Dietary supplementation of very long-chain n–3 fatty acids decreases whole body lipid utilization in the rat

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Abstract Rats were fed lard or n-3 fatty acid-supplemented diets ad libitum to study whole body oxidation of lipid and carbohydrate. One group of male rats was fed 21% fat (by weight) containing 19.5% lard and sufficient amounts of essential fatty acids (1.5%). Another group of rats had 6.5% of the lard replaced by ethyl esters of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The rats were fed these diets for 6–8 weeks. Body weight gain was similar for the two groups and absorption of fat was complete in animals fed both types of fatty acids. Indirect calorimetric measurements, after 3–5 weeks on these diets, by continuous registration of O_2 consumption and CO_2 formation showed no difference in mean energy expenditure during the experimental period. However, the mean respiratory quotient (RQ) was significantly increased for animals fed the n-3 fatty acid-supplemented diet. This was noted both under fasting conditions and after receiving a test meal of similar fatty acid composition for both feeding groups. Thus, mean substrate utilization demonstrated reduced oxidation of fat and increased oxidation of carbohydrate, during fasting as well as fed periods for the n-3 fatty acid group as compared to the lard group. After an additional 2–3 weeks, blood plasma, liver, and muscle samples were collected, and adipocytes and hepatocytes were isolated. Reduced postprandial plasma concentrations of triacylglycerol, phospholipids, unesterified fatty acids, and glycerol were promoted by the n-3 fatty acid diet as compared to lard. Plasma concentration of glucose was slightly increased, and liver and muscle content of glycogen were decreased in the n-3 fatty acid-fed rats. Experiments with isolated adipocytes showed decreased basal lipolysis after feeding n-3 fatty acids for 6–8 weeks for suspended epididymal adipocytes, whereas stimulated lipolysis by isoproterenol (0.1 μM) was higher in both epididymal and mesenteric adipocytes from rats fed n-3 fatty acids as compared to animals fed lard. In addition, epididymal adipocytes from rats fed n-3 fatty acids were significantly smaller than cells from animals fed lard. Hepatic peroxisomal fatty acid oxidation was significantly higher for n-3 fatty acid-supplemented animals, but total fatty acid oxidation was similar in both dietary groups. The hepatic content of triacylglycerol and phospholipids was similar for both diets. These results demonstrate that n-3 fatty acid replacement of a high-fat diet containing mostly saturates and monoenes for several weeks promotes reduced use of fat as energy source. This may be explained by decreased plasma concentration of unesterified fatty acids. Along with reduced plasma concentration of glycerol, our data could be interpreted as reduced lipolysis. These changes in plasma lipids may promote reduced triacylglycerol storage in certain adipose tissues, as well as decreased fatty acid availability to the liver for synthesis and secretion of VLDL, and increased carbohydrate utilization.—**Rustan, A. C., B-E. Hustvedt, and C. A. Drevon.** Dietary supplementation of very long-chain n-3 fatty acids decreases whole body lipid utilization in the rat. *J. Lipid Res.* 1993. **34:** 1299–1309.

Supplementary key words respiratory quotient • indirect calorimetry • EPA • DHA • adipocytes

Fish oils are enriched in very long-chain polyunsaturated fatty acids of the n-3 series, especially eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3). Dietary intake of these fatty acids is effective in lowering plasma concentration of triacylglycerol (1, 2). Metabolic experiments in humans and rats have indicated that these fatty acids promote reduced production of very low density lipoproteins (VLDL) and chylomicrons by the liver and intestine, respectively (2–4). The mechanism for this effect has been further evaluated using cell cultures (5, 6) and the results indicate that decreased triacylglycerol synthesis is a major cause for the reduced secretion of VLDL-triacylglycerol (7).

Availability of unesterified fatty acids in the liver plays a key role in regulation of VLDL synthesis and secretion (6–9). It has recently been observed that very long-chain n-3 fatty acids have the ability to decrease the level of unesterified fatty acids in plasma (10–13). This reduction

Abbreviations: L, lard, F, fish oil-derived n-3 fatty acids; VLDL, very low density lipoprotein; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; RQ, respiratory quotient; GLC, gas-liquid chromatography; TLC, thin-layer chromatography; BSA, bovine serum albumin; f-BSA, fatty acid-poor BSA.

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may contribute to reduced triacylglycerol formation within the liver due to lack of fatty acids as substrate for lipoprotein production. It is presently not clear why dietary n-3 fatty acids decrease plasma unesterified fatty acid levels. It is possible that mobilization of fatty acids from catabolism of triacylglycerol-rich lipoproteins (e.g., chylomicrons and VLDL) in the circulation and/or lipolysis from adipose tissues is decreased, or there might be a general increase in fatty acid oxidation both in the liver and muscle tissues. Reduced postprandial triacylglycerol levels after n-3 fatty acid supplementation to human subjects has been observed (3, 14, 15). A decrease in adipose tissue mass in fish oil-fed rats has recently been demonstrated to be linked to the reduction in triacylglycerol and unesterified fatty acids in plasma (13). Moreover, increased hepatic fatty acid oxidation after feeding n-3 fatty acids to rats and hamsters has been demonstrated (11, 16-20), although it is still unclear to what extent this contributes to decreased plasma unesterified fatty acids. Increased lipoprotein lipase activity in skeletal muscle has also been observed after fish oil-feeding to rats, suggesting an increased fatty acid oxidation in muscle tissue (12, 21). The present study was undertaken to further clarify mechanisms for reduced formation of triacylglycerol after dietary intake of long-chain n-3 fatty acids, and to focus particularly on plasma concentration of unesterified fatty acids. It was of special interest to examine whether changes in plasma lipids could be verified by measurements of whole body metabolism in vivo by indirect calorimetry.

