Dietary Fish Oil-induced Changes in Intrahepatic Cholesterol Transport and Bile Acid Synthesis in Rats

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Abstract

Hepatic cholesterol metabolism was studied in rats fed purified diets supplemented (9% wt/wt) with either fish oil (FO) (n-3 fatty acids) or corn oil (CO) (n-6 fatty acids) for 4 wk. Rats were equipped with permanent catheters in heart, bile duct, and duodenum to allow studies under normal feeding conditions. [^{3}H]-cholesteryl oleate-labeled small unilamellar liposomes, which are rapidly endocytosed by hepatocytes, were intravenously injected to label intrahepatic cholesterol pools, and plasma and bile were collected. FO as compared to CO induced a lowering of plasma cholesterol levels by 38% and of triglyceride levels by 69%. This reduction in plasma lipids in FO rats was accompanied by: (*a*) an increased bile acid pool size (28%); (*b*) a fourfold increase in the ratio cholic acid/chenodeoxycholic acid in bile; (*c*) increased biliary excretion of cholesterol (51%); (*d*) accelerated excretion of endocytosed free cholesterol into bile; (*e*) accelerated incorporation of endocytosed cholesterol in bile acids; (*f*) a significant increase in the bile acid-independent fraction of bile flow; and (*g*) a threefold increase in hepatic alkaline phosphatase activity. The results show that FO induces changes in transport and metabolic pathways of cholesterol in the rat liver, which result in a more rapid disposition of plasma-derived cholesterol into the bile. (*J. Clin. Invest.* 1991. 88:943-951.) Key words: enterohepatic circulation • lipids • polyunsaturated fatty acids • corn oil • alkaline phosphatase

Introduction

Consumption of fish oil (FO)[1] is associated with a reduced risk for coronary heart disease (1-4). This relation is probably of a multifactorial nature and may include changes in lipoprotein metabolism. A consistent effect of dietary FO on lipoprotein metabolism in man is a pronounced reduction in plasma triglyceride levels (5). However, plasma cholesterol is not systematically influenced by FO ingestion (see 5 for review). In a number of studies (6-9), in which dietary saturated triglycerides were replaced by FO, a decrease in LDL cholesterol levels was observed (5). In contrast, when FO was added as a supplement to the diet no change or even a slight increase in LDL cholesterol occurred (5). In rats, plasma cholesterol levels were greatly reduced after feeding of a diet containing FO (10-12). Recently, Ventura et al. (12) showed that this is mainly due to stimulation of hepatic lipoprotein receptor activity but the underlying mechanism remained unclear. The polyunsaturated n-3 fatty acids, eicosapentaenoic acid (C20:5 [n-3]) and docosahexaenoic acid (C22:6 [n-3]), which are present in relatively large amounts in FO, are supposed to be responsible for the observed effects of dietary FO.

Although it is recognized that the hepatobiliary pathway is the main route for removal of cholesterol from the body (13, 14), little information is available concerning dietary effects on intrahepatic cholesterol transport and bile formation. To gain further insight into the metabolic changes induced by dietary FO, we have investigated its effects on hepatic cholesterol metabolism and bile formation in the rat. The effects of purified diets containing either 9% (wt/wt) FO, which is rich in n-3 polyunsaturated fatty acids, or 9% (wt/wt) corn oil (CO), which is rich in n-6 polyunsaturated fatty acids, were compared. Changes in plasma lipids, hepatic cholesterol metabolism, bile formation, and hepatic plasma membrane composition were determined. In addition, intravenously injected [^{3}H]cholesteryl oleate-labeled liposomes (small unilamellar vesicles) were used as a tool to introduce labeled cholesteryl oleate into the liver. These small liposomes are known to be preferentially taken up by hepatocytes (15-17). After intralysosomal hydrolysis, the labeled cholesterol becomes available to the cell and can be used for bile acid synthesis (16, 17). The results show that a decrease in plasma cholesterol and triglyceride concentrations in rats fed FO is accompanied by quantitative and qualitative changes in hepatic sterol metabolism.

Methods

Materials. Tween-80, Triton WR-1339, glucose-6-phosphate, fatty acid-free BSA, oleoyl CoA, 3-hydroxy-3-methylglutaryl (HMG) CoA, and mevalonic acid lactone were purchased from Sigma Chemical Co., St. Louis, MO. Glucose-6-phosphate dehydrogenase, DTT, NADP, and ATP were from Boehringer Mannheim GmbH, Mannheim, Germany. Cholesterol, 7α-hydroxycholesterol, and 7β-hydroxycholesterol were from Steraloids, Inc., Wilton, NH. 3-Hydroxy-3-methyl[3-^{14}C]glutaryl CoA and [4-^{14}C]cholesterol were from the Radiochemical Centre, Ltd., Amersham, Bucks., UK. [5-^{3}H]-Mevalonic acid lactone and [1-^{14}C]oleoyl CoA were obtained from New England Nuclear, Boston, MA. All other chemicals used were of analytical grade.

Liposome preparation. Small unilamellar vesicles containing a trace amount of [1,2-^{3}H]cholesteryl oleate (48 Ci/mmol; Radiochemical Centre) were made as described previously (18, 19). The vesicles

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1. *Abbreviations used in this paper:* ACAT, acyl-CoA:cholesterol acyltransferase; BAIF, bile acid-independent fraction of bile flow; CO, corn oil; EHC, enterohepatic circulation; FO, fish oil; HMG, 3-hydroxy-3-methylglutaryl.

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were composed of cholesterol, phosphatidylcholine, and phosphatidylserine in a 5:4:1 molar ratio.

