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Reversibility of the changes induced by n-3 fatty acids in mouse plasma, liver and blood cell lipids

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Abstract

The changes induced by dietary n-3 fatty acids (FA) in the lipids and FA of plasma, liver and blood cells, and their reversibility, was studied in mice given a diet containing 9% fish oil (FO) for 2 weeks and then returned to, and kept for another 2 weeks on, the usual standard lab chow diet. In plasma, the concentrations of phospholipids (PL), mostly phosphatidylcholine (PC), triacylglycerols (TG), cholesterol and cholesterol esters (CE) decreased rapidly after starting the FO diet, and remained low from day 3 onwards. This decrease was concomitant with a remarkable reduction in the n-6 FA, especially 18:2n-6, not compensated for by the relative enrichment in n-3 FA induced by FO. In liver, TG and CE decreased and PL slightly increased, all of them showing reduced n-6/n-3 ratios. Sphingomyelin, which lacks polyunsaturated FA other than small amounts of 18:2 and 24:2n-6, showed altered ratios between its very long chain monoenes and saturates. In the washout phase, the most rapid event was an immediate increase in 18:2n-6 and after a few days in 20:4n-6 in plasma and liver, where most of the lipid and FA changes were reversed completely in about 10 days. In the case of blood cells even 2 weeks were insufficient for a reversal to the initial n-6/n-3 ratios. The lipid class responsible for this lack of reversibility was phosphatidylethanolamine, PC having returned to the initial fatty acid composition during the stated period.

Keywords: Fish oil; Hypolipidemia; Phospholipids; Cholesterol esters; Triglycerides; Washout

1. Introduction

Many of the beneficial effects of dietary long-chain n-3 polyunsaturated fatty acid (PUFA) have been associated with the changes they produce in the content and fatty acid (FA) composition of circulating lipids. In human intervention studies, an invaluable outcome of n-3 PUFA consumption is that they decrease the concentration of plasma triacylglycerols (TG), decreasing VLDL in both normolipidemic and hyperlipidemic subjects [1]. In plasma, as well as in blood cells and specific tissues in many animal species including man [2–4], decreased n-6/n-3 PUFA ratios result after dietary supplementation with fish oil (FO). Research following the time-course of the alterations induced by dietary n-3 fatty acids in plasma, blood cells and specific tissues has shown that both incorporation and washout are influenced by many factors [5–11]. Among these are the feeding period considered, the type and amount of dietary fat supplied, the form of administration, and the animal species under study. In this work we have examined, in mice, the shifts in the fatty acid profile in total lipid of plasma, liver, and blood cells, and those of their major lipid constituents, along several days on an n-3 PUFA-rich diet, and how they revert to normal after returning the animals to their usual diet. It is shown that in plasma and liver, maximum changes are attained rapidly during FO consumption and that most of these changes are completely reversible also within a few days, the two phases affecting different lipid classes to various extents. In blood cells in contrast, both the incorporation and the washout of n-3 PUFA are shown to be much slower, the duration of the changes being shorter for some phospholipids than for others.

Abbreviations: FO, fish oil; CD, chow diet; PL, phospholipids; TG, triacylglycerols; CE, cholesterol esters; PC, PE, SM: phosphatidylcholine; phosphatidylethanolamine, and sphingomyelin, respectively; FA, fatty acids; PUFA, polyunsaturated fatty acids.

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2. Materials and methods

Male albino mice of the CF1 strain, 8 months old, weighing 44.9 ± 4.7g, were housed under controlled temperature and light conditions and fed ad libitum. The experiment

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Table 1
Fatty acid composition of diets

	CD	FO
weight % of total fatty acids		
16:0	15.8	19.3
17:0	0.1	1.1
18:0	3.4	5.3
16:1	1.0	10.8
17:1	—	2.0
18:1	21.4	16.8
18:2n-6	50.5	9.4
18:3n-6	6.5	1.1
20:4n-6	0.2	2.0
22:4n-6	0.6	—
22:5n-6	0.1	1.2
18:3n-3	0.3	2.5
18:4n-3	—	0.2
20:4n-3	—	0.8
20:5n-3	—	15.5
22:5n-3	—	2.1
22:6n-3	0.1	9.9
n-6/n-3 PUFA	165	0.4

CD, chow diet; FO, fish oil duet; PUFA: polyunsaturated fatty acids.

consisted of changing their usual standard chow diet (CD) for an n-3 PUFA enriched one for 14 days, and then, from day 15 onwards, returning the animals to the standard lab chow diet for an additional 2 weeks. The diet consisted of 23% protein, 63% carbohydrates, 5% of a vitamin mixture and 9% fish oil (FO) (wt/wt). The fatty acid composition of CD and FO diets is shown in Table 1. The sums of major fatty acid types were as follows: saturated fatty acids (SFA): 19.3 and 25.8%; monounsaturated fatty acids (MFA) 22.4 and 29.6%; n-6 PUFA: 57.9 and 13.7%; and n-3 PUFA: 0.4 and 30.9%, respectively. Taking into account the amount of fat in each diet, and the above fatty acid percentages, the content (w/w) of n-6 PUFA was similar in CD and FO (~1.2%). The animals were killed after being anesthetized with acepromazine and ketamine. Blood was collected in heparinized material and centrifuged at 1500 rpm for 15 min to separate plasma and cells. Plasma samples were taken for the direct determination of total, HDL-, and LDL- cholesterol and triacylglycerols (TG), using the techniques and commercial kits employed in clinical laboratory settings for human samples. Blood cells were suspended in saline and centrifuged twice to wash out traces of plasma. Livers were dissected, rinsed, weighed, and cut into small pieces. Lipid extracts were prepared [12] from the three sources and aliquots were taken for the determination of total lipid phosphorus [13] and of the fatty acid composition of the total lipid.

The TG and cholesterol esters (CE) from plasma and liver were separated from the phospholipids (PL) by TLC using n-hexane : diethyl ether : acetic acid (80:20:1.5 by vol) up to the middle of the plates, followed by n-hexane : diethyl ether (95:5 by vol) up to the top. The PL from plasma, liver and blood cells were subjected to analytical TLC [14] for the determination of the PL class composition.

