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Absorption of Cholesterol Oxidation Products from Ordinary Foodstuff in Humans

Abstract

Background: Information on the absorption of cholesterol oxidation products (COP) from ordinary foodstuff in humans is scarce. **Methods:** Five healthy young men were offered a salami- and Parmesan-containing meal naturally rich in COP. Plasma and lipoprotein COP concentrations were measured over the following 9 h. **Results:** The mean plasma free (non-esterified) COP concentration showed its maximal increase 3 and 5 h after meal consumption. In contrast, the raise in plasma total COP concentration began 6 h after the meal with a maximum at 8 h and was statistically significant for 7α- and 7β-hydroxycholesterol and 7-ketocholesterol. The increase in plasma total cholesterol concentration was comparable to that of total COP. Comparing the COP composition of the chylomicrons and the test meal, cholestanetriol, 7-ketocholesterol, and to a lesser extent cholesterol-α-epoxide were underrepresented in the chylomicrons as was the opposite for 7β-hydroxycholesterol. In very-low-density lipoprotein, a steady increase in the COP:cholesterol ratio was observed from 6 h on. **Conclusion:** COP from ordinary foodstuff were absorbed in the human intestinal tract but differences in the bioavailability of the single COP compounds were found.

Introduction

Atherosclerotic lesions not only contain cholesterol but also a series of cholesterol oxidation products (COP) [1, 2]. In the widely accepted model for the pathogenesis of atherosclerosis the impact of oxidatively modified low-density lipoprotein (LDL) particles is very important [3, 4]. Besides polyunsaturated fatty acids and apolipoproteins, the choles-

terol moiety of LDL was also shown to undergo oxidative damage in vitro [5–7], resulting in different effects on target cells (e.g., macrophages, aortic smooth muscle cells) that may promote the development of atherosclerosis [8–10]. In vivo, COP are formed either enzymatically (e.g., 7α-hydroxycholesterol in bile acid synthesis) or via autoxidation/peroxidation of cholesterol [11, 12]. The contribution of dietary COP to the total amount of COP measurable in plasma and lipoproteins of humans is still unclear. However, the knowledge of their bioavailability is a necessary precondition for the toxicological evaluation of dietary COP in humans. In rabbits, feeding a high-cholesterol diet has been shown to increase plasma COP concentrations and COP content of aortic tissues in parallel [13]. As a most drastic example for the importance of dietary COP intake in humans, the prolonged consumption of ghee, a clarified butter product with high COP levels, is suspected to explain the high incidence of atherosclerotic complications in Indian immigrants in London [14]. Also other foodstuff containing cholesterol or prepared by use of cholesterol-containing food (e.g., deep-frying with animal fat) shows more or less high COP levels [15]. Recently, the importance of COP in the development of atherosclerotic lesions was underlined in a clinical study where the serum 7β-hydroxycholesterol concentration was identified as the strongest predictor of a rapid progression of carotid atherosclerosis in humans [16].

Several studies in rats, rabbits, and monkeys showed that different COP compounds can be absorbed from the intestine and were found in chylomicrons and other lipoproteins [13, 17–21]. In all these investigations pure cholesterol was taken to produce COP by different ways before administration to animals. Only one study reported the effect of feeding a powdered egg meal on the COP concentration of plasma and chylomicrons in humans [22]. Carrying on this subject, the aim of the present study was (1) to offer humans more common foodstuff like cheese or sausage rich in COP; (2) to distinguish plasma COP concentrations in free (nonesterified) compounds and COP acyl esters; (3) to use enzymatic hydrolysis of COP acyl esters instead of hot or cold alkaline saponification, and (4) to compare the COP pattern of meal and chylomicrons to look for discriminatory effects in the absorption of single COP compounds as described in animals [17].

Materials and Methods

Subjects

Five young normal-weight men (age 29–33 years, body weight 72–82 kg, body mass index 22.2–25.5 kg/m^2) free from acute or chronic disease and without any medication took part in the experiment. All subjects gave informed consent prior to the beginning of the study.