Animals. Male Wistar (Cpb:Wu) rats, aged 3 wk, were obtained from Harlan CPB, Zeist, The Netherlands. The animals were housed in groups in wire-topped polycarbonate cages with a layer of sawdust as bedding. The cages were placed in a temperature and light-controlled room (temperature 20°C; light on between 6 a.m. and 6 p.m.). The rats were maintained on commercial rat pellets (RMHB®, Hope Farms BV, Woerden, The Netherlands). At the age of 8 wk (day 0), the animals were divided into two dietary groups, consisting of 18 rats each, so that the distributions of plasma cholesterol concentrations and body weights of both groups were similar. Mean plasma cholesterol concentrations and body weights on day 0 were 2.69 mM and 264 g, respectively. The composition of the experimental diets, which were provided in powdered form, is given in Table I. The type of fat was the only variable. The diets were balanced for cholesterol in the FO (144 mg/100 g). CO was obtained from Knorr Caterplan GmbH, Heilbronn, FRG; FO (menhaden oil) was from Unilever Research Laboratory, Vlaardingen, The Netherlands. The animals received the experimental diets ad lib.; they had free access to tap water. From days 0 to 28, the rats were housed individually in stainless steel cages with wire mesh bases. As from day 20 on, the animals that were to be equipped with permanent catheters (see below) were housed in wire-topped polycarbonate cages with a layer of sawdust as bedding.

Table I. Composition of the Experimental Diets

	Dietary oil	
	Corn oil	Fish oil
Ingredients (g)		
Constant components	90	90
Corn oil	10	1
Fish oil	—	9
Cholesterol	0.08	0.07
Chemical analysis		
Cholesterol (g/100 g)	0.08	0.08
Crude fat (g/100 g)	10.1	10.1
Fatty acids (g/100 g fatty acids)		
C 14:0	nd	6.2
C 16:0	10.3	17.7
C 16:1	nd	7.5
C 18:0	1.9	3.0
C 18:1	29.5	15.0
C 18:2	55.8	7.6
C 18:4 (n-3)	nd	2.1
C 20:1 (n-9)	0.3	2.9
C 20:3 (n-3)	nd	1.0
C 20:5 (n-3)	nd	13.0
C 22:1 (n-9)	nd	2.2
C 22:5 (n-3)	nd	2.1
C 22:6 (n-3)	nd	8.4
Saturated total	12.2	26.9
Monounsaturated total	29.5	24.7
Polyunsaturated (n-6) total	55.8	7.6
Polyunsaturated (n-3) total		26.6

The constant components consisted of (in grams): casein, 17; starch, 21; dextrose, 19; molasses, 11; cellulose, 16; dicalcium phosphate, 0.6; calcium carbonate, 0.7; magnesium carbonate, 0.07; magnesium oxide, 0.03; potassium bicarbonate, 1.9; sodium chloride 0.5; vitamin premix, 1.2; mineral premix, 1.0. The composition of the mineral and vitamin premixes have been described elsewhere (20). nd, not detectable.

Experimental procedures. On day 0 blood was collected in the nonfasting state from the orbital plexus under diethylether anesthesia. On day 21, five rats of each group were equipped with permanent silastic catheters in bile duct, duodenum, and heart, as described earlier (21). These catheters were tunneled subcutaneously and fixed to the skull. The enterohepatic circulation (EHC) was restored immediately by connection of the bile and duodenal catheters. During bile diversion, which was started on day 30, bile was led through a polyethylene tubing with a swivel joint, thus permitting free movement of the rats, and allowing bile formation to be studied in conscious and unrestrained animals. The heart catheter allows intracardial administration as well as repeated blood sampling without the need to handle the animals (22). Animals of both groups rapidly recovered from surgery; food intake returned to normal within 2 or 3 d after the operation. Experiments were started on day 30.

On day 30, [^{3}H]cholesteryl oleate–labeled small unilamellar vesicles (2×10^6 dpm, 2 μmol lipid/100 g body wt) were injected via the heart catheter between 11:00 and 11:15 a.m. Immediately after injection, the artificially restored EHC was interrupted and bile was collected continuously for 24 h in tared test tubes, by using a fraction collector. Blood samples were taken at indicated time intervals after liposome injection.

Preparation of microsomes and assay of HMG-CoA reductase, acyl-CoA:cholesterol acyltransferase (ACAT), and cholesterol 7α-hydroxylase activity. For the isolation of microsomes on days 27 and 29, four additional rats of each group were used. The animals were anesthetized with diethylether and livers were perfused in situ with ice-cold 0.25 M sucrose until they became yellowish. Livers were immediately excised, cut into small pieces, and divided into three portions which were homogenized in different buffers (see below). All procedures were carried out at 4°C. After centrifugation at 12,000 *g* for 20 min, microsomes were isolated by centrifugation of the supernatant at 105,000 *g* for 60 min. The microsomes were resuspended and washed by another centrifugation step at 105,000 *g* for 60 min. The microsomes were resuspended (10–25 mg of microsomal protein per ml), frozen in liquid nitrogen in small portions, and stored at −60°C under nitrogen.

For the assay of cholesterol 7α-hydroxylase, microsomes were isolated in a 40-mM potassium phosphate buffer, pH 7.4, containing 100 mM sucrose, 50 mM potassium fluoride, 2.0 mM EDTA, and 5.0 mM DTT. After the first ultracentrifugation step, the pellet was resuspended and subsequently washed and stored as described above in the same buffer without potassium fluoride and with 100 mM potassium phosphate, pH 7.4. For the assay of HMG-CoA reductase, microsomes were isolated in a buffer containing 10 mM potassium phosphate, pH 6.8, 2.0 mM EDTA, and 250 mM sucrose. After the first ultracentrifugation step the pellet was resuspended and subsequently washed and stored as described above in a buffer containing 50 mM piperazine-*N,N'* bis(2-ethane sulfonic acid), pH 6.5, 10 mM EDTA, and 100 mM NaCl. For the assay of ACAT, microsomes were isolated in a buffer containing 100 mM potassium phosphate, pH 7.4, 1.0 mM glutathione, and 10 mM nicotinamide.

