3. Originalarbeiten

3.1 Effekt von Eisen und Fischöl auf die Oxysterolkonzentrationen in der Leber von Ratten

BRANDSCH C, RINGSEIS R, EDER K (2002) High dietary iron concentrations enhance the formation of cholesterol oxidation products in the liver of adult rats fed salmon oil with minimal effects on antioxidant status. *Journal of Nutrition* 132:2263-2269.

ABSTRACT The aim of this study was to investigate the effect of high dietary iron concentrations on the antioxidant status of rats fed two different types of fat. Four groups of male adult Sprague-Dawley rats were fed diets with adequate (50 mg iron supplemented per kg diet) or high (500 mg iron supplemented per kg diet) iron concentrations with either lard or salmon oil as dietary fat at 100 g/kg for 12 wk. The antioxidant status of the rats was profoundly influenced by the type of fat. Rats fed salmon oil diets had higher concentrations of thiobarbituric acid-reactive substances (TBARS) ($P<0.001$), various cholesterol oxidation products (COP) ($P<0.001$), total and oxidized glutathione ($P<0.05$) and a lower concentration of α-tocopherol ($P<0.05$) in liver and plasma than rats fed lard diets. The iron concentration of the diet did not influence the concentrations of TBARS, the activities of superoxide dismutase and glutathione peroxidase or the concentration of α-tocopherol in plasma or liver. The activity of catalase ($P<0.01$) and the concentrations of total, oxidized and reduced glutathione ($P<0.05$) in liver were slightly but significantly higher in rats fed high iron diets than in rats fed adequate iron diets, irrespective of the dietary fat. Rats fed the high iron diets with salmon oil, moreover, had higher concentrations of various COP in the liver ($P<0.001$) than rats fed adequate iron diets with salmon oil. These results suggest that feeding a high iron diet does not generally affect the antioxidant status of rats but enhances the formation of COP, particularly if the diet is rich in polyunsaturated fatty acids.

KEY WORDS: iron ● lard ● salmon oil ● antioxidant status ● cholesterol oxidation products ● rats

[2]Abbreviations used: COP, cholesterol oxidation products; FAME, fatty acid methyl esters; GSH, glutathione; GSH-Px, glutathione peroxidase; GSSG, oxidized glutathione; PUFA, polyunsaturated fatty acids; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances

Iron deficiency is a common nutritional problem (1), but there are some instances in which iron overload is considered a health issue as well. Iron overload occurs primarily in individuals with inherited disorders such as hereditary hemochromatosis, juvenile hemachromatosis, atransferrinemia and Friedreich´s ataxia (2). A high dietary intake of iron through meat or nutritional supplements is also a potential cause of iron overload. Iron promotes the generation of oxygen radicals (especially hydroxyl radicals via Fenton Chemistry), which may cause oxidative damage such as degradation of proteins and nucleic acids and peroxidation of polyunsaturated fatty acids (PUFA)[2] (3, 4). There are several animal and epidemiologic studies suggesting that a high iron status may increase the risk of heart disease (5, 6) and cancer, especially colon carcinoma (7, 8), whereas others did not confirm this (1, 9). One reason for the discrepancy between different feeding studies could be the fat used in the diet, because the effect of high dietary iron concentrations on the antioxidant system might be dependent on the type of fat. PUFA are a source of oxidative stress. The effect of high dietary iron is therefore likely to be strongest when diets with high

concentrations of PUFA are used. Little published information is available to date about the interactions between high dietary iron concentrations and different dietary fats. The present study was therefore carried out to determine the effect of high iron diets on the antioxidant status of rats fed two different dietary fats, either lard or salmon oil.

Several studies have investigated the effect of iron on lipid peroxidation (10-12). In many of these studies, the generation of thiobarbituric acid-reactive substances (TBARS) is a preferred method for estimation of lipid peroxidation. But in addition to PUFA, cholesterol may also undergo oxidative modifications. Cholesterol oxidation products (COP) have been demonstrated to possess a wide variety of biological effects and are especially implicated in the pathogenesis of atherosclerosis via oxides in LDL (13). The formation of COP may occur through enzymatic or nonenzymatic oxidation. Nonenzymatic processes include autoxidations involving several active oxygen species. It is believed that transition metal ions such as iron act as catalysts in tissue. Iron accumulation in tissues can therefore be expected to enhance the formation of COP. Studies about the relationship between the iron supply of animals and the formation of COP are lacking. Miyajima et al. (14) described enhanced concentrations of 7β-hydroxycholesterol and 7-ketocholesterol in brains and visceral organs of patients with aceruloplasminemia, a phenomenon characterized by excessive neurovisceral iron accumulation. The concentrations of these COP correlated with the amount of iron accumulated in various organs. The authors concluded that lipid peroxidation induced by the intracellular accumulation of iron is involved in the pathogenesis of aceruloplasminemia. It seems possible that raised iron levels in tissues as a result of dietary overload may also lead to enhanced lipid peroxidation and enhanced generation of COP. Our study therefore included the measurement of COP in the liver.

In general, animal studies allow standardized treatments, although many different regimens have been described. Iron overload can be achieved with different iron sources and different methods of administration. A feeding trial would be the method of choice for testing the effects of iron overload through supplementation. It is important to consider the possibility that high levels of iron may promote oxidation of the diet even before it has been fed to the animals. In preliminary studies, we prepared diets with high iron concentration (500 mg/kg diet) and PUFA-rich salmon oil (100 g/kg diet). After 7 d storage at 4°C, the diets had peroxide values >1200 mEq O_2/kg oil. Even when salmon oil was replaced with lard, the diets had peroxide values of ~260 mEq O_2/kg oil. Feeding diets with high concentrations of lipid peroxidation products has profound effects on the antioxidant system (15). Therefore, if diets with high concentrations of lipid peroxidation products are used, it is impossible to determine which effects are caused by high dietary iron concentrations per se and which are caused by dietary lipid peroxidation products. In the present study, such methodological problems were avoided by providing iron and fat in two separate dietary components.

MATERIALS AND METHODS

Animals. Adult male Sprague-Dawley rats (n = 40) with an average initial body weight of 244 ± 7.7 g (mean ± SD), obtained from Charles River GmbH (Sulzfeld, Germany), were randomly assigned to one of four groups of 10 rats each. They were housed individually in Macrolon cages in a room controlled for temperature (22 ± 2°C), humidity and light (12-h light:dark cycle). All animal procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the regional council of Saxony-Anhalt.

Diets. According to a bifactorial experimental design, four diets were used, differing in their iron supplement and their fat (**Table 1**). Iron was supplemented at a concentration of either 50 mg/kg diet ("adequate iron diets") or 500 mg/kg diet ("high iron diets"). Ferrous sulfate was used as the iron source because it is highly bioavailable and often used in iron supplements and for food fortification. The basal iron concentration of the diet was 10 mg/kg.

The total iron concentrations of the diets were 60 and 510 mg/kg in the iron-adequate and the high iron diets, respectively. The dietary fat was either lard (obtained from O. Stiegele Schmalzsiederei, Dresden, Germany) or salmon oil (obtained from Caelo GmbH, Hilden, Germany), at a concentration of 100 g/kg diet. The fatty acid composition and the tocopherol concentrations of the fats are shown in **Table 2**. We equalized the tocopherol concentrations of the fats by supplementing them individually with all-rac-α-tocopheryl acetate to a final concentration of 400 mg α-tocopherol equivalents/kg oil (allowing for the fact that the biopotency of all-rac-α-tocopheryl acetate in rats is 67% of that of α-tocopherol (16,17)). Both types of diet contained 40 mg α-tocopherol equivalents per kg. To avoid autoxidation of dietary PUFA, fat and iron were administered in two separate diet portions, which were administered in identical amounts. The two portions combined yielded the whole diet of the rats.

TABLE 1

Composition of the experimental diets fed to rats for 12 wk

	Diet			
Dietary fat	Lard	Lard	Salmon oil	Salmon oil
Dietary iron supplement (mg/kg)	50	500	50	500
Ingredient	*g/kg diet*			
Casein	200	200	200	200
Corn starch	408	408	408	408
Saccharose	200	200	200	200
Lard	100	100	-	-
Salmon oil	-	-	100	100
Cellulose	29.83	28.33	29.83	28.33
Mineral mixture[1]	40	40	40	40
Vitamin mixture[2]	20	20	20	20
DL-Methionine	2	2	2	2
Ferrous sulfate[3]	0.167	1.67	0.167	1.67

[1] Minerals supplemented (per kg diet): dicalcium phosphate, 8.92 g; calcium carbonate 8.44 g; potassium sulfate, 9.0 g; sodium chloride, 2.6 g; magnesium oxide, 166 mg; zinc oxide, 38 mg; cupric sulfate pentahydrate, 24 mg; manganous oxide, 16 mg; calcium iodate, 0.32 mg; sodium selenate, 0.33 mg.

[2] Vitamins supplemented (per kg diet): all-trans-retinol, 1.67 mg; cholecalciferol, 25 µg; menadione sodium bisulfate, 1 mg; thiamine-HCL, 5 mg; riboflavine, 6 mg; pyridoxine-HCL, 6 mg; nicotinic acid, 30 mg; Ca pantothenate, 15 mg; folic acid, 2 mg; biotin, 0.2 mg; cyanocobalamine, 0.025 mg; 1 g choline chloride.

[3] Ferrous sulfate hydrate contained 30 % iron.

TABLE 2

Fatty acid composition and α-tocopherol concentrations of the dietary fats

	Lard	Salmon oil
Fatty acids[1]	*g/100 g fatty acids*	
14:0	1.6	5.9
16:0	26.9	13.2
16:1 (n-7)	1.9	7.4
18:0	18.4	2.6
18:1 (n-9)	36.3	13.2
18:2 (n-6)	9.4	2.2
18:3 (n-3)	0.9	0.8
20:1 (n-9)	0.8	7.9
20:4 (n-6)	-	1.6
20:5 (n-3)	-	9.5
22:5 (n-3)	-	0.8
22:6 (n-3)	-	8.8
Tocopherols	*mg/kg*	
α-tocopherol	18	83
All-rac-α-tocopheryl acetate (supplemented)	569	476
Total tocopherol equivalents[2]	400	400

[1] Fatty acids in quantities <0.5 g/100g are not reported.

[2] Total tocopherol equivalents were calculated according to biological activities assessed in rat assays (16,17): mg total tocopherol equivalents = mg α-tocopherol + (mg all-*rac*-α-tocopheryl acetate X 0.67).

The diets were prepared weekly, freeze-dried and stored at 4°C. Lipid peroxidation was monitored by measuring the peroxide value. The peroxide value, determined in diets that were 8 d old, was 1.8 mEq O_2/kg diet in the salmon oil diets and 0.95 mEq O_2/kg diet in the lard diets.

Diets were administered in restricted amounts to standardize the feed intake. Feeding took place once daily at 0800 h. The amount of food offered daily was 18 g. Water was freely available from nipple drinkers. The diets were fed for 12 wk.

Sample preparation. Feces of five rats per group were collected from wk 8 to 12 to determine the digestibility of the iron. At the end of the feeding period, the rats were deprived of food overnight, anesthetized with diethyl ether and killed by decapitation. Blood was collected into heparinized polyethylene tubes and EDTA-treated tubes. Plasma was prepared by centrifuging the heparinized blood (1100 X g, 10 min), and the remaining red blood cells (RBC) were washed three times with 9 g/L sodium chloride solution. Liver and duodenum were removed. Identical sections of the duodenum were cut open lengthwise and mucosa was scraped from duodenal tissue with a clean microscope slide. All samples were immediately shock-frozen in liquid nitrogen and stored at -20°C pending analysis.

Hematological parameters. Whole EDTA blood was used immediately for hematocrit and hemoglobin determination as well as counting of different cell type numbers using "Buffy Coat" profiles with the VetAutoRead hematology system (Becton Dickinson, Wörrstadt, Germany). After uptake of the fluorescent dye acridine orange, different cell types were identified by their different fluorescent properties.

Iron analysis. Atomic absorption spectrometry (model # 3300, Perkin Elmer, Rodgau-Jügesheim, Germany) was used to determine the iron concentrations in diets, liver and feces. Aliquots of freeze-dried liver were dissolved in nitric acid and ashed under pressure at 170°C for 6 h. The ashes were dissolved in deionized water. Aliquots of diet and dried fecal samples were ashed at 550°C for 16h. The ashes were dissolved in hydrochloric acid at boiling temperature. All samples were filtered, measured at 248.3 nm and calculated with reference to standards.

Antioxidant status. Concentrations of α-tocopherol in plasma, liver, and dietary fats were determined by HPLC (HP 1100, Hewlett Packard, Waldbronn, Germany) (18). Samples (200 µL plasma, 50 mg liver) were mixed with 1 mL of 0.1 g/L pyrogallol solution (ethanol, absolute) and 150 µL of saturated sodium hydroxide solution. This mixture was heated for 30 min at 70°C, and tocopherols were extracted with n-hexane. Dietary fat samples were diluted 1:100 with n-hexane. Individual tocopherols of the extracts were separated isocratically using a mixture of n-hexane and 1,4 dioxane (96:4, v/v) as the mobile phase and a LiChrosorb Si-60 column (5-µm particle size, 250 mm length, 4 mm i.d., Merck, Darmstadt, Germany), and detected by fluorescence (excitation wavelength, 295 nm; emission wavelength, 330 nm).

Catalase activity was determined in liver homogenate at 25°C using hydrogen peroxide as the substrate by the method of Beers and Sizer (19). One unit of catalase activity is defined as the amount consuming 1 µmol hydrogen peroxide/min. Total superoxide dismutase (SOD) activities of duodenal mucosa homogenate and RBC were determined using the method of Marklund and Marklund (20) with pyrogallol as the substrate. One unit of SOD activity is defined as the amount of enzyme required to inhibit the autoxidation of pyrogallol by 50%. Glutathione peroxidase (GSH-Px) activity in plasma was measured with t-butyl hydroperoxide at 25°C according to the method of Paglia and Valentine (21) as modified by Levander et al. (22). One unit of GSH-Px activity is defined as 1 µmol NADPH oxidized/min. Total glutathione (GSH) concentration was determined in protein-free liver homogenates according to Griffith (23) with glutathione reductase and Ellman´s reagent. Calibration was performed using a standard curve. Oxidized glutathione (GSSG) was detected after derivatization of GSH with 2-vinylpyridine. The concentration of reduced GSH was calculated as total GSH – 2 X GSSG. Sample protein content was determined according to the

method of Lowry et al. (24). Enzyme activities of RBC, liver, and mucosa were expressed per milligram or gram protein.

Lipid analysis. The fatty acid composition of the dietary fats was determined by gas chromatography. Fats were methylated with trimethylsulfonium hydroxide (25). Fatty acid methyl esters (FAME) were separated by gas chromatography, using a system (HP 5890, Hewlett Packard GmbH, Böblingen, Germany) equipped with an automatic on-column injector, a polar capillary column (30 m FFAP, 0.53 mm i.d., Macherey and Nagel, Düren, Germany) and a flame ionisation detector. Helium was used as the carrier gas with a flow rate of 5.4 mL/min. FAME were identified by comparing their retention times with those of individually purified standards. Concentrations of total cholesterol and triglycerides in plasma were determined using commercially available enzymatic reagent kits (Merck; Cat. Nos. 1.14830, 1.14856).

Thiobarbituric acid reactive substances. TBARS were measured in plasma, liver, and mucosal homogenates using a modified version of the TBARS assay (26). Sample aliquots were mixed with thiobarbituric acid reagent (8 g/L thiobarbituric acid with 7% perchloric acid, 2:1,v/v) and heated for 60 min at 95°C. TBARS were extracted with n-butanol, and absorption was measured at 532 nm. Concentrations were calculated via a standard curve with 1,1,3,3,-tetraethoxypropan.

Cholesterol oxidation products. COP were determined in liver by gas chromatography/mass spectrometry in selected ion monitoring mode according to Mori et al. (27) with modifications. Total lipids of liver were extracted with a hexane/isopropanol mixture (3:2, v/v) (28). Aliquots of the extracts were mixed with 20 µg of the internal standard 5α-cholestane. The probes were dried under nitrogen, mixed with 2 mL of 1 mol/L methanolic potassium hydroxide, and incubated for 18 h at room temperature. Cholesterol oxides were extracted with 2 mL diethyl ether into the nonsaponifiable fraction, separated, dried under nitrogen, and dissolved in 100 µL pyridine. After derivatisation of the oxides with 100 µL 0.1 g/L bis(trimethylsilyl)-trifluoroacetamide, samples were separated using a nonpolar capillary column (DB-5, 30 m X 0.25 mm i.d., J&W Scientific, Folsom, CA) and detected by single ion monitoring. Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The cholesterol oxides 7β-hydroxycholesterol, 7-ketocholesterol, cholesterol-5α,6α-epoxide, cholesterol-5β,6β-epoxide, cholestanetriol, and 25-hydroxycholesterol were identified by comparing their retention times with those of authentic standards and quantified with the internal standard.

Statistics. Treatment effects were analyzed by two-way ANOVA using the Minitab statistical software (Release 13, Minitab, State College, PA). Classification factors were iron level and fat type, as well as their interaction. Means of the four treatment groups were compared by Fisher`s multiple range test. Differences were considered significant if $P<0.05$. Values in the text are means ± SD.

RESULTS

Body weight. Body weight gains during the experimental period were slightly, but significantly higher in the rats fed the high iron diets than in the rats fed the iron-adequate diets (n = 20; adequate iron diets, 212 ± 20.4 g; high iron diets, 227 ± 16.8 g; $P<0.05$). Rats fed salmon oil diets had higher body weight gains than rats fed lard diets (n = 20; salmon oil diets, 231 ± 16.1 g; lard diets, 208 ± 17.4 g; $P<0.05$).

Iron status. Liver iron concentration was significantly influenced by dietary iron concentration and dietary fat (**Table 3**). It was significantly higher in rats fed high iron diets than in rats fed adequate iron diets. The difference between rats fed the high iron diets and rats fed the adequate iron diets was ~20%. Rats fed the lard diet had significantly higher hepatic iron concentrations than rats fed the salmon oil diet. Fecal iron concentration, iron excretion, and apparent iron digestibility were significantly affected by the dietary iron

concentration, but not by the dietary fat type. The iron concentration of the feces and the amount of iron excreted with the feces were approximately four times higher in rats fed the high iron diets than in rats fed the adequate iron diets. In the rats fed the adequate iron diets, ~25% of the iron was apparently absorbed; in the rats fed the high iron diets, the apparent digestibility of iron was slightly negative, indicating that in a given period, they excreted more iron than they consumed.