Test Meal

After overnight fast the subjects were offered a meal consisting of 150 g salami, 150 g Parmesan, and 135 g white bread. Additional consumption of small amounts of coffee (with a maximum of two cups corresponding to 300 ml) was allowed. The test meal provided 2.09 μmol (0.84 mg) unesterified COP and 6.56 μmol (2.64 mg) COP acyl esters (table 1). Determination of COP in the chloroform/methanol (2/1, v/v) extract of minced food samples was performed as described below with and without prior incubation with buffered cholesterol esterase. Regarding the cholesterol content of the test meal, 24% of a total of 923 μmol (357 mg) cholesterol was found in esterified form (cholesterol colorimetric test combination, free cholesterol test combination; Boehringer Mannheim, Germany). The calculated contribution of fat, protein, and carbohydrates to the total energy content of the meal (1,512 kcal, 6.33 MJ) was 54, 26, and 21%, respectively. No further energy was provided before the end of the blood sampling period after 9 h.

Table 1. Free (nonesterified), esterified, and total COP (μmol/300 g) as well as cholesterol (μmol/300 g) in the test meal consisting of 150 g Parmesan and 150 g salami

	Free COP		Esterified COP in total meal (300 g)		Total COP	
	μmol	%	μmol	%	μmol	%
7α-Hydroxycholesterol	0.316	17.3	1.174	17.9	1.535	17.8
7β-Hydroxycholesterol	0.271	13.0	1.307	19.9	1.578	18.3
Cholesterol-β-epoxide	–	–	0.558	8.5	0.558	6.5
Cholesterol-α-epoxide	–	–	0.359	5.5	0.359	4.2
Cholestanetriol	0.059	2.8	0.737	11.2	0.796	9.2
7-Ketocholesterol	1.354	64.8	2.286	34.9	3.640	42.1
25-Hydroxycholesterol	0.035	1.7	0.145	2.2	0.180	2.1
Σ COP	2.088	100.0	6.558	100.0	8.646	100.0
Cholesterol	702		221		923	
COP:cholesterol ratio, %	0.297		2.974		0.937	

Blood Sampling and Plasma and Lipoprotein Preparation

Blood samples were taken before intake of the test meal as well as 2, 3, 4, 5, 6, 8, and 9 h afterwards. All blood samples for plasma preparation were drawn in tubes containing the antioxidants EDTA (1.6 mg/ml blood) and Trolox (1 μmol/l blood; both from Sigma, Deisenhofen, Germany). Chylomicrons, very-low-density lipoprotein (VLDL), LDL, and high-density lipoprotein (HDL) were separated from 5 ml plasma by stepwise ultracentrifugation [23] within density cut-off values <1.006 g/cm^3 (chylomicrons, VLDL) and <1.063 g/cm^3 (LDL). Chylomicrons were separated by centrifugation for 30 min at 20,000 rpm, VLDL for 16 h at 40,000 rpm, and LDL for 20 h at 40,000 rpm at 15°C. HDL are defined as lipoproteins with a density >1.063 g/cm^3. All aqueous solutions contained 1 mg EDTA/ml. Ultracentrifugation was performed by means of a Kontron TGA-50 centrifuge equipped with a TFT 65.13 fixed-angle rotor (Kontron Instruments, Neufahrn, Germany). Free and total cholesterol concentrations were determined enzymatically using test combinations (Monotest Cholesterin, Free Cholesterol; Boehringer Mannheim).

Analysis of COP

Analysis of free and total COP contents of plasma and lipoprotein samples was performed immediately. For total COP determination, cholesteryl esters were enzymatically hydrolyzed by cholesterol esterase (EC 1.1.1.13; Sigma). The plasma and lipoprotein samples were incubated for 2 h at pH 7.0 and 37°C with the enzyme buffered in 10 mmol/l Na-cholate. The enzyme activity added was about 12.5 U per 2 mmol/l cholesteryl ester. Free (nonesterified) COP were determined in 3-ml plasma samples without prior hydrolysis of cholesteryl esters.