Cholesterol 7α-hydroxylase activity was determined by measuring the synthesis of 7α-hydroxycholesterol from [^{14}C]cholesterol as described by Princen et al. (23, 24). HMG-CoA reductase was determined by measuring the conversion of [^{14}C]3-hydroxy-3-methylglutaryl CoA to mevalonic acid according to Philipp and Shapiro (25). ACAT activity was determined by measuring the incorporation of [^{14}C]oleoyl-CoA into cholesterol as described by Billheimer et al. (26).

Preparation of liver plasma membranes and enzyme assays. For the isolation of hepatic plasma membranes on day 28, eight animals per dietary group were used. The isolation method as described by Meier et al. (27) for Sprague-Dawley rats was modified for use in Wistar rats. All isolation steps were performed at 0–4°C. Rat livers were cut into small pieces, washed in 1 mM $NaHCO_3$ (pH 7.4), and homogenized and filtered. After centrifugation (5 min 500 *g* + 10 min 1,000 *g*), the crude nuclear pellet was resuspended in 1 mM $NaHCO_3$ and recentrifuged at 1,000 *g* (10 min). The pellet was resuspended in 5.5 vol of 56% sucrose (wt/wt) and stirred for 10 min to disrupt membrane aggregates. Sam-

ples of 15 ml of the suspension were transferred to centrifuge tubes (40 PA; Hitachi Ltd., Tokyo, Japan). The suspension was overlaid with 10 ml 43% sucrose, followed by 6 ml 36.5% sucrose. The tubes were filled to the top with 0.25 M sucrose and centrifuged for 120 min at 22,000 rpm (65,000 *g*) in a swing-out rotor (type SRP 28) in an ultracentrifuge (2331; LKB Instruments, Inc., Bromma, Sweden). The plasma membranes were recovered from the 36.5–43% interface and washed as described (27). The accuracy of the isolation procedure was checked by determining the activity of a number of plasma membrane marker enzymes. Na^+K^+-ATPase and Mg^{2+}-ATPase were determined by the kinetic assay described by Scharschmidt et al. (28), leucine aminopeptidase by the method of Goldbarg and Rutenberg (29), and alkaline phosphatase according to Keeffe et al. (30). As a test for the presence of intracellular organelles were determined: glucose-6-phosphatase for endoplasmic reticulum (31); succinate cytochrome c reductase for mitochondria (32); and acid phosphatase for lysosomes (33). Fluorescence depolarization of diphenylhexatriene, which gives an indication of membrane fluidity, was determined as described previously (34).

Analyses. Biliary bile acid concentration was determined by an enzymatic fluorimetric assay (Sterognost-Flu; Nyegaard Co., Oslo, Norway). Bile acid composition was analyzed by capillary gas chromatography and gas chromatography-mass spectrometry (GC-MS) as described previously (17). Analysis of radioactive bile acids was performed as described by Princen and Meijer (35). Cholesterol and phospholipids in bile were measured after lipid extraction (36), according to the methods of Gamble et al. (37) and Böttcher et al. (38), respectively. The mass of cholesterol and cholesteryl ester in liver was determined after lipid extraction (36) by a method using cholesterol oxidase, cholesterol esterase, and peroxidase (CHOD-PAP kit, catalogue no. 310328; Boehringer Mannheim). The same kit was used to determine plasma cholesterol concentrations. Triglyceride concentrations were determined enzymatically as described (39). Protein was determined according to Lowry et al. (40) with BSA as a standard.

For measurement of radioactivity, bile and liver homogenate samples were decolorized with an equal volume of 30% hydrogen peroxide, before administration of the scintillant (Hydroluma; J. T. Baker, Deventer, The Netherlands). Plasma samples were counted after addition of Plasmasol (Packard Instrument Company, Downers Grove, IL). Radioactivity was determined in a liquid scintillation counter (LKB Instruments) equipped with an external standard to correct for quenching.

Calculations and statistics. Values are presented as means±SEM. Significance of differences between dietary groups was assessed by Student's *t* test, at a $P < 0.05$ level of significance.

Results

Body weights, feed intake, and plasma lipids. The type of fat in the diets did not influence food intake and weight gain of the rats. The mean intake of food was 22.5 and 23.1 g/d in the CO and FO groups, respectively. The mean increase in body weight was 3.2 and 3.4 g/d in the CO and FO groups, respectively.

After 4 wk of feeding the FO diet, plasma cholesterol concentrations were on average reduced by 33% and triglyceride concentrations by 68% (Fig. 1). No significant effects with time on plasma cholesterol and triglyceride concentrations were observed after CO feeding.

Biliary excretion. Bile was collected in 1-h intervals during 4 h immediately after interruption of the EHC, and in two subsequent 10-h intervals. In the FO group, the amount of bile acids excreted during the first 4 h was increased by 28% compared to the CO group, indicating that dietary FO increases bile acid pool size (Table II). Bile flow in rats fed FO was increased by 37%, and cholesterol output by 51%. Phospholipid excretion was increased by 58%, but the difference did not reach

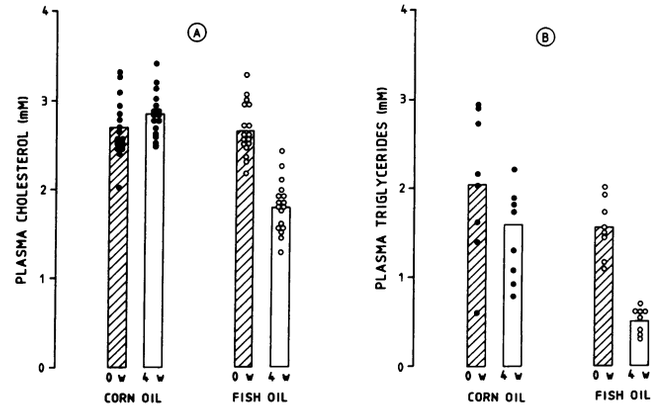

Figure 1. Plasma cholesterol (*A*) and triglyceride concentrations (*B*) of rats at the start of the experiment (0 w) and after 4 weeks (4 w) on a diet containing either 9% CO (●) or FO (○). Cross-hatched bars indicate group means at the start of the experiment. The clear bars indicate group means after 4 wk on the diet. Circles indicate individual values. Cholesterol concentration was determined in plasma samples of 16 and 17 animals of the CO and FO groups, respectively. Triglyceride concentrations were determined in eight plasma samples of each group.