EXPERIMENTAL

Materials

The ethyl ester concentrate of EPA and DHA (K85, batch 1020) was generously provided by Pronova AS, Oslo, Norway. The fatty acid composition determined by GLC was (in weight% of total): 18:4 n-3, 2.9; 20:3 n-3, 0.2; 20:4 n-6, 4.2; 20:4 n-3, 0.8; 20:5 n-3, 54.2; 22:4 n-3, 1.8; 22:5 n-3, 2.8; 22:6 n-3, 32.8; others, 0.3. Lard was obtained from Agro Fellesslakteri, Norges Kjøtt-og Fleskesentral, Oslo, Norway. Soybean oil was delivered by DeNoFa and Lilleborg Fabrikker, Oslo, Norway. Vitamin and salt mixtures for the semisynthetic diets were from ICN Pharmaceuticals, Cleveland, OH. Collagenase (type I), 4-(hydroxyethyl)1-piperazine-ethanesulfonic acid (HEPES), fatty acids (oleic and erucic), β-hydroxybutyrate dehydrogenase (H 5132), bovine serum albumin (BSA) (fatty acid-poor), amyloglucosidase (A 3514), and glycogen (type III from rabbit liver) were from Sigma Chemical Co. St. Louis, MO. Glycerokinase and glycerol-3-phosphate dehydrogenase, NAD, and ATP were from Boehringer-Mannheim, Mannheim, Germany. Thin-layer chromatography plates (Silicagel F 1500) were purchased from Schleicher & Schuell, Dassel, Germany. Other fine chemicals were from Sigma Chemical Co.

Animals

Male rats of the Wistar strain (SPF, Mol) were purchased from Møllegaard Breeding Center, Ejby, Denmark. The body weights of the animals were within the range 175-185 g at the start of the experimental feeding. They were randomly divided into two groups (n = 9) and immediately transferred to individual cages of wire mesh and given free access to experimental diets and tap water. Two other groups of rats (n = 8) were offered the same diets for 7-8 weeks and kept under the same conditions. Results from these animals are included in the study of adipocytes.

The temperature of the animal quarter was 24 ± 1°C, and the humidity was 55 ± 3%. The lighting was regulated to provide 12 h of light (0700 to 1900) and 12 h of darkness.

Diets

Each animal group was offered one of two semisynthetic diets, Lard: L (19.5% lard) or fish oil-derived n-3 fatty acids: F (13% lard and 6.5% K85), and an additional 1.5% soybean oil to both dietary groups. The composition was (weight% of total): sucrose, 20; cornstarch, 31.5; casein 20; cellulose, 1; vitamin mixture, 1.5; salt mixture 5; and fat 21. These diets provided 40% of the energy from fat. Diets were kept at −20°C in portions sufficient for 1 day supply.

The diets used for tube-feeding during the calorimetric measurements were prepared from a synthetic diet (Collett Spesialdiett, type 19 9414 containing 3 energy% fat from corn oil, Nycomed Pharma, Oslo, Norway), by addition of fat and water to a final suspension with an energy density of 12.6 kJ/g. The composition of the diet expressed as energy% was: carbohydrate 46.2, fat 42.4, and protein 11.4. The percent fatty acid composition of the lard (L) and n-3 fatty acid-supplemented (F) diets is shown in **Table 1.**

Experimental protocol for the indirect calorimetry

Food (from frozen stores) was given fresh each day and the intake was recorded. Body weight was registered twice each week. The calorimetric measurements were performed after 3-5 weeks on this regimen (**Fig. 1**). The protocol for the calorimetric measurement was as follows. The food was removed from the cage in the period of 14-16 h prior to the actual measurements. Between 0800 and 0900 the body weights of the rats were recorded. The complete animal cage, including the drinking bottle, was then transferred to a close-fitting calorimetric chamber. This chamber had an air inlet in the lower part of the walls and an outlet through a nozzle in the center of the air-tight lid. The O_2 consumption and CO_2 production in

TABLE 1. Fatty acid composition of semisynthetic diets

Fatty Acids	F	L
14:0	1.0	1.5
16:0	17.8	26.4
16:1 n-7	1.3	2.0
18:0	11.8	17.6
18:1 n-9	22.5	33.0
18:1 n-7	1.6	2.3
18:2 n-6	10.6	13.9
18:3 n-3	1.0	1.3
18:4 n-3	0.9	
20:1 n-9	0.7	1.0
20:3 n-3	0.1	
20:4 n-3	0.2	
20:4 n-6	1.3	
20:5 n-3	16.8	
22:4 n-3	0.6	
22:5 n-3	0.9	
22:6 n-3	10.3	0.2
Others	1.6	2.3

Fatty acid composition was determined by GLC as described in Experimental. F: 13% lard, 6.5% K85, and 1.5% soybean oil; L: 19.5% lard and 1.5% soybean oil.

the fasting state were then recorded for approximately 1 h. The measurements were then discontinued while the animals were tube-fed 6.8 g of diet L or F outside the chamber. Within 5 min the animals were returned to the calorimeter and the measurements continued for another 21 h. In total we have 22-h recordings from each animal.

During the next 2-3 weeks the rats were subjected to a slight change in feeding regimen. During this period they had access to food only in dark hours. This regimen was introduced prior to decapitation of the animals (for blood and tissue sampling, and cell preparation) to ensure that the animals were in a fed state between 0800 and 0900 the next day (Fig. 1).

Determination of digestibility

In a separate experiment, two groups of three rats each were placed in metabolic cages. They were offered the same diets and feeding regimen as described for the ad libitum situation. These cages permitted controlled food intake and quantitative and separate collection of urine and feces (22). Registration of food intake and complete feces and urine collection were performed during 3 days. The procedure for analysis of nitrogen and energy content of diet, urine, and feces was as described by Jeszka et al. (23). There was no difference in nitrogen or energy content either in urine or in feces between the two groups. The digestibility of the diets was therefore equal. The mean urine nitrogen content was close to 200 mg/day for each animal. This value was used for calculation of energy expenditure.

Indirect calorimetry

Air was drawn through the respiration chamber by a membrane pump and pushed through cylinders filled with granulated $CaCl_2$. The flowrate, 1.0-1.5 l/min, was measured on dried gas by a turbine flowmeter (Omniflow model FTM-NS-GJS) equipped with linearizer (model LIN-256, Flow Technology, Phoenix, AZ). The concentration of O_2 in the dried gas was continuously measured by an O_2 analyzer (model S-3A, Applied Electronics, Sunnyvale, CA) based on a high-temperature galvanic zirconia cell. The CO_2 concentration was determined by an infrared gas analyzer (Binos-IR, model 1, Leybold-Heraeus, Germany). The instruments were calibrated against dried outdoor air and a precision-analyzed gas mixture containing approximately 1% CO_2 and 20% O_2. Pure N_2 was used as zero gas. Care was taken to adjust the gas flow in the system so that the concentration difference for CO_2 and O_2 between air flowing in and out of the respiratory chamber was kept in the range 0.5-1.0%.

