Fasting-induced remodelling of hepatic triacylglycerols

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Rat liver triacylglycerol species are known to respond differentially to metabolic and nutritional manipulation but changes in triacylglycerols containing 20–22 carbon polyunsaturates have been difficult to determine. Liver total triacylglycerols were separated by high performance liquid chromatography into subclasses to determine whether their remodelling by fasting induced changes consistent with reacylation causing enrichment by long-chain polyunsaturated fatty acids. Triacylglycerol subclasses and their fatty acid composition were quantitatively determined. The fasting-induced changes in liver triacylglycerols were characterized by the appearance of new subclasses containing 20–22 carbon polyunsaturates at 18–30% but relative and quantitative depletion of more saturated subclasses. Previous studies have not been able to identify whether changes in the polyunsaturated fatty acid content of triacylglycerols during fasting were mainly a function of depletion of saturates and monounsaturates or whether extensive reacylation of subclasses/species specifically enriched in polyunsaturated fatty acids also occurred; our present data suggest that the latter is a significant component of the fasting-induced triacylglycerol remodelling process.

Keywords: fasting; triacylglycerols; HPLC; liver

Introduction

It has previously been shown that fasting causes changes in the fatty acid composition of hepatic and plasma triacylglycerols (TGs) involving proportional and quantitative accumulation of arachidonic (20:4n-6), docosahexaenoic (22:6n-3), and linoleic (18:2n-6) acids, but depletion of palmitic (16:0), palmitoleic (16:1n-7), and oleic acids (18:1n-9).[1–4] We previously used silver-nitrate thin layer chromatography[5] and high temperature gas liquid chromatography (GLC)[6,7] to characterize changes in individual TGs induced by partial energy restriction or complete 24–48 hr fasting. Based on these analytical methods, we suggested that fasting remodels hepatic TGs by increasing the 18:2n-6 content of linoleoyl-enriched hepatic TG species (two or three 18:2n-6 moieties) while simultaneously decreasing monolinoleoyl and nonlinoleoyl TG species.[6,7]

20:4n-6 and 22:6n-3 also increase in total hepatic TG during fasting, but TG species containing these and other highly unsaturated long-chain fatty acids cannot be recovered using GLC due to the degradation of highly unsaturated fatty acids at the elevated temperatures required to separate intact TGs. We have shown that hydrogenation increases the thermal stability of hepatic TG species enriched with polyunsaturated fatty acids, but it also prevents the full characterization of the original TG species.[6,7] High performance liquid chromatography (HPLC) has been used to analyze vegetable[8] and marine fish oil[9] TG structure but we are unaware of its use for the separation of animal organ TGs, especially after metabolic or nutritional manipulation. We describe here its application to the nondestructive monitoring of changes in hepatic TGs using the fasted rat model. This analytical approach provides more complete information on fasting-induced changes in hepatic TG subclasses containing 20–22 carbon polyunsaturates and their response to fasting.

Methods and materials

Animals and diet

Male Sprague-Dawley rats (240–250 g) were obtained from Charles River Canada (St. Constant, Quebec, Canada) and were randomly divided into three groups that were housed in the animal care facilities of the Division of Comparative

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Medicine, University of Toronto, Toronto, Canada. The rats were given a rodent chow diet (Ralston-Purina, St. Louis, MO USA) and tap water ad libitum for 7 days. One group continued to feed ad libitum (controls) while the other two groups were fasted for 24 hr or 48 hr. All rats were sacrificed under carbon dioxide anesthesia. The abdomen was then opened and the liver removed, washed with 0.9% saline, frozen at −20° C, and the total lipids extracted within 24 hr.

Thin layer chromatography

All solvents were obtained in bulk from Fisher Scientific (Toronto, Ontario, Canada) and glass-redistilled prior to use. Total liver lipids were extracted using chloroform : methanol (2:1, vol/vol) containing 0.02% butylated hydroxytoluene (Sigma Chemical Co., St. Louis, MO USA) as antioxidant. Lipid classes were separated by neutral lipid thin layer chromatography (20 × 20 cm plates precoated with 250 μm silica gel 60A, Chromatographic Specialties, Brockville, Ontario, Canada) using a developing solvent system of hexane : diethyl ether : acetic acid (80:20:1, vol/vol/vol). The silica band containing the total TGs was quantitatively recovered from the plates as previously described.[6] One aliquot of the hepatic TG mixture was converted to the corresponding methyl esters using 14% boron trifluoride in methanol (Sigma) under nitrogen gas[3] for determination of fatty acid composition. Another TG aliquot was redissolved in 500 μL chloroform for further separation by HPLC.