Lipid extraction with chloroform/methanol (2/1 v/v), solid-phase extraction of COP and quantification of COP, compounds with GC-MSD were performed according to a described methodology [5]. The following eight COP compounds were determined (trivial name in parentheses): 5-cholestene-3β,7α-diol (7α-hydroxycholesterol), 5-cholestene-3β,7β-diol (7β-hydroxycholesterol), 5,6β-epoxy-5α-cholestane-3β-ol (cholesterol-α-epoxide), 5,6β-epoxy-5β-cholestane-3β-ol (cholesterol-β-epoxide), 5α-cholestane-3β-ol-6-one (6-ketocholestanol), 5-cholestene-3β-ol-7-one (7-ketocholesterol), 5α-cholestane-3β,5α,6β-triol (cholestanetriol), and 5-cholestane-3β,25-diol (25-hydroxycholesterol). 5-Pregnene-3β-ol-7,20-dione (7-ketopregnenolone) was used as internal standard. All standard substances were delivered by Steraloids (New Barnett, UK) and Sigma. Identification of the sample peaks was made by retention times of the standard substances and the characteristic ion fragmentation. For quantification a selected ion-monitoring program was used. Recovery of free COP standard substances was in the range from 85% (cholestanetriol) to 123% (25-hydroxycholesterol); mean recovery for the sum of COP was 99%. The mean coefficient of variation for the determination of COP in plasma amounted to 2.3% with a range from

1.9% (7α-hydroxycholesterol) to 5.3% (25-hydroxycholesterol) [5].

Statistics

The results are given in terms of mean and standard error of the mean (SEM). Statistical analysis was performed with SPSS, version 6.0 (SPSS, Chicago, Ill., USA). For analysis of variance a two-factorial model with the factors 'person' and 'time point' was applied; comparisons of mean values were made with the paired t test.

Results

Intake of Parmesan and salami raised plasma COP concentrations (table 2). At first, plasma free COP concentrations increased, showing peaks 3 h (mean maximum concentrations of cholesterol-α-epoxide, 7-ketocholesterol, cholestanetriol) and 5 h (mean maximum concentrations of 7α-hydroxycholesterol, 7β-hydroxycholesterol, cholesterol-β-epoxide, 25-hydroxycholesterol) after the meal (fig. 1). All free COP measured increased by time and within every person; however, due to a very high interindividual variation (see table 2) no statistical significance was reached in the two-factorial model (analysis of variance) with the mean of 5 subjects.

In contrast to free COP, total COP levels in the plasma were up to 100-fold higher and showed a much lower variation (table 2). The intake of the COP-containing meal resulted in a significantly increased total COP concentration (sum of COP) after 6 and 8 h (fig. 1). Regarding single substances, mean plasma 7α- and 7β-hydroxycholesterol concentrations were significantly elevated 8 h after the meal, while the increase of 7-ketocholesterol reached statistical significance even after 6 h. These three substances were also the quantitatively dominating COP compounds. For cholesterol epoxides, cholestanetriol, and 25-hydroxycholesterol the mean increase was not statistically significant. Similar to total COP, the total cholesterol concentration in the plasma showed its maximum value 8 h after test meal ingestion (table 2); therefore, no distinct change in the total COP:total cholesterol ratio in the plasma could be observed.

Expressed in terms of percent of the sum of COP, the analysis of COP in the chylomicron fraction 3, 4, 5, and 6 h after meal intake gave nearly identical results. All COP contained in the test meal could be also found in chylomicrons (fig. 2). However, the contribution of 7-ketocholesterol, cholestanetriol, and choles-

Table 2. Concentrations (mean ± SEM) of plasma free (nonesterified) and total (free + esterified) COP as well as plasma free and total cholesterol after intake of a COP-rich meal (Parmesan, salami) in 5 healthy young men

Free COP concentrations nmol/l	7α-Hydroxycholesterol
	7β-Hydroxycholesterol
	Cholesterol-β-epoxide
	Cholesterol-α-epoxide
	Cholestanetriol
	7-Ketocholesterol
	25-Hydroxycholesterol
	Σ COP_{free}
Free cholesterol, mmol/l	
Total COP concentrations µmol/l	7α-Hydroxycholesterol
	7β-Hydroxycholesterol
	Cholesterol-β-epoxide
	Cholesterol-α-epoxide
	Cholestanetriol
	7-Ketocholesterol
	25-Hydroxycholesterol
	Σ COP_{total}
Total cholesterol, mmol/l	