statistical significance due to large interindividual variations. In the rat model employed, exhaustion of the endogenous bile acid pool occurs within 4 h (21, 41). Output of bile acids in the subsequent period therefore represents hepatic de novo synthesis. Time courses of bile flow and output rates of bile acids, phospholipids, and cholesterol are shown in Fig. 2. After exhaustion of the pool, no significant differences in output of bile acids, biliary phospholipids, and biliary cholesterol were observed between the groups, although output of all three bile constituents tended to be lower in rats fed FO (Table II). In contrast, bile flow was higher in these rats during the complete course of the experiment. Regression analysis of bile flow and bile acid output during the first 4 h after interruption, as first described by Berthelot et al. (42), revealed that the higher bile flow in rats fed FO was due to a significant ($P < 0.05$) increase in the so-called bile acid–independent fraction of bile flow (BAIF), which was 2.23 ml/kg per h in these animals compared

Table II. Bile Flow and Biliary Excretion of Bile Acids, Phospholipids, and Cholesterol during 0–4 h and 4–24 h after Interruption of the Enterohepatic Circulation

Interval, dietary oil	Bile flow	Bile acids	Phospholipids	Cholesterol
	ml/kg	*μmol/kg*	*μmol/kg*	*μmol/kg*
0–4 h				
Corn oil	9.16±0.56	303±8	31.6±5.9	2.93±0.39
Fish oil	12.57±0.95*	388±32*	49.9±7.8	4.42±0.32*
4–24 h				
Corn oil	29.88±1.29	288±21	74.6±12.0	10.01±0.76
Fish oil	37.80±1.37*	233±30	54.7±9.2	8.35±0.97

Two groups of animals were fed diets containing either CO or FO for 4 wk. Each value represents the mean±SEM for data obtained in five animals. * The values are significantly different at the $P < 0.05$ level from the corresponding values in the corn oil group.

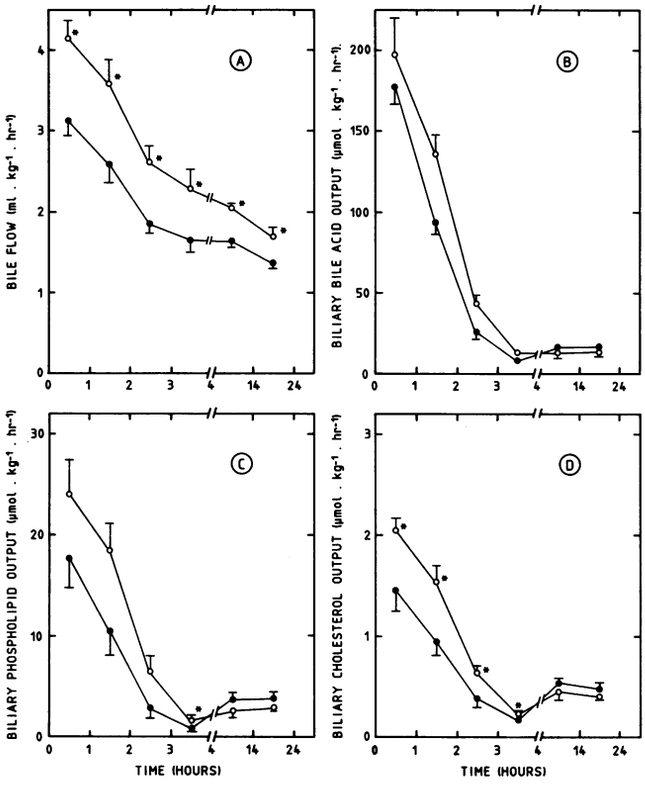

Figure 2. Bile flow (*A*) and excretion of bile acids (*B*), phospholipids (*C*), and cholesterol (*D*) during 24 h after interruption of the enterohepatic circulation of rats fed CO (●) or FO (○) for 4 wk. Data represent means±SEM for five animals. *Significant difference ($P < 0.05$).

to 1.61 ml/kg per h in rats fed CO (Fig. 3). In both groups the bile acid–dependent bile flow was similar; i.e., 9.38 μl/μmol in the FO group and 8.96 μl/μmol in the CO group.

Effects of FO on bile acid pool composition and synthesis of individual bile acid species. The composition of the bile acid pool and of newly synthesized bile acids was determined by gas chromatography and gas chromatography-mass spectrometry in bile samples in the period 0–2 h and 21–24 h after interruption of the EHC, respectively. This analysis revealed a significant difference between the two dietary groups (Table III). The pool of FO rats contained relatively more cholic acid and less chenodeoxycholic acid and muricholic acids. This difference was caused by altered hepatic synthesis rates of the latter bile acid species, as synthesis of chenodeoxycholic acid was markedly depressed in rats fed FO (Table III). The bile acid pool of both groups of rats contained a considerable amount, i.e., up to 20%, of a tertiary bile acid, which was tentatively identified as 3α,6α,7β,12α-tetrahydroxy-5β-cholanoic acid as based on its GC-MS spectrum (43). As this bile acid was found in much smaller amounts, i.e., ~ 4% in rats fed normal chow, it is tempting to speculate that the use of purified diet induces changes in bile acid metabolism.

