Effect of \( n \)-3 fatty acids on metabolism of apoB100-containing lipoprotein in type 2 diabetic subjects

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(Received 16 November 2005 – Revised 9 March 2006 – Accepted 14 March 2006)

The effect of long-chain \( n \)-3 PUFAs on the metabolism of apoB100-containing lipoprotein in diabetic subjects is not fully understood. The objective of the present study was to determine the effect of a daily intake of 1080 mg EPA and 720 mg DHA for diabetic subjects on the kinetics of apoB100-containing lipoprotein in the fasting state. A kinetic study was undertaken to determine the mechanisms involved in the effects of \( n \)-3 fatty acids in terms of a decrease in triacylglycerol level in type 2 diabetic patients. We have studied the effect of fish oils on the metabolism of apoB100 endogenously labelled by \([5,5,5-2H_3]\)-leucine in type 2 diabetic patients in the fasting state. The kinetic parameters of apoB100 in VLDL, intermediate-density lipoprotein and LDL were determined by compartmental modelling in five diabetic subjects before and 8 weeks after \( n \)-3 fatty acid treatment. Treatment did not change the plasma cholesterol level (0.801 (SD 0.120) v. 0.793 (SD 0.163) mmol/l) but lowered the plasma triacylglycerol level (1.776 (SD 0.280) v.1.356 (SD 0.595) mmol/l; \( P < 0.05 \)). Treated patients showed a decrease in VLDL apoB100 concentration (0.366 (SD 0.030) v.0.174 (SD 0.036) g/l; \( P < 0.05 \)) related to a decrease in VLDL 1 production (1.49 (SD 0.23) v.0.44 (SD 0.19) mg/kg per h; \( P < 0.05 \)) and an increase in the VLDL conversion rate (0.031 (SD 0.024) v.0.052 (SD 0.040) per h; \( P < 0.05 \)), with no change in fractional catabolic rates. Treatment led to a higher direct production of intermediate-density lipoprotein (0.02 (SD 0.052) mg/kg per h; \( P < 0.05 \)). In conclusion, the present study, conducted in the fasting state, showed that supplementation with \( n \)-3 fatty acids in type 2 diabetic patients induced beneficial changes in the metabolism of apoB100-containing lipoprotein.

\( n \)-3 fatty acids: apoB100: Kinetic analysis: Modelling: Diabetes

The relationship between increased plasma cholesterol concentration and the development of atherosclerosis has been demonstrated in many studies (Kannel et al. 1971). Although the role of hypertriacylglycerolaemia has been controversial, it is now accepted as a risk factor for CHD (Zilversmit, 1979; Gianturco et al. 1980), leading to their rapid conversion into foam cells. Hypertriacylglycerolaemia is frequently associated with type 2 diabetes and could contribute to the strong development of atherosclerosis in this disease (Goldberg, 2001). Previous studies have already underlined that the insulin resistance observed in type 2 diabetes is associated with an overproduction of apoB100-containing lipoproteins and reduced LDL and VLDL catabolism (Duvillard et al. 2000; Ouguerram et al. 2003).

Epidemiological studies in Inuits have suggested that marine oils rich in \( n \)-3 fatty acids are hypolipidaemic and anti-atherogenic (Kromann & Green, 1980). In addition to their anti-inflammatory, vasodilatory and antihypertensive effects (Lichtenstein et al. 1998), \( n \)-3 fatty acids are able to decrease triacylglycerol levels in animals (Harris, 1996) and human subjects (Connor et al. 1993; Harris, 1996). The effect on LDL cholesterol is more controversial depending on the dose used, the duration of treatment and the disease (Farmer et al. 2001).

Numerous \textit{in vivo} kinetic studies in healthy human subjects (Connor et al. 1993) and animals (Harris, 1996) have shown that this effect is associated with a decrease in VLDL apoB100 concentration related to a reduction in production. As in normolipidaemic subjects, the \( n \)-3 fatty acids in diabetic patients lead to a lower plasma triacylglycerol level, but the underlying mechanisms are not fully understood.

