The Metabolic Syndrome of ω3-Depleted Rats. VIII. Dietary Lipid-Induced Liver Steatosis

Willy J. Malaisse*[,1], Ying Zhang[1], Nurdan Bulur[1], Mirjam Hacquebard[2], Yvan Larondelle[3], Yvon A. Carpentier[2] and Abdullah Sener[1]

Laboratories of Experimental [1]Hormonology and [2]Surgery, Université Libre de Bruxelles, Brussels, Belgium; [3]Laboratory of Nutritional Biochemistry, Université Catholique de Louvain, Louvain-la-Neuve, Belgium

Abstract: The present study aims at investigating the determinants of the undesirable aggravation of liver steatosis observed in rats first deprived, for 7 months from the 6th week after birth onwards, of a dietary supply of long-chain polyunsaturated ω3 fatty acids by exposure to a 5% sunflower oil-containing diet and then given access for about 2 weeks to the same diet enriched with 5% flaxseed oil in order to restore a sufficient ω3 fatty acid content of tissue lipids. Control rats were exposed for 7 months to a 5% soybean oil-containing diet and then given access for about 2 weeks to the same diet enriched with either 5% flaxseed oil or another 5% soybean oil. In all cases, the increase in the lipid content of the diet provoked an increase in liver triglyceride content. The ratio between the daily increment in the C18:3ω3 content of liver triglycerides caused by the switch in diet and the C18:3ω3 relative content of the diet used after the switch averaged 0.035 in the control rats eventually exposed to the soybean-enriched diet, 0.051 in the control rats eventually exposed to the flaxseed oil-enriched diet and 0.120 in the ω3-deficient rats eventually also exposed to a flaxseed oil-enriched diet. Thus, under the present experimental conditions, the induction or aggravation of liver steatosis, and possibly also the parallel increase in adipose tissue mass, may correspond to the deposition of dietary lipids, also involving an increase in food intake, more pronounced in the ω3-depleted rats than in the control animals.

Keywords: Long-chain polyunsaturated ω3 fatty acid-depleted rats, soya, sunflower and flaxseed oils, liver phospholipid and triglyceride fatty acid pattern.

INTRODUCTION

Rats exposed from the 7th week after birth for the following 3 to 7 months to a diet deprived of long-chain polyunsaturated ω3 fatty acids were recently proposed as a suitable animal model for the study of changes in metabolic and hormonal variables resulting from such a deprivation [1, 2]. These rats were indeed found to display several features of the metabolic syndrome, such as insulin resistance, hepatic steatosis and visceral obesity, as previously described in second-generation ω3-depleted rats [3-5]. In our recent reports, exposure of the ω3-depleted rats for 2 to 4-5 weeks to a diet enriched with flaxseed oil was also found to result in a restoration of the ω3 fatty acid content in the phospholipids of liver, brain, duodenum, jejunum, caecum and colon [1, 6, 7]. However, under the same experimental conditions, no correction of liver steatosis and visceral obesity took place [1, 2]. On the contrary, when the ω3-depleted rats were exposed to the flaxseed oil-enriched diet, a further increase in both liver steatosis and the weight of parametrial adipose tissue was observed, this coinciding with a rapid and pronounced increase in body weight [2]. Such an undesirable situation could be due to an increase in food intake, conceivably attributable to the orexigenic action of long-chain polyunsaturated fatty acids, to the caloric enrichment of the diet, as resulting from the increase in its lipid content, and/or to a change in energy expenditure.

With the perspective of finding a suitable approach to prevent this undesirable situation, the major aim of the present study was to investigate the possible determinants of such increases in liver steatosis, adipose tissue mass and body weight. For such a purpose, after 7 months exposure to a diet containing 5% (w/w) sunflower oil, 6 ω3-depleted rats were exposed for 2 weeks to the same diet enriched with 5% (w/w) flaxseed oil. Two control groups of 6 rats each were first exposed for 7 months to a diet containing 5% (w/w) soya oil and then exposed for 2 weeks to the same diet enriched with 5% (w/w) of either soya oil or flaxseed oil. The inclusion of two control groups was motivated by the following considerations. The control rats eventually exposed to a flaxseed oil-enriched diet allowed to distinguish between the effects of the latter dietary manipulation in animals previously given access to a normal diet, as distinct from previously ω3-deficient rats. The control rats eventually exposed to a soybean-enriched diet permitted to assess the possible consequences of a mere increase in the lipid content of the diet from 5 to 10% (w/w).

The present report deals with the fatty acid content and pattern of the liver phospholipids and triglycerides in the three groups of rats. It provides evidence to incriminate both

*Address correspondence to this author at the Laboratory of Experimental Hormonology, Université Libre de Bruxelles, 808 Route de Lennik, B-1070 Brussels, Belgium; Tel: 32-2-5556237; Fax: 32-2-5556356;
E-mail: malaisse@ulb.ac.be

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the increase in the lipid content of the diet and an increase in food intake as determinants of the aggravation of liver steatosis found in the ω3-depleted rats when exposed to the flaxseed oil-enriched diet.

MATERIALS AND METHODS

Three groups of 6-week-old female normal rats (Iffa Credo, L'Arbresle, France) were housed separately, and given access for 7 months to tap water and either a control diet (AOE; SAFE, Villemoison-sur-Orge, France) or an ω3-depleted diet. The lipid composition of these two diets was given in a prior publication [1]. The control and ω3-depleted diet contained 5% (w/w) lipids from soya and sunflower, respectively, the C18:3ω3 weight percentage being below the limit of detection in the latter diet. Thereafter, the control rats were given access for the next 16 days to the control diet enriched with 5% (w/w) soybean oil (CS rats) or flaxseed oil (CF rats). Likewise, the ω3-depleted rats were eventually given access to their ω3-depleted diet enriched with 5% (w/w) flaxseed oil (DF rats). The diets used during the last 2 weeks of the present experiments were similar to those used in our prior study [1], but represented new preparations. Their lipid composition is given in Table **1**.