Hepatic cholesterol, enzyme activities, and plasma membranes. No differences between rats fed FO or CO were observed in the amount of free and esterified cholesterol in the liver (Table IV). The increased bile acid pool size in rats fed FO was apparently not caused by an increase in hepatic bile acid synthesis (see also Fig. 2): the activity of microsomal cholesterol 7α-hydroxylase, the rate-limiting enzyme in bile acid synthesis, was not altered by FO feeding (Table IV). The group mean activities of microsomal ACAT and HMG-CoA reductase, the rate-limiting enzymes in cholesterol esterification and cholesterol synthesis, respectively, were decreased in rats fed FO but the lowering failed to reach statistical significance.

No dietary effects were observed as to the relative amounts of phospholipids and cholesterol in total hepatic plasma membranes, or in membrane fluidity (Table V). No attempts were made to differentiate between sinusoidal and canalicular membrane fractions in this study. FO treatment induced an almost threefold increase in hepatic alkaline phosphatase activity. Activities of the other plasma membrane enzymes were not differentially influenced by dietary FO and CO.

Hepatic processing of liposome-derived [^{3}H]cholesteryl oleate. To gain insight into the effects of dietary fat on hepatic handling of endocytosed cholesteryl ester, rats of both dietary groups were intravenously injected with small unilamellar liposomes labeled with [^{3}H]cholesteryl oleate. By using liposomes as vehicle for cholesteryl oleate, we bypassed potential effects of the diet on receptor-mediated uptake processes, as recently described for LDL particles in FO-treated rats (12). No dietary effects were observed on plasma elimination of the liposomes

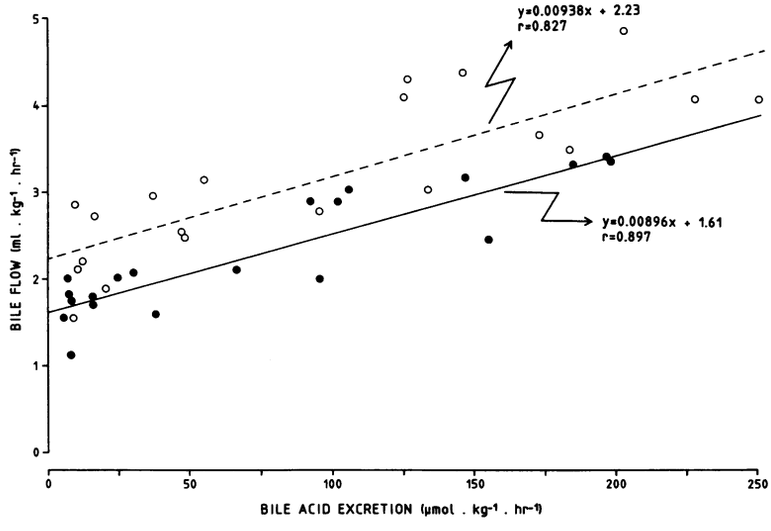

Figure 3. Relation between bile flow and biliary bile acid excretion measured in 1-h intervals during the first 4 h after interruption of the enterohepatic circulation of rats fed either with CO (●) ($n = 5$) or FO (○) ($n = 5$) for 4 wk. Regression analysis was carried out as first described by Berthelot et al. (42).

Table III. Bile Acid Pool Composition and Synthesis

	Bile acid pool		Bile acid synthesis	
	% of total bile acids		% of total bile acids	
	Corn oil	Fish oil	Corn oil	Fish oil
Cholic acid	42.8±2.9	57.5±2.6*	57.3±2.3	76.4±1.5*
Chenodeoxycholic acid	4.2±0.3	1.3±0.1*	24.6±2.8	8.1±1.0*
βMuricholic acid	10.5±3.8	5.8±1.6	12.9±1.0	11.0±0.8
αMuricholic acid	2.7±0.6	0.9±0.2*	nd	nd
Ursodeoxycholic acid	1.5±0.4	0.6±0.0	1.9±0.1	1.1±0.1*
Hyocholic acid	0.3±0.0	0.5±0.0*	0.5±0.1	0.8±0.1
3α,6α,7β,12α-Tetrahydroxy-5β-cholanoic acid	22.3±4.3	20.7±2.9	0.3±0.2	0.3±0.1
Hyodeoxycholic acid	5.5±0.3	3.4±0.6*	0.8±0.6	0.8±0.3
ωMuricholic acid	1.7±0.3	2.3±0.5	0.1±0.1	0.1±0.1
Deoxycholic acid	1.2±0.3	1.8±0.4	0.5±0.3	0.4±0.3
Unidentified	7.4±0.9	6.2±0.4	0.6±0.2	1.0±0.1

Two groups of animals were fed diets containing either CO or FO for 4 wk. Each value represents the mean±SEM for data obtained in five animals. Bile acid pool composition was measured in bile obtained during a period of 2 h immediately after interruption of the EHC. The absolute amounts of bile acids in the pool were 303±8 and 388±32 μmol/kg for the CO and FO group, respectively. Bile acid synthesis was measured in bile obtained during the period of 21–24 h after interruption of the EHC. The absolute amounts of bile acids synthesized during this period were 32.7±3.0 and 30.3±2.4 μmol/kg in the CO and FO group, respectively. * The values are significantly different at the $P < 0.05$ level from the corresponding values in the corn oil group.

(Fig. 4), indicating that the amount of labeled liposomes taken up by the liver was similar in the two experimental groups.

Biliary excretion of radioactivity occurred more rapidly in rats fed FO as compared to CO (Fig. 5). During the first 4 h after interruption of the EHC, output of radioactivity in the form of cholesterol was increased by 58% in rats fed FO. As described above, this effect was accompanied by a similar increase in biliary output of cholesterol mass. An increased incorporation of liposome-derived radioactivity into bile acids was also observed (+32%), at least during the initial 4-h period in which bile acids are drained from the pool (Fig. 5). After exhaustion of the bile acid pool, no significant differences were observed in biliary excretion of labeled bile constituents. The specific activities of bile acids and cholesterol of both dietary groups were similar (Fig. 6). The difference between the dietary groups in mass synthesis of individual bile acid species (see Table III) was also observed in the incorporation of the liposome-derived [^{3}H]cholesteryl ester into bile acids (Table VI): relatively more radioactivity was secreted in the form of cholic and less in chenodeoxycholic acid in FO-fed rats.