* $p < 0.05$; ** $p < 0.01$ versus 0 h (paired t test).

terol-α-epoxide to the sum of COP is distinctly lower in the chylomicrons than in the meal. Similar values were found for the proportions of 25-hydroxycholesterol and cholesterol-β-epoxide in chylomicrons and test meal, while 7α-hydroxycholesterol and particularly 7β-hydroxycholesterol were overrepresented in the chylomicrons when compared with the test meal COP composition. The total COP:total cholesterol ratio in the chylomicron fractions showed no distinct change by time with mean values of 0.6% (3 h) and 0.5 % (4, 5, 6 h).

Six hours after meal intake, the total COP:total cholesterol ratio in VLDL began to increase (fig. 3; no statistical significance), while the absolute amount of COP in VLDL remained constant or even slightly decreased

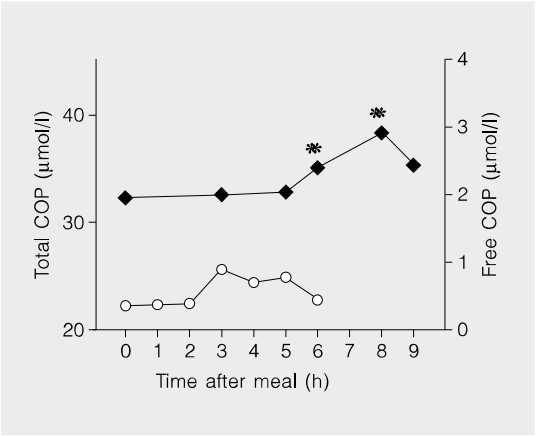

Fig. 1. Mean plasma concentrations (µmol/l) of free (○; nonesterified) and total (◆; free plus esterified) COP after intake of a COP-rich meal (Parmesan, salami) in 5 healthy young men.

Time after meal, h							
0	2	3	4	5	6	8	9[1]
70.2 ± 29.5	77.6 ± 28.4	101.8 ± 42.5	89.0 ± 30.2	148.6 ± 90.7	48.2 ± 9.8	–	–
40.6 ± 15.6	45.4 ± 14.7	87.6 ± 44.9	68.2 ± 27.2	94.0 ± 53.0	33.0 ± 7.4	–	–
116.8 ± 65.0	137.4 ± 59.5	131.8 ± 26.0	153.2 ± 53.2	234.0 ± 101.0	186.2 ± 91.0	–	–
39.2 ± 24.6	38.4 ± 24.7	140.4 ± 124.1	94.4 ± 76.6	66.0 ± 34.6	48.8 ± 33.6	–	–
8.2 ± 5.3	9.6 ± 5.9	196.8 ± 180.6	113.2 ± 91.2	38.0 ± 13.4	29.2 ± 9.0	–	–
75.4 ± 21.0	84.2 ± 19.5	235.2 ± 150.0	168.8 ± 79.4	179.8 ± 91.3	79.4 ± 16.9	–	–
2.8 ± 2.8	2.8 ± 2.8	7.6 ± 5.3	8.8 ± 7.6	14.8 ± 14.8	12.6 ± 12.6	–	–
353.2 ± 136.8	395.4 ± 123.9	907.0 ± 563.6	698.6 ± 349.6	775.6 ± 360.2	437.4 ± 157.6	–	–
1.17 ± 0.20	1.16 ± 0.19	1.16 ± 0.20	1.12 ± 0.19	1.08 ± 0.20	1.14 ± 0.19	–	–
7.65 ± 0.18	–	7.72 ± 0.24	–	7.60 ± 0.20	8.10 ± 0.12	9.31 ± 0.20*	8.55
9.06 ± 0.17	–	9.15 ± 0.26	–	9.09 ± 0.19	9.62 ± 0.17	10.58 ± 0.17*	9.93
2.51 ± 0.29	–	2.66 ± 0.21	–	2.59 ± 0.27	2.98 ± 0.19	2.27 ± 0.89	2.27
0.98 ± 0.23	–	0.81 ± 0.06	–	0.77 ± 0.02	0.84 ± 0.07	0.98 ± 0.14	0.81
0.39 ± 0.05	–	0.31 ± 0.03	–	0.43 ± 0.03	0.38 ± 0.07	0.62 ± 0.18	0.41
10.90 ± 0.32	–	11.28 ± 0.31	–	11.52 ± 0.32	12.43 ± 0.44*	13.65 ± 0.56*	12.45
0.77 ± 0.06	–	0.60 ± 0.09	–	0.76 ± 0.06	0.63 ± 0.17	0.76 ± 0.13	0.76
32.25 ± 0.89	–	32.54 ± 1.11	–	32.75 ± 0.85	34.97 ± 0.36**	38.17 ± 0.06**	35.18
4.09 ± 0.43	–	4.18 ± 0.49	–	4.16 ± 0.48	4.60 ± 0.22	4.63 ± 0.51	4.56

