:5 n–3 and 22:6 n–3. When rats were re-fed the original diet, Δ6D activity returned after 3 days to its initial level in the 9-month-old rats; in 24-month-old animals, recuperation was incomplete. The levels of 20:4 n–6 and 18:2 n–6 increased in the liver concurrently with a decrease in levels of 20:5 n–3, 22:5 n–3 and 22:6 n–3. In both age groups, the brain fatty acid profile remained unchanged 7 days after returning to the diet deficient in α-linolenic acid.

Introduction

It is of great interest to know how different proportions of n–6 and n–3 polyunsaturated fatty acid (PUFA) in the diet affect the metabolism of these fatty acids. Desaturation of PUFA is an important reaction for the synthesis of very-long-chain PUFA, and Δ6 desaturation is considered to be the rate-limiting step for these reactions [1, 2]. Fatty acid desaturation is regulated by a number of nutritional and hormonal factors [3]. Among nutrition-

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al factors, dietary fat has been shown to be an important factor [4–6].

The early studies by Brenner and Peluffo [7] and Actis Dato and Brenner [8] using in vitro incubation showed that long-chain (n–3) derivatives had a feedback inhibitory effect on Δ6 desaturase (Δ6D) activity. This hypothesis has been confirmed by some works devoted to the study of the effects of n–3 fatty acid diets on this enzyme activity [4, 9]. In a previous study [10], we also observed that hepatic Δ6D activity with linoleic acid as substrate was reduced by a fish oil diet containing long-chain (n–3) PUFA in rats at all ages. However, to our knowledge, no studies have been carried out on recuperation of enzyme activity when n–3 PUFA are eliminated from the diet. We have thus investigated whether Δ6D activity returns to its initial level after elimination of these feedback inhibitors. In addition, as Δ6D activity decreases with age [11–14], it was of interest to know whether adult and old rats showed similar Δ6D recovery.

In a previous work [10], we have shown that the activity of Δ6D on linoleic acid in the liver of n–3-deficient rats was unchanged during adult rat life (puberty to old age). When deficient animals were re-fed with either the 18:3 n–3 (peanut-rapeseed oil) or the 20:5 n–3 + 22:6 n–3 (fish oil) diets, Δ6D activity decreased at all ages; the decrease was more marked with fish oil. Thus fish oil was used in this work and administered for a long period to ensure that Δ6D activity decreased.

The aim of this work was to compare in adult and old rats the effect of withdrawal of fish oil on the specific activity of Δ6D on linoleic acid and on the fatty acid composition of total lipids in the liver. The same effect was also examined in the brain which is rich in lipids formed of PUFA from dietary essential linoleic and α-linolenic acids.

Materials and Methods

Animals and Diets

Female Wistar rats receiving a diet deficient in α-linolenic acid for two generations were raised in the animal house of our laboratory at a constant temperture (22 ± 2 °C) and with free access to food and water. The diet contained 5% (w/w) peanut oil and supplied 1,000 mg linoleic acid but only 5 mg α-linolenic acid/100 g of diet. The litters weaned at 21 days received the same diet as their mother. At the age of either 6 or 21 months, two groups of male rats were switched to a diet containing 10% fish oil (w/w) that supplied 400 mg linoleic acid and 3 g n–3 PUFA/100 g of diet for 3 months. At the end of this period, some of the animals were killed. The rest were returned to the original deficient diet containing peanut oil. For each age group, analyses were performed 3 or 7 days after the diet change. The results were compared with those of 9- or 24-month-old animals who had received only the diet deficient in α-linolenic acid and served as controls. The composition of the diets is given in tables 1 and 2.

Analysis of the Fatty Acid Composition of Total Lipids in Liver and Brain

Animals were sacrificed between 8 and 9 h a.m. to avoid variations in Δ6D activity due to circadian rhythm [15]. They had access to food up to the time of sacrifice to ensure optimal conditions for measurement of Δ6D activity [16].

The brain and liver were removed immediately. Both organs were then freeze dried. Lipids were extracted by sonication in a chloroform/methanol mixture (2:1, v/v) [17, 18] and then methylated [19]. Methyl esters were extracted with hexane and diethyl ether, then analysed by gas chromatography using a Carlo Erba chromatograph (Rueil-Malmaison, France) with an automatic on-column injector, a flame ionization detector and a Carbowax CP Wax 52 CB capillary column. Data were analysed using a microcomputer, and fatty acid levels were reported as percent of total fatty acid content.