Table IV. Hepatic Cholesterol Content and Microsomal Enzyme Activities

	Dietary oil	
	Corn oil	Fish oil
Cholesterol (*nmol/mg protein*)		
Free	25.81±0.76 (10)	27.11±4.55 (11)
Esterified	12.86±1.60 (10)	12.65±6.60 (11)
Enzymes (*pmol/min per mg protein*)		
ACAT	766±130 (4)	567±91 (4)
HMG-CoA reductase	41±8 (4)	30±10 (4)
Cholesterol 7α-hydroxylase	3.8±0.6 (3)	3.9±0.4 (3)

Two groups of animals were fed diets enriched with CO or FO for 4 wk. Each value represents the mean±SEM. Number of animals in parentheses.

Discussion

This study shows that dietary FO as compared to CO induces a number of changes in hepatic cholesterol and bile acid metabolism in rats and provides important new information concerning the mechanism by which dietary n-3 fatty acids influence hepatic sterol metabolism. The decrease in plasma triglyceride concentrations after FO feeding was the most pronounced effect on plasma lipids, confirming results of other studies (10–12). A number of investigators have shown that FO reduces VLDL secretion by the liver in both humans and experimental animals (44–46), which explains the observed decrease in plasma triglyceride concentrations. The reduction in hepatic VLDL synthesis and secretion in turn, has been shown to be related to inhibition of hepatic triglyceride synthesis by n-3 fatty acids (45, 47–51). Apart from plasma triglyceride concentrations, dietary FO also lowers plasma cholesterol in the rat (10, 12). Recently, Ventura et al. (12) analyzed the effects of FO on plasma lipids of rats in detail. These workers compared n-3 fatty acids (FO) with n-6 fatty acids (safflower oil) and found a 38% decrease in plasma cholesterol concentrations with n-3 fatty acids. The lowering occurred mainly in the VLDL, LDL, and apoprotein E-containing HDL_1 particles, i.e., $d < 1.095$ g/ml density fractions. It was shown that this effect of FO was mainly due to a relatively high LDL receptor activity in the liver, in combination with a low LDL production rate.

Hepatic lipoprotein metabolism and bile acid synthesis are known to be connected by complex intrahepatic pathways (13, 14). Increased intake of cholesterol results in increased bile acid synthesis in the rat. Changes in the size of the bile acid pool have been shown to influence LDL receptor activity and LDL

Table V. Characterization and Fluidity of Plasma Membranes

	Dietary oil	
	Corn oil	Fish oil
Marker enzymes	Homogenate (specific activity)	
Glucose-6-phosphatase	4.38±0.44	4.74±0.48
Succinaat cyt. c reductase	2.38±0.19	2.43±0.18
Leucine aminopeptidase	0.46±0.05	0.48±0.04
Alkaline phosphatase	0.13±0.05	0.36±0.11*
Mg^{2+}ATPase	2.49±0.19	2.72±0.18
Na^+K^+ATPase	1.13±0.28	1.16±0.25
	Plasma membranes (relative enrichment)	
Glucose-6-phosphatase	1.07±0.26	0.85±0.27
Succinaat cyt. c reductase	1.72±0.29	1.40±0.33
Leucine aminopeptidase	1.94±0.24	3.53±1.47
Alkaline phosphatase	3.9±0.9	4.2±0.4
Mg^{2+}ATPase	7.2±1.6	9.1±2.4
Na^+K^+ATPase	5.9±3.0	6.0±1.2
ATPases (mean)	6.8±1.8	8.2±1.8
Fluidity		
Total membranes	0.149±0.009	0.157±0.021
Plasma membranes	0.176±0.013	0.199±0.025
Lipids in plasma membranes		
Cholesterol	0.093±0.015	0.103±0.026
Phospholipids	0.227±0.016	0.262±0.021

Data are given as means±SEM of four preparations from eight livers (for each preparation two livers were combined). Specific activities of enzymes are expressed as μmol product formed · mg protein^{-1} · h^{-1}. The fluidity is measured as the degree of fluorescence polarization of diphenylhexatriene. Cholesterol and phospholipid concentrations are expressed as μmol · mg protein^{-1}. * Significantly different at $P < 0.05$ from the corresponding value in the CO group. Relative enrichment is defined as the ratio of specific activity in the plasma membranes to specific activity in the homogenate.

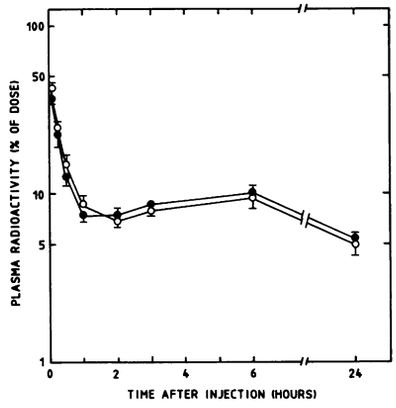

Figure 4. Elimination from plasma of intravenously injected liposomes in rats fed CO (●) or FO (○) for 4 wk. Data represent means±SEM for five animals.