High performance liquid chromatography

The hepatic TG samples were separated using a Hewlett-Packard 1050 HPLC (Palo Alto, CA USA) equipped with a quaternary pump solvent delivery system and two reverse phase C-18 columns in series (Zorbax ODS, 250 × 4.6 mm, 5 μm, Mandel Scientific Co., Guelph, Ontario, Canada). Liver TG mixtures (10–25 μg) were injected onto the lead column via the Rheodyne valve (15 μL capacity). A gradient of acetonitrile/chloroform was used at a flow rate of 0.8 mL/min (70:30 changing to 50:50 over 25 min, held for 10 min and then back to 70:30 over 10 min). A stream splitter separated the column eluent so that 45% went to the detector and 55% went to a fraction collector (Gilson FC 203, Mandel Scientific Co., Guelph, Ontario, Canada). The underivitized separated TG subclasses were monitored using an evaporative light scattering detector (Varex, model IIA, Burtonsville MD USA) and a Hewlett Packard 3396 Series II integrator. The detector settings were: nitrogen gas flow, 45 mL/min; tube temperature, 73.2° C; exhaust tube temperature, 46.5° C. Fatty acids in each TG subclass were converted to methyl esters using 14% boron trifluoride in methanol for determination of fatty acid composition by GLC.

Quantitative analysis of triacylglycerols by HPLC

The response of the mass detector was not linear but proportional to the concentration of TG.[10,11] For quantitation various concentrations of a standardized TG species mixture were injected under exactly the same operating conditions as liver TG samples *(Figure 1)*. The seven individual TG species (Sigma) exhibited a linear logarithmic relation between the amount of TG injected and the response of the detector *(Figure 2)*, with a power ranging from 1.86–1.96 (overall power of 1.90). The percentage of each peak in the liver TG sampled was determined according to equation 1:[12]

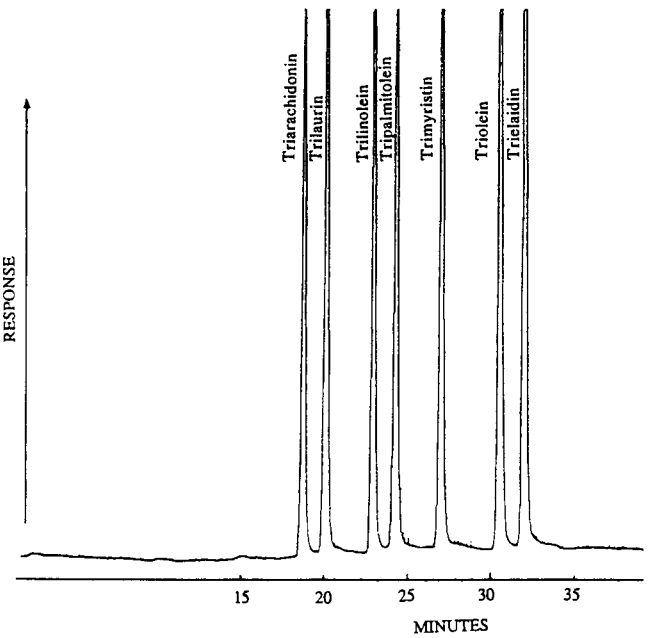

Figure 1 Separation of standard triacylglycerols by reversed-phase HPLC. Columns: 25 cm long, 4.6 mm i.d. Zorbax ODS 5 μm. Constant flow rate: 0.8 mL/min. Gradient elution, chloroform in acetonitrile. Starting conditions: 30:70, at 25 min: 50:50 then held for 10 min. At 45 min: 30/70. Detector conditions: nitrogen gas flow rate = 45 mm/min, drift tube temperature = 73.2° C.