TABLE 3

Iron concentration in liver and feces and apparent digestibility of iron of rats fed diets with two levels of iron (50 or 500 mg/kg supplemented) containing either lard or salmon oil at 100 g/kg diet for 12 wk [1,2,3]

Fat		Lard		Salmon oil		ANOVA (P<)		
Iron supplemented, *mg/kg diet*	n	50	500	50	500	Iron	Fat	Iron x Fat
Iron concentration								
Liver, *µmol/g DM*	10	8.04 ± 1.41[b]	9.68 ± 1.85[a]	6.63 ± 1.48[b]	7.89 ± 1.46[b]	0.01	0.01	NS
Feces, *µmol/g DM*								
wk 8	5	23.4 ± 2.8[b]	115 ± 8[a]	23.5 ± 2.1[b]	114 ± 8[a]	0.001	NS	NS
wk 12	5	24.4 ± 2.9[b]	113 ± 5[a]	23.7 ± 2.7[b]	111 ± 13[a]	0.001	NS	NS
Fecal excretion of iron, *mmol/7d*								
wk 8	5	0.24 ± 0.01[b]	1.25 ± 0.08[a]	0.25 ± 0.04[b]	1.17 ± 0.07[a]	0.001	NS	NS
wk 12	5	0.27 ± 0.01[b]	1.30 ± 0.09[a]	0.27 ± 0.01[b]	1.22 ± 0.17[a]	0.001	NS	NS
Apparent digestibility of iron, *%*								
wk 8	5	26.5 ± 3.7[a]	-5.70 ± 2.13[b]	28.4 ± 5.1[a]	-1.39 ± 6.09[b]	0.001	NS	NS
wk 12	5	22.1 ± 2.9[a]	-4.64 ± 7.61[b]	24.4 ± 3.6[a]	1.99 ± 1.37[b]	0.001	NS	NS

[1] Results are means ± SD.
[2] Means in a row without a common superscript letter differ, P<0.05.
[3] Abbreviations: DM, dry matter; NS, non significant (P≥0.05).

Hematological variables. Hematological variables did not differ among the four groups (overall means ± SD, n = 40; hematocrit, 0.44 ± 0.05; hemoglobin, 14.5 ± 1.93 g/L; reticulocytes, 2.84 ± 1.08%; leukocyte count, 7.46 ± 3.71 X 10^9/L; granulocyte count, 5.18 ± 2.88 X 10^9/L). The numbers of lymphocytes and monocytes, however, were higher in the rats fed salmon oil (2.82 ± 1.49 X 10^9/L) than in the rats fed lard (1.73 ± 0.58 X 10^9/L, P<0.05).

Antioxidant status. The concentrations of TBARS in plasma, liver, and mucosa were not influenced by the dietary iron concentration (**Table 4**), but the dietary fat affected the concentrations of TBARS in plasma and liver. Rats fed salmon oil diets had significantly higher concentrations of TBARS in plasma and liver than rats fed lard diets. The concentrations of TBARS in the mucosa did not differ among the four treatment groups.

TABLE 4

Concentration of thiobarbituric acid reactive substances in plasma, liver and mucosa of rats fed diets with two levels of iron (50 or 500 mg/kg supplemented) containing either lard or salmon oil at 100 g/kg diet for 12 wk [1,2,3]

Fat	Lard		Salmon oil		ANOVA (P<)		
Iron supplemented, *mg/kg diet*	50	500	50	500	Iron	Fat	Iron x Fat
Plasma, *µmol/l*	14.0 ± 1.4[b]	16.0 ± 2.5[b]	29.6 ± 7.7[a]	29.6 ± 8.5[a]	NS	0.001	NS
Liver, *nmol/g wet tissue*	38.0 ± 1.9[b]	42.1 ± 2.0[b]	62.4 ± 17.9[a]	64.8 ± 3.6[a]	NS	0.001	NS
Mucosa, *nmol/g protein*	92.8 ± 30.9	92.2 ± 34.6	95.8 ± 37.2	126 ± 30	NS	NS	NS

[1] Results are means ± SD, n=10.
[2] Means without a common superscript letter differ, P<0.05.
[3] Abbreviations: NS, non significant (P≥0.05).

The concentrations of hepatic COP were affected by the dietary iron concentration and fat **(Table 5)**. The concentrations of all COP measured were one- to threefold higher in rats fed salmon oil diets than in rats fed lard diets. The effect of the dietary iron on the concentrations of some COP depended on the type of dietary fat. Rats fed the salmon oil diet with high iron concentration had significantly higher concentrations of 7β-hydroxycholesterol, 7-ketocholesterol, and cholestanetriol in the liver than rats fed the salmon oil diet with adequate iron. In contrast, in rats fed the lard diet with high iron concentration, only the concentration of cholestanetriol was significantly higher than in rats fed the lard diet with adequate iron; the concentrations of 7β-hydroxycholesterol and 7-ketocholesterol did not differ between these two groups. The concentrations of cholesterol epoxides were not affected by the dietary iron concentration. There was a significant interaction between iron and fat on the concentrations of 7β-hydroxycholesterol and 7-ketocholesterol.

TABLE 5

Concentrations of various cholesterol oxidation products in liver of rats fed diets with two levels of iron (50 or 500 mg/kg supplemented) containing either lard or salmon oil at 100 g/kg diet for 12 wk[1,2,3]

Fat	Lard		Salmon oil		ANOVA (P<)		
Iron supplemented, *mg/kg diet*	50	500	50	500	Iron	Fat	Iron x Fat
	nmol/g wet liver						
7β-Hydroxycholesterol	9.81 ± 2.87[c]	11.1 ± 2.4[c]	22.2 ± 5.2[b]	42.8 ± 15.6[a]	0.001	0.001	0.01
7-Ketocholesterol	7.99 ± 4.69[b]	7.39 ± 2.78[b]	12.6 ± 3.5[b]	26.8 ± 10.7[a]	0.01	0.001	0.01
Cholesterol 5α,6α-epoxide	5.75 ± 1.59[b]	5.79 ± 0.40[b]	14.3 ± 6.5[a]	14.1 ± 4.2[a]	NS	0.001	NS
Cholesterol 5β,6β-epoxide	8.78 ± 3.38[b]	8.07 ± 2.56[b]	18.8 ± 9.9[a]	17.5 ± 4.9[a]	NS	0.001	NS
Cholestanetriol	0.59 ± 0.19[c]	1.24 ± 0.24[b]	1.47 ± 0.36[b]	1.97 ± 0.38[a]	0.001	0.001	NS
25-Hydroxycholesterol	0.17 ± 0.05[b]	0.24 ± 0.05[b]	0.30 ±0.10[ab]	0.37 ± 0.10[a]	0.05	0.001	NS

[1] Results are means ± SD, n=10.
[2] Means without a common superscript differ, P<0.05.
[3] Abbreviations: NS, non significant (P≥0.05).

Of the antioxidative enzymes measured, only the activity of hepatic catalase was affected by the dietary iron concentration **(Fig. 1)**. Rats fed high iron diets had significantly greater activity than rats fed adequate iron diets. Dietary fat had no effect on hepatic catalase activity. The activities of GSH-Px in plasma as well as of SOD in RBC and mucosa were not affected by either dietary iron or dietary fat (overall means ± SD, n = 40; GSH-Px, 2.51 ± 1.01 kU/L; RBC SOD, 9.97 ± 2.13 U/mg protein; mucosa SOD, 7.62 ± 2.23 U/mg protein).

The concentration of hepatic glutathione was influenced by the dietary iron and fat **(Fig. 2)**. Rats fed the high iron diets had significantly greater concentrations of total GSH, reduced GSH, and GSSG in the liver than rats fed adequate iron diets. Rats fed salmon oil diets had significantly higher concentrations of total GSH as well as GSSG than rats fed lard diets; the concentration of reduced GSH was not affected by dietary fat.

The concentrations of α-tocopherol in plasma and liver were not influenced by the dietary iron. However, rats fed the lard diets had higher concentrations of α-tocopherol in liver and plasma than rats fed the salmon oil diets (means ± SD, n = 20; liver, 1.71 ± 0.54 vs. 0.71 ± 0.21 µmol/mmol triglycerides plus cholesterol, $P<0.001$; plasma, 6.73 ± 1.64 vs. 5.08 ± 1.56 µmol/mmol triglycerides plus cholesterol, $P<0.05$).

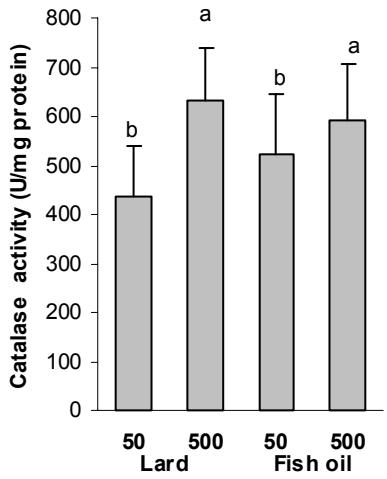

FIGURE 1 Hepatic catalase activity in rats fed diets with two levels of iron (50 or 500 mg/kg supplemented) containing either lard or salmon oil at 100 g/kg diet for 12 wk. Values are means ± SD, n=10. Means with different letters differ by Fisher´s multiple range test, $P<0.05$. Results of ANOVA: iron, $P<0.01$; fat, non significant; iron x fat, non significant.

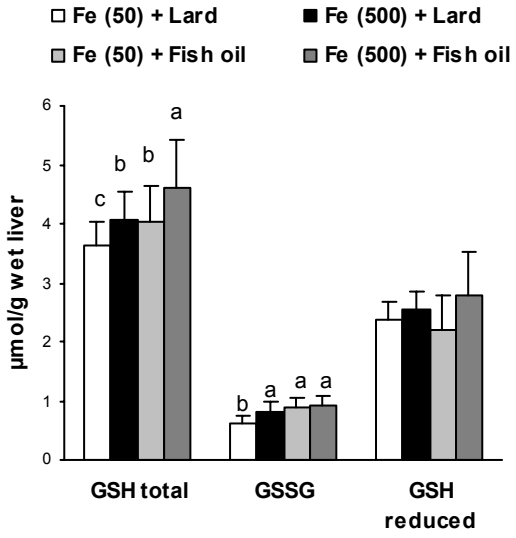

FIGURE 2 Concentrations of total glutathione (GSH total), oxidized glutathione (GSSG) and reduced glutathione (GSH reduced) in the liver of rats fed diets with two levels of iron (50 or 500 mg/kg supplemented) containing either lard or salmon oil at 100 g/kg diet for 12 wk. Values are means ± SD, n=10. Means with different letters differ, $P<0.05$. Results of ANOVA: GSH total: iron, $P<0.01$; fat, $P<0.05$; iron x fat, non significant. GSSG: iron, $P<0.05$; fat, $P<0.001$; iron x fat, non significant. GSH reduced: iron, $P<0.05$; fat, non significant; iron x fat, non significant.

DISCUSSION

We investigate the effect of high dietary iron concentrations on the antioxidant status of rats fed PUFA-rich fat compared with rats fed a fat low in PUFA. In addition to conventional components of the antioxidant system, we measured hepatic COP. To our knowledge, the effect of high iron diets on the formation of COP in association with various dietary fats has not previously been investigated.

Surprisingly, feeding the high iron diets (500 mg iron supplemented/kg) increased the hepatic iron concentration only 20% compared with feeding the iron-adequate diets (50 mg iron supplemented/kg). The hepatic iron concentrations in rats fed the high iron diets were lower than those observed in similar studies in which rats were fed high iron diets (9,10). The difference between those studies and our own results could be attributable to the age of the rats used; in our study, adult rats were used, whereas Bristow-Craig et al. (10) and Soyars and Fisher (9) studied growing rats. The moderate accumulation of iron in the livers of rats fed the high iron diets could also be attributed to a marked reduction in the apparent digestibility of

iron induced by a high iron intake. A net loss of iron was observed in the rats fed the high iron diets, suggesting a mechanism for preventing iron overload via increased excretion. The apparent digestibility of iron depends on the body's iron stores (1). In a study by Reddy and Cook (29), feeding a diet with 500 mg iron/kg lowered the rate of iron digestibility from 50 to 27% within only 1 wk; in a study by Rimbach and Pallauf (30) increasing the dietary iron concentration from 30 to 300 mg/kg reduced the apparent digestibility of iron from 25 to 8%. The observation that rats fed salmon oil have lower concentrations of hepatic iron than rats fed lard is consistent with other published studies that demonstrated that fish oil reduces the absorption of iron (31,32). The amounts of iron excreted with the feces in the wk 8 and 12 of the experiment, however, were not different between rats fed lard and those fed salmon oil. The differences in the absorption of iron may have occurred during the earlier phase of the experiment as a result of the dietary fat used, which would explain the different hepatic iron concentrations.

As expected, feeding the salmon oil diets caused a marked oxidative stress compared with feeding the lard diets. This was shown by greater concentrations of TBARS in liver and plasma, reduced concentrations of α-tocopherol in liver and plasma and greater concentrations of total and oxidized glutathione in the liver. This finding agrees with many other studies that also reported oxidative stress in animals fed diets containing marine oils (12,33). The present study, moreover, showed for the first time that dietary salmon oil also enhances the formation of COP in the liver. Cholesterol is susceptible to oxidation under a variety of conditions (13). In contact with air, cholesterol may autoxidize via various active oxygen species, whereas in cell membranes, cholesterol can be oxidized either enzymatically or via free radical-mediated peroxidation processes (13,34). We assume that the oxidation of cholesterol was initiated by the autoxidation of highly unsaturated fatty acids, which are very susceptible to oxidation. Feeding fish oil enriches cell membranes in highly unsaturated fatty acids such as eicosapentaenoic acid or docosahexaenoic acid (35).

The high iron diet did not dramatically stress the antioxidant system, irrespective of the dietary fat as shown by minor or no changes in TBARS, GSH-Px, SOD, GSH or α-tocopherol. This agrees with some other studies performed with rats that also did not find effects of a moderate dietary iron excess (≤400 mg iron/kg diet) on activities of antioxidant enzymes or concentrations of α-tocopherol, glutathione, and malondialdehyde in the liver (10,12). In contrast to the other antioxidant enzymes measured, the activity of catalase was increased by feeding high iron diets. This may have been because of increased iron concentrations in the liver rather than to induction by oxidative stress because the activity of catalase depends on iron status.

The finding that the concentrations of TBARS in intestinal mucosa were not elevated by feeding high iron diets suggests that the high iron concentrations in the chyme did not enhance lipid peroxidation in the intestine. Some other studies (36,37) suggest that high iron concentrations in the intestine enhance the formation of lipid peroxides in intestinal mucosa.

The present study showed for the first time that dietary iron excess could enhance the formation of COP in the liver, particularly in rats fed a diet rich in PUFA. The fact that the effect of high iron diets on the formation of COP was stronger in rats fed salmon oil than in rats fed lard also suggests that the formation of COP is a consequence of oxidation of PUFA. Interestingly, increased concentrations of 7-hydroxycholesterol and 7-ketocholesterol were also found in brains and visceral organs of aceruloplasminemic patients (14) who have extremely high concentrations of iron in brain and visceral organs, which are strongly prooxidative and enhance the formation of lipid peroxidation products. Our study shows that even a modest increase in dietary iron enhances the formation of COP in the liver when the antioxidant system is stressed by other factors, such as dietary highly unsaturated fatty acids.

Overall, the results of our research indicate that diets with high concentrations of highly unsaturated fatty acids stimulate the formation of COP in the liver and that simultaneous

dietary iron excess amplifies this effect. This is critical given that several unfavorable effects of COP have been described. They are associated with mutagenic and carcinogenic events (38-40) and are involved in the pathogenesis of atherosclerosis (41,42). COP are transported as constituents of lipoproteins (43) and may therefore leave the liver. Once they have entered the bloodstream, they could exert their toxic effects on various cell types, as described in *in vitro* studies of endothelial cells (44) and smooth muscle cells (45). Studies investigating the effects of PUFA and of high dietary iron concentrations should therefore include an investigation of hepatic cholesterol oxides.

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3.2 Effekt von Fischöl und oxidiertem Cholesterin auf die 7β-Hydroxycholesterol-Konzentrationen in der Ratte bei unterschiedlicher Vitamin E-Versorgung

RINGSEIS R, EDER K (2003) Effects of dietary fish oil and oxidized cholesterol on the concentration of 7β-hydroxycholesterol in liver, plasma, low density-lipoprotein and erythrocytes of rats at various vitamin E supply. *European Journal of Lipid Science and Technology* (im Druck).

ABSTRACT *Two experiments with rats were carried out to investigate the effect of dietary fish oil and oxidized cholesterol on the concentration of 7β-hydroxycholesterol, an oxysterol of mainly nonenzymatic origin, in liver, plasma, low density lipoproteins and erythrocytes of rats at different vitamin E supply. In Experiment 1, four groups of rats received diets with coconut oil or salmon oil (100 g/kg diet) and vitamin E concentrations of 40 or 240 mg α-tocopherol equivalents/kg. In Experiment 2, eight groups of rats received diets supplemented with pure or oxidized cholesterol (5 g/kg), coconut oil or salmon oil (100 g/kg diet) and vitamin E concentrations of 40 or 240 mg α-tocopherol equivalents/kg. Rats fed the salmon oil diets had significantly higher concentrations of 7β-hydroxycholesterol in liver, plasma, low density lipoproteins and erythrocytes than rats fed coconut oil diets. Rats fed the diets supplemented with oxidized cholesterol had significantly higher concentrations of 7β-hydroxycholesterol in all the samples analysed than rats fed pure cholesterol. Increasing the dietary vitamin E concentration from 40 to 240 mg α-tocopherol equivalents/kg diet reduced the concentration of 7β-hydroxycholesterol in plasma; the concentrations of 7β-hydroxycholesterol in liver, low density lipoproteins and erythrocytes were not influenced by the dietary vitamin E concentration. The study suggests that diets with fish oil or oxidized cholesterol are critical with respect to the formation of 7β-hydroxycholesterol.*

Keywords: 7β-hydroxycholesterol, fish oil, oxidized cholesterol, vitamin E, oxysterols, rat.

1 Introduction

As compared to 7α-hydroxycholesterol, 27-hydroxycholesterol, 24S-hydroxycholesterol and 4β-hydroxycholesterol, which are the dominating oxysterols in human plasma, 7β-hydroxycholesterol (7β-OH-Chol) is a minor oxysterol in the human circulation. However, 7β-OH-Chol is of scientific interest due to its potential involvement in the aetiology and progression of atherosclerosis [1, 2]. It has been shown that 7β-OH-Chol exerts cytotoxic effects towards the cells of the vasculature [3] and it appears that increased levels of 7β-OH-Chol are linked to a higher risk of atherosclerosis [4, 5]. In contrast to the aforementioned oxysterols, which are formed enzymatically through oxygenation by different cytochrome P-450 species, most of the 7β-OH-Chol in the circulation is formed nonenzymatically in the presence of reactive oxygen species (ROS) [6, 7]. Therefore, the amount of 7β-OH-Chol in the organism is the result of endogenous formation via peroxidation of cholesterol and dietary intake regarding that 7β-OH-Chol is readily absorbed in the intestine from oxysterol-containing foods [8, 9]. Thus, it is of great interest for practical nutrition wether concentrations of 7β-OH-Chol in plasma, lipoproteins and tissues can be influenced by dietary regimes. Therefore, several studies have been carried out to investigate the impact of various nutrients on the concentrations of 7β-OH-Chol in plasma and tissues [10-12].