production rates in the hamster, but the response in rats seems to be different; feeding of bile acids to rats did not affect hepatic LDL transport and plasma LDL cholesterol concentrations (52). It is known that the influx of lipoprotein cholesterol influences bile acid synthesis; recently, Junker and Davis (53) showed in isolated rat hepatocytes that receptor-mediated uptake of LDL increased bile acids synthesis, whereas receptor-independent uptake did not. The mechanism by which dietary FO enhances hepatic LDL receptor activity is not known. This effect may be mediated by a change in cholesterol balance across the liver, by a change in lipid environment of the receptor in the sinusoidal membrane, or by a change in compartmentation of cholesterol in the liver. In contrast to the situation in man, plasma cholesterol concentrations in the rat usually do not respond to changes in hepatic cholesterol balance, which makes the first option unlikely. For example, interruption of the EHC or feeding cholestyramine does not lead to higher hepatic receptor activity and consistently lower plasma cholesterol levels in this species (21, 41, 54). Our results suggest that FO does not induce gross changes in hepatic cholesterol balance, as we observed no change in cholesterol synthesis and bile acid synthesis. We found a 51% increase in biliary output of cholesterol (increase of 0.7 μmol/kg per h), but biliary output of cholesterol is relatively small in rats and can easily be compensated by a small decrease in bile acid synthesis (synthesis = ±12 μmol/kg per h). In addition, our results obtained with hepatic plasma membranes do not support the second possibility. In agreement with this, Ventura et al. found no dietary effects on receptor-mediated endocytosis of asialoglycoproteins (12). Our results, however, provide evidence for diet-induced changes in compartmentation of cholesterol in the liver.

We observed a 28% increase in bile acid pool size after FO feeding which was not due to an increased bile acid synthesis, as can be concluded from the in vivo bile acid production rates and the in vitro activity of cholesterol 7α-hydroxylase in isolated liver microsomes. The increased bile acid pool size in combination with an unaffected rate of de novo synthesis of bile acids suggests that intestinal absorption of the bile acids was more efficient in the FO-fed animals. The increase in pool size apparently did not result in a more effective feedback inhibition of bile acid synthesis although bile acid synthesis tended to be lower in the animals fed FO. These phenomena were accompanied by a considerable increase in the cholic acid/

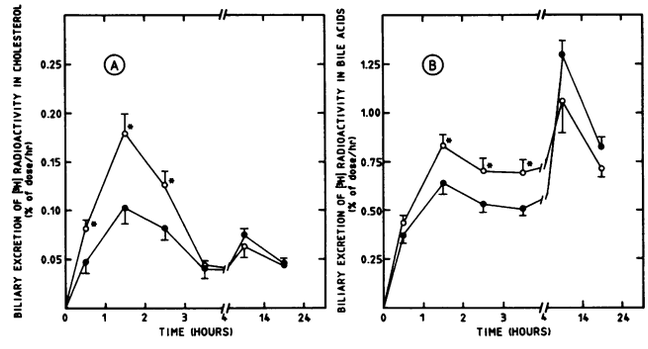

Figure 5. Biliary excretion of [^{3}H] radioactivity in cholesterol (*A*) and bile acids (*B*) after intravenous administration of [^{3}H]cholesteryl oleate–containing liposomes to rats fed CO (●) or FO (○) for 4 wk. Data represent means±SEM for five animals. *Significant difference ($P < 0.05$).

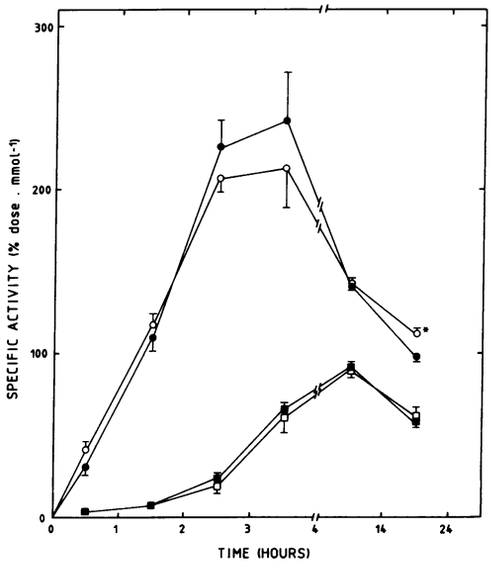

Figure 6. Specific radioactivity of biliary cholesterol (●, ○) and bile acids (■, □) in rats fed CO (*closed symbols*) or FO (*open symbols*) for 4 wk. Data represent means±SEM for five animals. *Significant difference ($P < 0.05$).

chenodeoxycholic acid ratio in the FO group, due to a diminished chenodeoxycholic acid synthesis. This effect was observed both in mass synthesis and in synthesis from a tracer of radiolabeled cholesterol (Tables III and VI). This difference in bile acid pool composition may account for the apparently less efficient feedback inhibition of cholesterol 7α-hydroxylase. More hydrophobic bile acids have been reported to be the most potent inhibitors (55–57). However, the intestines may also play a regulatory role in the EHC of bile salts as has recently been shown by Lilienau et al. (58). In rats, as in man, cholic acid is quantitatively the major bile acid synthesized in the liver. The observed change in cholic acid/chenodeoxycholic acid ratio suggests an effect of dietary FO on the regulation of the synthesis of the two primary bile acids in the liver. The molecular mechanism of this regulation is not fully understood, but it has been suggested that in rats the activity of the microsomal 12α-hydroxylase system plays a pivotal role or, alternatively, the activity of this enzyme relative to that of the microsomal and/or mitochondrial 26-hydroxylase (59). A lack of regulatory importance of 12α-hydroxylase in man was demonstrated by Björkhem (60). Whether FO induces changes in activities of the enzymes involved in bile acid synthesis is currently under investigation. In a number of other conditions changes in cholic acid/chenodeoxycholic acid ratio have been described. A patient with fish-eye disease, with high density lipoprotein levels less than 10% of normal, showed a reduced cholic acid/chenodeoxycholic acid ratio (61). Patients with alcoholic cirrhosis have a reduced cholic acid synthesis (62) and the thyroid hormone status has been shown to affect the cholic acid/chenodeoxycholic acid ratio in rats (63). It may be that the observed effects of FO on cholic and chenodeoxycholic acid synthesis reflect differences in the use of cholesterol from different precursor pools. Stange et al. (64) showed that in rats chenodeoxycholic acid is predominantly synthesized from preexisting cholesterol, whereas relatively more newly synthesized cholesterol is used for cholic acid synthesis. However, we found very similar changes in mass synthesis and synthesis from liposome-derived cholesterol, indicating no preferential incorporation of this preexisting cholesterol in any of the bile acid species.