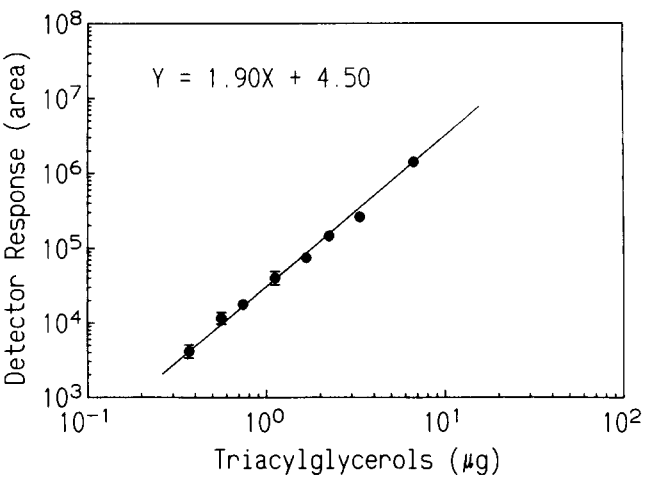

Figure 2 Log/log plot of mass detector response against amount of standard triacylglycerols (mean ± SD of seven standard triacylglycerols, see *Figure 1*).

$$\%C_i = \frac{(A_i)^{\frac{1}{1.90}}}{\sum_{i=1}^{n}(A_i)^{\frac{1}{1.90}}} \times 100 \qquad (1)$$

where C represents the concentration, A represents area of peak i, and n is the total number of peaks using Inplot GraphPad Version 3.1 (GraphPad Software, San Diego, CA USA). The term "subclass" is to designate a small cluster of TG species that reproducibly co-chromatograph as a single peak by HPLC, as verified

by very similar fatty acid composition after multiple reinjection of the same HPLC fraction.

Gas liquid chromatography

Fatty acid methyl esters of the separated TG subclasses were analyzed using a capillary column (Durabond 225, 30 m × 0.25 mm, I.D.) coated with 25 μm cyanopropylphenyl (J & W Scientific, Folson, CA USA) in a Hewlett-Packard 5890A GLC with automated sample delivery and injection (Hewlett-Packard 7671A) and peak integration (Hewlett-Packard 3393 integrator). The column temperature was programmed from 150° C–220° C in three stages with each run complete in 30 minutes.[6,7]

Statistics

Analysis of variance followed by Student's *t* test (two-tailed) was used for statistical evaluation of differences between groups.

Results

Fasting for 24 hr or 48 hr significantly decreased body weights by 5% and 11% and liver weight by 27% and 34%, respectively, as previously reported.[7] Although liver TG concentration was not significantly changed by 24 or 48 hr fasting (6.1 and 4.7 mg/g liver, respectively versus 5.2 mg/g liver in fed controls), total TG content decreased by 40% after 48 hr fasting (55.6–33.4 mg). Fasting caused characteristic changes in the fatty acid profile of total hepatic TGs including a proportional increase in stearic acid (18:0), 18:2n-6, 20:4n-6, and alpha-linolenic acid (18:3n-3) but a decrease in 16:0, 16:1n-7, and 18:1n-9 *(Figure 3)*. 18:2n-6 and 20:4n-6 increased the most, while 18:1n-9 decreased the most.

Reverse-phase HPLC resulted in 16 peaks (TG subclasses) being clearly and reproducibly resolved in the total hepatic TG samples of the fasted groups but only 12 TG subclasses in the fed controls *(Figure 4)*. Quantitative changes in each TG subclass have been calculated

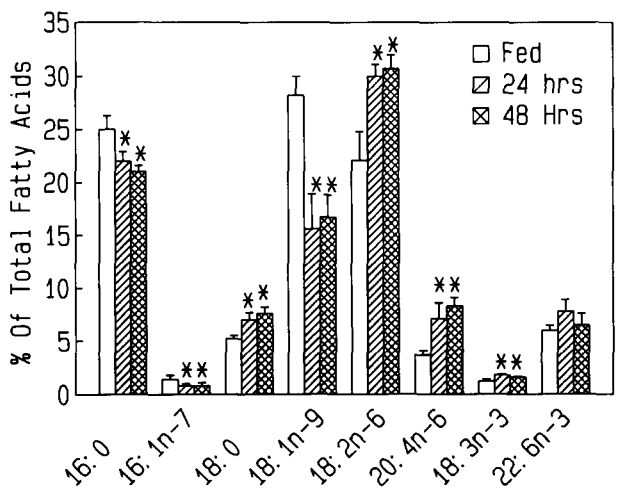

Figure 3 Percent change of individual fatty acids in hepatic TG induced by fasting for 24 or 48 hr fasting. Values are the mean ± SD (n = 5/group). $^*P < 0.05$; $^{**}P < 0.01$ versus fed controls.