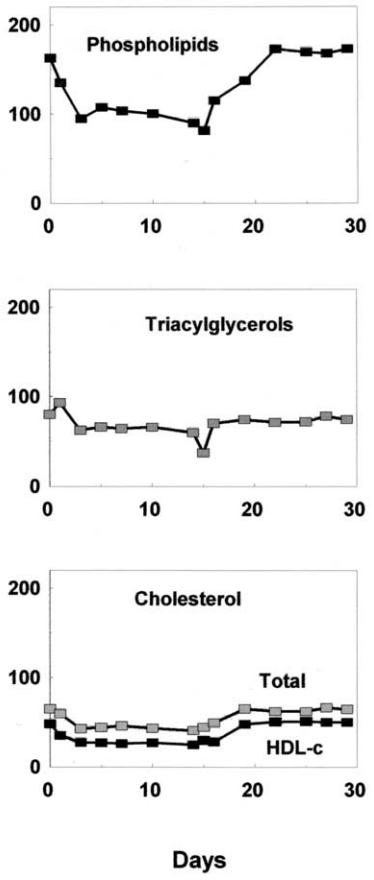

Fig. 1. Effect of n-3 PUFA administration and withdrawal on major lipid constituents of mouse plasma. The points represent the concentrations (mg/dL) of the depicted lipids in plasma along 2 weeks of administration of n-3 PUFA (days 0–14) and, following this, 2 weeks of consumption of the usual standard lab chow diet (days 15–30).

Major PL classes were also preparatively isolated to study their fatty acid composition. Diphosphatidylglycerol and PE were isolated with the same solvents [14] after thorough saturation of the TLC plates with solvent vapors. Sphingomyelin (SM) and PC were separated using chloroform : methanol : acetic acid : 0.15M NaCl, 50:25:8:2.5 [15], in this case without saturation. The spot of SM was eluted from the TLC plates [16], taken to dryness, and treated with 0.5 N NaOH in methanol at 50°C for 10 min. This was followed by another TLC using the same solvents, to ensure that the spot of SM was free of possible contamination with traces of glycerophospholipids.

Lipid fatty acids were analyzed by gas-chromatography of their fatty acid methyl ester derivatives [17]. Before GC, the latter were purified by TLC on silica gel G plates which had been pre-cleaned with methanol : ether (75:25), using hexane : ether (95:5). The esters were eluted from the silica by partition between water : methanol : hexane (1:1:2). All solvents used were of HPLC grade.

Four animals per group were used for the data corresponding to days 0 (controls), 14 (two weeks on the FO diet), and 30 (2 weeks on FO plus 2 weeks on the normal

Table 2
Lipid content and composition in mouse plasma and liver at day 0, after two weeks of fish oil supplementation, and after returning the animals to the standard lab chow diet for a period of 2 weeks

A) Plasma	Day 0	Day 14	Day 30
Lipid content, mg/dL (%)			
Phospholipids (PL)	165 ± 40 (53.3)	90 ± 17* (45.9)*	168 ± 30 (54.9)
Triacylglycerols (TG)	79 ± 9 (25.6)	65 ± 3* (33.2)*	74 ± 5 (24.2)
Free cholesterol (FC)	19 ± 3 (6.1)	10 ± 1* (5.1)	19 ± 3 (6.2)
Cholesterol esters (CE)	46 ± 4 (14.9)	31 ± 4* (15.8)	45 ± 2 (14.7)
Total cholesterol (TC)	65 ± 5	41 ± 4*	64 ± 1
HDL-Cholesterol (HDLc)	49 ± 2	25 ± 3*	50 ± 3
TC − HDLc	16	16	14
PL/FC	8.5	8.7	8.8
PL/TG	2.1	1.4	2.3
PL/CE	3.6	2.9	3.7
PL composition (mol %)			
PC	55.1 ± 1.0	45.4 ± 4.0*	64.3 ± 3.9*
LysoPC	30.3 ± 3.0	32.9 ± 2.7	19.7 ± 4.3*
PE	3.1 ± 0.4	5.9 ± 1.8	5.1 ± 1.7
PS+PI	5.1 ± 1.6	7.2 ± 2.0	6.3 ± 2.2
SM	3.5 ± 0.6	9.2 ± 1.0*	4.5 ± 1.2
PC/PE	17.8 ± 3.8	7.6 ± 2.4*	12.6 ± 3.2*
B) Liver	Day 0	Day 14	Day 30
Lipid content, mg/g (%)			
Phospholipids	18.6 ± 0.7 (67.1)	21.3 ± 1.1* (79.4)*	18.6 ± 0.9 (67.1)
Triacyglycerols	7.0 ± 1.5 (25.2)	3.0 ± 0.3* (11.2)*	7.0 ± 1.4 (25.3)
Free cholesterol	1.5 ± 0.1 (5.4)	2.0 ± 0.4 (7.5)	1.5 ± 0.1 (5.4)
Cholesterol esters	0.6 ± 0.2 (2.2)	0.5 ± 0.1 (1.9)	0.6 ± 0.1 (2.2)
PL/FC	12.4	10.7	12.4
PL/TG	2.7	7.1	2.7
PL/EC	31.0	42.6	31.0
PL composition (mol %)			
PC	50.7 ± 0.4	47.2 ± 0.5*	49.0 ± 0.9
Lyso PC	1.1 ± 0.2	1.4 ± 0.3	1.0 ± 0.1
PE	28.2 ± 0.3	31.0 ± 0.4*	29.8 ± 0.6
PS	3.9 ± 0.4	3.4 ± 0.1	3.9 ± 0.3
PI	8.4 ± 0.4	8.0 ± 0.2	8.6 ± 0.1
DPG	4.5 ± 0.2	5.2 ± 0.1*	4.5 ± 0.2
SM	3.2 ± 0.1	3.8 ± 0.03*	3.1 ± 0.1
PC/PE	1.8 ± 0.03	1.5 ± 0.02*	1.6 ± 0.07

Day 0 refers to mice initially fed on a lab chow diet. Day 14 refers to mice given a diet containing 9% fish oil (FO) for 2 weeks, and day 30 to animals of the FO group that were returned, from day 14 onwards, to the previous chow diet. Values are mean ± S.D. for 4 mice. The asterisks point to significant differences with respect to day 0.

chow diet). With the exception of these three days, each point in the time-course profiles shown in Figures 1 to 3 represents the average of two animals. Statistical analyses of the results were performed using the two-tailed Student's *t* test.

3. Results and discussion

3.1. Lipid content and composition

The time course of the changes induced by dietary FO supplementation and withdrawal in the concentrations of plasma phospholipids (PL), triacylglycerols (TG) and cholesterol, and their reversal upon return to the usual diet, is shown in Fig. 1. Dietary FO decreased the total lipid of plasma, especially affecting the PL, followed by the TG and cholesterol. Since mice are "HDL mammals" [18] and PL are major constituents of HDL, it is consistent that PL and the HDL-cholesterol fraction both be considerably reduced.