A study involving five type 2 diabetic patients was performed by Fisher et al. (1998) on apoB100-containing lipoproteins and reported that the response to \( n \)-3 fatty acids was mainly a decrease in VLDL apoB100 secretion and an increase in intermediate-density lipoprotein (IDL) and LDL production. In that study, the increase in LDL production with no change of fractional catabolic rate led to an increase in LDL mass. However,

Abbreviations: IDL, intermediate-density lipoprotein; LPL, lipoprotein lipase.

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the study was conducted in feeding subjects, which implies a possible acute effect of n-3-rich chylomicrons and their remnants on apoB100 metabolism (Zheng et al. 2001; Maldonado et al. 2003). Moreover, in that study (Fisher et al. 1998), the authors only compared the effect of n-3 to n-6 fatty acids, two acids known to influence lipid homeostasis (Mekki et al. 2002; Zheng et al. 2002). Such a comparison could mask some specific effects of n-3 fatty acids.

The purpose of the present study was to investigate the effect of fish oils on the metabolism of apoB100 in type 2 diabetic patients. This study was conducted in the fasting state to avoid the acute influence of chylomicrons and their remnants on apoB100 metabolism.

Methods

Subjects

Five patients with type 2 diabetes and dyslipidaemia (Table 1) were included in the study. Insulin treatment was an exclusion criterion. They had not been taking any medication that could affect carbohydrate or lipid metabolism for at least 1 month before the study. Patients had been treated with either sulphfonylureas or biguanides for at least 3 months and were instructed by a dietitian to follow a weight-maintenance diet, (composed of (% energy): carbohydrate 45; fat 35; protein 20) for at least 1 week prior to the study. Insulin resistance was assessed by the insulin sensitivity index (homeostasis model assessment; Matthews et al. 1985). The experimental protocol was approved by the ethical committee of Nantes University Hospital, and written informed consent was obtained before the study was started.

Experimental protocol

Five diabetic patients underwent a basal kinetic study the day before the start of the treatment. They then received six capsules/d MaxEPA (Pierre Fabre Santé, Castres, France), a concentrate of n-3, long-chain PUFA from fish oil, over 8 weeks. Each MaxEPA capsule contained 1000 mg methyl ester fatty acids, providing 180 mg EPA (20:5-n3), 120 mg DHA (22:6-n3) and 1·75 mg α-tocopherol acetate. The second kinetic study was carried out at the end of the treatment.

The kinetic protocol was similar to that previously described (Ouguerram et al. 2003). Briefly, the endogenous labelling of apoB100 was carried out by constant infusion of [5,5,5-2H3]-leucine in subjects fasted overnight for 12 h prior to the study who remained fasting during the entire procedure. Each subject received intravenously a prime of 10 μmol/kg tracer immediately followed by a constant tracer infusion (10 μmol/kg per h) for 14 h. Venous blood samples were drawn into EDTA tubes (Venoject, Paris, France) at baseline and at 15, 30 and 45 min, 1, 1·5, 2 and 2·5 h, and then hourly until 14 h. Sodium azide, an inhibitor of bacterial growth, and Pefabloc SC (Interchim, Montlucçon, France), a protease inhibitor, were added to blood samples at a final concentration of 1·5 and 0·5 mmol/l, respectively.

Analytical procedures

Isolation and measurement of enrichment of lipoprotein containing apoB100. Isolation of lipoproteins and measurement of leucine enrichment in apoB100 have previously been described (Ouguerram et al. 2003). Briefly, lipoproteins were separated by density-gradient ultracentrifugation, and apoB100 was isolated by sodium dodecylsulfate polyacrylamide gel electrophoresis. Apo bands were dried under a vacuum and then hydrolysed. The amino acids were purified by cation-exchange chromatography and then esterified and derivatised. Electron-impact GC–MS was performed on a 5891 A gas chromatograph connected with a 5971 A quadrupole mass spectrometer (Hewlett Packard Co., Palo Alto, CA, USA). The isotopic ratio was determined by selected ion-monitoring at m/z of 282 and 285. Calculations of apoB100 kinetic parameters were based on the tracer:tracee mass ratio (Cobelli et al. 1992).