Sixteen days after the switch in diet, the rats were eventually euthanized by carbon dioxide inhalation. A piece of liver was sampled for measuring the fatty acid content and pattern of hepatic phospholipids and triglycerides [8-10] by methods described in the cited references.

All results are presented as means (± SEM) together with either the number of individual observations (n) or degree of freedom (df). The statistical significance of differences between mean values was assessed using Student's *t*-test and confirmed by variance analysis with Bonferroni post-test.

RESULTS

Liver Phospholipids

The total fatty acid content of liver phospholipids was significantly lower ($p < 0.01$ or less) in DF rats than in either CF or CS rats (Table **2**).

In the liver phospholipids, the relative contents of C18:3ω3 and C20:5ω3 were much higher ($p < 0.001$) in the CF or DF rats than in the CS rats. Such was also the case ($p < 0.005$) when comparing the phospholipid C22:5ω3 relative contents of CS rats *versus* CF and DF rats. Only, the relative content of C22:6ω3 in liver phospholipids failed to differ significantly in the three groups of rats (Table **2**). The C20:5ω3/C18:3ω3 paired ratio was somewhat higher ($p < 0.02$) in DF rats than in the CF and CS rats. The C22:6ω3/C20:5ω3 ratio was higher ($p < 0.005$) in HCS rats than in either CF or DF rats. Inversely, the C22:5ω3/C22:6ω3 ratio was lower ($p < 0.05$ or less) in the CS rats than in either the CF or DF rats (Table **3**).

In the case of the long-chain polyunsaturated ω6 fatty

Table 1. Fatty Acid Composition of Diets (% of Total Fatty Acid Content)

Oil	Soya (5%)	Soya (10 %)	Sunflower (5%)
	Flaxseed (5%)		Flaxseed (5%)
C12:0	0.4	0.5	0.4
C14:0	4.3	4.4	3.1
C16:0	85.1	114.7	54.8
C16:1ω7	4.8	5.1	1.6
C18:0	25.0	24.1	32.5
C18 :1ω9	169.4	190.2	196.2
C18:2ω6	343.1	565.4	397.0
C20:0	0.8	2.9	0.9
C18:3ω3	343.1	67.1	310.1
C20:1ω9	7.0	5.9	0.0
C20:2ω6	1.4	1.3	0.0
C22:0	1.4	2.7	3.4
C20:4ω6	0.9	0.7	0.0
C22:1ω9	3.3	5.6	0.0
C20:5ω3	3.0	3.7	0.0
C22:5ω3	0.8	0.7	0.0
C22:6ω3	6.1	6.0	0.0

Table 2. Total Content and Relative Weight Percentage of Fatty Acids in Liver Phospholipids

Rats	CF	CS	DF
Total (mg/g wet wt.)*	27.32 ± 0.59^{e}	28.36 ± 0.54^{f}	$24.64 \pm 0.53^{e,f}$
C18:3ω3	0.9 ± 0.1^{f}	$0.2 \pm 0.0^{f,e}$	0.6 ± 0.1^{e}
C20:5ω3	3.6 ± 0.4^{f}	$0.7 \pm 0.2^{f,\zeta}$	$3.9 \pm 0.6^{\zeta}$
C22:5ω3	2.0 ± 0.1^{e}	$1.4 \pm 0.1^{e,a}$	2.2 ± 0.3^{a}
C22:6ω3	14.2 ± 1.0	14.9 ± 0.8	15.0 ± 0.6
C18:2ω6	15.6 ± 0.8^{e}	15.6 ± 0.5^{f}	$11.4 \pm 0.6^{e,f}$
C18:3ω6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
C20:2ω6	0.3 ± 0.0^{d}	0.4 ± 0.0^{f}	$0.2 \pm 0.0^{d,f}$
C20:3ω6	0.7 ± 0.0	0.7 ± 0.1	0.7 ± 0.1
C20:4ω6	24.8 ± 0.8^{d}	28.6 ± 0.8^{d}	26.5 ± 0.9
C22:4ω6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
C14:0	0.2 ± 0.0^{f}	0.2 ± 0.0^{d}	$0.4 \pm 0.0^{f,d}$
C16:0	12.6 ± 0.2	12.0 ± 0.3	11.9 ± 0.3
C16:1ω7	0.2 ± 0.1^{e}	0.2 ± 0.1^{d}	$0.5 \pm 0.1^{e,d}$
C18:0	22.0 ± 0.4^{e}	22.6 ± 0.3^{c}	$23.8 \pm 0.3^{e,c}$
C18:1ω9	1.6 ± 0.1^{a}	$1.2 \pm 0.1^{a,d}$	1.6 ± 0.1^{d}
C20:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
C20:1ω9	0.1 ± 0.0^{c}	0.1 ± 0.0^{f}	$0.0 \pm 0.0^{c,f}$
C22:0	0.2 ± 0.0^{f}	0.3 ± 0.0^{f}	0.3 ± 0.0
C22:1ω9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
C24:0	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0

*Mean values (± SEM) refer to 6 individual observations in all cases.
a: $p < 0.05$; c: $p < 0.02$; d: $p < 0.01$; e: $p < 0.005$; f and ζ: $p < 0.001$.

acids, the sole significant differences between CF and DF rats consisted in a lower values for the C18:2ω6 and C20:2ω6 relative weight contents ($p < 0.005$) in the latter than former animals (Table **2**). Likewise, the sole significant difference between CF and CS rats consisted in a higher C20:4ω6 relative content ($p < 0.01$) in the latter than former animals. In the DF rats, the low C20:3ω6/C18:3ω6 ratio and high C20:4ω6/C20:3ω6 and C22:4ω6/C20:4ω6 ratios otherwise found in ω3-depleted rats were all restored to values close to those found in the CS rats (Tables **2**). The access to the flaxseed oil-enriched diet even lowered significantly ($p < 0.02$) the C22:4ω6/C20:4ω6 ratio in the CF rats, below the level found in CS rats (Table **3**).