The lipid-lowering effect of dietary FO was already maximum at day 3, followed by a virtual plateau that was maintained for as long as FO feeding persisted. After withdrawal of the FO diet, total PL, TG and cholesterol increased again, although each with a different profile. PL and TG decreased below the steady-state levels the very first day of changing the food (day 15, Fig. 1), probably owing to the much lower fat content of the CD (2%), but a net trend of increase started thereafter. The return to a new steady state, in which the lipids reached similar concentrations to the initial ones, occurred firstly for TG, then for total cholesterol and lastly for PL, but was reached by the three lipids in about one week (day 21, Fig. 1). It is paradoxical that FO,

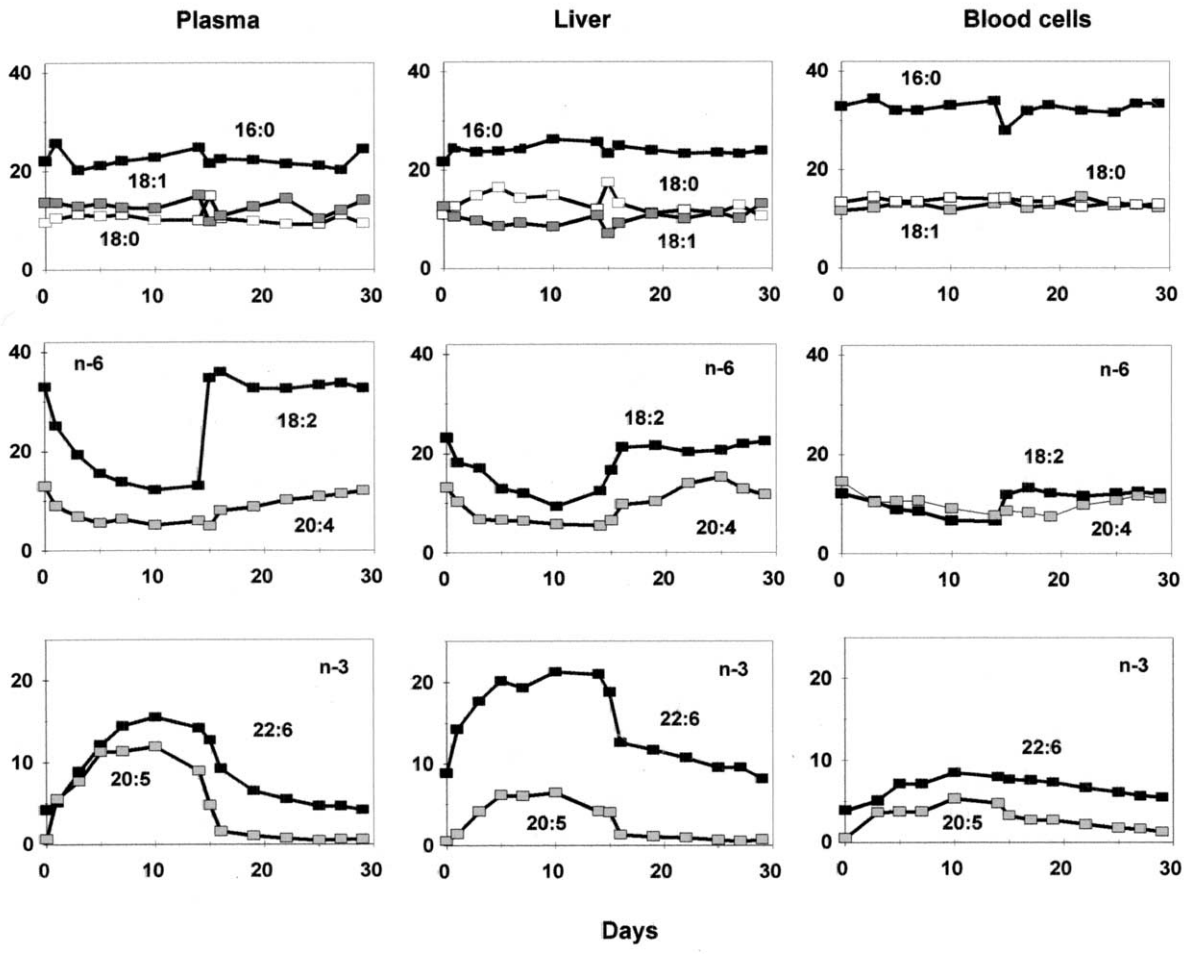

Fig. 2. Effect of n-3 PUFA administration and withdrawal on major fatty acid constituents of the total lipid from mouse plasma, liver and blood cells. The points represent the proportions of the depicted fatty acids (weight %), from the fatty acid composition of the total lipid at each point, analyzed by GC. Mice were administered a diet rich in n-3 fatty acids (days 0–14) and then were fed the usual standard lab chow diet (days 15–30).

a high-fat diet (9%), had a hypolipidemic effect, especially when considering that an important part of the fat provided by the salmon oil used contained saturated, monoenoic, and n-6 fatty acids in addition to the n-3 PUFA, whereas CD, with a lower fat content (2%), increased the lipids back to normal levels.

The quantitative data for the situation in plasma and in liver at three important time points of the experiment -day 0, after 2 weeks on FO (day 14), and when two weeks had elapsed into the washout phase (day 30)- are shown in Table 2. In plasma, although all lipids decreased, PL and free cholesterol (FC) decreased in parallel, to almost half the initial values, i.e., more than did the TG and CE after 2 weeks on FO. The PL/FC ratio remained the same, consistent with the fact that these two lipids form part of the outer surface of the lipoproteins that transport the lipids. The PL/TG and the PL/CE ratios decreased, showing that the circulating lipid of plasma under these conditions was relatively (although not absolutely) richer in lipoproteins carrying TG and CE. Consistently with the reduction of plasma PL, main constituent of HDL in HDL mammals [19], HDL (total) cholesterol was also reduced by half. If this result were to be extrapolated to LDL mammals like man it would certainly constitute an undesirable effect of a high n-3 PUFA diet, though this is generally not observed in human plasma, where the most conspicuous result of dietary n-3 PUFA is hypotriglyceridemia. However, most experimental studies on the effects of fish oil on human volunteers have used 1–12 g fish oil supplied as a single daily dose [9,20, 21], whereas in experiments with laboratory animals constant supplies of high percentages (5–20%) FO are attained, since it is usually mixed with the food. There is nevertheless one report [22] in which FO, furnishing in humans as much as 30% of the total energy ingested, resulted in reduction of HDL as well as VLDL.