Data logging and processing

The analog output from the gas analyzers and flowmeter was fed to a computer through a Lab-Master (Scientific Solutions Inc., OH) instrumental interface. Special software was developed to take care of the reading of the instruments, the immediate calculations, and storage of the results. The gas analyzers and the flowmeter were read 24 times in sequence each second, the average value for each second was temporarily stored, and the mean value for each minute was calculated. On the basis of the mean readings, O_2 consumption and CO_2 production were calculated and stored for each minute. The results were stored as sequential files and recalculated to obtain

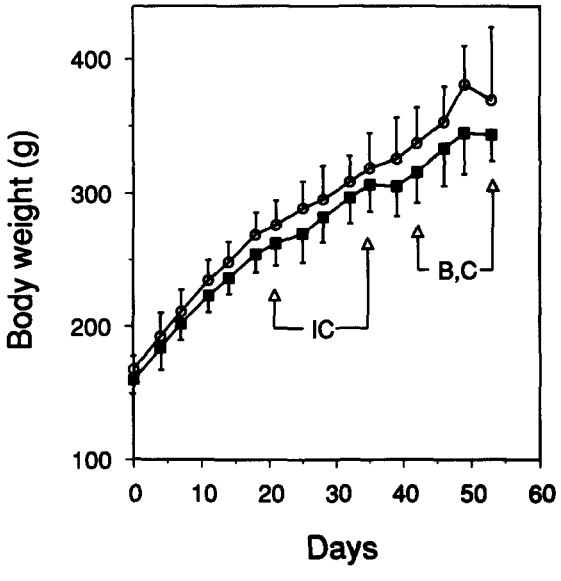

Fig. 1. Effect of n-3 fatty acid- (■) and lard- (○) supplemented diets on body weight. Data represent means ± SD for (initially) 9 rats in each dietary group, decreasing to 3 rats in each group at the end of the experiment. IC, indirect calorimetry; B, blood sampling; C, isolation of cells (adipocytes and hepatocytes).

mean values for 10-min intervals throughout the experiment. Calculation of energy expenditure and metabolic substrate utilization were performed as described by Hustvedt et al. (24). The calculations were based on the assumption that protein was oxidized at the same rate 24 h a day.

Plasma analysis

After decapitation of the rats, blood was collected with 0.1% EDTA (final conc.) and immediately chilled on ice. Plasma was prepared and stored at −70°C prior to analysis.

Triacylglycerol, phospholipid, and unesterified fatty acids were extracted (25) and measured by gas–liquid chromatography (GLC) as fatty acid methyl esters (FAME) (26). In short, the lipid spots on TLC plates were visualized by fluorescein, scraped into vials, redissolved in 1 ml benzene, and methylated by overnight incubation in 2 ml methanolic HCl (3 M) and 200 μl 2,2-dimethoxypropane. The mixtures were neutralized by 4 ml $NaHCO_3$ (0.7 M) and extracted twice with 2 ml n-hexane. After evaporation of the solvent, the lipids were redissolved in n-hexane, and an aliquot was injected into the gas–liquid chromatograph (Shimadzu, GC-14A), equipped with a polar capillary column (SGE BPX70, diameter 0.33 mm, length 25 m), and using helium as carrier gas. The oven temperature was programmed to rise from 160 to 220°C at 1°C/min. Retention times and peak areas were automatically computed. Identification of the individual methyl esters was performed by frequent comparisons with authentic standard mixtures analyzed under the same conditions. Triheptadecanoylglycerol, diheptadecanoylphosphatidylcholine, and heptadecanoic acid were used as internal standards for quantitation of triacylglycerol, phospholipids, and unesterified fatty acids, respectively. Hepatic content of triacylglycerol and phospholipid was also measured by GLC.

Glycerol concentration in plasma was measured fluorimetrically by the method of Laurell and Tibbling (27). Glucose was determined using the glucose dehydrogenase assay from E. Merck, Darmstadt, Germany.

Plasma insulin was determined by radioimmunoassay using a commercial kit for rat insulin (Linco Research Inc., St. Louis, MO).

Preparation of adipocytes

Epididymal and mesenteric adipose tissue samples (2–3 g) were taken, and isolated adipocytes were prepared by the collagenase method developed by Rodbell (28). Briefly, fat tissue was taken out, immediately washed in 145 mM NaCl buffer (with 5 mM KCl, 0.4 mM KH_2PO_4, 0.3 mM Na_2HPO_4, 0.8 mM $MgSO_4$, 1 mM $CaCl_2$, and 20 mM HEPES) containing 3% BSA, cut into small pieces and transferred to a polyethylene plastic tube. Two g tissue was added to 4 ml NaCl-buffer containing 1.5 mg collagenase/ml, 2 mM $CaCl_2$, and 3% BSA, pH 7.4, and incubated in a shaking water bath at 80 cycles/min for 1 h at 37°C. After incubation, the cells were filtered through 450 μm nylon mesh, and adipocytes were allowed to float for 3 min. The infranatant was removed and the adipocytes were washed twice with 10 ml NaCl-buffer containing 5 mM glucose and 3% fatty acid-poor BSA (f-BSA). The cells were finally resuspended in 5–10 ml NaCl-buffer with glucose and f-BSA. The size of the adipocytes was measured in aliquots of isolated cells using a microscope fitted with a graduated occular (29). In short, the cells were observed under low magnification (×40) with the aid of a calibrated grid and the mean diameter of 120–150 cells from each cell preparation was calculated. Since the fat cells float and rupture on contact with glass, no cover slide was used.

Lipolysis by adipocytes

Lipolysis in adipose cells was measured as glycerol released from freshly isolated fat cells after 2 h incubation at 37°C. Adipocytes corresponding to 50–100 mg lipid were incubated in a total volume of 2 ml buffer containing 5 mM glucose and 3% f-BSA, and either no isoproterenol (basal lipolysis) or 0.1 μM isoproterenol (total lipolysis). Incubations were terminated by a brief centrifugation to separate cells from the medium. The glycerol content in the medium was determined fluorimetrically as described above.

Lipid content of the cells was determined gravimetrically after extraction three times with 5 volumes of hexane. Lipid recovery by this procedure was greater than 95%. Based on cell average diameter, the volume and weight of the cells were calculated (specific gravity of triacylglycerol taken to be 0.9). Adipocyte concentration in the incubation system was then determined by dividing lipid content of the cells (obtained by gravimetry) by the weight of one cell (assumed to be 100% lipid) to obtain the number of cells in each incubation.