[1] n = 2.

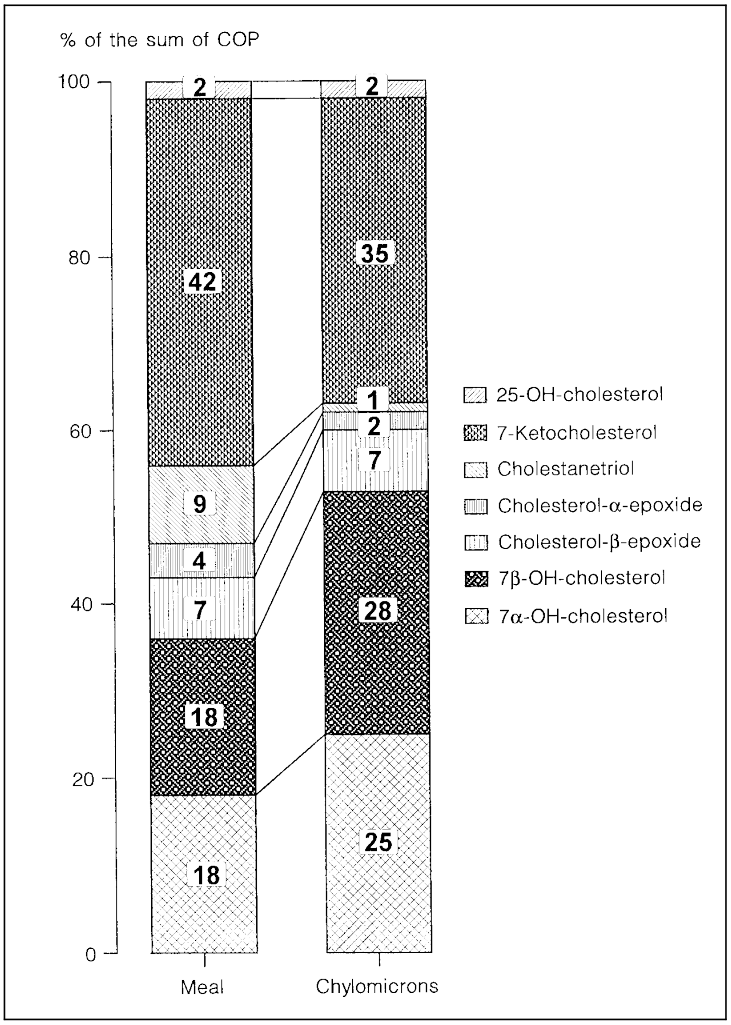

Fig. 2. Mean distribution of total COP in chylomicrons (mean of the results at 3, 4, 5, 6 h) of 5 healthy young men after intake of a COP-rich meal (Parmesan, salami) in comparison to the COP composition of that meal.

(not shown). No distinct effect of the test meal intake was found on the COP content or the COP:cholesterol ratio in LDL and HDL.