Table VI. Synthesis of [^{3}H]-labeled Bile Acids

	[^{3}H]-labeled bile acids (% of total)	
Bile acids	Corn oil	Fish oil
Cholic acid	55±3	77±2*
βMuricholic acid	13±1	11±1
Chenodeoxycholic acid	27±2	8±1*
Polar bile acids	5±1	4±2

Two groups of animals were fed diets enriched with corn oil or fish oil for 4 wk. Each value represents the mean±SEM for data obtained in five animals. Synthesis of labeled bile acids was measured in bile obtained during the period of 21–24 h after interruption of the EHC.
* The values are significantly different at the $P < 0.05$ level from the corresponding values in the corn oil group.

Despite similar rates of total bile acid synthesis in both experimental groups, the incorporation of liposome-derived labeled cholesterol into bile acids was higher (+30%) in the FO group during the first period after interruption. In this period bile acids are drained from the endogenous pool, resembling the natural "intact" situation in which large amounts of bile acids continuously pass through the liver. The results suggest that the higher flow of bile acids through the liver in the FO group somehow induces a shift of endocytosed liposome-derived cholesterol to the microsomal compartment for bile acid synthesis. In our study a 28% increase in bile acid transport resulted in a proportional 30% increase in microsomal incorporation of labeled cholesterol into bile acids. In agreement with this explanation is the observation that the increased incorporation of label in bile acids had disappeared after exhaustion of the bile acid pool. In this period the synthesis of bile acids from labeled cholesterol tended to be lower in the FO group, which is in line with the tendency towards lower hepatic bile acid synthesis rates. As a result, no difference in specific activity of the bile acids was observed between both dietary groups. From the present results, it cannot be determined whether the induced shift in cholesterol transport to the microsomal compartment is typical for an FO diet or represents a nonspecific secondary effect of the increased transport and processing of bile acids from the sinusoidal to the canalicular site of the liver.

We observed no significant differences between the dietary groups in hepatic cholesterol synthesis (HMG-CoA reductase activity), cholesterol esterification (ACAT activity), and in the amounts of free and esterified cholesterol in the liver. A decrease in hepatic cholesterol synthesis by dietary FO in comparison with n-6 polyunsaturated fatty acids has been observed by Topping et al. (65), whereas we and others observed no difference (this study, 12). This provides evidence again that cholesterol synthesis does not play a major regulatory role in determining plasma cholesterol levels in rats fed FO.

In our study, biliary excretion of cholesterol was increased by 51% in the FO group during the first period after interruption of the EHC in concert with the higher output of bile acids. The lithogenic index of bile was slightly higher in the FO group as compared to the CO group, but the difference was not significant. Biliary output of labeled cholesterol was increased by 58%

in the FO group. The resulting similar specific activities between both dietary groups indicates that the liposome-derived cholesterol was diluted to the same extent in intracellular pools destined for biliary excretion. Our results differ from those reported by Balasubramaniam et al. (10) who found no change in the amount of bile acids excreted and a fivefold increase in cholesterol excretion in rats fed FO. These investigators used much younger rats which might explain the different results.

In addition to the changes in hepatic cholesterol and bile acid metabolism, dietary FO induced increases in hepatic alkaline phosphatase activity and the BAIF. The BAIF was increased to 2.23 ml/kg per h in rats fed FO. In previous studies (66) we determined a value of 1.61 ml/kg per h in rats fed lab chow, which is similar to the value observed in the CO group. It has been proposed that the BAIF is to a large extent determined by excretion of glutathione (67). However, glutathione is probably not involved in the FO-induced increase in BAIF, since we did not find an increased biliary excretion of glutathione (results not shown). Also, no differences were observed in membrane cholesterol, phospholipids, fluidity, and NaK-ATPase activities, which suggests that these parameters are not involved in the observed diet-induced change in bile flow (68). Hepatic alkaline phosphatase activity was threefold increased in the FO group. This is compatible with results of Stenson et al. (69), who observed an increase in alkaline phosphatase activity in the small intestine of rats after feeding a FO diet. The authors suggested that changes in the local lipid environment of the enzyme caused the observed increase in activity. Brasitus et al. (70) concluded that in the small intestinal microvillus membrane the cholesterol/phospholipid molar ratio rather than total membrane fluidity affects alkaline phosphatase activity: a decrease of this ratio was associated with increased enzyme activity. In our experiments, however, no differences in cholesterol/phospholipid content were found, although of course local lipid microenvironments may have been changed in response to the different diets, thus modifying alkaline phosphatase activity. Our data on hepatic alkaline phosphatase are in perfect agreement with those recently reported by Ogawa et al. (71), who showed that an increased bile acid flux through the liver stimulates alkaline phosphatase activity, especially bile acids containing a 12α-hydroxyl group. Hence, the high hepatic activity of alkaline phosphatase in the FO rats is probably related to the increased flux of bile acids, especially that of cholic acid.

This study demonstrates that, in the rat, concomitant with the well-known FO-induced decrease in plasma cholesterol and triglyceride levels, a number of changes in hepatic cholesterol metabolism occur. Overall hepatic sterol fluxes were altered by an increased bile acid transport through the liver and a higher biliary excretion of cholesterol. Indicators of FO-induced shifts in intrahepatic pathways of cholesterol were the qualitative change in bile acid synthesis and preferential use of liposome-derived endocytosed cholesterol for bile acid synthesis. These effects were accompanied by an increase in BAIF and alkaline phosphatase activity, but not by changes in total hepatic cholesterol content, cholesterol synthesis or esterification, and total bile acid synthesis. The ultimate result was a more rapid disposition of endocytosed cholesterol into the bile. Whether there is a relation between these events and the regulation of hepatic lipoprotein receptor activity and lipoprotein synthesis remains to be established.

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