In the case of the weight percentage of saturated and monodesaturated fatty acids in liver phospholipids, the sole significant differences between CF and CS rats consisted in a higher value ($p < 0.05$) of C18:1ω9 in the former rats than in the latter animals and a lower value ($p < 0.001$) of C22:0 in CF rats (0.201 ± 0.049 %) than in CS rats (0.257 ± 0.072 %). The C18:1ω9/C18:0 ratio was also higher ($p < 0.05$) in CF rats than in CS rats (Table **3**). Several saturated (C14:0, C18:0 and C22:0) and monodesaturated (C16:1ω7) fatty acids yielded a higher relative weight content ($p < 0.01$ or less) in the liver phospholipids of DF rats as compared to CF rats. The opposite situation prevailed, however, in the case of C20:1ω9. The C16:1ω7/C16:0 ratio was also higher ($p < 0.01$) in the liver phospholipids of DF rats, as compared to CF rats (Table **3**).

Liver Triglycerides

The total fatty acid content of liver triglycerides was significantly higher ($p < 0.05$) in CS rats than in CF rats (Table **4**). In fair agreement with our prior study, the mean value was even somewhat higher, albeit not significantly so, in DF rats than in CS rats. As a matter of fact, when expressing the results relative to the latter mean values recorded in the CS rats and when pooling together the measurements made in the two series of experiments, the total fatty acid content of liver triglycerides averaged in the CF rats 86.5 ± 7.3 % ($n = 12$) as distinct ($p < 0.05$) from 115.7 ± 11.4 % ($n = 12$) in the DF rats of the reference value indeed found in CS rats (100.0 ± 7.2 %; $n = 12$).

Table 3. Ratio Between Selected Fatty Acids in Liver Phospholipids

Rats	CF	CS	DF
C20:5ω3/C18:3ω3*	4.31 ± 0.37^{c}	4.90 ± 0.78	6.80 ± 0.75^{c}
C22:6ω3/C20:5ω3	4.11 ± 0.52^{e}	$27.39 \pm 5.82^{e,\varepsilon}$	$4.33 \pm 0.68^{\varepsilon}$
C22:5ω3/C22:6ω3	0.144 ± 0.008^{e}	$0.094 \pm 0.011^{e,a}$	0.154 ± 0.024^{a}
C20:2ω6/C18:2ω6 (10^{-3})	15.62 ± 0.78^{e}	15.57 ± 0.48^{a}	$11.40 \pm 0.62^{e,a}$
C18:3ω6/C18:2ω6 (10^{-3})	$5.53 \pm 0.67^{a(x)}$	5.29 ± 0.57^{b}	$9.17 \pm 1.34^{a,b(y)}$
C20:3ω6/C18:3ω6	$8.03 \pm 0.68^{(x)}$	8.02 ± 0.49	$7.92 \pm 1.17^{(y)}$
C20:4ω6/C20:3ω6	37.80 ± 1.35	46.66 ± 4.71	38.45 ± 4.69
C22:4ω6/C20:4ω6 (10^{-3})	2.27 ± 0.76^{c}	4.71 ± 0.25^{a}	4.14 ± 0.39
C16:1ω7/C16:0 (10^{-3})	14.94 ± 3.16^{d}	15.48 ± 4.80^{b}	$38.81 \pm 5.97^{b,d}$
C18:1ω9/C18:0 (10^{-3})	74.7 ± 7.9^{a}	$52.9 \pm 4.0^{a,\alpha}$	$65.4 \pm 2.7^{\alpha}$

*Mean values (± SEM) refer to 6 individual observations, except otherwise indicated.
$^{(x)}$Only 4 individual observations.
$^{(y)}$Only 5 individual observations.
a and α: $p < 0.05$; b: $p < 0.025$; c: $p < 0.02$; d: $p < 0.01$; e and ε: $p < 0.005$.

Table 4. Total Content and Relative Weight Percentage of Fatty Acids in Liver Triglycerides

Rats	CF	CS	DF
Total (mg/g wet wt.)*	4.85 ± 0.38^{a}	6.00 ± 0.31^{a}	6.85 ± 0.90
C18:3ω3	$12.5 \pm 1.0^{f,b}$	$2.8 \pm 0.2^{f,\zeta}$	$9.4 \pm 0.6^{\zeta,b}$
C20:5ω3	$3.5 \pm 0.3^{f,\zeta}$	1.1 ± 0.1^{f}	$1.4 \pm 0.2^{\zeta}$
C22:5ω3	$4.1 \pm 0.3^{f,\zeta}$	1.6 ± 0.1^{f}	$1.3 \pm 0.2^{\zeta}$
C22:6ω3	$5.2 \pm 0.3^{a,\zeta}$	$4.0 \pm 0.4^{a,f}$	$1.2 \pm 0.3^{f,\zeta}$
C18:2ω6	$32.8 \pm 1.2^{e,a}$	$41.9 \pm 1.6^{e,f}$	$26.3 \pm 2.2^{a,f}$
C18:3ω6	$0.0 \pm 0.0^{c,f}$	0.4 ± 0.0^{f}	0.3 ± 0.1^{c}
C20:4ω6	$3.0 \pm 0.1^{b,c}$	$4.1 \pm 0.4^{b,e}$	$2.1 \pm 0.3^{c,e}$
C22:4ω6	0.3 ± 0.1^{c}	0.5 ± 0.1^{f}	$0.0 \pm 0.0^{c,f}$
C12:0	0.1 ± 0.0^{a}	0.0 ± 0.0	0.0 ± 0.0^{a}
C14:0	1.8 ± 0.3	1.4 ± 0.2	1.8 ± 0.2
C16:0	$17.6 \pm 0.3^{c,d}$	21.2 ± 1.2^{c}	23.4 ± 1.8^{d}
C16:1ω7	1.2 ± 0.1^{e}	1.7 ± 0.3^{c}	$3.7 \pm 0.6^{e,c}$
C18:0	2.4 ± 0.1^{c}	$2.0 \pm 0.1^{c,d}$	2.4 ± 0.1^{d}
C18:1ω9	15.5 ± 0.6^{f}	$17.1 \pm 0.9^{\zeta}$	$26.4 \pm 1.2^{f,\zeta}$