Discussion

As a natural COP source, the two food products Parmesan and salami were chosen in this experiment. For the production of both foods a long-lasting ripening period and a high microbial activity are needed and might be responsible for the high COP content [24–26]. In the offered test meal consisting of 150 g Parmesan and 150 g salami only 24% of the total amount of COP was nonesterified (free), contrasting to 76% of total cholesterol in free form (table 1). This indicates that in foodstuff with most of its cholesterol content in free form, the greatest part of COP may be found as acyl esters, pointing out the importance of a very careful and complete step of hydrolysis

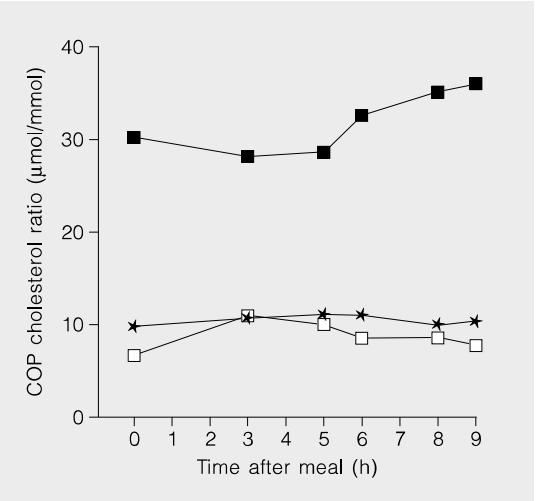

Fig. 3. Mean ratio of the sum of total COP (µmol/l) to total cholesterol (mmol/l) in VLDL (■), LDL (*), and HDL (□) of 5 healthy young men after intake of a COP-rich meal (Parmesan, salami).

in foodstuff and other biological material during analysis [27]. Like for other foods, the reported COP levels in Parmesan and salami are differing [15, 28, 29], apparently depending on the manufacturing technology and the method of COP analysis used. In spite of different antioxidative measures taken and an acceptable range of recovery of added standard substances (see Materials and Methods), also in the present study artifact formation during analysis procedure can not be completely excluded. This may be a point of criticism concerning the absolute amounts of COP measured but not concerning the observed relative increase in plasma levels after a COP-rich meal.

After ingestion of the COP-rich meal, more than 90% of the measured COP increase in plasma was COP acyl esters and less than 10% non-esterified COP. The maximal increase over prevalues amounted to 5.88 µmol/l (8 h) for total COP and 0.55 µmol/l (3 h) for free COP (table 2). Emanuel et al. [22] showed an increase in total plasma COP concentrations of 200–550 µg/dl (corresponding to about 5–12 µmol/l) in 5 males with individual maxima between 2.75 and 3.75 h after consumption of a powdered egg meal. Assuming a mean body weight of 70 kg, the estimated COP dosis was 28 µmol per subject and by that more than threefold higher than in the present investigation; moreover, with spray-dried powdered eggs a different COP source was chosen. In addition, COP analysis was limited to four COP compounds with blood sampling points between 0 and 6 h after the meal. Keeping in mind the differences in study design and analysis, the results of Emanuel et al. [22] fit well with the observed increase in plasma free COP concentrations in the present study. Also a very high variability between persons is documented by these authors. With 6 h as the last blood sampling point, the increase in total COP concentrations (with a maximum after 8 h in this study) could have been missed by Emanuel et al. [22].

In animal experiments (rats, squirrel monkeys, rabbits), plasma COP concentrations between 6 and 10 h after COP administration were not analyzed [13, 17–21]. In one study measuring lymphatic absorption of COP in rats, the increase (in % absorption) was highest between 6 and 9 h after COP adminstration [17]. Contrary results were reported by Vine et al. [18], showing peak maxima for the lymph content of 7β-hydroxycholesterol, 7-ketocholesterol and cholesterol-α-epoxide between 2 and 5 h after COP administration.

Inside the intestinal mucosal cells, the main part of (unmodified) cholesterol is esterified by means of acyl coenzyme A:cholesterol acyltransferase before incorporation in chylomicrons [30, 31]. Also COP were shown to be substrate of this enzyme [32, 33]. Concerning the mean plasma total cholesterol concentrations of the 5 men of our study, maximum val-

ues were reached 6 and 8 h after test meal intake (table 2). At the same time points, a statistically significant increase in total COP concentration was measured. Thus, COP absorption goes parallel with the absorption pattern of unmodified cholesterol. Accordingly, the calculated total COP:total cholesterol ratio in the plasma showed no distinct change over the study period.