The percent composition of the plasma PL fraction at the three extremes of the period considered is also included in Table 2. The sharp drop in the percentage of phosphatidylcholine (PC) under the dietary FO regime resulted in altered PL composition owing to relatively larger percent of PL classes other than PC. Since PC was the main component of the plasma PL fraction, and PC was reduced selectively

Table 3
Fatty acids of the total lipid from plasma, liver, and blood cells (%) at the beginning of the experiment, after administration of fish oil for a period of two weeks (day 14) and after 2 weeks of having returned to the usual lab chow diet (day 30)

	Plasma			Liver			Blood cells		
Days	0	14	30	0	14	30	0	14	30
14:0	0.2 ± 0.03	0.4 ± 0.1	0.2 ± 0.1	0.1 ± 0.01	0.4 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.04
15:0	0.3 ± 0.01	0.3 ± 0.05	0.2 ± 0.02	0.1 ± 0.01	0.2 ± 0.01	0.1 ± 0.02	0.2 ± 0.1	0.2 ± 0.02	0.2 ± 0.1
16:0	21.4 ± 0.7	23.8 ± 0.8*	22.7 ± 0.7	21.8 ± 0.6	25.8 ± 0.7*	23.5 ± 0.4	32.4 ± 1.0	34.5 ± 1.9	33.4 ± 1.6
17:0	0.4 ± 0.1	0.7 ± 0.1	0.5 ± 0.04	0.5 ± 0.03	0.6 ± 0.04	0.5 ± 0.1	0.7 ± 0.1	0.9 ± 0.1	0.8 ± 0.1
18:0	9.3 ± 0.3	9.7 ± 1.2	9.1 ± 0.4	11.0 ± 0.3	12.1 ± 0.6	10.7 ± 1.0	12.0 ± 0.5	13.2 ± 1.1	12.3 ± 0.9
16:1	1.6 ± 0.3	2.8 ± 0.5	1.5 ± 0.1	1.4 ± 0.3	1.9 ± 0.4	1.8 ± 0.8	1.3 ± 0.4	1.6 ± 0.1	1.1 ± 0.1
17:1	0.2 ± 0.1	0.4 ± 0.1	0.2 ± 0.02	0.2 ± 0.04	0.3 ± 0.04	0.2 ± 0.01	0.2 ± 0.1	0.2 ± 0.08	0.2 ± 0.03
18:1	13.3 ± 1.0	14.5 ± 1.8	12.7 ± 1.0	12.7 ± 1.2	11.0 ± 0.9	12.0 ± 0.4	13.5 ± 0.7	14.4 ± 0.4	13.1 ± 0.5
20:3n-9	1.3 ± 0.01	0.5 ± 0.01*	0.5 ± 0.1	0.7 ± 0.1	0.1 ± 0.03*	0.6 ± 0.1	0.3 ± 0.04	0.1 ± 0.03*	0.3 ± 0.1
18:2n-6	32.0 ± 1.9	15.6 ± 3.2*	31.5 ± 0.6	23.9 ± 1.3	12.5 ± 1.2*	22.8 ± 1.3	12.2 ± 1.0	6.7 ± 0.9*	12.2 ± 0.2
18:3n-6	1.0 ± 0.3	0.5 ± 0.01*	0.4 ± 0.01	0.4 ± 0.1	0.7 ± 0.1*	0.3 ± 0.1	0.3 ± 0.2	0.2 ± 0.1	0.5 ± 0.04
20:3n-6	1.4 ± 0.1	0.7 ± 0.1*	1.3 ± 0.1	1.7 ± 0.1	0.4 ± 0.02*	1.5 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
20:4n-6	12.6 ± 0.8	5.0 ± 0.5*	10.9 ± 0.5	13.5 ± 0.7	5.0 ± 0.4*	12.3 ± 0.2	14.1 ± 1.1	7.8 ± 0.3*	10.7 ± 1.1*
22:4n-6	0.3 ± 0.04	0.2 ± 0.04	0.2 ± 0.01	0.2 ± 0.04	0.1 ± 0.01	0.2 ± 0.04	3.1 ± 0.4	2.1 ± 0.1*	2.3 ± 0.3*
22:5n-6	0.7 ± 0.04	0.4 ± 0.1*	0.8 ± 0.1	0.3 ± 0.1	0.1 ± 0.01	0.3 ± 0.02	1.4 ± 0.1	1.2 ± 0.1	1.3 ± 0.1
18:3n-3	0.4 ± 0.1	0.7 ± 0.2*	0.6 ± 0.2	0.9 ± 0.3	0.4 ± 0.4*	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.1
20:5n-3	0.6 ± 0.1	8.6 ± 1.5*	0.9 ± 0.2	0.6 ± 0.1	4.2 ± 4.2*	0.7 ± 0.0	0.5 ± 0.1	4.8 ± 0.2*	1.6 ± 0.1*
22:5n-3	0.3 ± 0.1	1.4 ± 0.3*	0.5 ± 0.2	0.5 ± 0.1	2.0 ± 0.4*	0.8 ± 0.2	1.0 ± 0.1	1.7 ± 0.4*	1.6 ± 1.6*
22:6n-3	4.1 ± 0.4	13.3 ± 1.9*	3.7 ± 0.4	8.9 ± 0.5	21.0 ± 0.9*	8.9 ± 0.4	3.8 ± 0.3	8.3 ± 1.5*	5.7 ± 0.4*
n-6/n-3	8.8 ± 0.3	0.9 ± 0.2*	7.9 ± 0.4	3.7 ± 0.2	0.7 ± 0.1*	3.7 ± 0.2	5.6 ± 0.4	1.2 ± 0.1*	3.0 ± 0.2*
Saturates	31.1 ± 0.5	34.6 ± 0.8	33.4 ± 1.0	33.7 ± 1.1	39.1 ± 0.3*	35.0 ± 0.8	45.6 ± 0.6	48.9 ± 2.1	47.0 ± 2.0
Monoenes	15.6 ± 0.6	20.0 ± 2.2*	16.1 ± 1.0	15.0 ± 1.4	13.2 ± 0.7	15.8 ± 2.9	15.9 ± 0.9	16.6 ± 0.4	15.1 ± 0.5
n-6 PUFA	47.8 ± 1.2	22.5 ± 3.7*	45.2 ± 1.0	40.7 ± 1.2	20.1 ± 1.3*	38.6 ± 2.3	32.2 ± 0.7	18.9 ± 0.6*	27.9 ± 1.6*
n-3 PUFA	5.5 ± 0.3	24.0 ± 2.3*	5.7 ± 0.3	10.9 ± 0.4	27.6 ± 0.6*	11.1 ± 0.7	5.8 ± 0.6	15.3 ± 1.9*	9.4 ± 0.5*

The fatty acid composition was studied by GC. Other details, as in Table 1. All differences in major PUFA at day 14 were significantly different from day 0 and day 30. Except for blood cells, the fatty acid profiles for day 0 and day 30 did not differ significantly.

within a decreased mass of PL, it was the plasma lipid most affected by the FO diet.