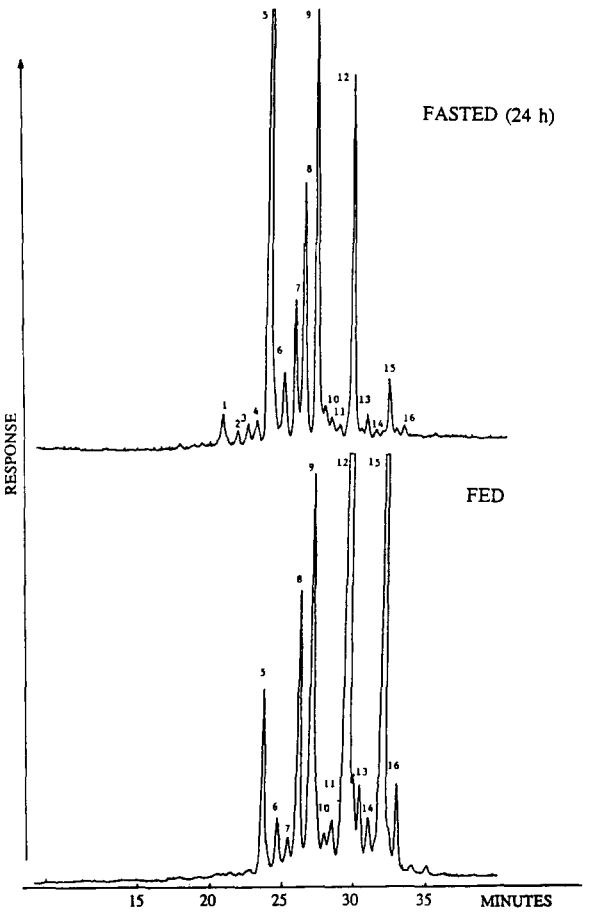

Figure 4 HPLC separation of liver triacylglycerols. See *Figure 1* for conditions and *Table 1* for percent composition.

according to the logarithm standard curve. Several distinct fasting-induced changes were observed *(Figure 4; Table 1)*: (1) Subclasses 1–4 were not detected in the fed group but, in total, accounted for 9–10% of the total TG in the fasted groups; (2) Subclasses 5, 7, and 9 increased significantly; (3) Subclasses 12, 15, and 16 were decreased markedly after fasting; (4) An additional 24 hr fasting (total 48 hr) did not cause much additional change in the proportion of each hepatic TG subclass *(Table 1)*. To simplify the presentation of the fatty acid data for the TG subclasses, comparison was made only between ad libitum-fed controls and the group fasted for 24 hr *(Table 2)*.

In some cases, the fatty acid profiles of the TG subclasses changed dramatically without a change in the proportion of the TG subclass itself, e.g., the tripling of 20:4n-6 in subclass 8 *(Table 2)*. Conversely, the proportion of subclass 12 decreased 35% with fasting, but its fatty acid profile was relatively unchanged. TG subclasses 1–4 were present only after fasting and had a content of 20–22 carbon polyunsaturated fatty acids of 18–30%. The fatty acid profile of TG subclass 5 changed quantitatively and qualitatively in rats fasted for 24 hr, particularly with respect to 18:2n-6, which increased from 20–35% after 24 hr fasting. The fasting-induced changes in TG subclasses 6–8 were generally character-

Table 1 Percent composition (%) of hepatic triacylglycerol subclasses isolated by HPLC in the rats fasted for 24 or 48 hr compared with ad libitum-fed controls.

Peak†	Fed	Fasted 24 hr	Fasted 48 hr
1	—	2.78 ± 0.64	3.41 ± 0.51
2	—	2.10 ± 0.29	1.37 ± 0.37
3	—	1.99 ± 0.83	2.60 ± 0.23
4	—	2.98 ± 0.95	2.82 ± 0.27
5	10.15 ± 1.92	18.22 ± 2.20*	15.63 ± 1.09*
6	5.54 ± 0.99	5.91 ± 0.52	3.89 ± 1.00
7	3.76 ± 0.62	7.32 ± 1.95*	8.08 ± 0.91*
8	12.11 ± 0.57	11.10 ± 0.50	12.23 ± 1.06
9	15.73 ± 0.90	19.79 ± 2.88*	19.15 ± 1.15*
10	2.32 ± 0.38	1.94 ± 0.55	3.98 ± 1.40
11	3.17 ± 0.90	2.31 ± 0.66	3.28 ± 1.02
12	23.45 ± 1.76	15.95 ± 3.05*	14.60 ± 0.90*
13	3.84 ± 1.11	3.21 ± 0.53	3.51 ± 0.73
14	2.85 ± 0.68	0.92 ± 0.23	1.07 ± 0.30
15	13.22 ± 1.85	3.64 ± 0.90*	3.01 ± 0.57*
16	3.23 ± 0.95	1.60 ± 0.27*	1.64 ± 0.47*

*$P < 0.01$, in contrast to fed controls.
†Peak number corresponds to that in *Figure 4*.
Values are the mean ± SD (n = 5/group).

ized by decreasing 16:0, 16:1n-7, 18:0, and 22:6n-3 (the latter in subclass 8 only) but increasing 18:2n-6 and 20:4n-6. TG subclass 15 was >80% saturated and monounsaturated and was >70% depleted after fasting with a tripling of the percent of 18:2n-6 but nearly a halving of 18:1n-9 *(Table 2)*.