The endogenous formation of 7β-OH-Chol is affected in particular by the concentration of cholesterol in membranes and lipoproteins. It was shown that feeding cholesterol-rich diets caused the concentrations of 7β-OH-Chol in plasma to increase markedly [12, 13]. As 7β-OH-Chol is formed in the presence of ROS its endogenous

formation in animal tissues is probably also influenced by the antioxidative status. Some studies suggest that the formation of 7β-OH-Chol from cholesterol in membranes and lipoproteins is promoted by oxidation of polyunsaturated fatty acids (PUFA). ROS formed during oxidation of PUFA can modify cholesterol as a secondary effect [14, 15]. The ingestion of fats containing a high proportion of oxidation-susceptible fatty acids, such as salmon oil, increases the susceptibility of lipids in tissues and lipoproteins to lipid peroxidation [16, 17]. It is therefore reasonable to assume that the endogenous formation of 7β-OH-Chol is also promoted by fats with a high proportion of PUFA. However, less studies exist about the effects of various dietary fats on the concentrations of 7β-OH-Chol in plasma, lipoproteins and tissues. Fat-soluble antioxidants like vitamin E on the other hand inhibit lipid peroxidation [16, 17]. It is therefore reasonable to assume that the endogenous formation of 7β-OH-Chol is suppressed by a high intake of vitamin E. In rabbits fed cholesterol-enriched diets supplementation with vitamins E and C [18] or probucol [6] reduced the concentrations of 7β-OH-Chol in plasma. In humans, dietary vitamin E supplementation reduced plasma 7β-OH-Chol in diabetic patients, but not in healthy controls [19]. Therefore, the effect of antioxidants on the concentration of 7β-OH-Chol in animal tissues requires further studies. Studies on the interaction between the type of dietary fat and vitamin E intake and its effect on the formation of 7β-OH-Chol are however lacking so far. Therefore, the first experiment of this study was conducted to investigate the interaction between dietary fats and vitamin E for the formation of 7β-OH-Chol in different tissues. Salmon oil was used as a source of highly unsaturated, oxidation-susceptible fatty acids. It is often used in practical nutrition for the prevention of coronary heart disease due to its high content of (n-3) PUFA. Coconut oil was used as a control fat with a very low concentration of PUFA.

On the other hand exogenous 7β-OH-Chol from the diet increases the concentration of 7β-OH-Chol in liver and plasma [20, 21]. However, there is less information whether the supply of vitamin E influences the incorporation of 7β-OH-Chol from the diet into lipoproteins and tissues. The second experiment of this study, therefore intended to investigate the interaction between dietary vitamin E and dietary oxidized cholesterol with regards to the concentrations of 7β-OH-Chol in tissues. As we expected that the concentrations of 7β-OH-Chol are influenced by the type of dietary fat we used two different dietary fats, salmon oil and coconut oil. Rats were used as model objects bearing in mind that rats show a different lipoprotein profile and lipoprotein metabolism than humans. However as they are susceptible to the induction of oxidative stress *in vivo* they are widely used to investigate the effect of different feeding regimes on markers of lipid peroxidation such as the formation of 7β-OH-Chol in tissues [10-12]. The concentrations of 7β-OH-Chol in plasma and low density lipoproteins (LDL) were of particular interest in this study with respect to its potential involvement in the development of atherosclerosis. Moreover, the concentrations of 7β-OH-Chol in the liver, the tissue which synthesizes and secretes plasma lipoproteins, and in erythrocytes, to study the effects of dietary treatment on the concentrations of 7β-OH-Chol in a pure membrane fraction, were analysed.

2 Materials and methods

2.1 Animals

Two experiments were carried out with male Sprague-Dawley rats supplied by Charles River (Sulzfeld, Germany). Experiment 1 included 36 rats with an initial body weight of 72 g (± 9 g, SD) which were assigned to four groups of nine rats each; Experiment 2 included seventy-two rats with an initial body weight of 70 g (± 8 g, SD) which were assigned to eight groups of nine rats each. The animals were kept individually in Macrolon cages in a room maintained at a temperature of 23°C and 50 to 60% relative humidity with lighting from 0600 to 1800 h. All experimental procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the council of Saxony-Anhalt.

2.2 Diets and feeding

Semisynthetic diets were used. The basal diet used in Experiment 1 consisted of (g/kg diet): corn starch (398), casein (200), saccharose (200), fat (100), cellulose (40), vitamins and minerals (60) and DL-methionine (2). Minerals and vitamins with the exception of vitamin E were supplemented in accordance with the recommendations of the American Institute of Nutrition [22] for rat diets. According to a bifactorial design, the dietary fat (coconut oil vs. salmon oil) and the vitamin E concentration of the diet (40 vs. 240 mg α-tocopherol equivalents per kg) were varied. The basal diet used in Experiment 2 was identical with that used in Experiment 1 with the exception that it was supplemented with 5 g/kg of either pure cholesterol or oxidized cholesterol at the expense of cellulose. According to a three-factorial design, besides the cholesterol supplement (5 g pure cholesterol/kg vs. 5 g oxidized cholesterol/kg), the dietary fat (coconut oil vs. salmon oil) and the vitamin E concentration of the diet (40 vs. 240 mg α-tocopherol equivalents per kg) were varied. Oxidized cholesterol was prepared by heating cholesterol (Sigma-Aldrich, Steinheim, Germany) placed as a thin film on a glass Petri dish at 115°C for 48 hours in an electric oven. To equalize the vitamin E concentrations of the diets irrespective of the dietary fats used, the native tocopherol concentrations of the two fats were analysed. Based on the native concentrations of the fats, diets were supplemented individually with all-*rac*-α-tocopheryl acetate, allowing for a biopotency of 67% compared to α-tocopherol. The fatty acid composition of coconut oil (Palmin, Hamburg, Germany) and salmon oil (Caelo, Hilden, Germany) are shown in Tab. 1. The peroxide values of the salmon oil and the coconut oil were 3.9 and <0.1 mEq O_2 per kg fat, respectively.

Tab. 1. Fatty acid composition of the dietary fats*

Fatty acid	Coconut oil	Salmon oil
	g/100g fatty acids	
Total saturated fatty acids	91.2	27.1
Total monounsaturated fatty acids	6.4	39.5
Total polyunsaturated fatty acids	2.0	29.1
(n-6) polyunsaturated fatty acids	1.9	3.1
(n-3) polyunsaturated fatty acids	0.1	23.8
Unsaturation index‡	0.10	1.73

*Fatty acids in quantities <0.05 g/100 g were not considered.
‡Average number of double bonds per mol of fatty acids

The diets were prepared weekly by mixing the fat with the dry components and water. Afterwards the diets were freeze dried and stored at –20°C to prevent autoxidation of lipids, e.g. PUFAs and cholesterol. The water content after freeze drying was below 5 g per 100 g of diet. To standardize the diet intake, the diets were fed daily in restricted amounts at 0800 h. The feeding schedule was identical in both experiments. The diet intake was recorded daily and increased from 7.0 g/d to 15.0 g/g during the experiment, resulting in an average daily diet intake of 14.4 g per rat in both experiments. Water was available ad libitum from nipple drinkers. The experimental diets were fed for 35 d in both experiments.

2.3 Sample collection

After 35 days of feeding the experimental diets the rats were starved overnight, anesthetized with diethyl ether and killed by decapitation. Blood was collected into heparinized polyethylene tubes (Sarstedt, Nürnbrecht, Germany). The liver was excised immediately and frozen with liquid nitrogen. Plasma was separated from blood by centrifugation (1100 g, 10 min) at 4°C and stored at –80°C. Erythrocytes were washed three times with physiological saline by centrifugation and resuspension. Lipoproteins were isolated by sequential

ultracentrifugation (900,000 g, 1.5 h) at 4°C. Until analysis, plasma, LDL, erythrocyte membrane, and liver samples were stored at –80°C.

2.4 Analyses

The fatty acid composition of the experimental fats was determined by gas chromatography of fatty acid methyl esters [23].

Liver, plasma, LDL and erythrocyte 7β-OH-Chol (Sigma-Aldrich) and oxysterol concentration of the diets were determined using a quantitative gas chromatography-mass spectrometry (GC-MS) method with selective ion monitoring [24]. Liver and erythrocyte lipids were extracted with a mixture of hexane and isopropanol (3:2, v/v) [25]. After aliquots of the liver and erythrocyte lipid extracts, respectively, were dried under a stream of nitrogen, 2 ml of 1 M potassium hydroxide solution and 5α-cholestane (Sigma-Aldrich) as internal standard were added and the tubes flushed with nitrogen. To 0.5 ml of plasma and 0.25 ml of LDL fraction, 2 ml of 1 M potassium hydroxide solution and 5α-cholestane were directly added. After saponification overnight in the dark, double-distilled water was added and lipids were extracted with (peroxide-free) diethyl ether. The lipid extract was dried under a stream of nitrogen and then derivatized with *bis*-(trimethyl-silyl)-trifluoroacetamide (BSTFA, Sigma-Aldrich) and pyridine by heating samples at 60°C for 60 min. A Shimadzu QP-5000 GC-MS (Shimadzu, Duisburg, Germany) fitted with a DB 5 fused silica column (30 m, 0.25 mm internal diameter, 0.25 µm film thickness, J&W Scientific, Folsom, CA) was used for GC-MS analysis operating with selected ion monitoring. Helium was used as the carrier gas with a flow rate of 1 ml/min (40 cm/s). 1 µl of the samples was injected with a split ratio of 1:20. Peak identification was performed by retention time and ion fragmentation comparison with external standards. Two characteristic ions per substance were used for each oxysterol. The quantification was conducted with calibration curves calculated with the internal standard. In order to control for the formation of 7β-OH-Chol during sample treatment we ran samples with cholesterol only. The results indicated no significant formation of 7β-OH-Chol during sample treatment. We further ran samples with spiked amounts of the oxysterols determined in the diets to gain insight into the stability of those oxysterols during the whole sample work-up. We found recoveries of more than 95% for those oxysterols indicating a high stability and low degradation during sample treatment.

Total cholesterol concentrations of liver, erythrocytes, plasma, and LDL were determined using an enzymatic reagent kit obtained from Merck Eurolab (Darmstadt, Germany). For the measurement of liver and erythrocyte total cholesterol, lipids of the extract were dissolved in Triton X-100 before enzymatic measurement as described by De Hoff et al. [26].

Concentrations of individual tocopherols in plasma, liver, LDL, and dietary fats were determined using a high-performance liquid chromatography (HPLC) method [27]. After saponification with saturated sodium hydroxide solution, individual tocopherols were extracted with n-hexane and separated isocratically on a HP 1100 HPLC station (Hewlett Packard, Waldbronn, Deutschland) using a mixture of n-hexane and 1,4 dioxane (96:4, v/v) as mobile phase and a LiChrosorb Si 60 column (5 µm particle size, 250 mm length, 4 mm internal diameter, Merck Eurolab) and detected by fluorescence.

2.5 Statistics

Data were subjected to ANOVA using the Minitab Statistical Software (Minitab, State College, PA, USA). In Experiment 1, the factors dietary fat (coconut oil vs. salmon oil), dietary vitamin E (240 vs. 40 mg/kg diet) and the interactions of those factors were included. In Experiment 2, ANOVA was performed separately for the rats fed coconut oil and for the rats fed salmon oil. Classification factors were cholesterol supplement (pure cholesterol vs. oxidized cholesterol), dietary vitamin E (240 mg vs. 40 mg/kg diet) and the interactions

between those factors. In the case of large differences in the variances of means, data were transferred into their logarithms prior to ANOVA. For statistically significant F values, individual means of the treatment groups were compared by Fisher´s multiple range test. Means were considered significantly different for $P<0.05$.

3 Results

3.1 Experiment 1

The growth of the rats was significantly influenced by the dietary fat. Animals whose diets contained salmon oil gained significantly more body weight than the animals whose diets contained coconut oil. Average daily body weight gains were 5.6 ± 0.4 g and 5.9 ± 0.3 g (means ± SD, n=18 for each dietary fat, $P<0.05$), that is for the rats fed coconut oil and the rats fed salmon oil. The dietary vitamin E concentration had no effect on the growth of the rats.

Among the various tocopherols, only α-tocopherol was detected in considerable concentrations in all analysed samples. The concentrations of α-tocopherol in liver, plasma and LDL were significantly influenced by dietary fat and dietary vitamin E concentration (Tab. 2). Rats fed the coconut oil diets had higher concentrations of α-tocopherols in those samples than rats fed the salmon oil diets. Increasing the dietary vitamin E concentration from 40 to 240 mg α-tocopherol equivalents/kg caused a significant increase of the α-tocopherol concentration in liver, plasma and LDL. There were, however, significant interactions between the dietary fat and the dietary vitamin E concentration regarding the α-tocopherol concentration in liver and LDL. The effect of the dietary vitamin E on the α-tocopherol concentration of the liver was stronger in rats fed coconut oil than in rats fed salmon oil; the effect of the dietary vitamin E on the α-tocopherol concentrations in LDL, in the opposite, was stronger in rats fed salmon oil than in the rats fed coconut oil. The effect of the dietary vitamin E concentration on the α-tocopherol concentration in plasma was independent of the dietary fat.

Tab 2. Concentrations of total tocopherols, total cholesterol and 7β-hydroxycholesterol in samples of rats fed diets with coconut oil or salmon oil and vitamin E concentrations of 40 or 240 mg/kg (Experiment 1)[†]

Oil	Coconut oil		Salmon oil		P values		
Vitamin E (mg/kg)	40	240	40	240	fat	vitamin E	fat x vitamin E
Total tocopherols							
Liver (nmol/g)	114 ± 22^{b}	352 ± 79^{a}	54 ± 15^{c}	145 ± 45^{b}	0.001	0.001	0.001
Plasma (µmol/L)	20 ± 2^{c}	35 ± 10^{a}	15 ± 2^{d}	28 ± 3^{b}	0.01	0.001	NS
LDL (µmol/L)	6 ± 1^{c}	11 ± 1^{b}	8 ± 2^{c}	18 ± 5^{a}	0.001	0.001	0.05
Total cholesterol							
Liver (µmol/g)	9 ± 1^{c}	9 ± 1^{c}	16 ± 4^{a}	13 ± 2^{b}	0.001	NS	NS
Plasma (mmol/L)	1.5 ± 0.2^{c}	1.5 ± 0.2^{c}	1.7 ± 0.2^{b}	1.9 ± 0.4^{a}	0.001	NS	NS
LDL (mmol/L)	0.22 ± 0.07^{b}	0.21 ± 0.08^{b}	0.55 ± 0.25^{a}	0.62 ± 0.28^{a}	0.001	NS	NS
Erythrocytes (µmol/g)	3.5 ± 1.0^{b}	4.0 ± 0.3^{ab}	4.2 ± 0.5^{ab}	4.5 ± 0.2^{a}	0.01	NS	NS
7β-hydroxycholesterol							
Liver (nmol/g)	1.4 ± 0.7^{b}	1.7 ± 0.8^{b}	4.4 ± 3.0^{a}	3.5 ± 3.2^{a}	0.05	NS	NS
Plasma (nmol/L)	98 ± 22^{c}	66 ± 9^{c}	232 ± 61^{a}	130 ± 24^{b}	0.001	0.001	0.01
LDL (nmol/L)	14 ± 7^{b}	17 ± 6^{b}	38 ± 6^{a}	33 ± 5^{a}	0.001	NS	NS
Erythrocytes (nmol/g)	0.15 ± 0.02^{b}	0.17 ± 0.03^{b}	0.28 ± 0.07^{a}	0.29 ± 0.04^{a}	0.001	NS	NS

[†]Results are means ± SD, n=9 for each treatment group. Values within one sample without a common superscript letter differ, $P<0.05$. NS - non significant.

The concentration of total cholesterol in liver, plasma, LDL and erythrocytes were significantly influenced by the dietary fat (Tab. 2). Animals whose diets contained salmon oil had significantly higher concentrations of total cholesterol in all those samples than the animals whose diets contained coconut oil. The dietary vitamin E concentration had no significant effect on the concentration of total cholesterol in the analyzed samples.

The concentrations of 7β-OH-Chol in liver, LDL and erythrocytes were significantly influenced by the dietary fat but not by the dietary vitamin E concentration. Rats whose diets contained salmon oil had significantly higher concentrations of 7β-OH-Chol in those samples than the animals whose diets contained coconut oil. The concentration of 7β-OH-Chol in plasma was not only significantly influenced by the dietary fat but also by the dietary vitamin E concentration; a significant interaction between both factors occurred there as well. Rats fed the salmon oil diets had significantly higher concentrations of 7β-OH-Chol in plasma than rats fed the coconut oil diet. Increasing the dietary vitamin E concentration from 40 to 240 mg α-tocopherol equivalents/kg significantly reduced the concentration of 7β-OH-Chol in plasma. The effect of the dietary vitamin E was, however, stronger in the rats fed the salmon oil diet than in rats fed the coconut oil diet.

3.2 Experiment 2

The diets supplemented with 5 g oxidized cholesterol/kg contained the following oxysterols (mg/kg): 7β-OH-Chol (2.7), α-epoxycholesterol (2.6), β-epoxycholesterol (6.9), cholestanetriol (0.1), 25-hydroxycholesterol (9.4), 7-ketocholesterol (1.7). The concentration of cholesterol was 4.63 g/kg diet in the diet supplemented with oxidized cholesterol. Other unidentified oxysterols have not been quantified. In the diets containing pure cholesterol the concentrations of all oxysterols were below the limit of detection of 0.025 mg/kg.

The average daily body weight gains of the rats in Experiment 2 were similar to those in Experiment 1 with 5.5 ± 0.3 g for the rats fed coconut oil and 6.0 ± 0.3 g for the rats fed salmon oil (means ± SD, n=36 for each dietary fat). The type of cholesterol and the dietary vitamin E concentration did not influence body weight gains of the rats fed both types of fat.

As in Experiment 1, among the various tocopherol isomers, α-tocopherol was the only one existing in considerable concentrations in all analysed samples. In the rats fed both types of fat, increasing the dietary vitamin E concentration from 40 to 240 mg α-tocopherol equivalents/kg led to a significant increase of the concentration of α-tocopherol in liver, plasma and LDL (Tab. 3). In the rats fed the salmon oil diets, the concentration of α-tocopherol in plasma and LDL was also influenced by the type of dietary cholesterol. Rats fed the diets containing oxidized cholesterol had lower concentrations of α-tocopherol in plasma and LDL than rats fed the diets containing pure cholesterol; the concentration of α-tocopherol in the liver was independent of the type of dietary cholesterol. In the rats fed the coconut oil diets, the type of cholesterol did not influence the concentration of α-tocopherol in any of the tissues investigated.