In mouse liver, the most dramatic effect of the n-3 PUFA rich diet was a decrease in TG concentrations to less than half after two weeks (Table 2), with slight decreases in CE and increases in PL and free cholesterol concentrations. There were also some changes in PL composition accompanying the n-6/n-3 PUFA ratio changes, such as a small relative decrease in PC and a concomitant increase in PE with the FO diet, reflected in a significant reduction of the PC/PE ratio with a constant value of the PC+PE sum in the intervals considered. Small increases in the percent and hence in the concentrations of DPG and SM were also observed after 14 days on FO (Table 2). This selective effect was also observed in previous work after one month on FO [17].

All changes described in plasma and liver lipids were completely reverted within two weeks of having returned to the standard chow diet, as shown by the almost identical lipid profiles at day 0 and day 30 in Table 2. An interesting exception was the percent of plasma PC, which remained somewhat higher, and that of lysoPC, somewhat lower, than the zero time values, with a similar value of the sum PC+LPC. The reasons for this particular effect are unknown at present. One determining factor could be normal animal aging, since one month is quite a long time in the life of a mouse. The PL percent composition of blood cells (not shown) was not affected at all by the diets.

3.2. Fatty acid changes in the total lipid of plasma, liver and blood cells

The shifts induced by the FO-rich diet and its withdrawal in the fatty acid (FA) profiles of plasma, liver and blood cells as a function of time are compared in Fig. 2. Table 3 shows the FA composition of the total lipid at day 0, after 2 weeks on the FO diet, and after 2 weeks on the CD. In all cases the percent of n-6 FA decreased considerably, whereas that of n-3 FA increased and that of major saturated and monoenoic FA changed less during the FO diet. During the washout interval studied, the diet-induced changes were completely reversed in plasma and liver, in contrast to blood cells, in which some of the diet-induced modifications in FA still persisted.

In plasma, the n-6/n-3 PUFA ratio changes began as early as the first day on the experimental diet. The individual fatty acid most reduced by the FO diet was 18:2n-6, initially the major circulating fatty acid, followed by 20:4n-6. The percent of plasma 20:5n-3 and 22:6n-3 increased rapidly, as evidenced by the slope of the curves, and reached their maximum proportions around day 10, after which the percent of both tended to plateau and then to decrease even

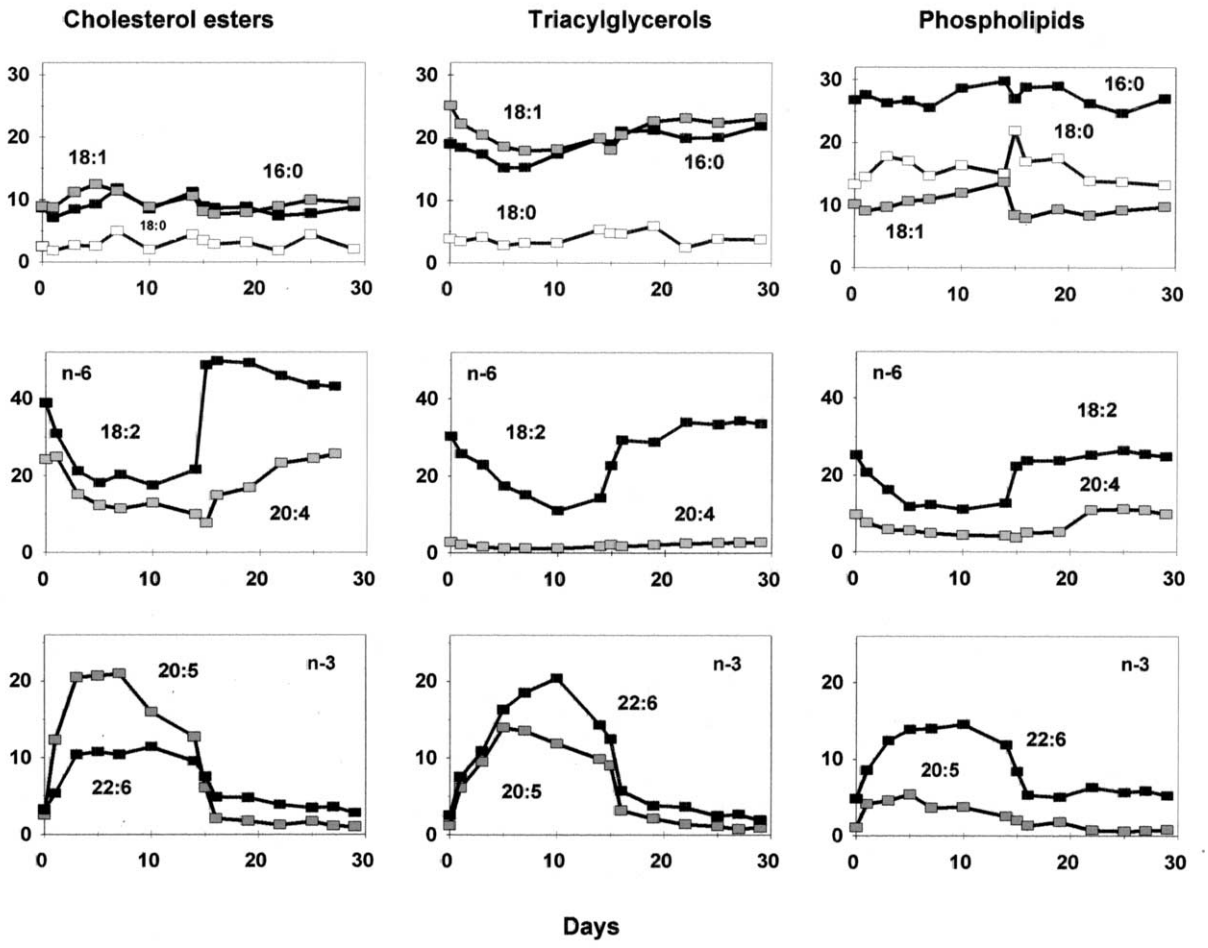

Fig. 3. Effect of n-3 PUFA administration and withdrawal on the percentages of major fatty acids of the three main lipid classes from mouse plasma. After separation of plasma phospholipids, cholesterol esters and triacylglycerols by TLC, their fatty acids were analyzed by GC. The points represent the percentages (weight %) of the depicted lipid fatty acids in lipids during administration of the FO diet (days 0–14) and after its suspension and replacement by the usual lab chow diet (days 15–30).

though the diet remained the same up to day 14. Plasma 18:2n-6 showed the fastest recovery after returning to the chow diet, its proportion even surpassing the zero time values the first days of the washout phase (Fig. 2). Comparatively, 20:4n-6 showed a more gradual pattern of reversal in plasma, needing a few more days to return to the initial levels. This difference is consistent with the fact that plasma 18:2n-6 is acquired pre-formed directly from the diet, whereas plasma 20:4n-6 has to be synthesized from the former, mainly in enterocytes and hepatocytes.