*Mean values (± SEM) refer to 6 individual observations in all cases.
a: $p < 0.05$; b: $p < 0.025$; c: $p < 0.02$; d: $p < 0.01$; e:$p < 0.005$; f and ζ: $p < 0.001$.

The relative content of liver triglycerides in all long-chain polyunsaturated ω3 fatty acids was lower ($p < 0.05$ or less) in CS rats than in CF rats. Such was also the case when comparing DF to CF rats. The DF rats differed, however, from the CS rats by a much higher C18:3ω3 relative content ($p < 0.001$) and much lower C22:6ω3 content ($p < 0.001$) in the former than latter animals. The C20:5ω3/C18:3ω3 and C22:6ω3/C20:5ω3 ratios were lower ($p < 0.005$ or less) in CF than in CS rats, whilst the opposite difference was observed in the case of the C22:5ω3/C22:6ω3 ratio ($p < 0.001$). The C20:5ω3/C18:3ω3 and C22:6ω3/C20:5ω3 ratios were further decreased ($p < 0.01$ or less) in DF rats, as compared to CF rats. Such was not the case ($p > 0.1$), however, when comparing the C22:5ω3/C22:6ω3 ratio in CF and DF rats (Table **5**).

Table 5. Ratio Between Selected Fatty Acids in Liver Triglycerides

Rats	CF	CS	DF
C20:5ω3/C18:3ω3*	$0.280 \pm 0.019^{d,f}$	$0.387 \pm 0.023^{d,\zeta}$	$0.143 \pm 0.014^{f,\zeta}$
C22:6ω3/C20:5ω3	$1.54 \pm 0.13^{f,d}$	$4.03 \pm 0.39^{f,\zeta}$	$0.93 \pm 0.12^{\zeta,d}$
C22:5ω3/C22:6ω3	0.80 ± 0.05^{f}	$0.43 \pm 0.03^{f,d}$	1.10 ± 0.19^{d}
C20:4ω6/C18:2ω6	0.093 ± 0.004	0.096 ± 0.006^{a}	0.077 ± 0.006^{a}
C16:1ω7/C16:0	0.069 ± 0.007^{f}	0.074 ± 0.011^{e}	$0.154 \pm 0.017^{f,e}$
C18:1ω9/C18:0	$6.74 \pm 0.53^{c,f}$	$8.65 \pm 0.28^{c,e}$	$11.02 \pm 0.56^{f,e}$

*Mean values (± SEM) refer to 6 individual observations in all cases.
a: $p < 0.05$; c: $p < 0.02$; d: $p < 0.01$; e: $p < 0.005$; f and ζ: $p < 0.001$.

The liver triglyceride content of C18:2ω6 remained lower ($p < 0.001$) in DF rats than in CS rats, as is also the case when comparing ω3-depleted rats to control animals before the switch in diet [1]. The CF rats yielded an in-between value significantly lower ($p < 0.005$) than that found in CS rats and significantly higher ($p < 0.03$) than that found in DF rats. The relative content of liver triglycerides in C18:3ω6, C20:4ω6 or C22:4ω6 was also higher ($p < 0.05$ or less) in CS rats than in either CF or DF rats. The overall efficiency of the stepwise generation of C20:4ω6 from C18:2ω6, as judged from the C20:4ω6/C18:2ω6 ratio, was lower ($p < 0.02$) in the DF rats than in the CS and CF rats.

The relative weight content of saturated and monodesaturated fatty acids in liver triglycerides also differed in the three groups of rats (Table **4**). A minor amount of C12:0 was only detected in 7 out of 18 rats. It did not exceed 0.1 ± 0.0 % (n = 7). The CF rats displayed a lower relative content of C16:0 ($p < 0.02$) but higher relative content of C18:0 ($p < 0.02$) than the CS rats. The relative content of C16:0, C16:1ω7 and C18:1ω9 was higher ($p < 0.01$ or less) in DF rats than in CF rats. Except for one CS rat, in which the C20:1ω9 relative weight content of liver triglycerides represented 1.8 %, no C20:0, C20:1ω9, C22:0, C22:1ω9 or C24:0 was detected in such triglycerides. The mean C16:1ω7/C16:0 and C18:1ω9/C18:0 ratios were lower in CF than CS rats, such a difference only achieving statistical significance ($p < 0.01$) in the case of the C18:1ω9/C18:0 ratio. These two ratios remained much higher ($p < 0.005$) in DF rats than in CS rats, as is also the case when comparing ω3-depleted rats to control animals before the switch in diet [1].

DISCUSSION

The present report extends prior observations on the design of a suitable animal model to simulate the situation found in human subjects suffering from a diet-induced depletion in long-chain polyunsaturated ω3 fatty acids and eventually oriented towards a compensatory access to an ω3-enriched diet [1, 2]. The following considerations underline the main contribution emerging from this study.