Table 1. Clinical characteristics of type 2 diabetic patients before (base) and after (post) supplementation with MaxEPA (Pierre Fabre Santé, Castres, France)

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (years)</th>
<th>BMI (kg/m²)</th>
<th>HbA1c (%)</th>
<th>Insulin MU/l</th>
<th>Glucose (mmol/l)</th>
<th>Homeostasis model assessment</th>
<th>Plasma cholesterol (mmol/l)</th>
<th>Plasma triacylglycerol (mmol/l)</th>
</tr>
</thead>
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<td>1·3</td>
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<td>22·0</td>
<td>7·1</td>
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<td>15·3</td>
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<td>8·9</td>
<td>0·662</td>
</tr>
<tr>
<td>6 Post</td>
<td>32·9</td>
<td>29·0</td>
<td>11·1</td>
<td>15·1</td>
<td>15·3</td>
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<td>0·662</td>
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<td>18·3</td>
<td>9·5</td>
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<tr>
<td>Mean post</td>
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<td>2·6</td>
<td>6·3</td>
<td>2·5</td>
<td>1·4</td>
<td>1·4</td>
<td>0·120</td>
</tr>
</tbody>
</table>

ND, not determined.

* P<0·05, post v. base.

For details of subjects and procedures, see this page.
Measurements of lipids and apoB100. Cholesterol and triacylglycerol concentrations were measured using commercially available enzymatic kits (Boehringer Mannheim GmbH, Mannheim, Germany) at three different sampling times. ApoB100 concentrations were obtained in lipoprotein fractions by combining selective precipitation and MS (Beghin et al. 2000). The percentage recovery of cholesterol, triacylglycerol and apoB100 after centrifugation was higher than 85.

Modelling. Kinetic analysis of tracer:tracee ratios was achieved using computer software for simulation, analysis and modelling. The model used (Fig. 1) is the same as that previously developed for apoB100 metabolism in diabetic patients (Ouguerram et al. 2003). This model takes into account heterogeneity in VLDL. In this model, a forcing function, corresponding to the time course of plasma leucine enrichment, was used to drive the appearance of leucine tracer in the apoB100 of the different lipoprotein fractions. ApoB100 enters into plasma through VLDL secretion and the direct production of IDL and LDL. Direct removal of apoB100 occurs from VLDL1 (k(0,10)), VLDL2 (k(0,20)), VLDL remnant (VLDLR) (k(0,11)), IDL (k(0,30)) and LDL (k(0,40)). ApoB100 transfer to higher-density lipoproteins occurs by conversion for VLDL1 (k(20,10), k(11,10)), VLDL2 (k(30,20), k(40,20)) and IDL (k(40,30)). The methods provided identified values and standard deviations as obtained by iterative least squares fitting for individual kinetic parameters. Standards deviations were less than 30 % for most of the parameters (data not shown). The use of more complex models did not provide a significant improvement in the fitting from the F test and Akaike information criterion (Pont et al. 1998).

For comparison between the two states (before and after n-3 treatment), the VLDL1, VLDL2 and VLDLR data were presented as the VLDL conversion rate and VLDL direct removal rate. The VLDL conversion rate was calculated as VLDL2 conversion flux divided by total VLDL mass. The direct removal of VLDL was calculated as the flux of VLDL1, VLDL2 and VLDLR direct removal divided by the total VLDL mass (Ouguerram et al. 2003). The VLDL fractional catabolic rate represented the sum of the conversion and direct removal rates.

As all our patients were obese, pools of apoB100 in the plasma or in VLDL, IDL and LDL were calculated by multiplying the apoB100 concentration by 0·037 (l/kg), assuming a plasma volume of 3·7 % of body weight (Dagher et al. 1965). The apoB100 production rate in mg/kg per h represented the product of fractional catabolic rate and pool size of apoB100 in the lipoprotein fractions.

Statistical analysis. Results are reported as means and standard deviations. Wilcoxon’s signed rank-test, performed with Statview F-4·5 (Abacus Concept, Berkeley, CA, USA) was used to compare data before and after treatment. A two-tailed probability level of 0·05 or less was accepted as statistically significant.