Measurement of Δ6D Activity

Determination of Δ6D-specific activity was carried out according to Blond and Lemarchal [20] as modified by Bourre et al. [14]. The liver was weighed and then homogenized at 4 °C in a buffer containing 0.25 *M* saccharose, 0.05 *M* Na_2HPO_4, 2 m*M* glutathione, pH 7.4, at 5 ml buffer/2 g fresh tissue. The homogenate was centrifuged at 12,000 *g* for 15 min to sediment cell debris, mitochondria and nuclei. The

Table 1. Composition of diets

Constituents g/kg	Peanut oil diet (5%)	Cod liver oil diet (10%)
Casein	220.0	220.0
DL-Methionine	1.6	1.6
Mineral mixture[1]	40.0	40.0
Vitamin mixture[2]	10.0	10.0
Cellulose	20.0	20.0
Oil	50.0	100.0
Starch	439.0	405.6
Saccharose	219.4	202.8

[1] Composition (g/100 g): $CaHPO_4 \cdot 2H_2O$, 38.0; K_2HPO_4, 24.0; $CaCO_3$, 18.0; NaCl, 6.9; MgO, 2.0; $MgSO_4 \cdot 7H_2O$, 9.0; $FeSO_4 \cdot 7H_2O$, 0.86; $ZnSO_4 \cdot H_2O$, 0.5; $MnSO_4 \cdot H_2O$, 0.5; $CuSO_4 \cdot 5H_2O$, 0.1; NaF, 0.08; $CrK(SO_4)_2 \cdot H_2O$, 0.05; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.002; KI, 0.004; $CoCO_3$, 0.002; $Na_2SeO_3 \cdot 5H_2O$, 0.002.

[2] Total vitamin supplement, United States Biochemical Corp., Cleveland, Ohio, USA.

Table 2. Fatty acid composition of the experimental diets

Fatty acids	Peanut oil diet (5%, n–3 deficient)	Cod liver oil diet (10%)
Content, %		
Σ SFA	19.2	23.5
Σ MUFA	60.7	41.0
18:2 n–6	19.9	2.3
18:3 n–3	0.1	0.7
20:5 n–3	–	12.2
22:6 n–3	–	12.0
Σ n–6	19.9	4.1
Σ n–3	0.1	31.4
Σ n–6 + Σ n–3	20.0	35.5
Content, mg/100 g of diet		
Σ n–6	935.0	385.0
Σ n–3	5.0	2,952.0
Σ n–6/Σ n–3	199.0	0.1
Percent of lipids	5.0	10.0

SFA = Saturated fatty acid; MUFA = monounsaturated fatty acid. Fatty acid composition was determined by gas chromatography as described in Materials and Methods.

supernatant contained both microsomes and cytosol. The latter increases the activity of acyl-CoA synthetase, an enzyme that favours the formation of acyl-CoA fatty acids [20], the substrate of desaturases. The supernatant was then diluted twofold in the same buffer. The concentration of proteins in the supernatant was determined by the method of Lowry et al. [21].

The supernatant was then incubated for 30 min at 37 °C under continuous agitation in a final volume of 2 ml containing 50 m*M* Na_2HPO_4, 7.5 m*M* ATP, 3.8 m*M* $MgCl_2$, 0.2 m*M* NADPH, 0.5 m*M* NADH, 0.2 m*M* CoA, 100 nmol [1-^{14}C] 18:2 n–6 (2 μCi, 20 μl) and in the presence of variable amounts of protein. The reaction was stopped by addition of 1 ml of 10% KOH/ethanol. Standard commercial free fatty acids (18:2 n–6, 18:3 n–6, 20:3 n–6, 20:4 n–6, 20:5 n–3, 22:6 n–3) were added at a concentration of 30 μg to aid subsequent identification of spots on the chromatograms and to provide standard Rf. After saponification at 100 °C for 30 min, 0.4 ml of 10 *N* HCl was added, then fatty acids were extracted with hexane and methylated with 1 ml of 14% boron trifluoride for 1 h at 100 °C. The methyl esters were then extracted with hexane and partially purified by an initial thin-layer chromatography with a mixture of petroleum ether and diethyl ether (80:20, v/v) as mobile phase. Methyl esters were located on the chromatographic plate by autoradiography, removed and extracted successively with hexane (twice 3 ml) and diethyl ether (twice 3 ml). The methyl esters were finally separated according to their degree of unsaturation by a second thin-layer chromatography on plates previously immersed for 15 min in a 10% $AgNO_3$ solution in acetonitrile and then dried at 100 °C for 20 min. The mobile phase consisted of a mixture of petroleum ether and diethyl ether (50:50, v/v). Spots were identified by autoradiography, and the distribution of radioactivity was determined by a plate reader (Automatic TLC Linear Analyser; Berthold, La Garenne-Colombes, France). Results were expressed as picomoles of 18:3 n–6 formed per minute of incubation per milligram of protein. Each value corresponds to the mean of at least 9 preparations each from 3 rats. Statistical analysis was carried out using a non-parametric test (Mann-Whitney). Differences were taken to be significant at $p < 0.05$.