Quantitation of the hepatic TG subclasses showed that each fatty acid was characteristically concentrated in 2–4 subclasses, and it was from these subclasses that the main fatty acids were especially depleted during fasting, e.g., 16:1n-7 and 18:1n-9 from subclasses 12 and 15, or were concentrated by fasting, e.g., 18:2n-6, 20:4n-6, and 22:6n-3 in subclasses 5, 8, and 9. Quantitative increase in 18:0 were mostly restricted to subclasses 1–4 *(Figure 5)*.

Discussion

The present results support previous observations that 18:2n-6 and 20:4n-6 in total liver TGs are proportionally increased, but 18:1n-9 in particular is depleted by short-term fasting, an effect that can occur without necessarily significantly changing the concentration of TGs[1–4] in the liver. While it has been previously speculated that hepatic TG species or subclasses enriched with 20–22

Table 2 Fatty acid composition (% of total) of triacylglycerol subclasses isolated by HPLC in the rat liver

	Peak 1*		Peak 2		Peak 3		Peak 4		Peak 5		Peak 6		Peak 7		Peak 8	
	Fed	24h†	Fed	24h	Fed	24h	Fed	24h	Fed	24h	Fed	24h	Fed	24h	Fed	24h
14:0	—	2.1	—	2.3	—	2.6	—	2.0	0.6	1.0	1.1	1.5	2.8	1.0	1.8	1.0
16:0	—	17.1	—	22.2	—	20.8	—	21.2	20.7	18.3	29.7	22.7	27.0	23.4	21.1	15.4
16:1n-7	—	1.7	—	2.3	—	2.0	—	1.8	0.5	0.8	2.5	0.4	1.7	1.3	1.3	0.2
18:0	—	8.7	—	12.6	—	11.4	—	9.4	5.8	3.7	11.4	7.7	9.1	7.2	6.2	7.7
18:1n-9	—	11.5	—	9.7	—	11.9	—	20.1	9.7	6.4	11.0	10.7	15.1	16.9	25.4	20.1
18:2n-6	—	26.8	—	20.1	—	18.1	—	25.7	20.2	35.0	17.9	23.5	16.1	20.4	16.6	21.1
20:3n-6	—	1.0	—	—	—	1.0	—	—	5.1	1.3	—	0.7	—	—	—	0.5
20:4n-6	—	6.5	—	6.5	—	10.4	—	4.6	6.2	7.0	7.9	9.6	19.5	19.1	6.7	18.2
18:3n-3	—	3.5	—	6.8	—	8.5	—	1.5	5.2	1.7	3.5	8.1	—	2.0	—	2.2
22:5n-3	—	2.5	—	6.0	—	1.6	—	—	5.0	1.8	9.6	7.5	2.9	1.8	1.6	0.9
22:6n-3	—	16.9	—	9.8	—	8.2	—	12.1	20.6	22.2	5.2	5.6	4.9	4.4	19.2	10.8

*Peak number corresponds to that in *Figure 4* (data normalized to 100%).
†24 h, 24-hour fasted.