Results
Concentrations of lipids and apoB100 in plasma and in lipoprotein fractions are shown in Tables 1 and 2 before and after n-3 administration. Compared with the basal state, a lower concentration of plasma triacylglycerol (1·776 (SD 0·280) mmol/l v. 1·356 (SD 0·595) mmol/l; P,0·05) was observed. This decrease was related to a fall in VLDL1 triacylglycerol (1·006 (SD 0·271) mmol/l v. 0·534 (SD 0·289) mmol/l; P<0·05). Treatment did not significantly affect plasma total cholesterol. A decrease of apoB100 in VLDL (0·366 (SD 0·360) v. 0·174 (SD 0·036) g/l; P<0·005) related to a fall in VLDL1 apoB (0·244 (SD 0·015) v. 0·075 (SD 0·050) g/l; P<0·005) was observed with treatment.

![Fig. 1. Model of apoB100-containing lipoprotein metabolism. For details of subjects and procedures, see p. 101. IDL, intermediate-density lipoprotein; VLDLR, very low-density lipoprotein R.](https://www.cambridge.org/core/cover)
The effect on LDL apoB100 was variable between subjects (a decrease in one subject and an increase in the others), leading to a slight but not significant increase in LDL apoB100. As shown in Table 1, no effect on BMI, fasting plasma glucose, homeostasis model assessment or HbA1c concentration was observed with treatment.

Lipoprotein kinetics

The time course of enrichment in VLDL, IDL and LDL apoB100 for subject 3 before and after n-3 treatment is shown in Fig. 2. Model fitted lines and experimental points showed close agreement. The masses of B100 calculated by SAAMII in each compartment were also similar to those chemically measured (data not shown). Kinetic parameters are shown in Table 2.

Compared with the basal situation (Table 3), treatment with n-3 significantly reduced the overall production of apoB100 (1·73 (SD 0·28) v. 0·97 (SD 0·13) mg/kg per h; P<0·05; Table 2). This decrease was related to VLDL1 apoB100 production (1·49 (SD 0·23) v. 0·44 (SD 0·19) mg/kg per h; P<0·05). In parallel to this decrease, treatment increased the direct production of IDL (0·02 (SD 0·01) v. 0·24 (SD 0·12) mg/kg per h; P<0·05) and LDL (0·07 (SD 0·05) v. 0·18 (SD 0·17) mg/kg per h; NS). No effect on the uptake of VLDL was observed with treatment, but the conversion rate was significantly increased (0·031 (SD 0·024) v. 0·052 (SD 0·04) per h; P<0·05), and VLDL were channelled more towards higher-density lipoproteins (data not shown). The fractional catabolic rates of IDL and LDL were not affected by the treatment.

Discussion

The aim of the present study was to examine, in type 2 diabetes, the effect of n-3 fatty acids (1·8 g/d) on the metabolism of apoB100-containing lipoprotein. Analyses were performed using stable isotopes on fasting subjects to avoid any acute influence of n-3-containing chylomicrons and chylomicron remnants on apoB100 metabolism. The model used in this study includes heterogeneity of VLDL and is consistent, as shown by the low CV of parameters. The present study showed that, in type 2 diabetes, n-3 fatty acids lowered triacylglycerolaemia by decreasing apoB100 VLDL production and by stimulating VLDL conversion to IDL. The heterogeneous response to n-3 fatty acids led to a non-significant increase

### Table 2. Lipoprotein composition in diabetic subjects before and during treatment with MaxEPA (Pierre Fabre Santé, Castres, France)

<table>
<thead>
<tr>
<th>Subject</th>
<th>VLDL1 TC (mmol/l)</th>
<th>VLDL1 TG (mmol/l)</th>
<th>VLDL1 ApoB (g/l)</th>
<th>VLDL2 TC (mmol/l)</th>
<th>VLDL2 TG (mmol/l)</th>
<th>VLDL2 ApoB (g/l)</th>
<th>IDL TC (mmol/l)</th>
<th>IDL TG (mmol/l)</th>
<th>IDL ApoB (g/l)</th>
<th>LDL TC (mmol/l)</th>
<th>LDL TG (mmol/l)</th>
<th>LDL ApoB (g/l)</th>
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<td>0·134</td>
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<td>0·372</td>
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<tr>
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IDL, intermediate-density lipoprotein; TC, total cholesterol; TG, triacylglycerol.

*P<0·05, **P<0·005.

For details of subjects and procedures, see p. 101.
of LDL in spite of a higher conversion rate of VLDL and a non-significant increase of direct LDL production. Treatment with n-3 acids did not affect diabetic control.