In liver, the PUFA of the total lipid behaved qualitatively in much the same way as that of plasma as a consequence of 2 weeks of FO intake and further replacement by the CD (Fig. 2). In the incorporation phase a decrease in nearly half the n-6 PUFA was compensated by an approximately two-fold increase in that of n-3 PUFA (Table 3). By the end of the washout period studied, the diet-induced changes in FA composition had been completely reversed in plasma and liver. In contrast to the rapid, large, and reversible changes in plasma and liver, in blood cells the proportions of n-3 fatty acids increased during the incorporation phase and decreased during the washout phase much more gradually (Fig. 2). On the last day of the washout period considered, the percent of PUFA like 20:5n-3 or 22:6n-3 still remained higher and those of 20:4n-6 lower than the initial values (Table 3).

The changes - mostly brought about by erythrocytes - affecting the fatty acids of blood cell lipids during and after the FO diet consumption periods are very interesting. At day 30, nearly two weeks after having abandoned the FO diet, and even when 18:2n-6 levels had been restored, blood cell total lipid FA were still only about half way to completing the reversal to initial conditions (Fig. 2, Table 3).

3.3. Fatty acid changes in individual lipid classes

Looking at the three main lipid classes carrying fatty acids in plasma (Fig. 3), it was clear that each contributed to the changes described in the total lipid, although to different extents. The PL fraction played the major role in the changes since this was the major plasma lipid constituent. Individually considered, and since its major components were initially 18:2n-6 and 20:4n-6, plasma CE was the lipid

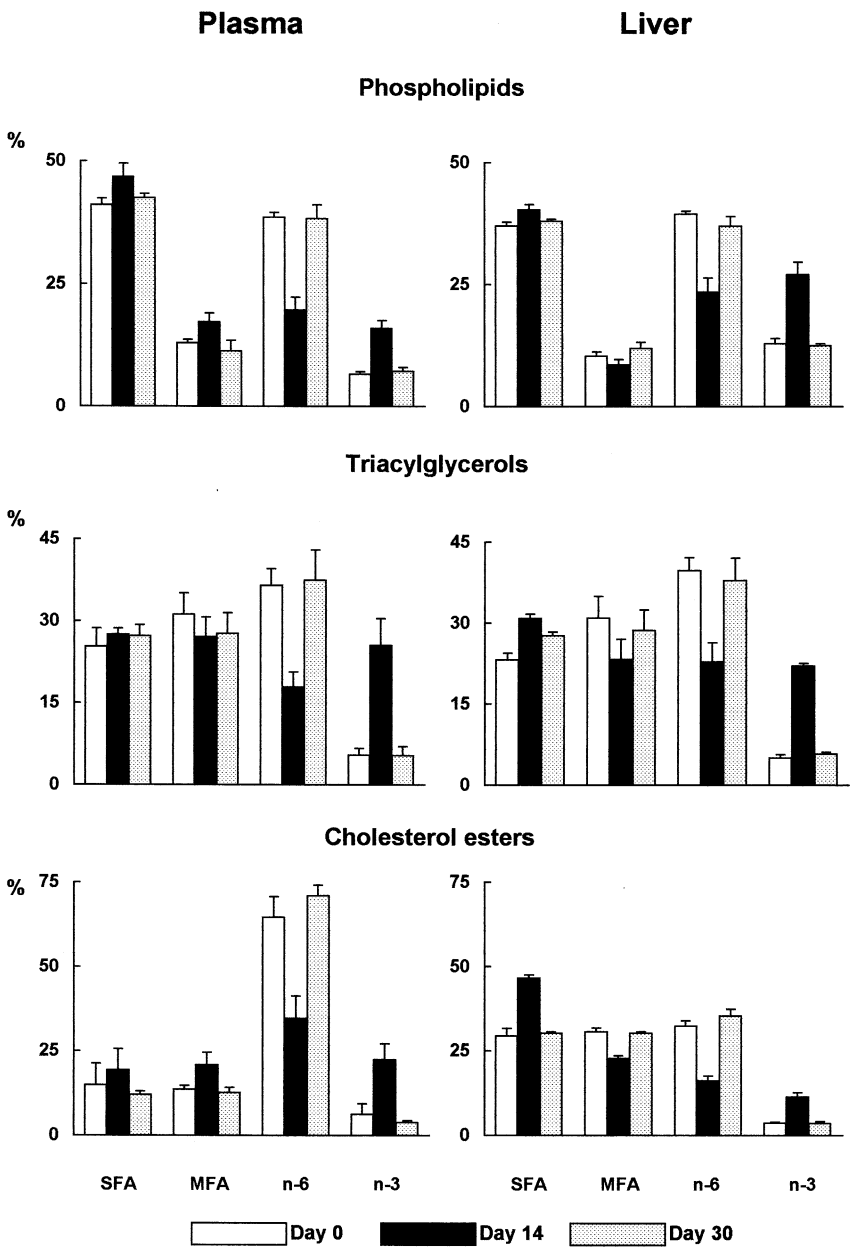

Fig. 4. Comparison with controls (day 0) of the effects, in plasma and liver lipids, of FO diet administration for 2 weeks (day 14), followed by 2 weeks without the diet (day 30). The bars represent the sums of saturated fatty acids (SFA), monoenoic fatty acids (MFA), n-6 PUFA and n-3 PUFA, respectively.

class changing the most with the n-3 diet, with such a large increase in the proportion of 20:5n-3 that it became the major highly unsaturated acyl group of this lipid. Also, CE was the lipid class showing the sharpest increase of 18:2n-6 upon return to the usual diet, followed by 20:4n-6. Plasma TG in turn had a higher 18:2/20:4 ratio than the other two lipids, since TG were much poorer in 20:4n-6, and incorporated similar amounts of 22:6n-3 and 20:5n-3 during the FO diet regime.

In liver, the pattern of changes produced in the FA of PL, CE and TG after 2 weeks on the n-3 diet was qualitatively similar to those observed in plasma lipids, as summarized in Fig. 4. In the incorporation period there was a considerable decrease in the percentages of n-6 PUFA concomitant to the enrichment in that of n-3 PUFA, and virtually unaltered proportions of SFA and MFA. Also, as it was the case in plasma, liver fatty acid changes were completely reverted during the washout period studied.