Table 2 *Continued*

Peak 9		Peak 10		Peak 11		Peak 12		Peak 13		Peak 14		Peak 15		Peak 16	
Fed	24h	Fed	24h	Fed	24h	Fed	24h	Fed	24h	Fed	24h	Fed	24h	Fed	24h
0.7	0.6	1.9	3.1	3.0	1.5	0.9	1.0	1.8	1.3	4.0	3.2	1.3	4.6	3.2	4.6
25.3	26.0	36.0	26.6	28.0	26.9	26.2	25.3	36.8	31.8	31.7	30.1	26.9	34.2	40.6	40.9
1.9	0.8	2.3	0.1	2.9	0.3	1.1	0.4	1.9	1.0	0.9	0.6	1.2	0.8	1.6	1.9
4.5	4.8	13.8	17.7	9.4	5.7	3.4	7.7	10.1	14.1	11.0	14.5	5.3	13.7	11.0	18.8
15.0	8.2	18.3	18.3	32.3	29.7	37.6	29.3	26.6	24.5	37.1	35.1	59.0	31.4	35.4	21.6
37.2	47.6	16.6	21.3	20.4	30.8	29.9	31.7	20.4	20.7	11.9	15.3	6.3	18.0	7.6	9.4
—	1.9	—	1.2	—	—	—	0.9	—	—	—	—	—	—	—	—
4.0	3.5	9.2	7.2	2.8	1.8	0.5	1.8	—	3.7	—	—	—	—	—	—
2.2	1.7	—	—	—	—	0.2	—	—	—	—	—	—	—	—	—
6.1	1.5	—	1.0	—	1.2	—	0.3	—	—	—	—	—	—	—	—
3.5	3.0	0.9	1.4	—	1.2	0.3	0.8	—	—	—	—	—	—	—	—

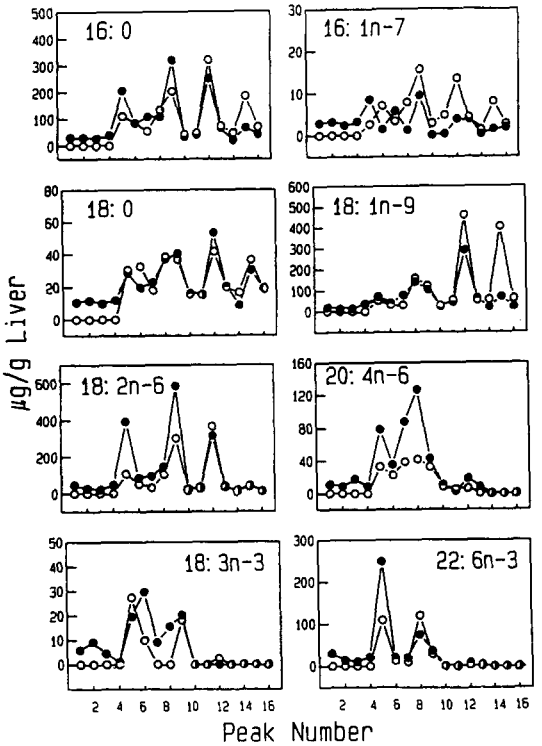

Figure 5 Quantitative changes in composition of main fatty acids of hepatic triacylglycerol subclasses separated by reversed-phase HPLC. Open circle (ad libitum-fed). Filled circle (fasted for 24 hr). Peak numbers correspond to those in *Figure 4*.

carbon polyunsaturated fatty acids were probably quantitatively increased by fasting,[6,7] we believe this is the first report in which nondestructive separation of hepatic TG subclasses using HPLC and mass detection clearly shows the selective retention and/or reacylation of TGs enriched in 20–22 carbon polyunsaturated fatty acids as a result of fasting.

Using this methodology, it seems clear that fasting differentially remodels hepatic TGs, e.g., some TG subclasses are reacylated and enriched with polyunsaturated fatty acids (peak 1–4), while others are selectively depleted (peaks 12 and 15), without necessarily changing total hepatic TG concentration. In the subclasses in which individual fatty acids changed without the subclass changing in amount, e.g., 20:4n-6 in subclass 8, this seems to indicate highly selective reacylation. Conversely, the change in subclass proportion without much change in fatty acid composition, e.g., subclass 12, suggests its specific utilization as a fatty acid source during fasting.

Our present results confirm the hypothesis that TG species containing 20–22 carbon polyunsaturates are quantitatively increased by fasting.[6,7] Furthermore, our present data show that, even among the subclasses enriched with 20–22 carbon polyunsaturated fatty acids, fasting differentially remodels TGs by selective reacylation, e.g., the 2–3-fold increase in 20:4n-6 but 44% reduction in 22:6n-3 in subclass 8, which occurred without changing the overall amount of the TG subclass involved. Despite the improvement in recovery and separation of TGs provided by reverse phase HPLC and mass detection, it is still difficult to make better than approximate TG species assignments. Nevertheless, it seems clear that the hepatic TG subclasses/species that are reacylated or preferentially retained during fasting are unusual in containing at least 40% 18:2n-6, 20:4n-6, and 22:6n-3. The location and function of such highly unsaturated TGs is of considerable interest, especially as they become more prominent in response to nutritional manipulation.

Acknowledgments

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