Preparation of rat hepatocytes

Hepatocytes were prepared according to Seglen (30). In short, the rats were anesthetized with barbiturates, hepatocytes were isolated by collagenase perfusion, washed, and used immediately as suspended cells for determination of fatty acid oxidation.

Fatty acid oxidation: ketone bodies and hydrogen peroxide

Determination of ketone bodies and hydrogen peroxide generation were used as indicators for mitochondrial and peroxisomal fatty acid oxidation, respectively.

Hepatocytes were placed in polyethylene plastic bottles and 145 mM NaCl buffer (5 mM KCl, 0.4 mM KH_2PO_4, 0.3 mM Na_2HPO_4, 0.8 mM $MgSO_4$, and 1 mM $CaCl_2$) containing glucose (12 mM), HEPES (20 mM), f-BSA

(0.2 mM), and L-carnitine (1 mM) was added to a final cell concentration of 6×10^6/ml. The cells were preincubated for 30 min at 37°C in a shaking water bath at 60 rev/min. The cells were then suspended in NaCl buffer (145 mM) containing glucose (12 mM), HEPES (20 mM), L-carnitine (1 mM), semicarbazide (10 mM), methanol (50 mM), f-BSA (0.2 mM), and fatty acids (0.6 mM of either oleic or erucic) and incubated for another 30 min at 3×10^6 cells/ml. After incubation, 0.8 ml 2.1 M perchloric acid was added, and after cooling on ice, the supernatant was collected by centrifugation (2000 rpm/5 min). H_2O_2 was measured in 1.0 ml of the supernatant. The supernatant excess was neutralized with KOH (2 M) and potassium phosphate buffer (0.5 M, pH 6.5), and stored at −70°C prior to analysis of ketone bodies.

Ketone bodies (β-hydroxybutyrate plus acetoacetate) were measured enzymatically according to Williamson and Mellanby (31). The generation of H_2O_2 was estimated from the peroxidatic generation of formaldehyde trapped as its semicarbazone (32, 33). Values for the production of ketone bodies and hydrogen peroxide correspond to measurements after incubation in the presence of substrate, corrected for blank values of nonincubated vials containing substrate.

The protein content of each preparation of hepatocytes was determined using bovine serum albumin as standard (34).

Measurement of glycogen content of liver and skeletal muscle

Liver and muscle samples were washed in cold saline and frozen in liquid nitrogen immediately after decapitation of the rats. They were stored at −70°C prior to measurement of glycogen content.

Glycogen content in the liver and muscle samples (quadriceps femoris) was determined by enzymatic hydrolysis with amyloglucosidase (35). The glucose formed was measured using the glucose dehydrogenase assay from E. Merck. A standard curve was prepared by hydrolyzing samples of pure rabbit liver glycogen using the same procedure. Each sample was corrected for the presence of free glucose by treating unhydrolyzed aliquots with the glucose dehydrogenase reagent.

Presentation of data and statistics

Indirect calorimetry. Different time intervals of the RQ curve were examined statistically according to the following model. The RQ curve representing the n−3 fatty acid group was assumed to be equal to the RQ curve for the lard group plus a constant. The constant was assumed to be the same for all time intervals. Mathematically this can be expressed as log (n−3 fatty acid RQ) = log (lard RQ) + constant. To decide whether this constant was different from zero, the average log (RQ) for all animals in each group within the actual time interval was calculated and the groups were compared using a two-sample *t*-test. Substrate utilizations (means ± SD) calculated from the indirect calorimetric data were statistically evaluated by the Mann-Whitney nonparametric test (Minitab, Minitab Inc., State College, PA).

Plasma and cells. Data are shown as means ± SD of triplicate measurements from three or more animals in each group as described in legends to figures and tables. Data were statistically evaluated by the Mann-Whitney non-parametric test.

RESULTS

Animals and diets

The rats were fed the experimental diets ad libitum. There was no significant difference in food consumption between the two dietary groups. The average food intake during the last week prior to indirect calorimetry was 16.1 ± 1.8 and 16.6 ± 1.4 g/day for n−3 fatty acid- and lard-fed animals, respectively; $P = 0.3$). Furthermore, no difference in body weight gains was noted during the feeding period (Fig. 1). In addition, this feeding protocol was repeated with another eight rats in each group to increase the number of animals at the end of the feeding period (7–8 weeks). Their body weights were 413.4 ± 13.2 g and 402.2 ± 25.0 g for n−3 fatty acid- and lard-fed animals, respectively (n = 8).

Indirect calorimetry

The rats were used in calorimetric experiments as indicated (Fig. 1) after the experimental diets were fed for 3–5 weeks. They were placed individually in the calorimeter, and oxygen consumption and carbon dioxide production were recorded during an initial fasting period (0–1 h) and then for 21 h after the animals were administered a test diet by tube-feeding. The mean energy expenditures for each 10-min period for the two groups (n = 9) throughout the experiment are shown in **Fig. 2.** Due to the respiratory disturbances introduced by the handling of the animals during the tube-feeding procedure, no data are presented for the time period 1–2 h. The intragastric feeding time is indicated by an arrow. There was no difference in energy expenditure between the groups. The energy expenditure immediately increased in both groups when the light was turned off in the animal room.

The mean respiratory quotients (RQ) for each 10-min period for the two groups are shown in **Fig. 3.** During the whole registration period, except for the time immediately after feeding, the RQ of the group fed n−3 fatty acids was significantly increased compared to the rats fed lard ($P < 0.003$ during the fasting period prior to feeding, and $P < 0.01$ for the period from 3 to 22 h).

The mean substrate utilization for two different time periods during the experiment was calculated from the

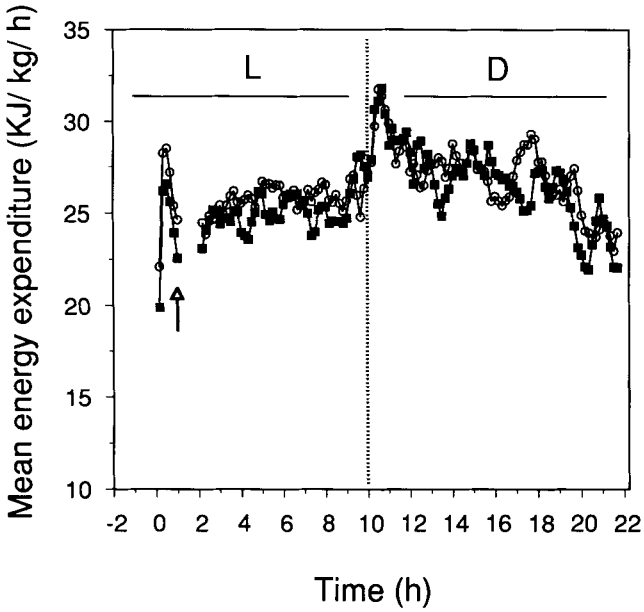

Fig. 2. Mean energy expenditure (kJ/kg/h) (n = 9) for each 10-min period for the n-3 fatty acid- (■) and lard- (○) supplemented rats throughout the registration period (22 h). The intragastric feeding time is indicated by the arrow. No data are shown in the time interval 1–2 h due to disturbances introduced by the tube-feeding. L, light and D, darkness in the animal quarter.