The present results relative to the fatty acid content and pattern of liver lipids were, as a rule, in close agreement with those of our prior study [1]. As a matter of fact, the mean relative weight content of the 20 fatty acids measured in the liver phospholipids of the 3 groups of rats examined in the present experiments, when compared to the corresponding values measured in our prior study, yielded a coefficient of correlation equal to unity (1.0000; n = 60). Likewise, for the 13 fatty acids measured in the liver triglycerides of the 3 groups of rats, the coefficient of correlation between the present results and those of our prior study amounted to + 0.9933 (n = 39; $p < 0.001$). When the mean values found in CF, CS and DF rats for each of the ten ratios between the relative weight content of selected fatty acids in liver phospholipids were expressed relative to the overall means value for the same ratio in the three groups of rats, a highly significant correlation ($r = + 0.9062$; n = 30; $p < 0.001$) was also observed between the results of the present and prior experiments. Such was also the case for the six ratios between selected fatty acids in liver triglycerides ($r = + 0.9414$; n = 18; $p < 0.001$). Even for those triglyceride ratios which could not be assessed in all three groups of rats, namely the C18:3ω6/C18:2ω6, C20:4ω6/C18:3ω6 and C22:4ω6/C20:4ω6 ratios, the mean values recorded in only two groups of rats yielded, for the quotient between the present data and those collected in our prior study, a mean percentage of 96.0 ± 6.9 % (df = 51; $p > 0.5$).

The present results confirm the lower total content of fatty acids in the phospholipids of DF, as compared to either CS or CF rats.

The higher content of C18:3ω3 and C20:5ω3 and C22:5ω3 in the phospholipids of CF and DF rats, as distinct from CS rats, coincides with the 4 to 5 times higher content of C18:3ω3 in the diet offered to the former animals, as compared to the latter ones. This situation may also account for the much lower C22:6ω3/C20:5ω3 ratio in the CF and DF rats, than in the CS ones. Our data also suggest a more efficient conversion of C22:6ω3 to C22:5ω3 in CF and DF rats, than in CS animals. Last, the higher C20:5ω3/C18:3ω3 ratio in DF rats, as compared to CS and CF rats, may well reflect an accelerated generation of C20:5ω3 from its precursor in the previously ω3-deficient animals. These several changes coincided with the restoration of the phospholipid C22:6ω3 content in DF rats to a mean value comparable to that found in either CS or CF rats.

In the case of phospholipid long-chain polyunsaturated ω6 fatty acids, the present data confirm both the decrease in

the C18:2ω6 content in DF rats when compared to CS and CF animals and the lower C20:2ω6/C18:2ω6 ratio found in DF rats than in CS rats.

Last, as far as saturated and monodesaturated fatty acids are concerned, the lower value for the C16:1ω7/C16:0 ratio in CF and CS rats than in DF rats, as observed in this study, also duplicates our prior observation [1].

Likewise, in addition to the close analogy already mentioned between our prior and present results in terms of the total fatty acid content of triglycerides in the three groups of rats, both studies document a higher triglyceride content in C18:3ω3 , C20:5ω3 and C22:5ω3 in CF than CS rats and a lower triglyceride content of C18:3ω3 , C20:5ω3, C22:5ω3 and C22:6ω3 in DF than CF rats. The triglyceride relative weight content of the four long-chain polyunsaturated ω6 fatty acids listed in Table **4**, when expressed relative to their respective mean values in the three groups of rats, yielded a correlation coefficient ($r = + 0.7925$; $n = 12$; $p < 0.003$) between the present and prior results documenting the close analogy of the two series of experiments in terms of the dietary effects on the variables under consideration. Such was also the case for the triglyceride content in the saturated and monodesaturated fatty acids C16:0, C16:1ω7, C18:0 and C18:1ω9 ($r = + 0.9711$; $n = 12$; $p < 0.001$) and for the C16:1ω7/C16:0 and C18:1ω9/C18:0 ratio ($r = + 0.9913$; $n = 6$; $p < 0.001$).

In our opinion, an interesting finding consists in the fact that the C22:6ω3 relative weight content of liver phospholipids failed to differ significantly in the 3 groups of rats (Table **2**), whilst, in liver triglycerides, it remained much lower ($p < 0.001$) in DF rats than in either CF or CS rats (Table **4**). The latter situation is likely to be attributable to the fact that the diet eventually offered to the DF rats contained C18:3ω3 as the sole long-chain polyunsaturated ω3 fatty acid and that, even in the other two diets eventually offered to CF and CS rats, the total amount of C20:5ω3, C22:5ω3 and C22:6ω3, provided by the soya oil, did not exceed 1.0 ± 0.3 % ($n = 2$) of the total fatty acid content of the diet (Table **1**). These findings are in close agreement with those of our prior study [1]. Their interpretation must take into account the fact that, in all 3 groups of rats, the total fatty acid content of liver triglycerides is significantly higher after than before the switch in diet [1]. Hence, it appears that, under the present experimental conditions, the increase in the liver triglyceride content after the switch in diet mainly reflects a further accumulation of triglycerides with the same fatty acid pattern as that prevailing in the diets eventually offered to the CF, CS and DF rats. A further support to this proposal resides in the finding that the increase in the C18:3ω3 relative weight content of liver triglycerides recorded after the switch in diet was much higher ($p < 0.001$) in the CF rats (+ 11.3 ± 1.2 %; $df = 9$) and DF rats (+ 10.6 ± 1.3 %; $n = 6$) than in the CS rats (+ 0.6 ± 0.2 %; $df = 9$), in fair agreement with the difference in the C18:3ω3 relative weight content of the corresponding diets eventually offered to each of these groups of rats. The observation that both the C18:3ω3 and C22:6ω3 relative contents of liver triglycerides did not differ significantly in DF rats exposed for only 2 weeks or 4-5 weeks to the flaxseed oil-enriched di*et al*so provides further support to the present proposal. The above mentioned difference in terms of the C22:6ω3 relative weight content of liver terms of the C22:6ω3 relative weight content of liver phospholipids *versus* liver triglycerides would then imply a selective incorporation of C22:6ω3 generated in the liver from C18:3ω3 into phospholipids, as distinct from triglycerides.