The changes in fatty acid composition of liver PL were contributed mainly by phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (data not shown). In both these lipids, 20:4n-6 decreased more than did 18:2n-6 after two weeks on FO, and PE admitted a considerably larger proportion of 22:6n3, 22:5n3, and 22:6n3 than did PC. In both

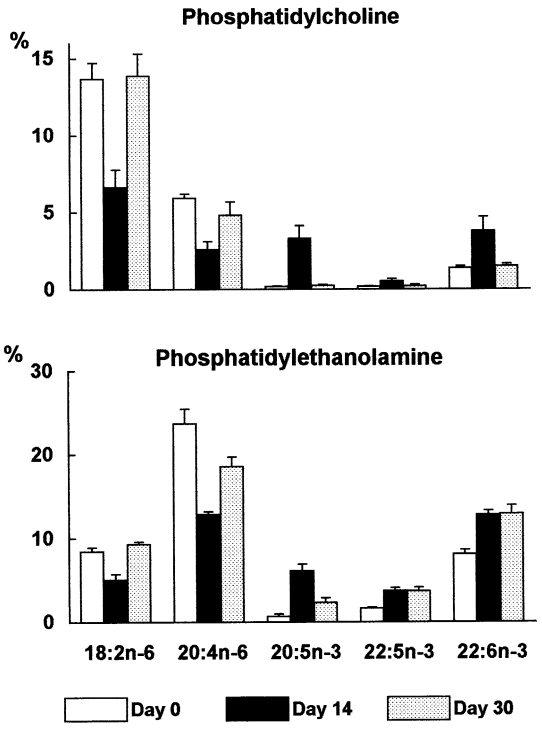

Fig. 5. Percentages of major polyunsaturated fatty acids of PC and PE of blood cells in mice fed FO for 2 weeks (day 14) and 2 weeks after having returned to the CD (day 30)

lipids the n-6/n-3 PUFA ratios, significantly decreased by FO, were restored to normal within two weeks (4.8 ± 1.3, 1.0 ± 0.3 and 4.7 ± 0.1 for PC, and 2.0 ± 0.9, 0.3 ± 0.1, and 2.0 ± 0.9 for PE at days 0, 14 and 30, respectively).

Diphosphatidylglycerol, a relatively minor glycerophospholipid, exceedingly rich in 18:2n-6 (54.4 ± 5.2% of the FA), remained an 18:2n-6-rich PL (43.6 ± 0.5% of the FA) even when it incorporated n-3 PUFA under the pressure of the FO diet (from a total of 8.2% to 14.8% of the FA after 14 days on FO). The total n-6/n-3 PUFA ratio in this lipid changed from 7.7 ± 1.5 to 3.4 ± 0.8 and from this value to 9.1 ± 0.2 at days 0, 14, and 30 respectively. The small increase in the amount of this PL observed here (Table 2), and especially after one whole month on FO supplementation [17] may correlate to the proliferation of mitochondria that is induced by excessive PUFA consumption [23–27]. Conversely, the changes DPG undergoes in fatty acid composition could influence the activity of enzymes immersed in mitochondrial membranes.

When analyzing the FA of the PL classes of blood cells, it became apparent that the reason for the lack of complete reversibility of the changes induced by 2 weeks of FO (Fig. 2, Table 3) was a different rate of restoration to the initial fatty acid composition of PC versus PE (Fig. 5). Thus, blood cell PC behaved in the same manner as liver or plasma PC, with a complete reversal of the diet-induced FA changes, whereas PE still remained significantly altered at the end of the 2-week washout period considered.

In blood cells, in this case mainly erythrocytes, the n-3 PUFA are likely to be incorporated into the lipids of precursor cells present in hemopoyetic tissues, and into the membrane phospholipids of maturing cells until, in time, these cells are released into the circulation. The changes in fatty acid composition of the blood cells observed here during the FO supplementation period were thus mostly brought about by these newly formed blood cells enriched in n-3 fatty acids plus the pre-existing ones, rich in n-6 PUFA, which explains the apparent slowness of the dietary induced changes. Similarly, since the washout period surveyed (15 days) was shorter than the average lifetime of mouse erythrocytes (20 days), it is possible that even at the end of this period not all the cells formed during the first two weeks of n-3 PUFA abundance had the opportunity to be removed from circulation. This was not the only factor involved, however, as suggested by the fact that this could selectively apply to PE and not to PC. Phosphatidylethanolamine is known to be located mainly in the inner leaflet of the red blood cell membrane [28,29] and is thought to be renewed by acylation of lysoPE, which is apparently a slow process [30], whereas PC is mainly located in the outer leaflet, and could in part be renewed by other mechanisms such as phospholipid transfer proteins exchanging PC with that of circulating lipoproteins.

Another intriguing effect of the n-3 PUFA-rich diet, also observed previously after one month FO consumption [17], was that the concentration of sphingomyelin (SM) tended to decrease in plasma and to increase slightly in liver with the n-3 dietary oil (Table 2). This relatively minor lipid of plasma and liver virtually lacks PUFA altogether, except for small amounts of 18:2n-6 and 24:2n-6 (together, nearly 3.5% of total fatty acids of this lipid). Apart from a reduction in these two n-6 PUFA in animals on the FO diet (added up, to 2.0% and to 1.0% of the FA in plasma and liver SM, respectively) plasma and liver SM became significantly poorer in 24:1 and somewhat richer in 24:0, thus showing a considerably decreased 24:1/24:0 ratio (2.2 to 1.4 in plasma, and 1.4 to 0.6 in liver after 2 weeks on FO). In liver only, a significant increase in 22:0 also occurred after two weeks with FO (from 18.8% to 29.4% of the FA). Neither of these qualitative and quantitative changes was observed in the SM of blood cells, which remained rich in 24:0 and 24:1 (25.2% and 15.4% respectively) throughout the experiment. To our knowledge, the only previous reports showing that the intake of PUFA can affect the fatty acids of liver SM is that by Bettger et al. [31] and that by Pennacchiotti et al. [17]. The mechanism(s) involved and the possible physiological effects of such diet-induced SM modification are still unknown.