Tab 3. Concentrations of total tocopherols in samples of rats fed diets with pure cholesterol or oxidized cholesterol at two different dietary fats (coconout oil vs. salmon oil) and two different vitamin E concentrations (40 vs.240 mg/kg) (Experiment 2)†

Cholesterol	Cholesterol		Oxidized cholesterol		P values		
Vitamin E (mg/kg)	40	240	40	240	cholesterol	vitamin E	cholesterol x vitamin E
			Coconut oil				
Liver (nmol/g)	125 ± 40^{b}	545 ± 92^{a}	163 ± 37^{b}	534 ± 159^{a}	NS	0.001	NS
Plasma (µmol/L)	29 ± 7^{b}	50 ± 7^{a}	27 ± 2^{b}	47 ± 9^{a}	NS	0.001	NS
LDL (µmol/L)	11 ± 4^{b}	16 ± 4^{a}	9 ± 3^{b}	15 ± 5^{a}	NS	0.001	NS
			Salmon oil				
Liver (nmol/g)	96 ± 21^{c}	434 ± 83^{a}	83 ± 16^{c}	360 ± 96^{b}	NS	0.001	NS
Plasma (µmol/L)	11 ± 2^{c}	24 ± 4^{a}	9 ± 2^{c}	19 ± 5^{b}	0.01	0.001	NS
LDL (µmol/L)	7 ± 1^{b}	15 ± 3^{a}	5 ± 1^{b}	12 ± 4^{a}	0.05	0.001	NS

†Results are means ± SD, n=9 for each treatment. Values within a row without a common superscript letter differ, $P<0.05$. NS - non significant.

In the rats fed the coconut oil diets, the concentration of total cholesterol in all the analysed tissues was not influenced by the type of dietary cholesterol and the dietary vitamin E concentration (Tab. 4). In the rats fed the salmon oil diets, dietary oxidized cholesterol significantly reduced the concentrations of total cholesterol in plasma and erythrocytes as compared with pure cholesterol; the cholesterol concentrations in liver and LDL were not influenced by the type of dietary cholesterol. In the rats fed the salmon oil diets, the dietary vitamin E concentration also had no effect on the cholesterol concentration in any of the analysed samples.

Tab 4. Concentrations of total cholesterol in samples of rats fed diets with pure cholesterol or oxidized cholesterol at two different dietary fats (coconout oil vs. salmon oil) and two different vitamin E concentrations (40 vs.240 mg/kg) (Experiment 2)†

Cholesterol	Cholesterol		Oxidized cholesterol		P values		
Vitamin E (mg/kg)	40	240	40	240	cholesterol	vitamin E	cholesterol x vitamin E
			Coconut oil				
Liver (µmol/g)	103 ± 29	101 ± 21	111 ± 25	104 ± 23	NS	NS	NS
Plasma (mmol/L)	4.8 ± 0.5	4.7 ± 0.7	4.5 ± 0.6	4.4 ± 0.9	NS	NS	NS
LDL (mmol/L)	0.67 ± 0.27	0.65 ± 0.31	0.85 ± 0.42	0.77 ± 0.34	NS	NS	NS
Erythrocytes (µmol/g)	4.0 ± 0.4	3.8 ± 0.8	4.3 ± 0.6	3.6 ± 0.6	NS	NS	NS
			Salmon oil				
Liver (µmol/g)	125 ± 22	135 ± 32	133 ± 25	142 ± 24	NS	NS	NS
Plasma (mmol/L)	1.9 ± 0.3^{ab}	2.3 ± 0.4^{a}	1.7 ± 0.2^{b}	1.9 ± 0.5^{b}	0.01	NS	NS
LDL (mmol/L)	0.43 ± 0.10	0.47 ± 0.19	0.39 ± 0.121	0.33 ± 0.13	NS	NS	NS
Erythrocytes (µmol/g)	4.4 ± 0.4^{ab}	4.5 ± 0.5^{a}	3.9 ± 0.6^{b}	3.7 ± 0.9^{b}	0.01	NS	NS

†Results are means ± SD, n=9 for each treatment. Values within a row without a common superscript letter differ, $P<0.05$. NS - non significant.

In the rats fed both types of fats, the concentrations of 7β-OH-Chol in liver, LDL and erythrocytes were, as in Experiment 1, independent of the dietary vitamin E concentration (Tab. 5). In contrast, the concentration of 7β-OH-Chol in plasma was significantly reduced by increasing the dietary vitamin E concentration from 40 to 240 mg α-tocopherol equivalents/kg in the rats fed both types of fat. In the rats fed the coconut oil diets, oxidized cholesterol caused a significant increase of the concentration of 7β-OH-Chol in all the tissues analysed as compared with pure cholesterol. In the rats fed the salmon oil diets, dietary oxidized

cholesterol increased the concentrations of 7β-OH-Chol in liver and plasma; the concentrations of 7β-OH-Chol in LDL and erythrocytes were not influenced by the type of dietary cholesterol.

Tab 5. Concentrations of 7β-hydroxycholesterol in samples of rats fed diets with pure cholesterol or oxidized cholesterol at two different dietary fats (coconout oil vs. salmon oil) and two different vitamin E concentrations (40 vs.240 mg/kg) (Experiment 2)†

Cholesterol	Cholesterol		Oxidized cholesterol		P values		
Vitamin E (mg/kg)	40	240	40	240	cholesterol	vitamin E	cholesterol x vitamin E
			Coconut oil				
Liver (nmol/g)	7 ± 2^{b}	9 ± 3^{b}	14 ± 2^{a}	14 ± 5^{a}	0.001	NS	NS
Plasma (nmol/L)	374 ± 122^{c}	206 ± 92^{d}	770 ± 90^{a}	500 ± 110^{b}	0.001	0.001	NS
LDL (nmol/L)	63 ± 17^{b}	60 ± 11^{b}	94 ± 10^{a}	96 ± 32^{a}	0.05	NS	NS
Erythrocytes (nmol/g)	0.24 ± 0.07^{ab}	0.20 ± 0.10^{b}	0.31 ± 0.07^{a}	0.33 ± 0.12^{a}	0.01	NS	NS
			Salmon oil				
Liver (nmol/g)	26 ± 4^{b}	21 ± 3^{b}	40 ± 13^{a}	39 ± 13^{a}	0.001	NS	NS
Plasma (nmol/L)	558 ± 136^{b}	326 ± 78^{c}	707 ± 156^{a}	497 ± 176^{b}	0.01	0.001	NS
LDL (nmol/L)	99 ± 22^{ab}	74 ± 6^{b}	120 ± 26^{a}	94 ± 15^{ab}	NS	NS	NS
Erythrocytes (nmol/g)	0.32 ± 0.06	0.28 ± 0.10	0.33 ± 0.20	0.34 ± 0.10	NS	NS	NS

†Results are means ± SD, n=9 for each treatment. Values within a row without a common superscript letter differ, $P<0.05$. NS - non significant.

4. Discussion

The primary aim of this study was to investigate the effects of dietary PUFA, oxidized cholesterol and vitamin E on the concentration of 7β-OH-Chol in rat tissues. Implementation of a restrictive feeding regime enabled us to standardize the feed intake, thus eliminating secondary effects which might result from differences in the feed intake. In both experiments, the animals whose diet contained salmon oil unexpectedly gained more weight than the animals whose diet contained coconut oil. This effect might be due to the low levels of linoleic and α-linolenic acid in coconut oil. There were, however, no signs of a deficiency of essential fatty acids, as evidenced by the low concentration of 20:3 (n-9) in the erythrocyte membrane. We do not consider that the lower weight gains of the rats whose diet contained coconut oil jeopardize the results of the study as a whole.

One main finding of this study was that feeding a diet containing fish oil leads to higher concentrations of 7β-OH-Chol in liver, plasma, LDL, and erythrocytes as compared with feeding a diet containing coconut oil. Increased concentrations of 7β-OH-Chol in liver, plasma and LDL of rats fed fish oil compared with rats fed coconut oil, observed in Experiment 1, might be partially due to the fact that fish oil feeding increased the concentrations of total cholesterol in those samples. The higher hepatic cholesterol concentration in rats fed salmon oil compared with rats fed coconut oil might be due to an increased activity of acyl CoA:cholesterol acyl transferase caused by fish oil [28, 29]. The main reason for increased concentrations of 7β-OH-Chol may be that feeding fish oil causes an incorporation of highly unsaturated fatty acids such as eicosapentaenoic acid and docosahexaenoic acid in tissues and lipoproteins, which are highly susceptible to lipid peroxidation [16, 17]. We assume that in rats fed salmon oil the oxidation of cholesterol in liver, lipoproteins and erythrocyte membranes was promoted by an increased rate of oxidation of PUFA. In a recent study with rats, we also found higher concentrations of 7β-OH-Chol and other oxysterols in the liver of rats fed salmon oil than in rats fed lard [30].

The vitamin E requirement of man and animals depends mainly on the intake of dietary PUFA. Assuming a specific vitamin E requirement for unsaturated fatty acids as

suggested by Muggli [31], we estimated the vitamin E requirement of the rats on the salmon oil diets at 51 mg all-*rac*-α-tocopheryl acetate/kg diet, and that of the rats on the coconut oil diets at 3 mg all-*rac*-α-tocopheryl acetate/kg diet. As expected, increasing the dietary vitamin E concentration from 40 to 240 mg per kg markedly increased the concentration of α-tocopherol in the tissues investigated. Lower tocopherol concentrations, after feeding the salmon oil diets as compared with feeding coconut oil, are due to enhanced use and depletion of vitamin E by highly unsaturated PUFA [16, 17]. The finding that feeding oxidized cholesterol reduces the concentrations of α-tocopherol in plasma and LDL in rats fed salmon oil as compared with feeding pure cholesterol could be the result of oxidative stress due to oxidized cholesterol. There is some indication from *in vitro* studies that oxidized cholesterol is a source of oxidative stress [32, 33]. Increased concentrations of α-tocopherol in plasma and LDL of rats fed the coconut oil diets supplemented with pure or oxidized cholesterol in Experiment 2 relative to rats fed the coconut oil diets without cholesterol in Experiment 1 may be due to their higher lipid concentrations in plasma and LDL. A strong relationship exists between the concentration of tocopherols and the concentration of total lipids in plasma [34]. The finding that an increase of the dietary vitamin E concentration from 40 to 240 mg/kg failed to reduce the concentrations of 7β-OH-Chol in liver, LDL and erythrocytes suggests that a dietary vitamin E concentration of 40 mg/kg provided maximum protection of cholesterol in these samples against oxidation even in the case of fish oil as dietary fat. The observation that increased dietary vitamin E reduced the concentrations of 7β-OH-Chol in plasma but not in the liver suggests that vitamin E is able to prevent cholesterol in lipoproteins against oxidative modification during their circulation in the blood. However, because the concentration of 7β-OH-Chol in LDL was not reduced by increased dietary vitamin E concentrations, vitamin E may have protected cholesterol only in very low density lipoproteins or high density lipoproteins. The role of vitamin E in the protection of cholesterol against oxidation in lipoproteins requires further investigation.

Comparing the concentrations of 7β-OH-Chol in the liver as found in Experiment 1 with those found in Experiment 2 shows that feeding a diet enriched with cholesterol largely enhances the formation of 7β-OH-Chol in the liver. This is probably due to cholesterol accumulation in the liver by cholesterol feeding. Other studies also demonstrated that there is a relationship between the concentration of cholesterol and the concentrations of 7β-OH-Chol in the liver [35, 36]. Oxidative modification of cholesterol is favoured by free radicals released from the autoxidation of PUFA as shown in *in vitro* studies with phospholipid membrane bilayers [14, 15]. This might explain why simultaneous feeding of salmon oil strongly enhanced the formation of 7β-OH-Chol in the liver of rats fed cholesterol.

In Experiment 2, diets were supplemented with an oxidized cholesterol preparation. To provide a rough estimate of the composition and the degree of oxidation of the oxidized cholesterol preparation used in this study we measured six of the dominating oxysterols knowing to be formed under the applied oxidizing conditions and cholesterol. Cholesterol contributed to about 93% in the oxidized cholesterol preparation. This indicates that we, in contrast to other investigators using oxidized cholesterol preparations which were almost completely oxidized, used a moderately oxidized cholesterol preparation [35, 36]. This is by far more relevant with respect to human diets concerning the estimation that about 1% of the cholesterol consumed in a mixed Western diet is oxidized [37]. The study showed that the concentration of 7β-OH-Chol in the liver was significantly increased by feeding oxidized cholesterol as compared with feeding pure cholesterol. This demonstrates that dietary oxysterols like 7β-OH-Chol are incorporated into the liver. Other studies also reported increased concentrations of hepatic oxysterols like 7β-OH-Chol after feeding diets supplemented with oxidized cholesterol [21, 35, 36]. It was remarkable that the concentration of 7β-OH-Chol in erythrocyte membranes, in opposite to those in liver and plasma, was not significantly increased by feeding diets with oxidized cholesterol. This suggests that less

oxidative modified cholesterol was transferred from lipoproteins to erythrocytes during the renewal of the erythrocyte membrane. Relatively low concentrations of 7β-OH-Chol in erythrocytes, when expressed per mmol of cholesterol, generally suggest that erythrocytes in comparison to liver or plasma are well protected against oxidative modification of cholesterol located within the membrane.

It has been shown that hepatic oxysterols are incorporated into lipoproteins [38, 39]. We, therefore, assume that most of 7β-OH-Chol present in plasma and lipoproteins derives mainly from the liver. Hence, it was not surprising that the concentrations of 7β-OH-Chol in plasma and LDL, when related to the concentration of total cholesterol, within the treatment groups reflected those of the liver. The contribution of dietary oxysterols to the development of atherosclerosis is still discussed controversially [21, 40]. The present study, however, suggests that dietary regimes rich in oxysterols could be critical because they increase the concentration of 7β-OH-Chol in LDL.

From view of pathophysiology, the 7β-OH-Chol concentrations in plasma and LDL might be relevant. It has been shown that 7β-OH-Chol in plasma and lipoproteins has cytotoxic effects on cells of the arterial wall [3]. Plasma concentrations of 7β-OH-Chol have been used as a possible marker of oxidative stress [4, 5]. However, it is not clear whether raised 7β-OH-Chol will increase the risk of atherosclerosis. Although the results found in rats cannot be directly transferred to humans due to a different lipoprotein profile and lipoprotein metabolism (smaller LDL fraction and lacking of cholesterol ester transfer protein in rats), the study suggests that consumption of fish oil, with respect to the formation of 7β-OH-Chol, must be considered critically. Highly unsaturated fatty acids enhance the formation of 7β-OH-Chol in the liver which is secreted into the blood by lipoproteins. Diet regimes with high concentrations of 7β-OH-Chol must be considered cautiously regarding the potential incorporation of dietary 7β-OH-Chol into plasma lipoproteins. Supplementation of vitamin E above the animals´ requirement reduced the concentrations of 7β-OH-Chol only in plasma but not in liver, LDL and erythrocytes and thus did not provide an efficient protection against enhanced formation of 7β-OH-Chol by dietary fish oil, cholesterol and oxidized cholesterol.

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3.3 Effekt von Fischöl und variierender Vitamin E-Versorgung auf die 7β-Hydroxycholesterol-Konzentrationen in der Ratte

RINGSEIS R, EDER K (2002) Insufficient dietary vitamin E increases the concentration of 7β-hydroxycholesterol in tissues of rats fed salmon oil. *Journal of Nutrition* 132:3732-3735.

ABSTRACT This study was conducted to determine the interaction between the type of dietary fat (coconut oil vs. salmon oil) and the vitamin E concentration of the diet (10 vs. 20 vs. 40 vs. 240 mg α-tocopherol equivalents (α-toc)/kg) in relation to the concentration of 7β-hydroxycholesterol (7β-OH) in liver, plasma, LDL and erythrocytes of rats. In the rats whose diet contained salmon oil, the concentration of 7β-OH was dependent on the dietary vitamin E concentration. Rats whose diet contained 10 mg α-toc/kg had significantly higher concentrations of 7β-OH in all samples studied than those whose diet contained 20, 40 or 240 mg α-toc/kg. Increasing the dietary vitamin E concentration from 40 to 240 mg α-toc/kg did not reduce the concentration of 7β-OH in any samples. In the rats whose diet contained coconut oil, the concentration of 7β-OH was independent of the dietary vitamin E concentration in all samples. The study shows that insufficient vitamin E in the diet increases the formation of 7β-OH in rats fed salmon oil, whereas a dietary vitamin E supply in excess of the requirement does not lower 7β-OH concentrations compared with an adequate vitamin E supply.

KEY WORDS: ● 7β-hydroxycholesterol ● vitamin E ● oxysterols ● fish oil ● rats

[2]Abbreviations used: α-toc, α-tocopherol equivalents; 7β-OH, 7β-hydroxycholesterol; GC-MS, gas chromatography-mass spectrometry; PUFA, polyunsaturated fatty acids.
[3]Diet composition: Experimental diets contained (g/kg): casein, 200; sucrose, 200; fat, 100; cellulose, 40; corn starch, 398; mineral mixture, 40 (consisting of calcium carbonate, 7.56; dicalcium phosphate, 8.67; potassium chloride, 6.87; sodium bicarbonate, 3.77; magnesium oxide, 1.01; ferrous sulfate hydrate, 0.116; zinc oxide, 0.038; manganese oxide, 0.016; copper sulfate pentahydrate, 0.024; calcium iodate, 0.0032; sodium selenite pentahydrate, 0.0033); vitamin mixture 20 (consisting of all-trans-retinol, 1.34 mg; cholecalciferol, 25 μg; menadion sodium bisulfite, 7.5 mg; thiamine hydrochloride, 5 mg; riboflavine, 6 mg; pyridoxine hydrochloride, 6 mg; biotin, 0.2 mg; calcium pantothenate, 15 mg; nicotinic acid, 30 mg; vitamin B12, 0.025 mg; folic acid, 2 mg; choline chloride, 1,000 mg); DL-methionine, 2.

Oxysterols, which are either ingested with the diet or formed endogenously in mammals, are of great pathophysiologic importance (1) and are involved in the pathogenesis of arteriosclerosis (2). The endogenous formation of oxysterols from cholesterol is promoted especially by peroxidation of polyunsaturated fatty acids (PUFA)[2] (3, 4). The susceptibility of membranes and lipoproteins to oxidation is determined primarily by the fatty acid composition of lipids and the concentrations of antioxidants, in particular tocopherols. Incorporation of highly unsaturated fatty acids from fish oil into lipids increases the susceptibility of tissues to oxidation, whereas an increased concentration of tocopherols lowers it (5-7). There is some evidence that the endogenous formation of oxysterols is influenced by the type of dietary fat and the ingestion of antioxidants (8-12). We are not aware of any studies investigating the interactions between the type of dietary fat and the level of the vitamin E supply in relation to the concentrations of oxysterols. The objective of our study was to determine the effect of the dietary vitamin E supply on the formation of oxysterols in tissues of rats fed fish oil or coconut oil. We considered a wide spectrum of vitamin E supply, from inadequate to excessive. Fish oil was chosen because it is often used

in the prevention of arteriosclerosis due to its many favourable properties; coconut oil was used as a control fat with a very low PUFA content. From among the oxysterols formed in the body, we considered 7β-hydroxycholesterol (7β-OH) as a marker for the formation of oxysterols. The concentration of 7β-OH in tissues reflects the endogenous, nonenzymatic formation of oxysterols fairly accurately (13, 14). We determined the concentrations of 7β-OH in plasma and LDL because this is where oxysterols are of particular pathophysiologic relevance. To gain further insight into the formation of oxysterols, we also measured the concentrations of 7β-OH in liver and erythrocytes.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (n = 72) with an initial body weight of 63 ± 5 g (mean ± SD) were obtained from Charles River (Sulzfeld, Germany). They were assigned to eight groups of nine rats each and housed in Macrolon cages in groups of 3 rats/cage in a room maintained at a temperature of 23°C and 50-60% relative humidity with lighting from 0600 to 1800 h. All experimental procedures described followed guidelines for the care and handling of laboratory animals and were approved by the council of Saxony-Anhalt.