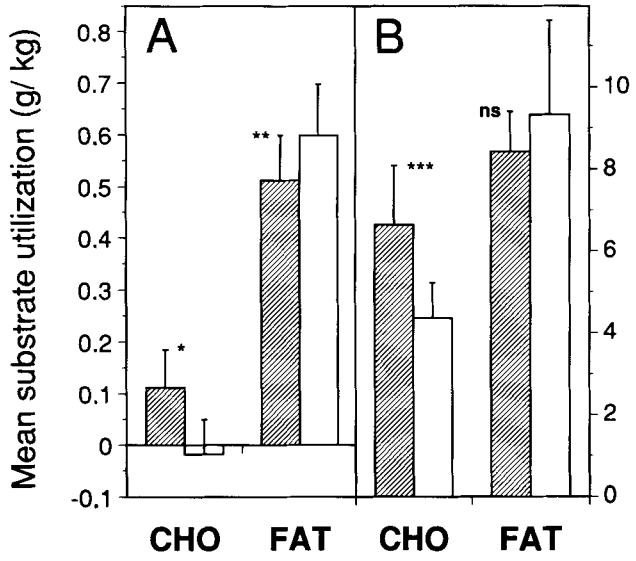

Fig. 4. Mean integrated substrate utilization (g/kg body weight) for two periods during the experiment, calculated from the calorimetric data. Panel A present the substrates oxidized, fat and carbohydrate (CHO) during the last hour prior to feeding, 0–1 h (n–3 fatty acids, hatched bars; lard, open bars). Panel B present results for the period 3–22 h. Data represent means ± SD (n = 9). Significant difference between dietary groups, $^{*}P < 0.003$; $^{**}P < 0.03$; $^{***}P < 0.001$; ns, not significant (Mann-Whitney).

calorimetric data (**Fig. 4**). Panel A presents the substrate oxidized (fat and carbohydrate) during the last hour prior to feeding, after a 14–16 h fast. Panel B presents results for the period 3–22 h after zero time. There was a difference in substrate utilization between the two groups. In the fasting state the oxidation of fat was significantly reduced ($P < 0.003$), whereas that of carbohydrate was increased ($P < 0.03$) for animals fed n-3 fatty acids. In the postprandial period the carbohydrate oxidation was significantly increased ($P < 0.001$), whereas the reduction in fat oxidation for n-3 fatty acid-fed rats did not reach significance.

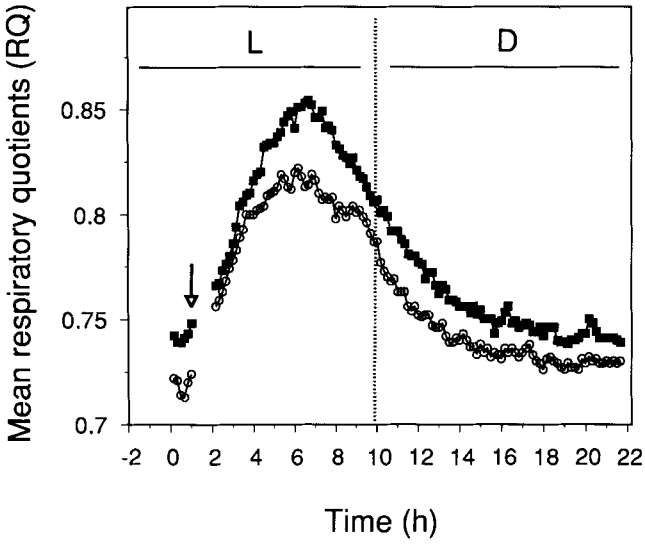

Fig. 3. Mean respiratory quotients (RQ) (n = 9) for every 10-min period for the n-3 fatty acid- (■) and lard- (○) supplemented rats throughout the registration period (22 h). Due to respiratory disturbances introduced by the handling of the animals during the tube-feeding procedure no data are shown between 1 and 2 h. L, light and D, darkness in the animal quarter. The results are tested for difference during two time periods, 0–1 h ($P < 0.003$) and 3–22 h ($P < 0.01$) (two-sample *t*-test).

Postprandial plasma

After an additional 2–3 weeks on the same diets, the rats were killed by decapitation for blood sampling and preparation of adipocytes and hepatocytes (Fig. 1). Intake of the n-3 fatty acid-supplemented diet markedly reduced nonfasting plasma concentrations of triacylglycerol (75% reduction, $P < 0.004$), phospholipids (30% reduction, $P < 0.004$), unesterified fatty acids and glycerol (both reduced by 40–50%, $P < 0.004$) as compared to lard-fed rats (**Fig. 5**).

Plasma glucose concentration was slightly increased by 0.7 mM for the n-3 fatty acid diet (6.82 ± 0.44 mM as compared to the lard diet, 6.13 ± 0.21 mM, $P < 0.025$). There was no significant difference in insulin concentration between the two dietary groups (7.12 ± 0.73 and 8.13 ± 0.9 ng/ml for the n-3 fatty acid and lard diets, respectively, $P = 0.08$).