In the light of these considerations, a further analysis of the liver data provided information on possible differences in food intake in the CS, CF and DF rats after the switch in diet. In our prior study, the increase in the C18:3ω3 content of liver triglycerides in the CS and CF rats recorded after 4-5 weeks exposure to the soya oil- or flaxseed oil-enriched diet, above the mean value measured prior to the switch in diet, averaged, respectively, 79.8 ± 28.3 and 565.1 ± 97.3 μg/g liver wet wt. ($df = 9$ in both cases). It was thus much higher ($p < 0.001$) in the CF rats than in the CS rats. However, when expressed relative to the corresponding C18:3ω3 relative weight content in the fatty acids of the diet (expressed as ‰), the difference between CS rats (1.115 ± 0.396; $df = 9$) and CF rats (1.610 ± 0.277; $df = 9$) did no more achieve statistical significance ($p > 0.3$). In the DF rats, the increase in the C18:3ω3 content of liver triglycerides, above the zero value recorded just before the switch in diet, averaged 611.9 ± 80.0 and 959.3 ± 184.8 μg/g liver wet wt. ($n = 6$ in both cases) after, respectively, 2 and 4-5 weeks exposure to the flaxseed oil-enriched diet. Although the latter two values were not significantly different from one another, they parallel the changes in body weight [2], in that the main daily increase in the C18:3ω3 content of liver triglycerides recorded over the first 2 weeks of exposure to the flaxseed oil-enriched diet (43.7 ± 5.7 μg/g per day) was higher than that found over 4-5 weeks exposure to the same diet (30.5 ± 5.9 μg/g per day). Moreover, when the overall mean value for the daily increase in the C18:3ω3 content of liver triglycerides in the DF rats (37.1 ± 4.4 μg/g per day) was divided by the relative weight content of C18:3ω3 in the fatty acids of their flaxseed oil-enriched diet (again expressed as ‰), the resulting quotient (0.120 ± 0.014; $n = 12$) was much higher ($p < 0.001$) than the corresponding quotients found in either CS rats (0.035 ± 0.013; $df = 9$) or CF rats (0.051 ± 0.088; $df = 9$). The increase in the total fatty acid content of liver triglycerides, if indeed attributable to the accumulation of ingested triglycerides, would then be expected to yield a comparable hierarchy (CS ~ CF < DF).

A comparable analysis could not be achieved in the case of C22:6ω3, since the latter fatty acid is absent from the flaxseed oil-enriched diet eventually offered to the DF rats. Nevertheless, the two following observations merit to be mentioned. First, the increase in the C22:6ω3 content of liver triglycerides above the mean value measured prior to the switch in diet did not differ significantly ($p > 0.5$) in CS and CF rats, with an overall mean value of 80.6 ± 34.7 μg/g liver wet wt. ($df = 18$; $p < 0.04$ *versus* zero). If divided by the relative weight content of C22:6ω3 in the fatty acid of the diets (6.05 ± 0.05 ‰), such an increase would yield a quotient (13.326 ± 5.738) one order of magnitude higher ($p < 0.05$) than the overall mean value for the same quotient found in the case of C18:3ω3 in the CS and CF rats (1.362 ± 0.241), again documenting the generation of C22:6ω3 from C18:3ω3. Second, in the DF rats, the time course for the accumulation of C22:6ω3 in liver triglycerides after the switch in diet was comparable to that mentioned above for the

C18:3ω3 enrichment of the same triglycerides. Relative to the mean increments found 4-5 weeks after the switch in diet (100.0 ± 15.3%; n = 12), those recorded only 2 weeks after such a switch indeed failed to differ significantly ($p > 0.3$) in the case of C18:3ω3 (63.8 ± 8.3%; n = 6) and C22:6ω3 (78.4 ± 10.5%; n = 6), yielding an overall mean value of 71.1 ± 6.7% (n = 12). Hence, if expressed relative to the length of the period after the switch in diet, the mean daily enrichment of liver triglycerides occurred more rapidly ($p < 0.02$) over the first 2 weeks (5.08 ± 0.48%; n = 12) than over the entire period of 4-5 weeks (3.17 ± 0.48%; n = 12). In other words, the main daily enrichment of liver triglycerides in the DF rats would be thrice higher ($p < 0.02$) during the first 2 weeks after the switch in diet (5.08 ± 0.48%; n = 12) than during the ensuing 2-3 weeks (1.65 ± 0.96%; df = 22). This time-related pattern is superimposable to that characterizing the time course for the increase in the total fatty acid content of the liver triglycerides (Fig. **1**, lower panel).

Likewise, the hierarchy found for the C18:3ω3 enrichment of liver triglycerides after the switch in diet (CS ~ CF < DF) is similar to that characterizing the increase in the total fatty acid content of liver triglycerides over the same period (Fig. **1**, upper panel), with mean values of 1.90 ± 0.49 mg/g