Diets and feeding. We used purified diets that were formulated according to the recommendations of the AIN (15) for rat diets.[3] The dietary fat, 100 g/kg coconut oil (Palmin, Hamburg, Germany) or 100 g/kg salmon oil (Caelo, Hilden, Germany) and the vitamin E concentration of the diet (10 vs. 20 vs. 40 vs. 240 mg α-tocopherol equivalents (α-toc)/kg) were varied according to a bifactorial design. The major fatty acids in coconut oil were (g/100 g total fatty acids): caprylic acid (8:0), 8.1; capric acid (10:0), 6.3; lauric acid (12:0), 45.6, myristic acid (14:0), 17.0; palmitic acid (16:0), 9.7; stearic acid (18:0), 4.5; oleic acid (18:1), 6.3; linoleic acid (18:2), 1.9. The major fatty acids in salmon oil were (g/100 g total fatty acids): myristic acid, 6.1; palmitic acid, 14.5; palmitoleic acid (16:1), 8.2; stearic acid, 3.0; oleic acid, 12.9; linoleic acid, 2.2; α-linolenic acid (18:3(n-3)), 0.8; eicosanoic acid (20:1), 4.3; eicosapentaenoic acid (20:5(n-3)), 12.7; docosanoic acid (22:1), 4.5; docosapentaenoic acid (22:5(n-3)), 3.1; docosahexaenoic acid (22:6(n-3)), 10.0.

Vitamin E (as all-*rac*-α-tocopheryl acetate, Merck Eurolab, Darmstadt, Germany) was added to the diets at the expense of dietary fibre. To equalize the vitamin E concentrations of the diets, the native tocopherol levels of the two fats were analysed. On the basis of the native concentrations of the fats, diets were supplemented individually with all-*rac*-α-tocopheryl acetate, allowing for a biopotency of 67% compared with α-tocopherol. The diets were prepared weekly by solubilizing the all-*rac*-α-tocopheryl acetate in the fat and mixing it with the dry components and water. The diets were freeze-dried and stored at –20°C to prevent autoxidation of lipids, e.g., PUFA. The peroxide values of the dietary fats extracted from the diets with a mixture of hexane and isopropanol (3:2, according to (16)), were determined at various times during storage of the diets. They were independent of the dietary vitamin E concentration and did not significantly increase during storage. Average peroxide values of the four vitamin E concentrations, measured according to official methods (17), were 0.8 mEq O_2/kg fat in the coconut oil diets and 8.6 mEq O_2/kg fat in the salmon oil diets. The water content after freeze drying was <5 g/100 g diet. The experimental diets were fed for 42 days. Rats consumed the experimental diets and water, from nipple drinkers, ad libitum.

Sample collection. After completion of the feeding period, the rats were starved overnight, anesthetized with diethyl ether and killed by decapitation. Blood was collected into heparinized polyethylene tubes (Sarstedt, Nürnbrecht, Germany). The liver was excised immediately and frozen with liquid nitrogen. Plasma was separated from blood by centrifugation (1100 X g, 10 min) at 4°C and stored at –80°C. Erythrocytes were washed three times with physiologic sodium chloride solution (9 g/L). Plasma lipoproteins were separated by step-wise ultracentrifugation (Mikro-Ultracentrifuge, Sorvall Products, Bad Homburg, Germany) at 900,000 X g at 4°C for 1.5 h. In the first step, the plasma density was

adjusted to 1006 g/L by adding 0.3 mL of a solution containg 0.195 mol/L sodium chloride and 2.44 mol/L sodium bromide to 0.6 mL of plasma. After centrifugation, the upper portion of 0.3 mL, which contained the VLDL fraction, was removed by suction and discarded. Then the density was adjusted to 1063 g/L by adding 0.3 mL of a solution containing 0.195 mol/L sodium chloride and 2.44 mol/L sodium bromide. After centrifugation, the upper portion of 0.3 mL, which contained the LDL fraction, was removed by suction and used for analysis of tocopherols and 7β-OH. Concentrations of tocopherols and 7β-OH in LDL were expressed per L of plasma. Plasma, LDL, erythrocytes and liver were stored at –80°C until analysis.

Analytical methods. The fatty acid composition of the experimental fats was determined by gas chromatography of fatty acid methyl esters (18, 19). Liver, plasma, LDL and erythrocyte membrane 7β-OH (Sigma-Aldrich, Steinheim, Germany) was determined using a quantitative gas chromatography-mass spectrometry (GC-MS) method with selective ion monitoring (20). Liver and erythrocyte lipids were extracted with a mixture of hexane and isopropanol (3:2, v/v) (16). After aliquots of the liver and erythrocyte lipid extracts were dried under a stream of nitrogen, 1 mol/L potassium hydroxide solution and 5α-cholestane (Sigma-Aldrich, Steinheim, Germany) as internal standard were added, respectively, and the tubes flushed with nitrogen. To 0.5 mL of plasma or 0.25 mL of LDL fraction, 1 mol/L potassium hydroxide solution and 5α-cholestane were added directly. After saponification overnight in the dark, double-distilled water was added and lipids were extracted with (peroxide-free) diethyl ether. The lipid extract was dried under a stream of nitrogen and then derivatized with *bis*-(trimethyl-silyl)-trifluoroacetamide (Sigma-Aldrich) and pyridine by heating samples at 60°C for 60 min. A Shimadzu QP-5000 GC-MS (Shimadzu, Duisburg, Germany) fitted with a DB 5 fused silica column (30 m, 0.25 mm i.d., 0.25 µm film thickness, J&W Scientific, Folsom, CA) was used for GC-MS analysis operating with selected ion monitoring. Helium was used as the carrier gas with a flow rate of 1 mL/min (40 cm/s). The sample (1 µL) was injected with a split ratio of 1:20. Peak identification was performed by retention time and ion fragmentation comparison with external standards. Two characteristic ions were used for 7β-OH. Quantification was performed using calibration curves calculated with the internal standard. To control for the generation of 7β-OH during treatment of the samples, controls that contained only pure cholesterol (2.4 µmol/assay) were assayed in parallel. The amount of 7β-OH formed during treatment of the sample was below the detection limit of 0.02 nmol, indicating that <0.001% of the cholesterol was converted into 7β-OH during sample treatment.

Concentrations of individual tocopherols in plasma, liver, LDL, erythrocytes and dietary oils were determined by HPLC (21). Samples were mixed with 1 mL of 1% pyrogallol solution (in ethanol, absolute) and 150 µL of saturated sodium hydroxide solution. This mixture was heated for 30 min at 70°C, and tocopherols were extracted with n-hexane. Individual tocopherols of the extracts were separated isocratically using a mixture of n-hexane and 1,4 dioxane (96:4, v/v) as mobile phase and a LiChrosorb Si 60 column (5 µm particle size, 250 mm length, 4 mm i.d., Merck, Darmstadt, Germany) and detected by fluorescence (excitation wavelength: 295 nm; emission wavelength: 320 nm).

Statistics. Data were subjected to ANOVA including the factors fat and vitamin E and the interactions between fat and vitamin E using the Minitab Statistical Software (Minitab, State College, PA). When variances were heterogeneous, data were transformed into their logarithms before ANOVA. For statistically significant F-values, individual means of the treatment groups were compared by Fisher´s multiple range test. Means were considered significantly different at $P<0.05$.

RESULTS

Food intake and final body weights. In rats fed coconut oil, food intake was independent of the dietary vitamin E concentration. In those fed salmon oil, intake of the rats whose diet contained 10 mg α-toc/kg was significantly lower than that of the groups whose diet contained 20, 40 or 240 mg α-toc/kg (**Table 1**). Daily body weight gains were affected by the interaction between the type of dietary fat and the dietary vitamin E supply. In rats fed coconut oil, daily body weight gains were independent of the dietary vitamin E concentration. In rats fed salmon oil, the rats whose diets contained 10 mg α-toc/kg had significantly lower body weight gains those whose diets contained 20, 40 or 240 mg α-toc/kg.

TABLE 1

Food intake, body weight gain and concentrations of total tocopherols in liver, plasma, LDL and erythrocytes of rats fed diets with coconut oil or salmon oil and four dietary vitamin E concentrations[1, 2]

		Diet Vitamin E (mg α-tocopherol equivalents/kg)			
	Fat	10	20	40	240
Food intake,[*] *g/d*	Coconut oil	17.6 ± 0.9^{bc}	18.1 ± 0.6^{b}	19.0 ± 0.5^{ab}	18.5 ± 1.4^{ab}
	Salmon oil	16.7 ± 0.9^{c}	17.8 ± 0.6^{b}	19.1 ± 1.0^{a}	18.3 ± 1.7^{ab}
Body weight gain,[*†] *g/d*	Coconut oil	7.0 ± 0.5^{bc}	7.0 ± 0.5^{bc}	7.3 ± 0.7^{b}	7.3 ± 0.6^{b}
	Salmon oil	6.5 ± 0.4^{c}	7.3 ± 0.5^{b}	8.1 ± 0.9^{a}	7.7 ± 0.8^{ab}
		Total tocopherols			
Liver,[#*†] *nmol/g*	Coconut oil	26 ± 4^{d}	55 ± 6^{c}	67 ± 13^{c}	248 ± 128^{a}
	Salmon oil	1.0 ± 0.3^{f}	7.6 ± 2.1^{e}	29 ± 9^{d}	104 ± 21^{b}
Plasma,[#*†] *µmol/L*	Coconut oil	6.6 ± 1.1^{d}	13 ± 2.9^{b}	15 ± 3.8^{b}	21 ± 5.1^{a}
	Salmon oil	0.5 ± 0.2^{g}	1.7 ± 0.5^{f}	4.3 ± 0.6^{e}	8.5 ± 1.5^{d}
LDL,[#*†] *µmol/L*	Coconut oil	1.2 ± 0.2^{c}	2.0 ± 0.5^{b}	2.5 ± 0.6^{ab}	2.8 ± 0.5^{a}
	Salmon oil	0.2 ± 0.1^{e}	0.5 ± 0.2^{d}	1.2 ± 0.4^{c}	2.3 ± 0.5^{ab}
Erythrocytes,[#*†] *nmol/g*	Coconut oil	5.2 ± 3.1^{c}	6.7 ± 1.8^{bc}	11 ± 5.9^{b}	16 ± 4.5^{a}
	Salmon oil	0.7 ± 0.3^{e}	2.3 ± 0.6^{d}	5.1 ± 1.7^{c}	15 ± 7.2^{ab}

[1] Results are means ± SD, n=9.
[2] Values without a common superscript letter differ, $P<0.05$.
Significance of factors ($P<0.05$): [#] Fat, [*] vitamin E, [†] fat x vitamin E.

Tocopherol concentrations. In the rats fed diets containing 10 mg α-toc/kg, the concentrations of tocopherols in liver, plasma, LDL and erythrocytes were 5-26 times higher in those whose diet contained coconut oil rather than salmon oil (**Table 1**). Increasing the dietary vitamin E supply caused greater proportional increases in the tocopherol concentrations in all samples studied in rats fed salmon oil than in those fed coconut oil. Increasing the dietary vitamin E concentration from 10 to 240 mg α-toc/kg led to 12- to 100-fold increases in the tocopherol concentrations in liver, plasma, LDL and erythrocytes in rats fed salmon oil, whereas in rats fed coconut oil, the increases in tocopherol concentrations of these tissues were only 2- to 9-fold. At the highest vitamin E concentration of 240 mg α-toc/kg diet, the tocopherol concentrations of LDL and erythrocytes did not differ between rats whose diet contained salmon oil and those whose diet contained coconut oil. The tocopherol concentrations in liver and plasma, on the other hand, were more than twice as high in the rats whose diet contained coconut oil than in the rats whose diet contained salmon oil and a vitamin E concentration of 240 mg α-toc/kg.

Concentrations of 7β-OH. At the lowest vitamin E concentration, the rats whose diet contained salmon oil had 2.4-6 times higher concentrations of 7β-OH in the samples studied than those whose diet contained coconut oil (**Table 2**). In the rats fed salmon oil, increasing the vitamin E concentration from 10 mg α-toc/kg lowered the concentration of 7β-OH in all samples studied. The lowest concentrations of 7β-OH were found in liver and plasma of rats

fed diets comtaining vitamin E levels of 40 and 240 mg α-toc/kg and in LDL and erythrocytes in rats fed diets containing vitamin E levels of 20, 40 and 240 mg α-toc/kg. In rats fed coconut oil, the concentrations of 7β-OH in all samples studied did not differ due to vitamin E concentration. At vitamin E concentrations of 10 and 20 mg α-toc/kg, the rats fed salmon oil had higher concentrations of 7β-OH in all samples studied than those fed coconut oil. At vitamin E concentrations of 40 and 240 mg α-toc/kg, concentrations of 7β-OH differed between the rats fed coconut oil and salmon oil only in the liver, not in plasma, LDL or erythrocytes.

TABLE 2

Concentrations of 7β-hydroxycholesterol in liver, plasma, LDL and erythrocytes of rats fed diets with coconut oil or salmon oil and four dietary vitamin E concentrations[1, 2]

Sample	Fat	Diet Vitamin E (mg α-tocopherol equivalents/kg) 10	20	40	240
		7β-hydroxycholesterol			
Liver,[#*†] *nmol/g*	Coconut oil	0.31 ± 0.06^{d}	0.43 ± 0.11^{d}	0.37 ± 0.05^{d}	0.44 ± 0.04^{d}
	Salmon oil	1.73 ± 0.66^{a}	1.36 ± 0.42^{b}	0.92 ± 0.23^{c}	1.06 ± 0.31^{bc}
Plasma,[#*†] *nmol/L*	Coconut oil	25 ± 7^{c}	22 ± 3^{c}	29 ± 5^{c}	35 ± 5^{c}
	Salmon oil	123 ± 45^{a}	58 ± 8^{b}	33 ± 16^{c}	34 ± 11^{c}
LDL,[#*] *nmol/L*	Coconut oil	20 ± 5^{bc}	14 ± 6^{bc}	16 ± 4^{bc}	11 ± 3^{c}
	Salmon oil	47 ± 19^{a}	26 ± 5^{b}	20 ± 12^{bc}	18 ± 5^{bc}
Erythrocytes,[#*†] *nmol/g*	Coconut oil	0.10 ± 0.05^{bc}	0.07 ± 0.02^{c}	0.09 ± 0.02^{bc}	0.10 ± 0.02^{bc}
	Salmon oil	0.26 ± 0.08^{a}	0.12 ± 0.04^{b}	0.11 ± 0.03^{bc}	0.10 ± 0.03^{bc}

[1] Results are means ± SD, n=9.

[2] Values without a common superscript letter differ, $P<0.05$.

Significance of factors ($P<0.05$): [#] Fat, [*] vitamin E, [†] fat x vitamin E.

DISCUSSION

In this study diets, containing coconut oil or salmon oil and different vitamin E concentrations were fed to rats. As expected, the vitamin E concentrations in the tissues were distinctly lower after feeding salmon oil than after feeding coconut oil. This is because PUFA, which are incorporated into membranes and lipoproteins, increase the use and depletion of tocopherols (22). The extremely low tocopherol concentrations in the tissues of rats whose diet contained the lowest vitamin E concentration and salmon oil suggest a deficiency of vitamin E, which would also explain their slower growth compared with the rats from all other groups.

Low vitamin E concentrations in tissues and LDL, combined with ingestion of highly unsaturated fatty acids as found in fish oil, markedly increased concentrations of 7β-OH. This is probably due to increased peroxidation of PUFA. High levels of highly unsaturated fatty acids and low tocopherol concentrations promote lipid peroxidation in tissues and lipoproteins (5, 6). The observation that the concentrations of 7β-OH in tissues were reduced by increasing the vitamin E supply confirms the importance of vitamin E as a protective factor against the formation of oxysterols. This is in agreement with several other studies that also suggested that the supply of antioxidants affects the formation of oxysterols (9-13). The study also shows very clearly, however, that an excessive supply of vitamin E does not further decrease the concentration of 7β-OH. The study indicates that even at a very high intake of highly unsaturated fatty acids, relatively moderate vitamin E concentrations of 20-40 mg α-tocopherol/kg diet are sufficient to protect the cholesterol in membranes and lipoproteins from oxidation.

The study also showed that the amount of vitamin E required to protect cholesterol from oxidation is distinctly lower in rats fed a fat with a low PUFA content than in rats fed a fat high in PUFA. At any given vitamin E supply, it is likely that fats low in PUFA lead to

higher vitamin E concentrations, lower concentrations of PUFA in tissues and lipoproteins and therefore a reduced susceptibility to oxidation than do fats high in PUFA (23).

Oxysterols in plasma and LDL are of particular pathophysiologic relevance. It has been shown that oxysterols as constituents of LDL are cytotoxic toward endothelial cells (24, 25). What is more, a correlation has been established between the concentration of 7β-OH in plasma and the etiology of arteriosclerosis (26, 27). The observation that at adequate vitamin E levels of 40 or 240 mg α-toc/kg diet, the concentrations of 7β-OH in plasma and LDL were not greater in rats fed salmon oil than in those fed coconut oil suggests that fish oil likely does not influence the formation of oxysterols when the supply with vitamin E is sufficient.

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3.4 Effekt oxidierten Cholesterins auf Parameter des antioxidativen Schutzsystems bei unterschiedlichen Diätfetten bei der Ratte

RINGSEIS R, EDER K: Effects of dietary oxidized cholesterol on the antioxidant defence system in rats fed coconut oil or salmon oil. *International Journal for Vitamin and Nutrition Research* (Manuskript: eingereicht zur Publikation 2002)

Summary: An experiment was conducted with rats to investigate the effect of dietary oxidized cholesterol on the antioxidant status. Four groups of male, growing Sprague-Dawley rats received diets containing pure or oxidized cholesterol (5 g/kg diet) with either coconut oil or salmon oil as dietary fat (100 g/kg diet) for 5 weeks. Rats fed diets containing oxidized cholesterol had significantly higher concentrations of 7β-hydroxycholesterol and 7-ketocholesterol in the liver than rats fed diets containing pure cholesterol. Moreover, rats fed the diets containing oxidized cholesterol had significantly higher mRNA concentrations of glutathione peroxidase and superoxide dismutase, a significantly higher activity of glutathione peroxidase and significantly lower concentrations of total and reduced glutathione in the liver than rats fed diets containing pure cholesterol. These effects were independent of the dietary fat. The study suggests that dietary oxidized cholesterol stresses the antioxidant defence system in rats.