Lipolysis by epididymal and mesenteric adipocytes

Adipocytes were isolated from epididymal and mesenteric fat, and both basal and β-adrenergic-stimulated

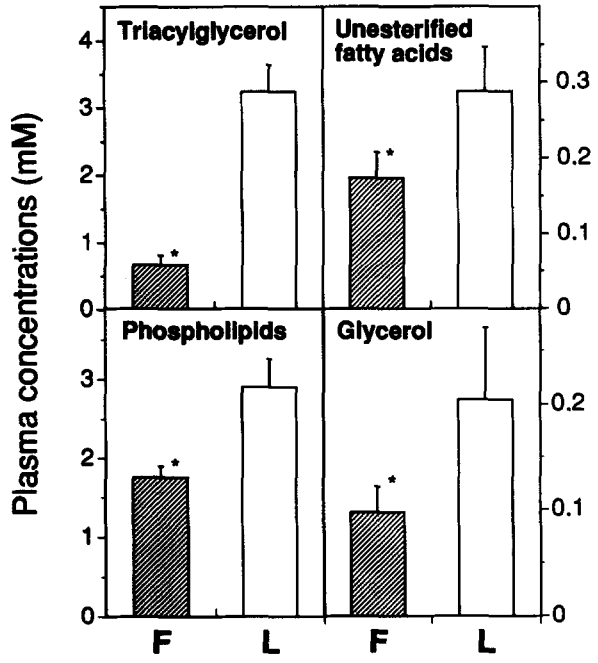

Fig. 5. Effect of n-3 fatty acid- (F) (hatched bars) and lard- (L) (open bars) supplemented diets on rat postprandial plasma lipids and glycerol. Between 6 and 8 weeks on the respective diets, blood was collected after decapitation, and plasma analyses of lipids and glycerol were performed as described in Experimental. Data represent means ± SD (n = 6 animals from each dietary group). *Significantly different at $P < 0.004$ (Mann-Whitney).

lipolysis was measured. The n-3 fatty acid diet significantly decreased basal lipolysis (glycerol release) by 50-70% ($P < 0.004$) from isolated epididymal adipocytes (**Fig. 6A and B**), whereas no change in basal lipolysis was observed with mesenteric adipocytes (Fig. 6C and D). Addition of isoproterenol (0.1 μM) markedly increased lipolysis as compared to unstimulated cells. This concentration of isoproterenol was chosen as it was found to be optimal (data not shown). Stimulated lipolysis was increased by 7-55% for epididymal adipocytes from n-3 fatty acid-treated rats when compared to lard-fed animals (Fig. 6A and B). The increase in lipolysis with isoproterenol was even higher (80% increase) for mesenteric cells from the n-3 fatty acid group in comparison with lard (Fig. 6C and D).

The volume of epididymal adipocytes from n-3 fatty acid-fed animals was on average 0.57 ± 0.32 nl, whereas cells from lard fed rats were 0.83 ± 0.46 nl ($P < 0.0001$). No difference in size of mesenteric adipocytes was observed; the average cell-volume was 0.36 ± 0.18 and 0.37 ± 0.17 nl for n-3 fatty acid- and lard-fed animals, respectively.

Fatty acid oxidation by rat hepatocytes

Suspended hepatocytes were incubated in the presence of fatty acids (0.6 mM of either oleic or erucic acid) and their capacity to produce hydrogen peroxide and ketone bodies (acetoacetate and β-hydroxybutyrate) was determined. The n-3 fatty acid diet increased hydrogen peroxide production by 75-105% ($P < 0.004$) in the presence of both oleic and erucic acid (**Fig. 7**). No significant difference in formation of ketone bodies from either oleic or erucic acid was observed (Fig. 7). Thus, total β-oxidation (ketone bodies plus hydrogen peroxide) was similar for both dietary groups.

Moreover, no difference in liver content of triacylglycerol and phospholipids was observed (0.13 ± 0.04 and 0.13 ± 0.03 μmol triacylglycerol/mg cell protein and 1.16 ± 0.10 and 0.89 ± 0.22 μmol phospholipid/mg cell protein for the n-3 fatty acid and lard diets, respectively).

Liver and muscle glycogen

Samples from liver and skeletal muscle were collected and their content of glycogen was measured. Liver and muscle glycogen were decreased by 26 and 41%, respectively, after feeding n-3 fatty acids when compared to lard-fed animals (**Fig. 8**).

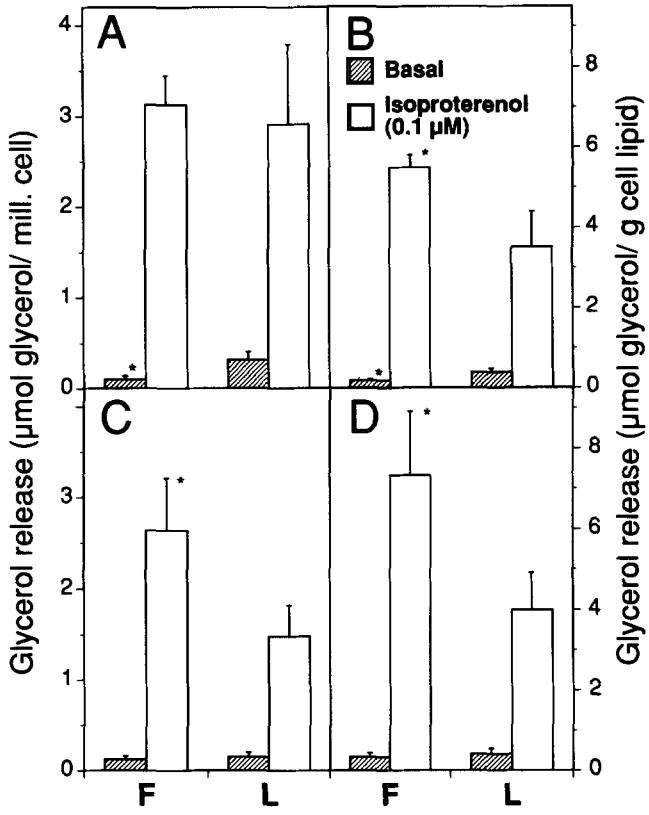

Fig. 6. Effect of n-3 fatty acid- (F) and lard- (L) supplemented diets on basal and isoproterenol-stimulated lipolysis in isolated epididymal (A, B) and mesenteric (C, D) adipocytes. Adipocytes were isolated after 6-8 weeks on the respective diets. The cells were incubated in the absence or presence of 0.1 μM isoproterenol, and glycerol released to the medium was measured enzymatically as described in Experimental. Data represent means ± SD of triplicates (n = 6-8 animals for epididymal and mesenteric fat in each dietary group, respectively). The results are shown either as μmol glycerol/10^6 cells (A, C) or as μmol glycerol/g cell lipid (B, D). *Significantly different at $P < 0.004$ (Mann-Whitney).