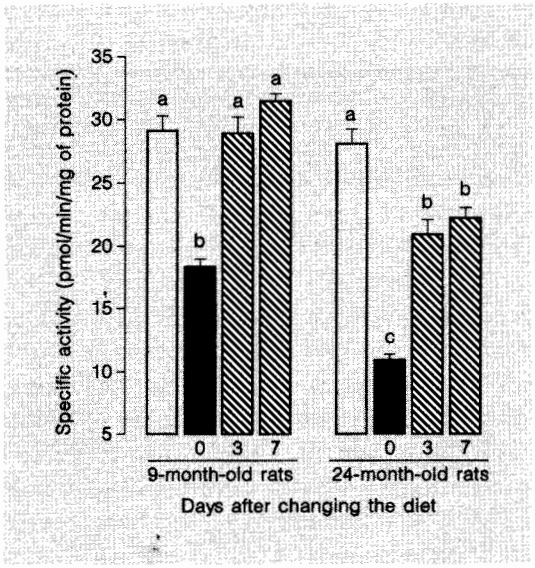

Fig. 1. Recuperation of Δ6D-specific activity due to elimination of n–3 PUFA from the diet after 3 months with a diet enriched in fish oil. Experimental conditions are described in Materials and Methods. □ = 5% peanut oil (n–3 PUFA deficient); ■ = 10% cod liver oil; ▨ = 5% peanut oil refeeding. Values are the means ± SD of at least 9 different preparations each from 3 rats/experiment. Values that are significantly different ($p < 0.05$) are indicated by different letters.

Results

Δ6D-Specific Activity in Liver

After 3 months of the fish oil diet, in 9-month-old rats the Δ6D activity was decreased by 40% and in 24-month-old rats it was decreased by 65% compared with animals deficient in n–3 PUFA used as controls (fig. 1).

For both age groups, 3 days after changing the diet, Δ6D-specific activity rose rapidly: in 9-month-old rats it increased from 18 to 28 pmol/min/mg of protein and in 24-month-old rats from 10 to 19 pmol/min/mg of protein. Between 3 and 7 days after changing the diet, the increase in enzyme activity was less rapid and reached a level of 32 pmol/min/mg in 9-month-old and 22 pmol/min/mg in 24-month-old rats. Δ6D-specific activity in the liver of control rats fed a diet deficient in n–3 PUFA was of the order of 29 pmol/min/mg of protein in 9- and 24-month-old rats.

Fatty Acid Composition of Total Lipids in Liver and Brain

After 3 months of the fish oil diet, the n–3 PUFA were eliminated and an n–3-deficient diet was given. In brain and liver and at both ages (tables 3, 4), the n–3 PUFA deficiency had no effect on the levels of saturated and monounsaturated fatty acids. In contrast, analysis of the n–6 and n–3 fatty acid composition showed considerable variations.

After 3 months of fish oil feeding, for both ages, the level of 18:2 n–6 in the liver was decreased by about 10%. Concomitantly, 18:3 n–3 was detected and represented about 0.5%. The amount of n–3 PUFA was 30-fold higher. The amount of 18:2 n–6 in the brain was not or only slightly altered. In parallel, the level of n–3 PUFA was 2-fold higher.

After 3 days of n–3 PUFA deficiency, in 9-month-old rats the level of 18:2 n–6 in the liver was increased by 35% (table 3). In 24-month-old rats, 18:2 n–6 levels were increased by 35% after 7 days. Consequently, the sum of n–6 PUFA was increased by 35% in 9-month-old rats and by 40% in 24-month-old rats. In the first group, the sum of n–3 PUFA was decreased by 20%, but in the second it was practically unchanged. In all cases, the level of n–6 and n–3 PUFA did not return to the level observed in deficient rats used as controls. At both 3 and 7 days after changing the diet from fish oil and for both age groups, brain fatty acid profiles were the same as after 3 months of the 10% fish oil diet (table 4).

Table 3. Effect of dietary n–3 PUFA elimination on the fatty acid composition (%) of total liver lipids from adult and old rats

Fatty acids	9-month-old rats				24-month-old rats			
	D	0	3	7	D	0	3	7
18:2 n–6	7.6	6.6	8.9	9.0	7.4	6.6	8.4	8.9
Σ n–6	24.1	12.5	16.9	16.3	22.9	9.8	12.8	13.7
18:3 n–3	–	0.6	0.4	0.3	–	0.5	0.4	0.4
Σ n–3	0.8	22.9	19.2	19.2	1.1	21.7	18.4	16.6

Values are the means of 2 animals and are expressed as percent of total fatty acids. D = Deficient diet used as control diet; 0, 3, 7 = days after changing the diet from fish oil feeding.