Introduction

Numerous studies indicate that oxysterols, being constituents of LDL, are involved in the pathogenesis of atherosclerosis [1, 2]. Oxysterols are formed not only endogenously by enzymatic or non-enzymatic processes, but also enter the circulation and the liver as a result of consuming cholesterol-rich foods [3, 4]. In vitro studies in human macrophages and monocytes and in rat hepatocytes showed that oxysterols such as 7β-hydroxycholesterol (7β-OH) and 7-ketocholesterol (7-K) have cytotoxic properties and induce oxidant stress which manifests itself by glutathione (GSH) depletion and increased activity of antioxidant enzymes [5-8]. There are, however, no studies investigating the potential effect of oxysterols on the antioxidant status in vivo. The purpose of the present study was therefore to ascertain whether dietary oxysterols affect the antioxidant defence system in the liver.

It is known that the type of dietary fat also affects the antioxidant status. Fats with a high proportion of polyunsaturated fatty acids (PUFA) like fish oil for example stress the antioxidant defence system because of the high susceptibility of these fatty acids to oxidation. It is therefore reasonable to assume that the effect of oxidized cholesterol on the antioxidant defence system will also depend on the type of dietary fat. We expected that the effects of oxidized cholesterol on the antioxidant defence system would be magnified if a fat with a high proportion of PUFA was fed at the same time. Studies on potential interactions between oxysterols and the type of dietary fat are lacking so far.

In order to consider the effect of oxysterols in isolation, we selected a study design where the effect of an oxidized cholesterol (oxChol) preparation was compared with untreated cholesterol. In order to simulate a practical human diet we used an oxChol preparation that was only moderately oxidized. Most other studies investigating the effects of oxysterols used oxChol preparations that were almost completely oxidized [9, 10]. But in an average Western mixed diet only about 1% of the ingested cholesterol is present in oxidized form [11]. Rats were the model objects in our study. The concentrations of tocopherols and GSH in the liver and the gene expression and activities of several antioxidant enzymes in the liver were determined as parameters for the antioxidant defence system. We know from the literature that these parameters reflect the antioxidant status and expose nutritive oxidant stress [12-15].

Materials and methods

Animals: 36 male Sprague-Dawley rats (Charles River, Sulzfeld, Germany) with an initial body weight of 72 g (± 7 g, SD) were assigned to four groups of nine rats each. The animals were kept individually in Macrolon cages in a room maintained at a temperature of 23° C and 50 to 60% relative humidity with lighting from 0700 to 1900 h. All experimental procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the council of Saxony-Anhalt.

Diets and feeding: Semisynthetic diets were used. The basal diet consisted of (g/kg diet): corn starch (398), casein (200), saccharose (200), fat (100), cellulose (35), vitamins and minerals (60) and DL-methionine (2). Minerals and vitamins with the exception of vitamin E were supplemented in accordance with recommendations by the American Institute of Nutrition AIN-93G [16] for rat diets. The dietary fat (coconut oil vs. salmon oil) and cholesterol obtained from Sigma-Aldrich (Steinheim, Germany) (5 g pure cholesterol per kg vs. 5 g oxidized cholesterol per kg) were varied according to a two-factorial design. Oxidized cholesterol was prepared by heating cholesterol (Sigma-Aldrich, Steinheim, Germany) placed as a thin film on a glass Petri dish at 115°C for 48 hours in an electric oven.

To equalize the vitamin E concentrations of the diets irrespective of the dietary fats used, the native tocopherol concentrations of the two fats were analysed. Based on these native concentrations, diets were supplemented individually with all-*rac*-α-tocopheryl acetate (Merck Eurolab, Darmstadt, Germany), allowing for a biopotency of 67% compared to α-tocopherol. After supplementation the vitamin E concentrations of both types of diets were 40 mg α-tocopherol equivalents/kg. The fatty acid composition of coconut oil (Palmin, Hamburg, Germany) and salmon oil (Caelo, Hilden, Germany) is shown in *Table I*.

Table I: Fatty acid composition of the dietary fats (g/100g fatty acids)

Fatty acid	Coconut oil	Salmon oil
Caprylic acid (8:0)	8.1	-*
Capric acid (10:0)	6.3	-*
Lauric acid (12:0)	45.6	0.1
Myristic acid (14:0)	17.0	6.1
Palmitic acid (16:0)	9.7	14.5
Palmitoleic acid (16:1)	-*	8.2
Hexadecadienoic acid (16:2)	-*	1.4
Hexadecatetraenoic acid (16:4)	-*	1.4
Stearic acid (18:0)	4.5	3.0
Oleic acid (18:1 n-9)	6.3	12.9
Octadecenoic acid (18:1 n-7)	-*	2.7
Linoleic acid (18:2)	1.9	2.2
α-Linolenic acid (18:3 n-3)	0.08	0.8
Eicosanoic acid (20:1)	0.05	4.3
Eicosatrienoic acid (20:3 n-6)	-*	0.3
Arachidonic acid (20:4 n-6)	-*	0.8
Eicosatetraenoic acid (20:4 n-3)	-*	1.4
Eicosapentaenoic acid (20:5 n-3)	-*	12.7
Docosanoic acid (22:1)	-*	4.5
Docosapentaenoic acid (22:5 n-3)	-*	3.1
Docosahexaenoic acid (22:6 n-3)	-*	10.0

* - not detected (<0.05g/100g fatty acids)

The peroxide values of the dietary fats, which were extracted from the diets with a mixture of hexane and isopropanol (3:2, as described in [17]) and measured according to official methods [18], were 3.9 and <0.1 mEq O_2 per kg salmon oil and coconut oil, respectively.

The diets were prepared weekly by solubilizing the all-*rac*-α-tocopheryl acetate and pure cholesterol/oxidized cholesterol preparation in the fat and mixing with the dry

components and water. The diets were then freeze dried and stored at –20°C to prevent autoxidation of lipids, e.g. polyunsaturated fatty acids and cholesterol. The water content after freeze drying was below 5 g per 100 g of diet.

To standardize the feed intake, the diets were fed daily in restricted amounts at 0800 h. The feed intake was increased from 7.0 g per day to 15.0 g per day during the experiment resulting in an average daily feed intake of 14.4 g. Water was provided ad libitum from nipple drinkers. The experimental diets were fed for 35 days.

Sample collection: After 35 days of feeding the experimental diets the rats were starved overnight, anesthetized with diethyl ether and killed by decapitation. The liver was excised immediately, frozen with liquid nitrogen and stored at -80°C pending analysis.

Lipid analysis: The fatty acid composition of the experimental fats was determined by gas chromatography of fatty acid methyl esters (FAMEs) [19]. FAMEs were separated in a HP 5890 gas chromatographic system (Hewlett-Packard, Taufkirchen, Germany) fitted with a polar capillary column (FFAP, 30 m, 0.53 mm internal diameter, Machery and Nagel, Düren, Germany), detected by flame ionization and identified by comparing their retention times with those of individually purified standards [20].

Oxysterols: 7β-OH and 7-K in the liver and oxysterols (Sigma-Aldrich, Steinheim, Germany) in the diets were determined using a quantitative GC-MS method with selective ion monitoring [21], which has been described in detail in a previous paper [22].

Antioxidant status: Concentrations of individual tocopherols in the liver and dietary fats were determined using a HPLC method [23].

The activity of glutathione peroxidase (GSH-Px) in the liver was determined with t-butyl hydroperoxide at 25°C according to the method of Paglia and Valentine [24]. One unit of GSH-Px activity is defined as one µmol reduced β-nicotinamide adenine dinucleotide phosphate oxidized per min. Determination of total (GSH_{tot}) and oxidized glutathione (GSSG) was performed in protein-free liver homogenates according to Tietze [25] with glutathione reductase and Ellman´s reagent. Reduced glutathione (GSH_{red}) was calculated by subtracting two times the GSSG value from the GSH_{tot} value. Calibration was performed using a standard curve. The activity of glucose-6-phosphate dehydrogenase (G6PDH) in the liver was determined by the method of Deutsch [26]. One unit of G6PDH is defined as one µmol reduced β-nicotinamide adenine dinucleotide phosphate oxidized per min.

Measurement of relative mRNA concentrations: To quantify the gene expression of GSH-Px, SOD and GSH reductase the total RNA was isolated from liver by Trizol^{TM} reagent (Invitrogen, Karlsruhe, Germany). The relative quantity of GSH-Px mRNA compared to glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA was determined by means of semiquantitative RT-PCR using a commercially available one step RT-PCR kit (Amersham Pharmacia Biotech, Freiburg, Germany) and specific primers coding for GSH-Px and GAPDH. The PCR mixtures were subjected to 30 cycles of amplification at 95°C 30 s, 60°C 30 s and 72°C 45 s. The PCR products were then separated by ethidium bromide-containing agarose gel electrophoresis, digitalized with a digital camera (SynGene, Cambridge, England) using SynGene-Software (GeneSnap and GeneTools) for identification and quantification. The relative quantities of SOD mRNA and GSH reductase mRNA compared to GAPDH mRNA were determined by quantitative real-time RT-PCR. First strand cDNA was prepared using the Omniscript RT Kit (200) from QIAGEN (Hilden, Germany) and Oligo dT-Primer $pd(T)_{12-18}$ from Amersham Pharmacia Biotech (Freiburg, Germany). The real-time PCR was performed with a Taq DNA Polymerase from Promega (Mannheim, Germany) and specific primers coding for GAPDH, SOD and GSH reductase. 35 Cycles of amplification (95°C 20 s, 60°C 30 s and 72°C 40 s) were performed using a real-time RCR cycler (Rotorgene, Corbett Research, LTF Labortechnik, Wasserburg, Germany). The primers used for PCR were as follows: 5´-GCATGGCCTTCCGTGTTCC-3´ (forward) and 5´-GGGTGGTCCAGGGTTTCTTACTC-3´ (reverse) for rat GAPDH; 5´-

CACCACGACCCGGGACTACACC-3´ (forward) and 5´-CACCAAGCCCAGATACCAGGAATG-3´ (reverse) for rat GSH-Px; 5´-TCCGGTGCAGGGCGTCATTC-3´ (forward) and 5´-ACACATTGGCCACACCGTCCTT-3´ (reverse) for rat SOD; 5´-ACGCTGGCGGTGTTGAGGTTCT-3´ (forward) and 5´-TGAGCCCCACTGTCCCGATAGG-3´ (reverse) for rat GSH reductase.

Statistics: Data were subjected to ANOVA using the Minitab Statistical Software (Minitab, State College, PA, USA). Classification factors were dietary fat, cholesterol and the interaction of both factors. In cases where the differences between variances and means were large, data were transformed to logarithms prior to ANOVA. For statistically significant F values, individual means of the treatment groups were compared by Fisher´s multiple range test. Means were considered significantly different for $P<0.05$.

Results

Concentrations of oxysterols in the diets: The diets supplemented with 5 g oxidized cholesterol/kg contained the following oxysterols (mg/kg) which are known to be formed at the highest concentrations during heating of cholesterol: 7β-OH, 2.7; α-epoxycholesterol, 2.6; β-epoxycholesterol, 6.9; cholestanetriol, 0.1; 25-hydroxycholesterol, 9.4; 7-ketocholesterol, 1.7. The concentration of cholesterol was 4.63 g/kg diet in the diet supplemented with oxidized cholesterol. Other minor, unidentified oxysterols have not been quanified. In the diets containing pure cholesterol the concentrations of all oxysterols were below the limit of detection of 0.025 mg/kg.

Food intake and final body weight of the rats. The final body weight of the rats was significantly affected by the type of dietary fat. Rats fed the salmon oil diets had significantly higher final body weights than rats fed the coconut oil diets (salmon oil: 284 ± 10 g; coconut oil: 263 ± 11 g, means ± SD, n=18 for each group, $P<0.05$). The type of cholesterol (oxidized vs. pure cholesterol) did not affect the rats' final body weight.

Concentrations of 7β-OH and 7-K in the liver (Table II). The concentrations of 7β-OH and 7-K were affected by the dietary fat and the type of cholesterol. Rats fed the salmon oil diets had 3.3 times higher concentrations of 7ß-OH and 3.5 times higher concentrations of 7-K in the liver than rats fed the coconut oil diets. Rats fed the diets containing oxidized cholesterol had 1.7 higher concentrations of 7ß-OH and 2.3 times higher concentrations of 7-K than rats fed the diets containing pure cholesterol. The concentrations of both oxysterols were not affected by interactions between dietary fat and type of cholesterol.

Antioxidant status of the liver (Table II). The concentration of α-tocopherol in the liver was not different between rats fed diets containing pure cholesterol and rats fed diets containing oxidized cholesterol. However, rats whose diet contained coconut oil had significantly higher concentrations of α-tocopherol than rats whose diets contained salmon oil. Rats fed diets containing oxidized cholesterol had a significantly higher activity of GSH-Px than rats fed diets containing pure cholesterol. Rats fed the diets containing oxidized cholesterol, moreover, had significantly lower concentrations of total and reduced glutathione in the liver than rats fed the diets containing pure cholesterol. The activity of G6PDH was markedly lower in rats fed salmon oil diets than in rats fed coconut oil diets but was not affected by the type of cholesterol. The activities of antioxidant enzymes and concentrations of total and reduced glutathione were not affected by interactions between dietary fat and type of cholesterol.

Table II: Concentrations of 7β-hydroxycholesterol and 7-ketocholesterol, α-tocopherol and total and reduced glutathione and activities of antioxidant enzymes in the liver of rats fed a diet containing 0.5% cholesterol or 0.5% oxidized cholesterol at two different dietary fats (coconut oil vs. salmon oil)[1,2]

Fat	Coconut oil		Salmon oil	
Cholesterol	Cholesterol	oxidized Cholesterol	Cholesterol	oxidized Cholesterol
7β-hydroxycholesterol (nmol/g) [3,4]	6.5 ± 1.9[d]	13.6 ± 1.9[c]	25.7 ± 3.7[b]	40.2 ± 13.0[a]
7-ketocholesterol (nmol/g) [3,4]	24.1 ± 6.1[d]	112 ± 38[c]	164 ± 43[b]	312 ± 60[a]
α-tocopherol (nmol/g) [3]	138 ± 35[a]	149 ± 28[a]	96 ± 21[b]	83 ± 16[b]
GSH-Px (U/mg protein) [4]	1.02 ± 0.13[b]	1.39 ± 0.17[a]	1.15 ± 0.20[b]	1.43 ± 0.17[a]
G6PDH (U/mg protein) [3]	6.94 ± 2.38[a]	8.21 ± 3.93[a]	1.07 ± 0.24[b]	1.22 ± 0.28[b]
GSH_{tot} (µmol/g) [4]	3.15 ± 0.50[ab]	2.75 ± 0.50[b]	3.24 ± 0.43[a]	2.99 ± 0.38[ab]
GSH_{red} (µmol/g) [4]	2.20 ± 0.32[ab]	1.87 ± 0.18[b]	2.36 ± 0.31[a]	2.02 ± 0.30[b]

[1]Results are means ± SD, n=9 for each treatment group. Means within a row not sharing the same superscript letters differ significantly by Fisher´s multiple range test (P<0.05).
[2]Significance of Factors (P<0.05): [3]Fat, [4]Cholesterol, [5]Fat x cholesterol.

mRNA concentration of antioxidant enzymes (Table III). Rats fed diets containing oxidized cholesterol had significantly higher mRNA concentrations of GSH-Px and SOD than rats fed diets containing pure cholesterol. Rats fed the diets containing oxidized cholesterol also showed a tendency (P<0.15) towards higher mRNA concentrations of GSH reductase as compared to rats fed diets with pure cholesterol. The concentrations of mRNA of all the enzymes studied were not affected by the dietary fat and no interactions occurred between dietary fat and type of cholesterol.

Table III: Relative mRNA concentrations (% of GAPDH) of antioxidative enzymes in the liver of rats fed a diet containing 0.5% cholesterol or 0.5% oxidized cholesterol at two different dietary fats (coconut oil vs. salmon oil)[1,2]

Fat	Coconut oil		Salmon oil	
Cholesterol	Cholesterol	oxidized Cholesterol	Cholesterol	oxidized Cholesterol
	(% of GAPDH)			
mRNA GSH-Px [3]	206 ± 20.1[b]	286 ± 14.1[a]	225 ± 48.7[b]	298 ± 41.9[a]
mRNA SOD [3]	30.4 ± 4.41[b]	38.8 ± 9.77[a]	32.6 ± 4.39[ab]	38.0 ± 6.10[a]
mRNA GSH reductase	0.44 ± 0.18[b]	0.53 ± 0.18[ab]	0.53 ± 0.18[ab]	0.68 ± 0.21[a]

[1]Results are means ± SD, n=9 for each treatment group. Means within a row not sharing the same superscript letters differ significantly by Fisher´s multiple range test (P<0.05).
[2]Significance of Factors (P<0.05): [3]Cholesterol.

Discussion

In this study we investigated whether dietary oxysterols affect the antioxidant status of rats. The experimental animals were fed a restrictive diet in order to exclude secondary effects which might result from different feed intakes. Yet to our surprise, the rats whose diet contained salmon oil had higher body weights at the end of the experiment than the rats

whose diet contained coconut oil. This might be due to the low concentrations of linoleic and α-linolenic acid in the coconut oil diets, although raised concentrations of 20:3 (n-9) in liver lipids, which would indicate a deficiency of essential fatty acids, were not observed (data not shown). The differences in body weights between the rats are however unlikely to jeopardize the results of our study with regard to the effects of oxidized cholesterol.

As expected, the consumption of oxidized cholesterol vs. pure cholesterol lead to increased concentrations of 7β-OH and 7-K in the liver. This is due to the fact that oxysterols are absorbed from the diet and carried to the liver where they are partially incorporated [3, 4]. The observation that rats whose diet contained fish oil had higher concentrations of 7β-OH and 7-K in the liver than rats whose diet contained coconut oil is probably due to the fact that incorporation of highly unsaturated fatty acids into liver lipids makes them much more prone to oxidation. This is consistent with the assumption that 7β-OH and 7-K, unlike other oxysterols, are secondary products of peroxidation of PUFA [27]. Other studies have also shown that oxidation of PUFA can secondarily lead to oxidation of cholesterol [22, 28].

The study also shows that consumption of oxidized cholesterol as opposed to pure cholesterol leads to increased expression and activity of antioxidant enzymes in the liver and a reduced concentration of glutathione. Induction of the expression of antioxidant enzymes and a reduced concentration of glutathione in the liver have both been described as a consequence of oxidant stress [15, 29]. The present study also suggests that consumption of oxidized cholesterol as opposed to pure cholesterol leads to oxidant stress in the liver. It was noted that the effects of oxidized cholesterol on expression and activity of the named enzymes were as strong after feeding coconut oil as after feeding fish oil. This shows that oxidized cholesterol stresses the antioxidant defence system even if the susceptibility of tissues to oxidation is low. The present study in rats is therefore consistent with studies in cell systems, where oxysterols such as 7β-OH and 7-K also induced oxidant stress. Oxysterols led to increased activities of antioxidant enzymes and depletion of glutathione also in human macrophages and monocytes and in rat hepatocytes [5-8]. In studies with monocytes or macrophages the depletion of glutathione through oxysterols was described as having great pathophysiological significance [5, 30, 31].

The study also shows that the vitamin E status of the liver was not significantly affected by oxidized cholesterol, irrespective of the type of dietary fat. This suggests that oxidized cholesterol, unlike oxidized fatty acids [32], does not raise the vitamin E consumption in tissues, which leads us to conclude that ingestion of oxidized cholesterol does not significantly increase the requirement for vitamin E.