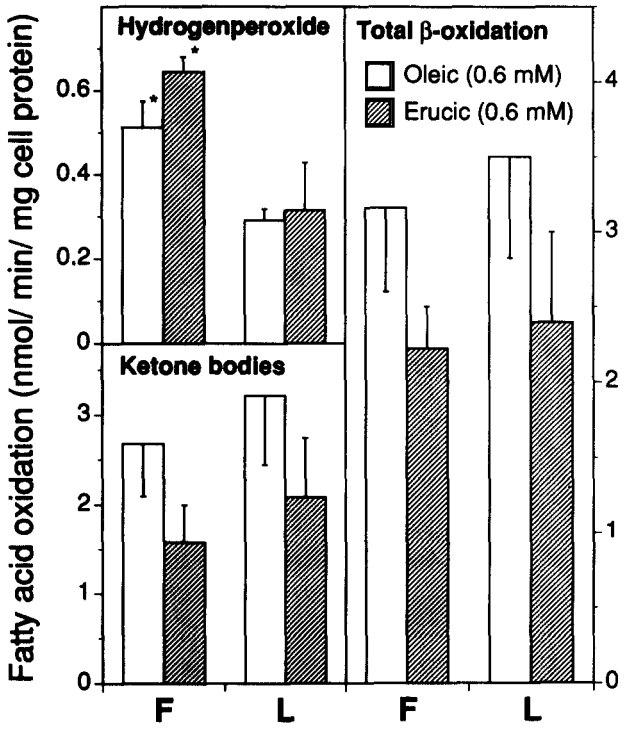

Fig. 7. Effect of n-3 fatty acid- (F) and lard- (L) supplemented diets on fatty acid oxidation in isolated rat hepatocytes. Hepatocytes were isolated after 6-8 weeks on the respective diets. The cells were incubated in the presence of 0.6 mM of either oleic or erucic acid, and hydrogen peroxide and ketone bodies released from the cells were measured enzymatically as described in Experimental. Data represent means ± SD of triplicates from three animals in each dietary group. *Significantly different at $P < 0.004$ (Mann-Whitney).

DISCUSSION

This study shows that a high intake of very long-chain polyunsaturated n-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), lowers nonfasting plasma concentrations of triacylglycerol, phospholipids, unesterified fatty acids, and glycerol in rats compared to a diet containing mostly saturated and monounsaturated fat (Fig. 5). At the same time, plasma concentration of glucose is slightly, but significantly, increased. These changes in postprandial plasma lipids, glycerol, and glucose may explain the observed effects on whole body energy metabolism measured by indirect calorimetry (Figs. 3 and 4). Increased respiratory quotient (RQ) after dietary n-3 fatty acids demonstrates increased carbohydrate utilization, whereas there was reduced lipid oxidation in fasted as well as in fed animals. Nitrogen balance studies showed that there was no difference between the two diets, thus the increased RQ for n-3 fatty acid-fed animals must be caused by increased utilization of carbohydrate and not by increased oxidation of protein. Furthermore, the total energy consumption (Fig. 2) and digestibility of the diets were equal in the two groups, suggesting that the changes in metabolism were not caused by differences in food intake or by fat malabsorption. Such differences in substrate utilization could change body composition over time. In this study we observed a decrease in epididymal adipose tissue after 7-8 weeks, together with decreased content of liver and muscle glycogen for n-3 fatty acid-treated animals (Fig. 8). However, despite these changes, the body weight of the animals was similar for both groups. Other studies have also shown that dietary fish oil limits growth of adipose tissues without significantly changing the body weight (12, 36, 37). Parrish et al. (36) have reported that adipose tissue (epididymal and perirenal) was the only tissue whose mass decreased after fish oil supplementation, whereas mass of liver and spleen increased.

The present study shows reduced level of plasma unesterified fatty acids after n-3 fatty acid supplementation. This is in agreement with our recent observation of reduced postprandial plasma unesterified fatty acid concentration in rats fed a fish oil-enriched diet (11). Reduced plasma unesterified fatty acids after dietary fish oil to rats has been observed by others (12, 13). It has also been shown that n-3 fatty acid supplementation, as compared to other polyunsaturated fatty acids, reduces serum unesterified fatty acids in hyperlipidemic patients (10).

It is not clear how dietary intake of fish oil decreases plasma concentrations of unesterified fatty acids. Mobilization of fatty acids from adipose tissue, as well as from

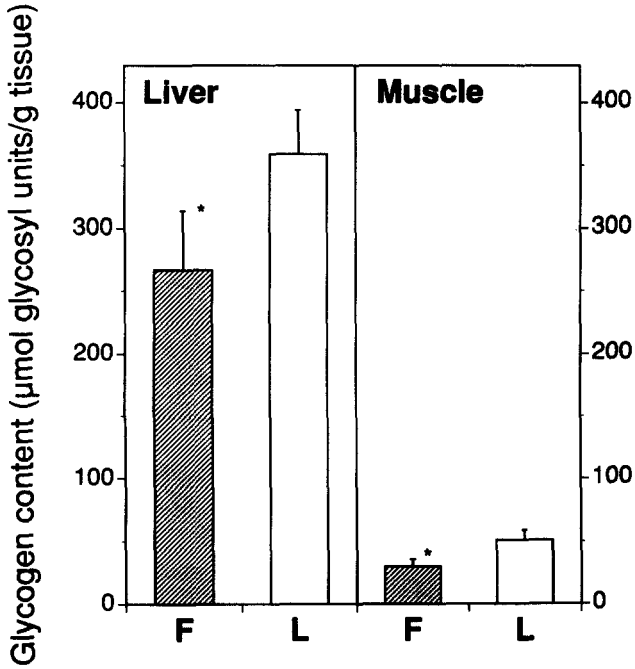

Fig. 8. Effect of n-3 fatty acid- (F) and lard- (L) supplemented diets on glycogen content in liver and muscle. Glycogen was measured as total glucose released after enzymatic hydrolysis with amyloglucosidase as described in Experimental. Data represent means ± SD of triplicates from four animals in each dietary group. *Significantly different at $P < 0.004$ (Mann-Whitney).

catabolism of triacylglycerol-rich lipoproteins, may be decreased, or there may be an increased removal of fatty acids from the circulation. As the liver plays a major role in the removal of fatty acids from the circulation, differences in hepatic lipid metabolism may be of great importance. Feeding experiments with 10–20% (by weight) dietary fat cause reduced fatty acid esterification and increased peroxisomal β-oxidation in subcellular liver fractions for fish oil-fed rats, as compared to other polyunsaturated high-fat diets (11, 17, 19). Furthermore, we have also measured mitochondrial and peroxisomal fatty acid oxidation by isolated hepatocytes in this study (Fig. 7). We observed a twofold increase in peroxisomal fatty acid oxidation (measured as H_2O_2 production), whereas no significant change in mitochondrial β-oxidation (measured as ketone body production) was found. Thus, total hepatic fatty acid oxidation was not higher in hepatocytes from n-3 fatty acid-fed rats, when compared to lard-fed animals. Other authors have demonstrated increased ketone body production in perfused livers from rats fed fish oil (16), and that intake of highly purified eicosapentaenoic acid (EPA) stimulated the activities of carnitine palmitoyltransferase, fatty acyl-CoA oxidase, and peroxisomal β-oxidation in subcellular fractions of rat liver (18). Moderate dietary intake of polyunsaturated n-3 fatty acids in Syrian hamsters has recently been shown to affect only mitochondrial fatty acid oxidation and hepatic triacylglycerol synthesis, but not peroxisomal fatty acid oxidation (20).