Table 4. Effect of dietary n–3 PUFA elimination on the fatty acid composition (%) of total brain lipids from adult and old rats

Fatty acids	9-month-old rats				24-month-old rats			
	D	0	3	7	D	0	3	7
18:2 n–6	0.6	0.3	0.4	0.5	0.7	0.3	0.4	0.6
Σ n–6	18.7	9.9	9.9	10.2	20.9	11.0	11.3	11.1
Σ n–3	6.3	14.6	14.6	14.8	6.4	15.7	15.9	14.6

Values are the means of 2 animals and are expressed as percent of total fatty acids. D = Deficient diet used as control diet; 0, 3, 7 = days after changing the diet from fish oil feeding.

Discussion

The Δ6D-specific activity of 18:2 n–6 in livers of n–3-deficient rats remained constant during adult life (9–24 months old). When rats were fed the diet containing 10% of fish oil rich in long-chain (n–3) fatty acids, the Δ6D activity was decreased. Thus, the altered Δ6D activity is due to the type of fat and to the quantity of fat. Moreover, this down-regulation of the Δ6D activity was more marked in old rats. These results are in agreement with previous studies [4, 10] which showed that fish oil diet reduced Δ6 desaturation of 18:2 n–6. Interestingly, we found that this reduction was significantly greater for old animals than for adult animals.

Elimination of n–3 PUFA from the diet for a short period induced rapid change in Δ6D-specific activity in the liver. In 9-month-old rats, enzyme activity recovered rapidly within 3 days after elimination of n–3 PUFA and continued to increase slightly at 7 days. In 24-month-old rats, the rate of recuperation of activity was comparable to 9-month-old animals at 3 days but was slower than in young animals at 7 days.

This result provides new information concerning Δ6D: it shows for the first time that elimination of long-chain (n–3) PUFA, inhib-

itors of Δ6 desaturation of linoleic acid, restores enzyme activity to its initial level. This recuperation is more rapid in adult and not complete in old animals. According to Hrelia et al. [22], there is a linear correlation between the loss of enzyme affinity for its substrate, linoleic acid, and the age of the animal. On the other hand, the affinity of Δ6D for α-linolenic acid does not change as a function of age. In view of these results, it could be supposed that the presence of long-chain (n–3) derivatives inhibits Δ6D activity in the liver, and that this inhibition is greater when the affinity of the enzyme for its substrate is lower, i.e. in old rats. For the same reason, when these inhibitors are eliminated from the diet, younger animals in whom enzyme affinity for its substrate is still intact recuperate more readily than older animals.

To explain the mechanism of fish oil and of recovery of Δ6D activity, further investigations will need to be carried out to determine whether these changes resulted from changes in the level of enzyme activity or enzyme synthesis.

Concomitantly with Δ6D activity alterations, there was a fall in the level of n–3 PUFA in the liver. This decrease in the n–3 fatty acid level was compensated by the increase in n–6 fatty acid levels resulting from the recovery in the 18:2 n–6 level that had been lowered by the high concentration of fish oil. This shows that the provision of large quantities of long-chain n–3 derivatives in the diet profoundly alters the levels of n–3 and n–6 fatty acids. Seven days of changed diet were not sufficient to reverse the effect of the previous diet. The brain was more resistant since after 7 days of n–3 deficiency it conserved the same fatty acid profile as during the high fish oil diet. The liver responded more rapidly than the brain to the change in diet, but 7 days of n–3 PUFA deficiency were not sufficient for the fatty acid levels to recover the same composition of that observed in n–3-deficient animals. This result is in agreement with our recent studies which showed that in the 60-day-old rat the nervous structures maintain the same level of 22:6 n–3 for at least 31 weeks despite a deficiency in α-linolenic acid, whereas the liver showed variations [23]. These changes in fatty acid composition of total lipids suggest that the fatty acid composition of membrane phospholipids needs to be studied.

In conclusion, results of this study show that the hepatic Δ6D activity decrease due to long-chain (n–3) derivatives can be reversed by elimination of n–3 fatty acids from the diet. In this case, Δ6D activity recovers rapidly and completely in 9-month-old rats, but 24-month-old rats do not completely recuperate initial activity. Changes in Δ6D-specific activity due to the mode of feeding are accompanied by an alteration in the fatty acid composition of total lipids in the liver. On the other hand, elimination of n–3 PUFA from the diet for 3 months has no effect on the fatty acid profile in the brain.

These results require further investigations to follow recuperation of Δ6D activity progressively during adult life and not only at two ages. It would be of interest to study the relation between membrane structure and enzyme activities by examining phospholipid fatty acid composition.

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