After cholesterol intake, great differences in the reaction of plasma levels between individuals were described (hypo- and hyperresponders) [34, 35]. Among the subjects studied, neither in plasma total cholesterol nor in plasma total COP concentrations a great variability in response to the test meal existed.

In rats measuring the lymphatic absorption of COP after a gastric bolus over the following 24 hours, the absorption rate of 7-ketocholesterol (12%) and cholestanetriol (15%) were found to be significantly lower than those of the other ingested COP: 7α-hydroxycholesterol (30%), cholesterol-α-epoxide (27.5%), and cholesterol-β-epoxide (32%); 7β-hydroxy-cholesterol was absorbed at a higher rate (42%) than were the other COP [17]. These results are in good accordance with the data of the present investigation in humans: the comparison of the COP composition of the chylomicrons and of the test meal indicates a discrimination of 7-ketocholesterol, cholestanetriol and cholesterol-α-epoxide, while 7α-hydroxycholesterol and particularly 7β-hydroxycholesterol were found at higher proportions in the chylomicrons (fig. 2). An explanation of the different absorption rates by differences in the chemical structure of the COP molecules may be practical for 7-ketocholesterol and cholestanetriol as the more polar COP compounds, but it does hardly work when a preferred transport of 7β-hydroxycholesterol (e.g., versus 7α-hydroxycholesterol) should be explained. As reasons for the observed differences in absorption rates of COP and cholesterol, Osada et al. [17] suspected a lower solubility of COP in bile salt micelles, a lesser susceptibility of COP to esterification in intestinal mucosal cells, and direct toxic effects of COP on mucosal cells.

In the literature no data for mean COP absorption in humans exist. For rats, very different COP absorption rates of 90% for cholestanetriol and cholesterol-α-epoxide [21, 36], 30% for the sum of COP (versus 67% for cholesterol) [17], and 6% for the sum of COP [18] were reported.

Once absorbed, free (albumin-bound) and acylated COP in the plasma were substrates for lecithin:cholesterol acyltransferase and cholesteryl ester transfer protein [32]. Therefore, an exchange between lipoproteins seems likely, and radiolabelled COP compounds administered were found to a more or less large extent in all plasma lipoproteins of animals [19, 20]. In the observed time frame of 9 h a nonsignificant increase in VLDL COP enrichment was visible, while the other lipoproteins remained unaffected. This may also hint to an active role of the liver for the incorporation of chylomicron COP into VLDL. The possibility of elimination of dietary COP via bile acid synthesis or direct COP secretion into bile [8, 10, 12] may at least in part lower the dietary COP concentration in plasma. However, with administration of radiolabelled cholesterol-α-epoxide in rats it was demonstrated that excretion of the absorbed α-epoxide is a relatively long-lasting process [21]. Cholesterol-α-epoxide was almost completely cleared from the blood within 2 days, but its excretion in the stool continued for several days with a rapid elimination within the first 3 days followed by a slow and linear elimination on the following days. By means of the slow excretion data, Bascoul et al. [21] estimated a half-life of cholesterol-α-epoxide of 27 days.

On the other hand, in rats oxidatively modified chylomicrons (without COP deter-

mination) were shown to reveal an altered clearence with less lipolysis, less hepatic uptake, and an inreased uptake in spleen [37]. Moreover, the remnants of oxidized chylomicrons may serve as substrates for arterial macrophages and by this way increase the atherogenicity of postprandial remnants [38–40]. It seems likely that oxidized chylomicrons also contain a rather high amount of COP, and for COP-loaden LDL an enhanced uptake and accumulation in macrophages was already shown [41]. As found for COP-loaden LDL [42, 43], direct toxic effects towards aortic endothelial cells cannot be excluded in the case of highly COP-enriched chylomicrons.

To get exact data on the further fate of dietary COP in the human organism, another study design as well as other methods (stable isotopes) have to be applied. In combination with the knowledge of COP absorption rates in humans, an evaluation of the adverse health effects of dietary COP and probably the definition of an acceptable daily intake level of COP can be performed.

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