4. Concluding remarks

Our results show that although more time may be required for tissues with a slow rate of cell formation and/or

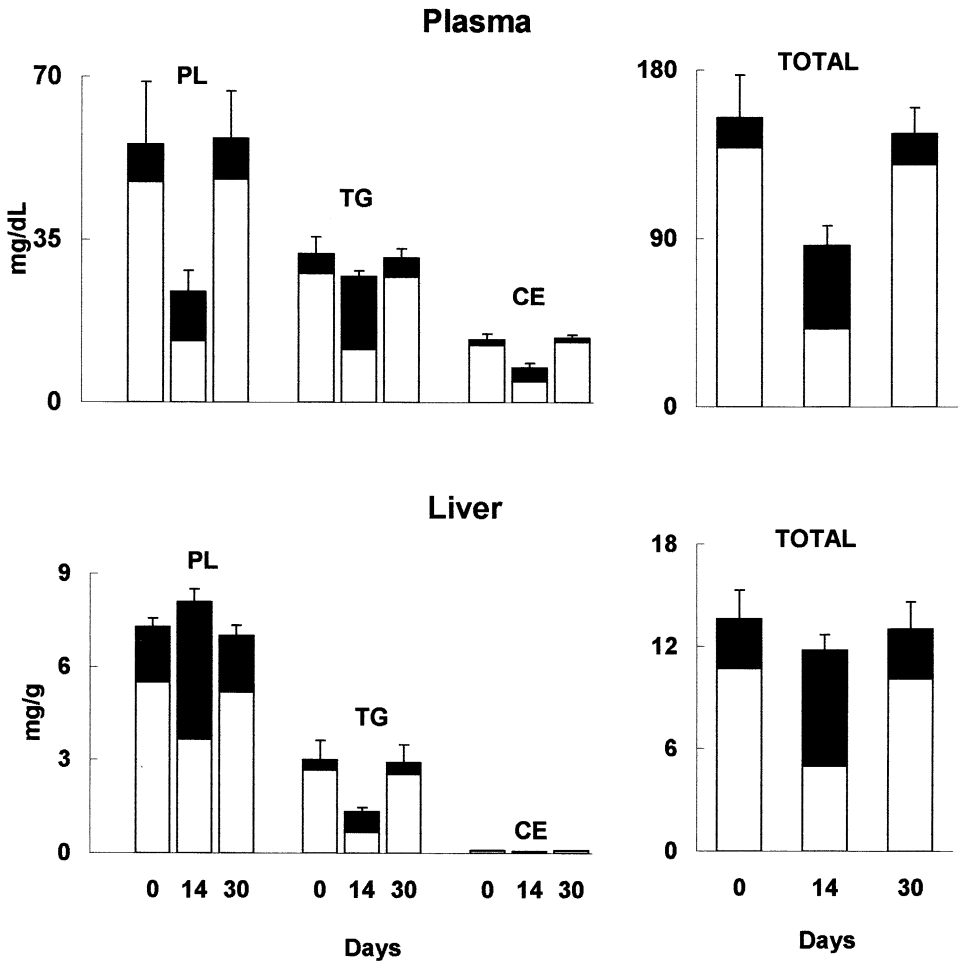

Fig. 6. Amounts of n-6 PUFA and n-3 PUFA (white and black portion of the bars, respectively) in plasma and liver lipids at day 0, after two weeks on an n-3 fatty acid rich diet (day 14), and after 15 days of having returned the animals to the usual chow diet (day 30). Results were calculated considering the lipid class concentrations (Table 1) and their corresponding fatty acid compositions. "Total", represents the sums of PL, CE and TG.

lipid synthesis or turnover, in mice, 10 days of fish oil supplementation are apparently sufficient to reach maximum proportions of n-3 PUFA in plasma and liver lipids. Irrespective of the high fat proportion in the fish oil diet, after sustained administration, the animals seem to adapt to the over-abundance of n-3 PUFA by becoming more efficient at dispensing with the excess fat. Maintaining a constant body and liver weight, our mice showed a decrease in the concentration of TG in liver, and in that of major lipids circulating in plasma. This is because the experimental tool we are using for the purpose of increasing the n-3 PUFA in tissues may promote per se its own consumption, since it induces FA oxidation in liver. It has been shown that 15 days on a FO-rich diet (10% wt/wt) results in a strong stimulatory effect of mitochondrial and peroxisomal oxidative metabolism in mouse liver [24–27]. Prolonging administration of the same 9% FO diet used here to a period of one month did not significantly alter the outcome [17]. Thus, liver TG, naturally designed to accumulate extra fatty acids from the diet, increased from ~0.5% to 1.4% of the liver weight from the CD to the condition of having eaten the FO diet for 30 days, respectively, a modest increase considering the high amount of total fat eaten by the animals over such a long period of their lives. As in the present case, even when a different strain of mice was used, the body weight remained constant and the level of plasma lipids decreased.

Even though the percentages of n-3 PUFA increased several fold in plasma and liver lipids during the FO feeding period, quantitatively the most remarkable change FO produced was not the increase in these fatty acids but the drastic *decrease* of n-6 PUFA (Fig. 6). In plasma, this led in fact to an overall reduction in the total amount of circulating PUFA (n-6 + n-3) (from a total of 154.6 to 86.4 mg/dl between days 0 and 14, Fig. 6). In liver, the concentrations of n-3 PUFA increased somewhat, but not beyond a certain limit, while the total amount of (n-6 + n-3) PUFA did not change significantly (from 13.6 to 11.8 mg/g of tissue between days 0 and 14, Figure 6). This occurred despite the facts that the FO diet was substantially richer in fat than the CD, and that it supplied much more n-3 PUFA and similar

amounts of n-6 PUFA (2.54 versus 2.49% of the calories as n-6 PUFA). In the second phase, fish oil withdrawal allowed the rapid restoration in plasma lipids of n-6 PUFA percentages, and at the same time reverted to normal (i. e., increased again) the concentrations of the plasma lipids in which these fatty normally abound (Table 1, Fig. 6). Again, this occurred despite the fact that the dietary fat content was smaller and that the amount of n-6 PUFA in the diet did not increase. It is worth mentioning that in the present experiments, except for the TG, lipids of tissues like brain, testes or adrenals (all of them n-6 PUFA-rich tissues in mice) either did not change at all, or changed much less than those of plasma or liver their fatty acid composition between CD and FO (days 0 and 14) and between FO and CD (days 14 and 30). Taken together, these results suggest that, since FO is a relatively poor source of n-6 PUFA, those available from plasma lipids may in part have been held in peripheral tissues for esterification into lipids and used in preference to the relatively more abundant n-3 PUFA. It is not unreasonable that tissues like brain and testes take precedence over plasma and liver as far as the maintenance of a constant n-6/n-3 PUFA ratio in their lipids is concerned.

Concerning the n-3 PUFA incorporation phase, comparing our results using mice as an experimental model with those in humans [9] and dogs [10] it would appear that for a given animal species and dose of n-3 PUFA probably there is an optimum period required for a new steady state to be reached, a plateau beyond which it is not worth prolonging administration or increasing the dose to attain a maximum of n-3 PUFA incorporation into tissue lipids. Something similar may happen for the washout phase: whereas humans [9] and dogs [10] may need at least 3 to 6 weeks, a period of less than two weeks without the FO-rich diet suffices in mice for the lipid compositional alterations in mouse plasma and liver to be completely reversed, even in the present case in which high FO doses were used in the uptake phase. This underscores the usefulness of determining the required duration of FO doses in order for other effects of dietary n-3 PUFA, such as their beneficial properties in preventive or therapeutic applications, to be sustained over time.

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