Increased lipoprotein lipase activity in muscle tissue after feeding fish oil to rats has also been observed. This may indicate increased fatty acid utilization in these tissues, thereby promoting lowering of plasma triacylglycerol (12, 21). In the present study, however, a reduced total fatty acid oxidation in vivo was demonstrated by indirect calorimetry (increased RQ) after n-3 fatty acid supplementation. Skeletal muscles contribute approximately 45% of the body weight of a rat, and energy consumption by this tissue would be of substantial importance in determining the respiratory quotient. This suggests that the observed reduction of fatty acid oxidation and the increased carbohydrate oxidation in muscle tissues may be caused by decreased availability of unesterified fatty acids and increased availability of glucose for oxidation.

Decreased concentration of plasma glycerol after n-3 fatty acid consumption may be explained by decreased triacylglycerol lipolysis. This effect may be caused by reduced basal adipose tissue lipolysis, and/or by decreased production of triacylglycerol-rich lipoproteins, presumably chylomicrons from the intestine in this study, as the blood samples were collected in the postprandial state. It has been observed in human studies that dietary intake of n-3 fatty acids decreases postprandial chylomicron levels (2, 3, 14, 15). This reduction in chylomicron levels must be due either to reduced rate of formation and/or secretion, or to an increased rate of triacylglycerol removal from the circulation. Most evidence indicates that chylomicron clearance by lipoprotein lipase is unaltered after fish oil supplementation (2, 3, 13, 15, 38). This suggests that decreased postprandial plasma triacylglycerol most likely is caused by reduced chylomicron production and/or secretion by the intestine, and that catabolism of triacylglycerol-rich lipoproteins contributes little to the triacylglycerol-lowering effect of fish oil (3, 13).

Parrish et al. (36, 37) have recently shown that dietary fish oil modifies adipose tissue trophic growth as a function of animal age, diet, and feeding period. They observed decreased basal lipolysis from epididymal adipocytes, whereas β-adrenergic-stimulated lipolysis by epididymal and perirenal adipocytes was increased for fish oil-fed rats as compared to a lard-enriched diet. Stimulation of lipolysis with isoproterenol (100 μM) was greatest for n-3 fatty acid-fed rats. Their observations are in agreement with our results (Fig. 6). This may reflect increased activity of any step along the cAMP cascade from cell surface receptor to hormone-stimulated lipase, and may be mediated by diet-induced changes in fatty acid composition of membrane phospholipids as discussed (36). Furthermore, the reduction in mass of epididymal and perirenal fat pad after n-3 fatty acid supplementation may be due to reduced plasma triacylglycerol concentration in conjunction with increased hormone-stimulated lipolysis (36). In the present study we have also measured plasma levels of unesterified fatty acids and glycerol. The cause of reduced size of epididymal adipocytes after dietary n-3 fatty acids could be explained by the low concentrations of circulating plasma triacylglycerol and unesterified fatty acids, thus providing less fat for storage in adipose tissues. An increased lipolysis (isoproterenol stimulation) will counteract the lowering of plasma unesterified fatty acids. Therefore, we suggest that the primary action of very long-chain n-3 fatty acids on plasma unesterified fatty acids is not mediated initially by changes in lipid metabolism in fat tissues. The cell size or basal lipolysis of mesenteric adipocytes did not change after n-3 fatty acid supplementation (Fig. 6). Thus, the effect of n-3 fatty acids on adipose tissue varies for different fat depots.

Decreased basal lipolysis by adipose tissues after n-3 fatty acid supplementation could be mediated by the increase in plasma glucose observed for these animals. In humans, it has been shown that plasma glucose regulates lipid metabolism, independently of hormones, by suppression of lipolysis (39).

The tendency for decreased plasma insulin in n-3 fatty acid-fed animals in this study, together with increased carbohydrate oxidation, suggest an improvement in insulin sensitivity. It has been observed in rats that fish oil feeding prevents the insulin resistance induced by very

high-fat diets (40). Moreover, Storlien et al. (41) has reported increased insulin sensitivity (insulin-stimulated glucose metabolism) in skeletal muscle in rats fed fish oil. We also observed a reduced glycogen content in liver and muscle in the present study, further supporting increased use of carbohydrate as substrate for oxidation.

The reduced plasma concentrations of triacylglycerol caused by fish oil consumption may also be due to decreased formation of VLDL by the liver (2, 4). From short-term experiments with cultured rat hepatocytes we know that synthesis and secretion of triacylglycerol are reduced by purified EPA and DHA (5–7). It has also been shown that EPA is diverted to phospholipid synthesis and thereby decreases VLDL secretion in cultured rabbit hepatocytes (42). In addition, it is possible that the decreased plasma concentration of unesterified fatty acids after n-3 fatty acid supplementation together with increased peroxisomal oxidation (Fig. 7) reduces hepatic VLDL formation by providing less substrate for this process.

In conclusion, the lowering of plasma triacylglycerol after n-3 fatty acid supplementation to rats, could in part be caused by decreased plasma concentrations of unesterified fatty acids and thereby less flux of fatty acids to the liver for lipoprotein production. Moreover, the reduced size of epididymal adipocytes and decreased lipid oxidation in vivo suggest that less fatty acid is available both for storage in adipose tissues and as an energy source. Based on these observations, we suggest that the reduced level of plasma unesterified fatty acids and glycerol after feeding n-3 fatty acids in the postprandial state to a large extent is caused by reduced production/or secretion of triacylglycerol-rich lipoprotein particles by the intestine.

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