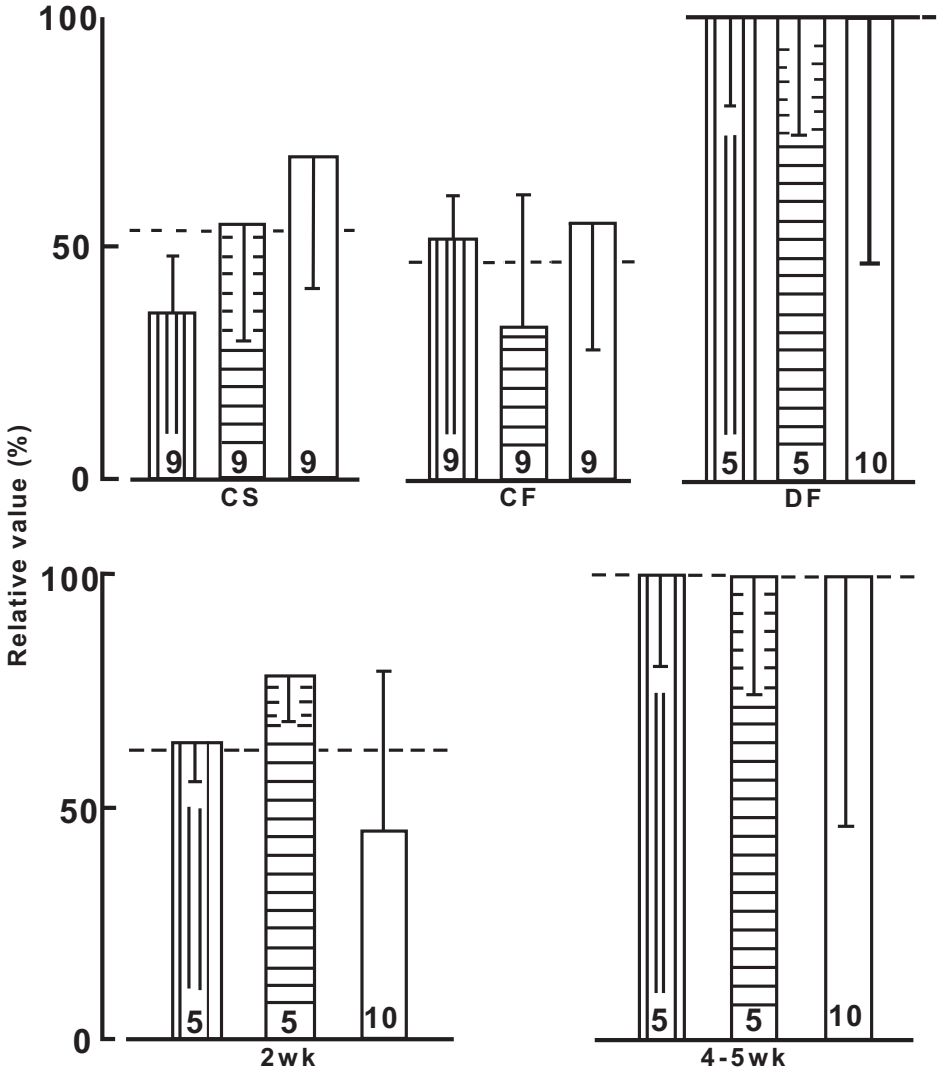

Fig. (1). Indirect estimation of food intake in CS, CF and DF rats after the switch in diet. Upper panel: comparison between CS (left), CF (middle) and DF (right) rats in terms of the enrichment of liver triglycerides in C18:3ω3 relative to its content in the diet (vertically hatched columns), C22:6ω3 (horizontally hatched columns) and total fatty acids (open columns) 4-5 weeks after the switch in diet. The results for C18:3ω3 and total fatty acids are expressed relative to the mean corresponding values found in DF rats. Since the diet eventually offered to the DF rats contains no C22:6ω3, the experimental values for this fatty acid recorded in the CS and CF rats were recalculated to yield an overall mean value identical to that found for C18:3ω3. Lower panel: comparison between the enrichment of liver triglycerides observed after either 2 weeks or 4-5 weeks exposure of the DF rats to the flaxseed oil-enriched diet, as judged from the increments in C18:3ω3 (vertically hatched columns), C22:6ω3 (horizontally hatched columns) and total fatty acids (open columns), the mean results collected after 4-5 weeks being taken as the 100% reference value. In both panels, mean values (± SEM) refer to the df shown at the bottom of each column, the horizontal dashed lines indicating the overall mean values derived from the 3 variables under consideration.

wet wt. (df = 18) in CS and CF rats and 3.05 ± 1.39 mg/g wet wt. (df = 20) in DF rats. As illustrated in Fig. (**1**) (upper panel), when pooling together the results relative to the indirect estimation of food intake and derived from 3 independent variables (C18:3ω3, C22:6ω3 and total fatty acid content of liver triglycerides), the results recorded in the CS and CF rats 4-5 weeks after the switch in diet averaged respectively 53.5 ± 13.2% and 47.0 ± 13.3% (df = 27 in both cases) of those recorded at the same time in DF rats (100.0 ± 19.3%; df = 20). Thus, the overall mean value found in the CS and CF rats (50.3 ± 9.3%; df = 54) was significantly lower ($p < 0.03$) than that recorded in the DF rats.

It should be stressed that, in the DF rats examined in our prior experiments, the individual values for the liver triglyceride C22:6ω3/C18:3ω3 ratio did not differ significantly ($p > 0.3$) after 2 weeks (0.150 ± 0.015; n = 6) or 4-5 weeks (0.125 ± 0.023; n = 6) exposure to the flaxseed oil-enriched diet. These mean values also failed to differ significantly ($p > 0.4$ or more) from that recorded in the present experiments (0.131 ± 0.020; n = 6), with an overall mean value of 0.135 ± 0.011 (n = 18). In other words, as judged from the C22:6ω3/(C18:3ω3 + C22:6ω3) ratio in liver triglycerides or DF rats, the fractional conversion of C18:3ω3 to C22:6ω3 averaged, in these two series of experiments 11.8 ± 0.9% (n = 18).