To summarise, the results of this study support the conclusion that consumption of oxidized cholesterol causes oxidant stress in the liver of rats. We can therefore assume that the prooxidant effects described probably contribute to the pathophysiological effects of oxysterols.

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3.5 Effekt oxidierten Cholesterins auf Parameter des Lipidstoffwechsels und der VLDL-Synthese und -Sekretion bei unterschiedlichen Diätfetten bei der Ratte

RINGSEIS R, EDER K: Dietary oxidized cholesterol decreases expression of hepatic microsomal triglyceride transfer protein in rats. *Journal of Nutritional Biochemistry* (Manuskript: eingereicht zur Publikation 2003)

ABSTRACT The aim of this study was to compare the effects of dietary oxidized cholesterol and pure cholesterol on plasma and VLDL lipids and on some parameters of VLDL assembly and secretion in rats fed two different dietary fats. Four groups of male growing Sprague-Dawley rats were fed diets containing pure or oxidized cholesterol (5 g/kg diet) with either coconut oil or salmon oil as dietary fat (100 g/kg diet) for 35 days. Rats fed oxidized cholesterol supplemented diets had significantly lower concentrations of triglycerides and cholesterol in plasma and VLDL than rats fed pure cholesterol supplemented diets irrespective of the type of fat. In addition, rats fed oxidized cholesterol supplemented diets had significantly lower relative concentrations of microsomal triglyceride transfer protein mRNA than rats fed pure cholesterol supplemented diets. In contrast, hepatic lipid concentrations and the relative concentration of apolipoprotein B mRNA were not influenced by the dietary factors investigated. Parameters of hepatic lipogenesis (relative mRNA concentration of sterol regulatory element binding protein-1c and activity of glucose-6-phosphat dehydrogenase) were significantly reduced by feeding fish oil compared to coconut oil, but were not affected by the type of cholesterol. In conclusion, the data of this study suggest, that dietary oxidized cholesterol affects VLDL assembly and/or secretion by reducing the synthesis of MTP but not by impairing hepatic lipogenesis or synthesis of apolipoprotein B.

Introduction

Results from animal studies exhibited that dietary oxidized cholesterol influences lipid metabolism [1-3]. Several studies showed that dietary oxidized cholesterol leads to decreased concentrations of triglycerides and/or cholesterol in plasma as compared to pure cholesterol [3-5].

Plasma lipids mainly derive from the liver, which are secreted by the liver within apolipoprotein B (apoB) containing lipoproteins (very low density lipoprotein, VLDL). Dietary cholesterol enhances VLDL synthesis and secretion through an increase in the transcription of microsomal triglycerid transfer protein (MTP) [6]. MTP is essential for the assembly of apoB containing lipoproteins by translocation of apoB and component lipids (cholesterol, cholesterol esters, triglycerides, and phospholipids) across the endoplasmatic reticulum [7]. Transcription of the MTP gene is regulated by cholesterol through a sterol response element located in its promoter [8]. Specific oxysterols are potent regulators of lipid metabolism through activation of liver X receptor α (LXRα), a nuclear transcription factor involved in the regulation of genes important for cholesterol homeostasis [9]. Therefore, it appears to be possible that dietary oxidized cholesterol alters the synthesis and secretion of VLDL by affecting the transcription of MTP and/or apoB, too. However, the effects of dietary oxidized cholesterol on VLDL synthesis or secretion have not yet been investigated. Hence, this study was conducted to determine the effects of oxidized cholesterol on plasma and VLDL lipids and the gene expression of apoB and MTP in rats. Rats fed pure cholesterol were used as controls.

Concentrations of VLDL lipids are strongly influenced by hepatic lipid concentrations, too. Concerning, that dietary oxidized cholesterol was shown to affect hepatic cholesterol biosynthesis and lipogenesis [1, 2, 5], we proposed to measure the concentrations of

cholesterol, triglycerides and phospholipids in the liver. As parameters of hepatic lipogenesis we determined the gene expression of the sterol regulatory element binding protein-1c (SREBP-1c) and the activity of the lipogenic enzyme glucose-6-phosphate dehydrogenase (G-6-PDH). SREBP-1c, a target gene of LXRα, is a transcription factor that regulates expression of various lipogenic genes. Therefore, SREBP-1c not only coordinates the regulation of hepatic lipogenesis but also controls the assembly and secretion of apoB-containing lipoproteins [10]. As the type of fat shows a marked influence on hepatic lipid synthesis and plasma lipid concentrations we planned to use two dietary fats, salmon oil and coconut oil, with a very different fatty acid composition. Fish oil shows a suppressive effect on hepatic lipogenesis, which is mediated by a reduced transcription of lipogenic enzymes, which are controlled by SREBP-1c [11]. Dietary oxidized cholesterol was shown to modulate desaturation of fatty acids within liver phospholipids [1, 2, 12], which might influence the concentration of VLDL component lipids. Therefore, we further determined the fatty acid composition of hepatic phospholipids and triglycerides to assess possible effects of oxidized cholesterol on the desaturation of fatty acids.

Materials and methods

Animals and diets

36 male Sprague-Dawley rats (Charles River, Sulzfeld, Germany) with an initial body weight of 72 g (± 7 g, SD) were assigned to four groups of nine rats each. The animals were kept individually in Macrolon cages in a room maintained at a temperature of 23° C and 50 to 60% relative humidity with lighting from 0700 to 1900 h. All experimental procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the council of Saxony-Anhalt.

Semisynthetic diets were used. The composition of the basal diets is shown in Table 1. Minerals and vitamins with the exception of vitamin E were supplemented in accordance with recommendations by the American Institute of Nutrition AIN-93G [13] for rat diets. The dietary fat (coconut oil vs. salmon oil) and cholesterol obtained from Sigma-Aldrich (Steinheim, Germany) (5 g pure cholesterol per kg vs. 5 g oxidized cholesterol per kg) were varied according to a two-factorial design. Oxidized cholesterol was prepared by heating cholesterol (Sigma-Aldrich, Steinheim, Germany) placed as a thin film on a glass Petri dish at 115°C for 48 hours in an electric oven. Oxysterols (Sigma-Aldrich, Steinheim, Germany) in the diets were determined using a quantitative GC-MS method with selective ion monitoring [14], which has been described in detail in a previous paper [15].

Table 1 Composition of the basal diets

Ingredient	Cholesterol (g/kg diet)	Oxidized Cholesterol (g/kg diet)
Corn starch	398	398
Casein	200	200
Saccharose	200	200
Fat*	100	100
Mineral mixture†	40	40
Vitamin mixture‡	20	20
Cellulose	35	35
Cholesterol	5	-
Oxidized Cholesterol	-	5
DL methionine	2	2

*100 g/kg coconut oil or 100 g/kg salmon oil

†Mineral mixture supplied the following (per kg diet): 7.56 g calcium carbonate; 8.67 g dicalcium phosphate; 6.87 g potassium chloride; 3.77 g sodium bicarbonate; 1.01 g magnesium oxide; 0.116 g

ferrous sulfate hydrate; 0.038 g zinc oxide; 0.016 g manganese oxide; 0.024 g copper sulfate pentahydrate; 0.0032 g calcium iodate; 0.0033 g sodium selenite pentahydrate; saccharose to 40 g.
‡Vitamin mixture supplied the following (per kg diet): 1.34 mg all-*trans*-retinol; 25 µg cholecalciferol; 7.5 mg menadion sodium bisulfite; 5 mg thiamine hydrochloride; 6 mg riboflavine; 6 mg pyridoxine hydrochloride; 15 mg calcium pantothenate; 30 mg nicotinic acid; 1,000 mg choline chloride; 2 mg folic acid; 0.2 mg biotin; 0.025 mg vitamin B12; saccharose to 20 g.

To equalize the vitamin E concentrations of the diets irrespective of the dietary fats used, the native tocopherol concentrations of the two fats were analysed. Based on these native concentrations, diets were supplemented individually with all-*rac*-α-tocopheryl acetate (Merck Eurolab, Darmstadt, Germany), allowing for a biopotency of 67% compared to α-tocopherol. The final vitamin E concentrations of both types of diets were 40 mg α-tocopherol equivalents/kg. The fatty acid composition of coconut oil (Palmin, Hamburg, Germany) and salmon oil (Caelo, Hilden, Germany) is shown in Table 2.

Table 2 Fatty acid composition of the dietary fats*

Fatty acid	Coconut oil (g/100 g fatty acids)	Salmon oil (g/100 g fatty acids)
8:0	8.1	-†
10:0	6.3	-†
12:0	45.6	-†
14:0	17.0	6.1
16:0	9.7	14.5
16:1	-†	8.2
18:0	4.5	3.0
18:1 (n - 9)	6.3	12.9
18:1 (n - 7)	-†	2.7
18:2 (n - 6)	1.9	2.2
20:1	-†	4.3
20:4 (n - 3)	-†	1.4
20:5 (n - 3)	-†	12.7
22:1	-†	4.5
22:5 (n - 3)	-†	3.1
22:6 (n - 3)	-†	10.0

*The table contains fatty acids with amounts greater than 1 g/100 g fatty acids only.
†Fatty acid exists in amounts smaller than 1 g/100 g fatty acids.

The peroxide values of the dietary fats, which were extracted from the diets with a mixture of hexane and isopropanol (3:2, as described in [16]) and measured according to official methods [17], were 3.9 and <0.1 mEq O_2 per kg salmon oil and coconut oil, respectively.

The diets were prepared weekly by solubilizing the all-*rac*-α-tocopheryl acetate and pure cholesterol/oxidized cholesterol preparation in the fat and mixing it with the dry components and water. The diets were then freeze dried and stored at –20°C to prevent autoxidation of lipids, e.g. polyunsaturated fatty acids and cholesterol. The water content after freeze drying was below 5 g per 100 g of diet.

To standardize the feed intake, the diets were fed daily in restricted amounts at 0800 h. The feed intake was increased from 7.0 g per day to 15.0 g per day during the experiment resulting in an average daily feed intake of 14.4 g. Water was provided ad libitum from nipple drinkers. The experimental diets were fed for 35 days.

Sample collection

At the end of the feeding period the rats were starved overnight, anesthetized with diethyl ether and killed by decapitation. The liver was excised immediately, frozen with liquid

nitrogen and stored at -80°C until analysis. Plasma was separated from blood by centrifugation (1,100 g, 10 min) at 4°C. For separation of VLDL the plasma density was adjusted to δ=1006 g/L by adding 0.3 mL of a solution containing 0.195 mol/L sodium chloride and 2.44 mol/L sodium bromide to 0.6 mL of plasma and centrifuged using a Mikro-Ultracentrifuge (Sorvall Products, Bad Homburg, Germany) at 900,000 g for 1.5 h. Plasma and VLDL were stored at –80°C until analysis.

Lipid analysis
Liver lipids were extracted with a mixture of hexane and isopropanol (3:2, v/v) [16]. Total cholesterol and triglyceride concentrations of liver, plasma, and VLDL were determined using enzymatic reagent kits obtained from Merck Eurolab (Darmstadt, Germany). For the measurement of liver total cholesterol and liver triglycerides, lipids of the extract were dissolved in Triton X-100 before enzymatic measurement as described by De Hoff et al. [18]. Hepatic cardiolipin (CL), phosphatidyl ethanolamine (PE), phosphatidyl inositol (PI), phosphatidyl choline (PC) and sphingomyelin (SM) were separated using a HPLC station (Hewlett Packard, Waldbronn, Germany) fitted with a Supelcosil column (25 cm, 4.6 mm, 5 µm; Supelco, Bellefonte, USA) and quantified using an evaporative light scattering detector (Sedex 55; SEDERE, Alfortville Cedex, France) and external standards. For analysis of fatty acids of liver lipids triglycerides, PE and PC were collected with a fraction collector (FC 203B; Abimed, Langenfeld, Germany). The fatty acid composition of experimental fats and liver TG, PE and PC was determined by gas chromatography (GC) of fatty acid methyl esters (FAME) as described previously in detail [19]. Briefly, fats were methylated with trimethylsulfonium hydroxide according to [20]. FAME were separated using a GC system (HP 5890, Hewlett-Packard GmbH, Böblingen, Germany) fitted with an automatic on-column injector, a polar capillary column (30 m FFAP, 0.53 mm i.d., Macherey and Nagel, Düren, Germany) and a flame ionization detector.

Measurement of G6PDH activity
The activity of glucose-6-phosphate dehydrogenase (G6PDH) in the liver was determined by the method of Deutsch [21]. One unit of G6PDH is defined as one µmol reduced β-nicotinamide adenine dinucleotide phosphate oxidized per min.

Measurement of relative mRNA concentrations
To quantify the gene expression of apoB, MTP and SREBP-1c the total RNA was isolated from liver by Trizol^{TM} reagent (Invitrogen, Karlsruhe, Germany). The relative quantities of apoB mRNA, MTP mRNA and SREBP-1c mRNA compared to GAPDH mRNA were determined by quantitative real-time RT-PCR. First strand cDNA was prepared using the Omniscript RT Kit (200) from QIAGEN (Hilden, Germany) and Oligo dT-Primer pd$(T)_{12-18}$ from Amersham Pharmacia Biotech (Freiburg, Germany). The real-time PCR was performed with a Taq DNA Polymerase from Promega (Mannheim, Germany) and specific primers coding for GAPDH, apoB, MTP and SREBP-1c. Amplification of first strand cDNA was performed using a real-time RCR cycler (Rotorgene, Corbett Research, LTF Labortechnik, Wasserburg, Germany). The primers used for PCR were as follows: 5′-GCATGGCCTTCCGTGTTCC-3′ (sense) and 5′-GGGTGGTCCAGGGTTTCTTACTC-3′ (antisense) for rat GAPDH; 5′-GGAAAGGGGAGGGAAAAGGTT-3′ (sense) and 5′-TTAGGTAGGGGCTCACATTATTGG-3′ (antisense) for rat apoB; 5′-GGAGCCATGGATTGCACATT-3′ (sense) and 5′-AGGAAGGCTTCCAGAGAGGA-3′ (antisense) for rat SREBP-1c; mouse specific 5′-CGCGAGTCTAAAACCCGAGTG-3′ (sense) and 5′-CCCTGCCTGTAGATAGCCTTTCAT-3′ (antisense) for rat MTP.

Statistical analysis
Data were subjected to ANOVA using the Minitab Statistical Software (Minitab, State College, PA, USA). Classification factors were dietary fat, cholesterol and the interaction of both factors. In cases where the differences between variances and means were large, data were transformed to logarithms prior to ANOVA. For statistically significant F values, individual means of the treatment groups were compared by Fisher´s multiple range test. Means were considered significantly different for $P<0.05$.

Results
Concentrations of oxysterols in the diets. The diets supplemented with 5 g oxidized cholesterol/kg contained the following oxysterols (mg/kg) which are known to be formed at the highest concentrations during heating of cholesterol: 7β-OH, 2.7; α-epoxycholesterol, 2.6; β-epoxycholesterol, 6.9; cholestanetriol, 0.1; 25-hydroxycholesterol, 9.4; 7-ketocholesterol, 1.7. Other minor, unidentified oxysterols have not been quanified. In the diets containing pure cholesterol the concentrations of all oxysterols were below the limit of detection of 0.025 mg/kg.
Body weights of the rats. The initial body weights of the rats were similar within the four treatment groups (Table 3). However, rats fed the salmon oil diets gained significantly more body weight during the feeding period and had significantly higher final body weights than the rats fed coconut oil. The type of cholesterol (oxidized vs. pure cholesterol) did not affect the body weight gain and the final body weights of the rats.

Table 3 Body weights, body weight gain and concentrations of plasma and VLDL lipids of rats fed diets with two different dietary fats (coconut oil vs. salmon oil) and with 5 g/kg of either pure or oxidized cholesterol

Dietary Fat	Coconut oil		Salmon oil	
Dietary Cholesterol	Pure Cholesterol	Oxidized Cholesterol	Pure Cholesterol	Oxidized Cholesterol
Parameter	(*n* = 9)	(*n* = 9)	(*n* = 9)	(*n* = 9)
Initial body weight (g)	72.0 ± 8.2	72.2 ± 8.0	72.3 ± 8.3	71.9 ± 9.1
Final body weight (g)*	261 ± 13^{b}	265 ± 10^{b}	284 ± 10^{a}	283 ± 11^{a}
Body weight gain (g/d)*	5.41 ± 0.34^{b}	5.52 ± 0.21^{b}	6.05 ± 0.22^{a}	6.04 ± 0.36^{a}
Triglycerides (mmol/L)				
Plasma*†	0.79 ± 0.25^{a}	0.59 ± 0.12^{b}	0.54 ± 0.13^{b}	0.44 ± 0.19^{b}
VLDL*†	0.21 ± 0.08^{a}	0.13 ± 0.04^{b}	0.12 ± 0.04^{bc}	0.07 ± 0.03^{c}
Cholesterol (mmol/L)				
Plasma*†	4.80 ± 0.47^{a}	4.45 ± 0.63^{a}	1.94 ± 0.25^{b}	1.68 ± 0.17^{b}
VLDL*†‡	3.18 ± 0.38^{a}	2.40 ± 0.66^{b}	0.45 ± 0.14^{c}	0.34 ± 0.16^{c}

Results are expressed as means ± SD.
Results of analysis of variance: *Significant effect of factor fat ($P < 0.05$); †Significant effect of factor cholesterol ($P < 0.05$); ‡Significant interaction between factors fat and cholesterol ($P < 0.05$). Mean values in a row without a common superscript letter differ ($P < 0.05$).
VLDL-very low density lipoprotein.

Plasma and VLDL lipids. The lipid concentrations of plasma and VLDL were significantly influenced by both dietary factors, the type of cholesterol and the type of fat (Table 3). The results of ANOVA showed that rats fed the diets containing oxidized cholesterol had significantly lower concentrations of triglycerides and cholesterol in plasma and VLDL than

rats fed diets containing pure cholesterol. Rats whose diets contained coconut oil had significantly higher concentrations of triglycerides and cholesterol in plasma and VLDL than rats whose diets contained salmon oil.

Parameters of hepatic lipogenesis and concentrations of liver lipids. The relative concentrations of SREBP-1c mRNA and the activities of G-6-PDH were both affected by the type of dietary fat (Table 4); rats fed the salmon oil diets had markedly lower relative concentrations of SREBP-1c mRNA and activities of G-6-PDH than rats fed the coconut oil diets. The type of cholesterol did not influence the relative concentrations of SREBP-1c mRNA and the activities of G-6-PDH. In contrast, neither the type of fat nor the type of cholesterol influenced the concentrations of total cholesterol, triglycerides and phospholipids in the liver.