In type 2 diabetic patients, the kinetics of apoB100 metabolism is well documented (Duvillard et al. 2000; Ouguerram et al. 2003). Briefly, in mild diabetes, an overproduction of VLDL and impaired lipolysis leads to hypertriacylglycerolaemia (Duvillard et al. 2000), whereas in moderate or severe diabetes, a delay in LDL catabolism is also reported, explaining the combined hyperlipidaemia (Kissebah et al. 1983; Ouguerram et al. 2003).

The present study is the first kinetic study on diabetic subjects performed in the fasting state that has compared the effect of n-3 fatty acid treatment to a basal diet. To distinguish a chronic effect of n-3 fatty acids and an acute effect of n-3 absorption, the study was performed in the fasting state, in contrast to a previous study by Fisher et al. (1998). It is well known that chylo-microns and their remnants enriched in n-3 fatty acids (Zheng et al. 2001; Maldonado et al. 2003) or a direct delivery of dietary n-3 PUFA to the liver (Wang et al. 1993) reduces hepatic VLDL synthesis. A study design performed in the feeding state did not allow a discrimination between a short-term effect consequent on absorptive flux and a long-term effect consequent on a change in cell membrane composition. To avoid such confusion, the present kinetic study was conducted in the fasting state.

The effect of fish oil on plasma lipid and apo concentration has been well documented in animal (Harris, 1996) and in healthy (Connor et al. 1993) and hypertriacylglycerolaemia (Fisher et al. 1998; Chan et al. 2003) human subjects. In these studies, dramatic reductions in plasma triacylglycerol levels occurred, with an inconsistent response in plasma total cholesterol that varied from study to study depending on the diet previously administrated and mainly with the nature of the fatty acids added, although also with the dose administered and the duration of treatment (Fisher et al. 1998; Farmer et al. 2001). The mechanisms underlying these effects were largely reported in animals (Harris, 1996), and healthy human subjects (Connor et al. 1993; Harris, 1996), but data on diabetic subjects have been missing, particularly in terms of the lack of a kinetic study. Only one study had previously been performed on diabetic subjects (Fisher et al. 1998), but the data compared n-6 and n-3 treatments without making any comparison with the basal state.

In the present study, n-3 acids significantly decreased triacylglycerolaemia. This fall is related to lower VLDL and especially VLDL1 production, as previously reported in other studies (Fisher et al. 1998; Chan et al. 2003). This was not related to a change in the abundance of mRNA for apoB100 (Zheng et al. 2001). However, an enhanced intracellular degradation of apoB100 was shown in primary rat hepatocytes (Wang et al. 1993). One other major effect of n-3 is the inhibition of triacylglycerol synthesis by decreasing the activity of acyl-CoA:1,2-diacylglycerol acyltransferase (Rustan et al. 1988) and increasing their triacylglycerol oxidation (Yamazaki et al. 1987). Several studies have also showed that n-3 fatty acids regulate the expression of numerous genes (Clarke, 2004) involved in the suppression of fatty acid synthesis. As VLDL secretion depends on the available synthesised triacylglycerol (Wu et al. 1996), the inhibition of fatty acid and triacylglycerol synthesis could result in an increased degradation of apoB100. It also has to be emphasised that the present study was performed in the fasting state without making any comparison with the basal state.

Table 3. Kinetic parameters in diabetic subjects before and during treatment with MaxEPA (Pierre Fabre Santé, Castres, France)

<table>
<thead>
<tr>
<th>Subject</th>
<th>V1PR (mg/kg per h)</th>
<th>V2PR (mg/kg per h)</th>
<th>VPR (mg/kg per h)</th>
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<tr>
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<td>0.59</td>
<td>0.44*</td>
<td>0.52</td>
</tr>
</tbody>
</table>

For details of subjects and procedures, see p. 101.

IP address: 54.191.40.80, on 20 Apr 2017 at 08:34:50, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. https://doi.org/10.1079/BJN20061806
state, suggesting that VLDL synthesis was not due to an acute inhibition by n-3-enriched chylomicrons but probably to profound and chronic changes in n-3 concentration in the hepatocytes after long-term treatment.