Our findings are also relevant to the modality of brain phospholipid enrichment in long-chain polyunsaturated ω3 fatty acids in the DF rats [6]. In a recent series of articles, Igarashi *et al.* proposed that C22:6ω3 generated in the liver from circulating C18:3ω3 is the source of brain C22:6ω3 when the latter fatty acid is absent from the diet [11-13]. With this information in mind, the present findings strongly suggest that the enhancement of brain phospholipids in long-chain polyunsaturated ω3 fatty acids taking place in the DF rats after the switch in diet is linked to the export from the liver of ω3-enriched phospholipids, e.g. by lipoprotein secretion, rather than triglycerides.

Some differences between the changes in fatty acid pattern of liver and brain phospholipids, as recorded after the switch in diet, should not be ignored, however. First, no C18:3ω3 was detected in brain phospholipids, even in the CF or DF rats. Second, in the CF rats, only the C22:5ω3, but not C22:6ω3, relative weight content was increased in the brain phospholipids after the switch in diet. Third, in the DF rats, a further increase in the brain phospholipid relative weight content of both C22:5ω3 and C22:6ω3 was recorded when the length of the period of exposure to the flaxseed oil-enriched diet was increased from 2 to 4-5 weeks, whilst such was not the case in liver phospholipids [1, 6].

A modest contribution of circulating triacylglycerols, as distinct from phospholipids, to the supply of long-chain polyunsaturated ω3 fatty acids to the brain should not be ignored. For instance, within 60 to 120 min after the bolus intravenous injection of a medium-chain triglycerides fish:oil emulsion to second-generation ω3-depleted rats, the absolute value for the brain phospholipid content in C22:5ω3 averages 15.3 ± 1.7 μg/g liver wet weight (n = 29), as distinct ($p < 0.05$) from only 10.4 ± 1.6 μg/g liver wet weight (n = 48) in the second-generation ω3-depleted rats either not injected with any lipid emulsion or injected with a medium-chain triglyceride:olive oil control emulsion [14]. Such a proposal is also compatible with the finding that, in the CF, CS and DF rats examined after the switch in diet, the C22:5ω3 relative weight content of brain phospholipids tightly correlates ($r = + 0.691$; n = 24; $p < 0.001$) with the C18:3ω3 relative weight content of liver triglycerides, the paired ratio between the former and latter variable being much lower ($p < 0.001$), however, in the CF rats (2.74 ± 0.18 %; n = 6) or DF rats (3.46 ± 0.29 %; n = 12) than that found in the CS rats (7.49 ± 0.33 %; n = 6). As a matter of fact, even in the control and ω3-depleted rats examined before the switch in diet, the C22:5ω3 relative weight content of brain phospholipids also displayed a significant positive correlation ($r = + 0.864$; n = 23; $p < 0.001$) with the C18:3ω3 relative weight content of liver triglycerides, but with a slope for the regression line (0.0700 ± 0.0089) about five times higher ($p < 0.001$) than that recorded after the switch in diet (0.0147 ± 0.0033). The overall pattern of such a relationship thus suggested an exponential rule [$y = S(1 - e^{kx})$], tending towards saturation (S) (Fig. **2**). Taken as a whole, these findings indeed support the view that the long-chain polyunsaturated ω3 fatty acids found in brain phospholipids, i.e. C22:5ω3 and C22:6ω3, may, to a limited extent at least, be generated in brain cells from C18:3ω3 provided by circulating triglycerides and/or phospholipids.

In conclusion, the present study affords three major pieces of information. First, whilst not ignoring the role of the increase in the lipid content of the diet, it strongly suggests that an increase in food intake, twice higher in DF rats than in either CS or CF rats, participates in the undesirable aggravation of liver steatosis and visceral obesity found when ω3-deprived rats first exposed for 7 months to a diet containing 5% (w/w) sunflower oil are then exposed to the same diet enriched with another 5% flaxseed oil. It also

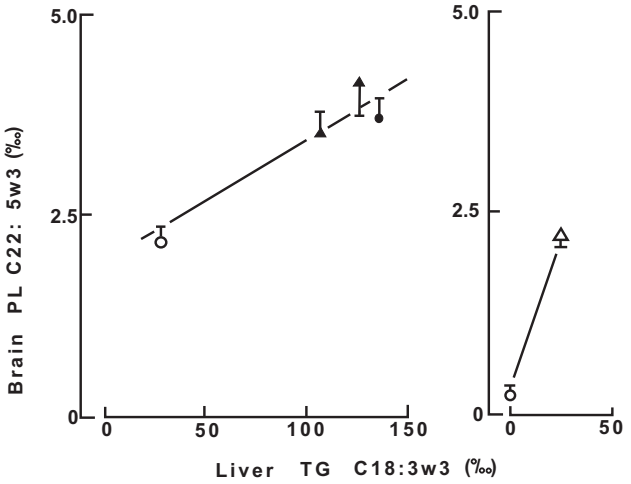

Fig. (2). Left: relationship between the liver triglyceride C18:3ω3 relative weight content and brain phospholipid C22:5ω3 relative weight content in CS rats (open circle), CF rats (closed triangle), and DF rats exposed for either 2 or 4-5 weeks to the flaxseed oil-enriched diet (closed triangles). Right: same relationship in control (open triangle) and ω3-depleted (open circle) rats examined before the switch in diet. Mean values (± SEM) refer to 6-12 rats [1, 6].

documents that this postulated increase in food intake is about 3 times higher during the first 2 weeks of exposure to the flaxseed oil-enriched diet than over the ensuing 2-3 weeks. Second, it indicates that, during exposure to the latter diet, C18:3ω3 provided by a diet deprived of C22:6ω3 is converted in the liver to C22:6ω3 with a fractional conversion extent, as judged from the liver triglyceride data, close to 11 %. Last, whilst not excluding a modest role of circulating triglycerides, it supports the view that, in ω3-depleted rats deprived of an exogenous supply of C22:6ω3, its generation in the liver from circulating C18:3ω3 and the export from the liver of C22:6ω3-enriched phospholipids represent the main source of brain C22:6ω3.

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