Table 4 Parameters of hepatic lipogenesis and concentrations of liver lipids of rats fed diets with two different dietary fats (coconut oil vs. salmon oil) and with 5 g/kg of either pure or oxidized cholesterol

Dietary Fat	Coconut oil		Salmon oil	
Dietary Cholesterol	Pure Cholesterol	Oxidized Cholesterol	Pure Cholesterol	Oxidized Cholesterol
Parameter	(*n* = 9)	(*n* = 9)	(*n* = 9)	(*n* = 9)
SREBP-1c mRNA (% of GAPDH*100)*	8.15 ± 4.03[a]	7.26 ± 3.70[a]	1.90 ± 0.91[b]	1.27 ± 0.45[b]
G-6-PDH activity (U/mg protein)*	6.94 ± 2.38[a]	8.21 ± 3.93[a]	1.07 ± 0.24[b]	1.22 ± 0.28[b]
		(µmol/g)		
Total cholesterol	103 ± 28	111 ± 24	125 ± 22	133 ± 25
Triglycerides	26.6 ± 5.6	35.6 ± 11.4	38.0 ± 11.5	41.7 ± 19.7
Cardiolipin	0.77 ± 0.15	0.80 ± 0.12	0.68 ± 0.10	0.69 ± 0.09
PE	11.9 ± 0.7	12.3 ± 0.6	11.7 ± 1.1	12.0 ± 1.2
PI	4.98 ± 0.41	4.72 ± 0.46	4.62 ± 0.50	4.34 ± 0.77
PC	17.7 ± 1.5	17.6 ± 1.2	18.5 ± 1.4	17.5 ± 2.0
Sphingomyelin	3.07 ± 1.05	2.86 ± 0.44	2.33 ± 0.32	2.65 ± 0.65

Results are expressed as means ± SD.
Results of analysis of variance: *Significant effect of factor fat ($P < 0.05$). Mean values in a row without a common superscript letter differ ($P < 0.05$).
SREBP-1c-sterol regulatory element binding protein-1c. G-6-PDH-glucose-6-phosphat dehydrogenase. PE-phosphatidylethanolamine. PI-phosphatidylinositol. PC-phosphatidylcholine.

Fatty acid composition of liver lipids. The fatty acid compositions of liver TG, PE and PC were strongly influenced by the type of dietary fat (Tables 5-7). Rats fed salmon oil diets had significantly higher proportions of long chain n-3 PUFA and total PUFA, but significantly lower proportions of total MUFA in TG, PE and PC than the rats fed coconut oil diets. Proportions of total SFA were slightly elevated in PE and PC, but strongly reduced in TG of the rats fed salmon oil compared to the rats fed coconut oil. Oxidized cholesterol showed only slight effects on the fatty acid composition of hepatic TG, PE and PC as compared to pure cholesterol. The only notable effect of dietary oxidized cholesterol was exhibited on the proportion of 20:4 n-6 in hepatic TG, PE and PC. Rats fed oxidized cholesterol had significantly higher proportions of 20:4 n-6 in TG, PE and PC than rats fed pure cholesterol.

Table 5 Fatty acid composition of liver triglycerides of rats fed diets with two different dietary fats (coconut oil vs. salmon oil) and with 5 g/kg of either pure or oxidized cholesterol

Dietary Fat	Coconut oil		Salmon oil	
Dietary Cholesterol	Pure Cholesterol	Oxidized Cholesterol	Pure Cholesterol	Oxidized Cholesterol
Parameter	(*n* = 9)	(*n* = 9)	(*n* = 9)	(*n* = 9)
Total SFA*	33.7 ± 2.0^{a}	33.3 ± 1.1^{a}	16.0 ± 1.3^{b}	16.6 ± 1.3^{b}
16:0*	28.1 ± 2.0^{a}	27.5 ± 1.4^{a}	13.1 ± 1.4^{b}	13.8 ± 1.3^{b}
18:0*	1.76 ± 0.28^{a}	1.71 ± 0.17^{ab}	1.50 ± 0.15^{b}	1.48 ± 0.22^{b}
Total MUFA*	46.9 ± 2.9^{a}	48.9 ± 1.7^{a}	34.7 ± 1.2^{b}	34.1 ± 1.8^{b}
16:1*	12.0 ± 1.3^{a}	12.9 ± 0.6^{a}	7.84 ± 1.15^{b}	7.53 ± 0.99^{b}
18:1*†‡	34.1 ± 1.8^{b}	37.0 ± 2.5^{a}	25.8 ± 1.4^{c}	25.7 ± 1.2^{c}
Total PUFA*	2.94 ± 0.50^{b}	2.98 ± 0.36^{b}	35.3 ± 1.3^{a}	34.2 ± 1.3^{a}
18:2n-6*†	1.35 ± 0.25^{c}	1.57 ± 0.16^{c}	4.15 ± 0.43^{b}	4.63 ± 0.34^{a}
20:2n-6*	0.36 ± 0.08^{a}	0.34 ± 0.05^{a}	0.13 ± 0.10^{b}	0.14 ± 0.11^{b}
20:3n-6*	0.09 ± 0.03^{b}	0.09 ± 0.03^{b}	0.22 ± 0.03^{a}	0.24 ± 0.04^{a}
20:4n-6*†	0.41 ± 0.06^{b}	0.47 ± 0.09^{b}	0.62 ± 0.07^{a}	0.69 ± 0.12^{a}
20:5n-3*	0.14 ± 0.07^{b}	0.10 ± 0.05^{b}	6.61 ± 0.86^{a}	6.87 ± 1.01^{a}
22:5n-3*	0.21 ± 0.12^{b}	0.13 ± 0.05^{b}	4.81 ± 0.69^{a}	4.50 ± 0.60^{a}
22:6n-3*	0.26 ± 0.14^{b}	0.12 ± 0.03^{b}	18.2 ± 1.0^{a}	17.4 ± 1.4^{a}

Results are expressed as means ± SD.
Results of analysis of variance: *Significant effect of factor fat ($P < 0.05$); †Significant effect of factor cholesterol ($P < 0.05$); ‡Significant interaction between factors fat and cholesterol ($P < 0.05$). Mean values in a row without a common superscript letter differ ($P < 0.05$).
SFA-saturated fatty acids. MUFA-unsaturated fatty acids. PUFA-polyunsaturated fatty acids.

Table 6 Fatty acid composition of liver phosphatidylethanolamine of rats fed diets with two different dietary fats (coconut oil vs. salmon oil) and with 5 g/kg of either pure or oxidized cholesterol

Dietary Fat	Coconut oil		Salmon oil	
Dietary Cholesterol	Pure Cholesterol	Oxidized Cholesterol	Pure Cholesterol	Oxidized Cholesterol
Parameter	(*n* = 9)	(*n* = 9)	(*n* = 9)	(*n* = 9)
Total SFA*†	31.8±1.0^{b}	32.4±0.7^{b}	32.2±1.6^{b}	33.7±1.0^{a}
16:0	14.4±0.8	14.5±0.9	15.5±1.05	14.9±0.9
18:0	16.9±1.3	17.5±1.2	16.9±1.5	18.4±1.0
Total MUFA*†	13.3±0.8^{a}	12.8±1.2^{a}	11.5±0.7^{b}	10.4±1.2^{c}
16:1*†	1.95±0.25^{a}	1.73±0.13^{b}	1.38±0.17^{c}	1.15±0.20^{d}
18:1*	5.46±0.57^{a}	5.38±0.64^{a}	4.94±0.24^{ab}	4.62±0.74^{b}
Total PUFA*†	39.8±0.7^{c}	40.6±0.9^{bc}	41.5±1.6^{b}	42.7±0.7^{a}
18:2n-6*	5.15±0.53^{a}	5.30±0.43^{a}	2.29±0.27^{b}	2.53±0.21^{b}
20:2n-6*	2.91±0.55^{a}	3.11±0.45^{a}	0.25±0.22^{b}	0.22±0.22^{b}
20:3n-6*	1.21±0.13^{a}	1.24±0.13^{a}	0.47±0.07^{b}	0.51±0.07^{b}
20:4n-6*†	20.6±1.4^{b}	21.9±0.6^{a}	7.18±0.73^{d}	8.09±0.79^{c}
20:5n-3*	0.60±0.09^{b}	0.66±0.11^{b}	11.2±0.60^{a}	11.5±0.93^{a}
22:5n-3*	0.34±0.15^{b}	0.33±0.07^{b}	3.01±0.33^{a}	2.86±0.48^{a}
22:6n-3*	7.59±0.78^{b}	7.16±0.47^{b}	16.4±1.50^{a}	15.8±1.04^{a}

Results are expressed as means ± SD.
Results of analysis of variance: *Significant effect of factor fat ($P < 0.05$); †Significant effect of factor cholesterol ($P < 0.05$). Mean values in a row without a common superscript letter differ ($P < 0.05$).
SFA-saturated fatty acids. MUFA-unsaturated fatty acids. PUFA-polyunsaturated fatty acids.

Table 7 Fatty acid composition of liver phosphatidylcholine of rats fed diets with two different dietary fats (coconut oil vs. salmon oil) and with 5 g/kg of either pure or oxidized cholesterol

Dietary Fat	Coconut oil		Salmon oil	
Dietary Cholesterol	Pure Cholesterol	Oxidized Cholesterol	Pure Cholesterol	Oxidized Cholesterol
Parameter	(*n* = 9)	(*n* = 9)	(*n* = 9)	(*n* = 9)
Total SFA*	36.8±0.9^{b}	37.2±1.1^{b}	38.0±1.2^{ab}	38.3±0.8^{a}
16:0*†‡	16.7±0.7^{c}	17.1±0.9^{c}	21.7±1.1^{a}	20.3±0.9^{b}
18:0*†‡	19.5±1.2^{a}	19.4±1.2^{a}	15.6±1.2^{c}	17.4±1.0^{b}
Total MUFA*†	20.0±1.2^{a}	19.3±1.2^{a}	17.4±0.8^{b}	16.0±1.2^{c}
16:1*†	3.77±0.46^{a}	3.44±0.39^{ab}	3.16±0.37^{b}	2.66±0.35^{c}
18:1*	10.3±0.5^{a}	10.5±0.4^{a}	9.43±0.52^{b}	9.21±0.93^{b}
Total PUFA*	33.1±0.7^{b}	33.8±1.0^{b}	37.2±0.8^{a}	37.5±0.8^{a}
18:2n-6*	9.43±0.65^{a}	9.53±0.71^{a}	3.98±0.41^{b}	4.32±0.29^{b}
20:2n-6*	6.20±0.88^{a}	6.29±0.66^{a}	0.26±0.12^{b}	0.27±0.12^{b}
20:3n-6*	2.56±0.19^{a}	2.50±0.22^{a}	1.21±0.12^{b}	1.28±0.11^{b}
20:4n-6*†	11.5±0.5^{ab}	12.0±1.1^{a}	9.83±0.50^{c}	11.1±0.3^{b}
20:5n-3*†‡	0.31±0.10^{c}	0.34±0.05^{c}	11.3±0.6^{a}	10.3±0.7^{b}
22:5n-3*	0.11±0.02^{b}	0.13±0.02^{b}	2.06±0.19^{a}	1.90±0.26^{a}
22:6n-3*	2.44±0.31^{b}	2.34±0.09^{b}	7.80±0.49^{a}	7.61±0.51^{a}

Results are expressed as means ± SD.
Results of analysis of variance: *Significant effect of factor fat ($P < 0.05$); †Significant effect of factor cholesterol ($P < 0.05$); ‡Significant interaction between factors fat and cholesterol ($P < 0.05$). Mean values in a row without a common superscript letter differ ($P < 0.05$).
SFA-saturated fatty acids. MUFA-unsaturated fatty acids. PUFA-polyunsaturated fatty acids.

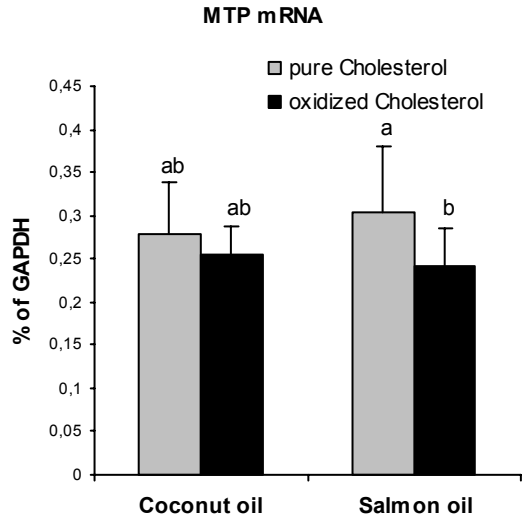

Figure 1 Relative concentration of microsomal triglyceride transfer protein (MTP) mRNA (MTP mRNA in % of GAPDH) of rats fed diets with two different dietary fats (coconut oil vs. salmon oil) and with 5 g/kg of either pure or oxidized cholesterol. Results are means ± SD of nine rats per group. Results of analysis of variance: Significant effect of factor cholesterol ($P < 0.05$). Bars marked without a common superscript letter differ ($P < 0.05$).

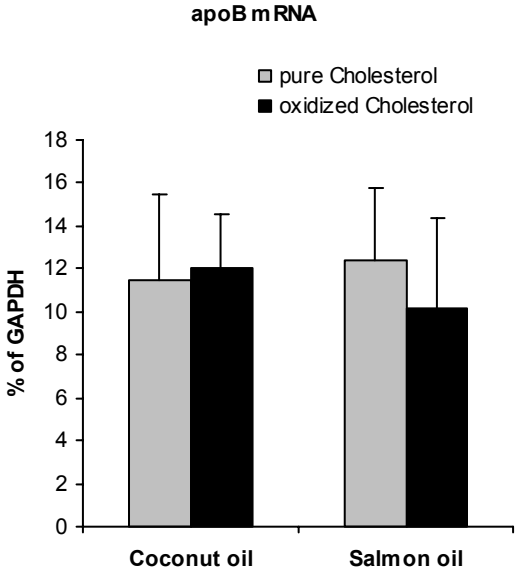

Figure 2 Relative concentration of apolipoprotein B (apoB) mRNA (apoB mRNA in % of GAPDH) of rats fed diets with two different dietary fats (coconut oil vs. salmon oil) and with 5 g/kg of either pure or oxidized cholesterol. Results are means ± SD of nine rats per group.

Relative mRNA concentrations of apoB and MTP. According to ANOVA, the relative concentrations of MTP mRNA were significantly influenced by the factor cholesterol (Figure

1). Rats receiving oxidized cholesterol had lower relative concentrations of MTP mRNA than rats receiving pure cholesterol. The type of fat showed no effect on the relative concentrations of MTP mRNA. The relative concentrations of apoB mRNA were not affected by both dietary factors investigated, the type of fat and type of cholesterol (Figure 2).

Discussion

In this study the effect of dietary oxidized cholesterol compared to pure cholesterol on lipid metabolism in rats was investigated with special regard to parameters of VLDL synthesis and secretion. To gain insight into the composition and the degree of oxidation of the oxidized cholesterol preparation we measured six of the dominating oxysterols knowing to be formed under the applied oxidizing conditions. The measured oxysterols in the oxidized cholesterol preparation are the quantitatively dominating oxysterols in Western foods [22]. Regarding the low concentrations of those oxysterols suggests that we used a moderately oxidized preparation. This is relevant with respect to human diets concerning the estimation that about 1% of the cholesterol consumed in a mixed Western diet is oxidized [22]. The experimental animals were fed a restrictive diet in order to exclude secondary effects which might result from different feed intakes. However, the rats fed salmon oil showed a higher body weight gain than the rats fed coconut oil. This effect might be attributed to the low concentrations of essential fatty acids (linoleic, α-linolenic acid) in the coconut oil, although we could not observe any signs of a deficiency of essential fatty acids e.g. raised concentrations of 20:3 (n-9). The differences in body weights between the rats are however unlikely to jeopardize the results of our study with regard to the effects of oxidized cholesterol.

It is well documented in rats and other animal species that oxysterols of an oxidized cholesterol preparation are readily absorbed from the intestine and transported to the liver, where they exert various biological effects [4, 23]. Our study showed that feeding oxidized cholesterol lowered the concentrations of plasma cholesterol and triglycerides as observed in feeding experiments from other investigators [3-5]. The experiment further revealed that dietary oxysterols reduced the concentrations of VLDL cholesterol and triglycerides possibly indicating a reduced VLDL synthesis and/or secretion. It is known that a decreased expression of apoB and MTP reduces VLDL assembly and secretion [7]. Thus, we measured the expression of these two genes and found that the expression of MTP was significantly reduced in the rats fed oxidized cholesterol, whereas the expression of apoB was not influenced. In patients with the recessive disorder abetalipoproteinemia the genetic loss of the expression of the MTP gene is responsible for the abnormally lowered VLDL concentrations [24]. Therefore, it appears to be possible, that the reduced transcription of the MTP gene is at least in part responsible for the lowered plasma and VLDL lipid concentrations in the rats fed oxidized cholesterol.

Dietary fish oil, as compared to coconut oil, is a source of oxidative stress due to the incorporation of highly oxidation susceptible n-3 PUFA into liver lipids [25]. According to ANOVA, the effect of oxidized cholesterol on the relative gene transcription of MTP was independent of the dietary fats used. This suggests that the effect of oxidized cholesterol on MTP gene transcription is not due to the induction of oxidative stress.

The observation that hepatic concentrations of lipids and the parameters of hepatic lipogenesis (SREBP-1c mRNA, activity of G-6-PDH) were not influenced by the dietary oxidized cholesterol varies from results from other investigators [1, 2, 5]. However, this difference might be attributed to the fact that we, in contrast to those investigators, used a moderately oxidized cholesterol preparation. Oxysterols are potent inhibitors of hepatic HMG-CoA reductase and the high amount of oxysterols in the diets from those investigators might have strongly suppressed cholesterol biosynthesis in the liver [26]. The increased proportion of 20:4 n-6 in liver TG, PE and PC in the rats fed oxidized cholesterol possibly indicates a raised desaturation of linoleic acid. This is consistent with the observation from

other studies, that dietary oxidized cholesterol increases the activity of Δ6-desaturase [1, 2, 12].

Therefore, the data of our study suggest, that dietary oxidized cholesterol possibly affects VLDL assembly and/or secretion by reducing the transcription of MTP but does not impair the synthesis of component lipids. The reduced transcription of MTP after feeding oxidized cholesterol may be in part explained by the results from *in vitro* studies, which showed that the expression of cholesterol-7α-hydroxylase (CYP7A1) also affects VLDL assembly and secretion due to a positive correlation between CYP7A1 mRNA and MTP mRNA [10]. The activity of CYP7A1 was reported to be decreased by dietary oxidized cholesterol as compared to pure cholesterol [1, 2, 5]. As the activity of CYP7A1 is highly correlated with the abundance of CYP7A1 mRNA it seems possible that a decreased transcription of CYP7A1 is responsible for the reduced concentrations of MTP mRNA in this study. In addition, results from *in vitro* study showed, that the oxysterols 25-hydroxycholesterol and 7-ketocholesterol, both of which were found in the oxidized cholesterol-supplemented diets, reversed the CYP7A1-induced changes on VLDL assembly and secretion [27]. However, this explanation is speculative and has to be confirmed by following studies.

In conclusion, the study showed that dietary oxidized cholesterol significantly reduced plasma and VLDL lipid concentrations. It was further shown that dietary oxidized cholesterol lowered the expression of MTP, which is essential for VLDL assembly and secretion. However, further studies are necessary to confirm a causal linkage between dietary oxidized cholesterol and a decreased expression of MTP and lipid lowering in plasma.

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