We did not observe any effect of n-3 fatty acids on the VLDL, LDL and HDL fractional catabolic rates. Although the absence of a significant effect on VLDL and IDL fractional catabolic rate has previously been reported (Fisher et al. 1998; Chan et al. 2003), data on the LDL fractional catabolic rate are conflicting (Ventura et al. 1989; Spady et al. 1995; Schectman et al. 1996; Vasandani et al. 2002; Theobald et al. 2004). Feeding fish oil stimulated the clearance of LDL in rats (Ventura et al. 1989) and mice (Vasandani et al. 2002) but decreased the receptor-mediated clearance of LDL in hamsters (Spady et al. 1995), primates (Schectman et al. 1996) and human subjects (Theobald et al. 2004). This decrease could be a result of either decreased binding of LDL to the LDL receptor or decreased expression of the LDL receptor. The decrease in the number of specific binding sites for acetyl LDL in peritoneal macrophages in rat by EPA was reported by Saito et al. (1992). LDL undergo a change in physical properties in response to n-3 fatty acids (Bell et al. 1996), which renders them more susceptible to oxidation (Whitman et al. 1994; Nestel et al. 1997). Although LDL generated after n-3 supplementation are susceptible to oxidation (Whitman et al. 1994; Nestel et al. 1997), they are not atherogenic. In the present study, as in others (Fisher et al. 1998), LDL have been analysed as a one homogenous pool, so the fractional catabolic rate measured is a mean of that for all LDL subpopulations. Although Rivellese et al. (2003) observed no modification in LDL size on n-3 supplementation, it might be interesting to examine their effect on the kinetics of small and large LDL.

In the present study, we showed that n-3 fatty acids increased the conversion of VLDL to LDL but not the conversion of IDL to LDL. The effect of n-3 fatty acids on lipoprotein lipase (LPL) activity was reported in animals (Huff & Telford, 1989), healthy human subjects (Kasim-Karakas et al. 1995; Harris et al. 1997) or insulin-resistant human subjects (Chan et al. 2003). Dietary n-3 fatty acids have been also shown to accelerate chylomicron triacylglycerol clearance in rats (Rahman et al. 2000). Supplementation with n-3 enhances chylomicron triacylglycerol clearance by increasing LPL and hepatic lipase activity in healthy subjects but only LPL activity in hypertriglyceridaemia patients (Harris et al. 1997). An increase in post-heparin LPL activity and the level of LPL mRNA in the adipose tissue of subjects with an atherogenic lipoprotein phenotype has been reported with n-3 acids (Khan et al. 2002). In parallel to a decrease of VLDL production, n-3 fatty acids shift the channelling of apoB100 from large VLDL to small VLDL, LDL and HDL. This could be explained by the secretion of small VLDL with a lower triacylglycerol:apoB ratio, which are rapidly converted to LDL (Lu et al. 1999).

In the present study, we did not observe any difference in the triacylglycerol:apoB ratio between the two states (data not shown). This discrepancy could be explained by the lower dose of n-3 used in our study. In Fisher et al.’s (1998) study, the authors did not observe any change in lipoprotein conversion. In that study, the comparison was against a diet enriched in n-6 fatty acids, and any comparison with a basal diet was missing. Although the major effect of n-6 fatty acids is to lower plasma cholesterol level (Kasim-Karakas et al. 1995), they also increase plasma triacylglycerol hydrolysis as n-3 fatty acids (Mekki et al. 2002). Therefore, the comparison between n-3 and n-6 fatty acids does not allow an assessment of the actual magnitude of the effect of n-3. Because n-3 fatty acids affect VLDL conversion into IDL, but not the conversion of IDL into LDL, we suggest that LPL and hepatic lipase are not regulated by similar pathways (Peinado-Onsurbe et al. 1992; Ougueram et al. 2003).

In conclusion, n-3 fatty acids were effective in lowering triacylglycerolaemia in individuals with type 2 diabetes patients via the suppression of VLDL production and the stimulation of its conversion into LDL. As diabetic subjects also respond to statins known to stimulate LDL uptake (Ougueram et al. 2003), combined therapy could be recommended in case of mixed dyslipidaemia.

Acknowledgements

This work was supported by Pierre Fabre Santé. The authors are grateful to Carole Le Valegant for her technical assistance.

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