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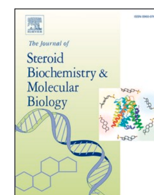

Plasma oxyphytosterols most likely originate from hepatic oxidation and subsequent spill-over in the circulation

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ABSTRACT

We evaluated oxyphytosterol (OPS) concentrations in plasma and various tissues of two genetically modified mouse models with either increased cholesterol (apoE KO mice) or increased cholesterol and plant sterol (PS) concentrations (apoExABCG8 dKO mice). Sixteen female apoE KO and 16 dKO mice followed the same standard, low OPS-chow diet. Animals were euthanized at 36 weeks to measure PS and OPS concentrations in plasma, brain, liver and aortic tissue. Cholesterol and oxysterol (OS) concentrations were analyzed as reference for sterol oxidation in general. Plasma campesterol (24.1 ± 4.3 vs. 11.8 ± 3.0 mg/dL) and sitosterol (67.4 ± 12.7 vs. 4.9 ± 1.1 mg/dL) concentrations were severely elevated in the dKO compared to the apoE KO mice ($p < 0.001$). Also, in aortic and brain tissue, PS levels were significantly elevated in dKO. However, plasma, aortic and brain OPS concentrations were comparable or even lower in the dKO mice. In contrast, in liver tissue, both PS and OPS concentrations were severely elevated in the dKO compared to apoE KO mice (sum OPS: 7.4 ± 1.6 vs. 4.1 ± 0.8 ng/mg, $p < 0.001$). OS concentrations followed cholesterol concentrations in plasma and all tissues suggesting ubiquitous oxidation. Despite severely elevated PS concentrations, OPS concentrations were only elevated in liver tissue, suggesting that OPS are primarily formed in the liver and plasma concentrations originate from hepatic spill-over into the circulation.

1. Introduction

Plant sterols are naturally occurring constituents of plants and found in our diet in products like vegetable oils, such as corn oil, soybean oil, and rapeseed oil, as well as cereals, nuts, fruit and vegetables. The average daily intake of plant sterols in Western countries is approximately 300 mg, but can be as high as 600 mg in vegetarians [1]. Since plant sterols share structural similarities with cholesterol, they compete in the gastrointestinal tract with cholesterol for incorporation into mixed micelles, which lowers the bioavailability of cholesterol for absorption. This results in an up-regulation of low-density lipoprotein (LDL)-receptor expression and a compensatory increased endogenous cholesterol synthesis, which ultimately results in a net reduction in plasma LDL-cholesterol (LDL-C) concentrations [2]. Besides this mixed micellar theory as underlying mechanism, there are also strong indications that plant sterols activate the trans intestinal cholesterol excretion pathway [3,4]. Consumption of functional foods enriched with plant sterols can, via the above-mentioned mechanisms, reduce LDL-C concentrations up to 10 % and consumption of plant sterol enriched products is therefore recommended in current dietary guidelines [5,6]. Interestingly, in contrast to cholesterol, plant sterols are hardly absorbed [7] and together with a high biliary secretion, this explains their relatively low plasma concentrations. On average, circulating plant sterol concentrations (~6.9 μmol/L for sitosterol and ~13.1 μmol/L for campesterol) are ~300 fold lower as compared to cholesterol concentrations (~6.0 mmol/l) [8]. Plant sterols have a typical double bond between C5-C6 in their steroid nucleus and are, just like cholesterol, susceptible to reactive oxidative species (ROS)-mediated oxidation which can result

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in the formation of oxidized plant sterols [9]. These so-called oxyphytosterols (OPS) are found in the circulation and we have previously demonstrated that not only the plant sterol concentrations, but also OPS concentrations increased during the postprandial phase after the intake of a plant sterol-enriched meal [10]. These meals contained plant sterols (3 g) and additionally a very low amount (0.68 mg) of naturally present OPS. An important remaining question was whether the observed increase in circulating OPS was derived from intestinal OPS uptake or from endogenous OPS formation. Recently, Lin et al. [11] quantified changes in plasma OPS concentrations after six weeks' dietary intake of three different very high doses of OPS (range: 9–37 mg/d), i.e approximately 13–50 times higher than habitual intakes. This well-controlled randomized placebo-controlled dose-response study demonstrated that plasma OPS concentrations increased nonlinearly and clearly reached plateau concentrations (range: 50−166 nmol/L increase from baseline) already after one week, indicating that diet-derived OPS are indeed absorbed. However, these meals not only contained higher amounts of OPS, but also of plant sterols (2.7–3.2 g/d). This implies that it is still unknown whether the observed increase in circulating OPS was only derived from dietary uptake or whether there was also endogenous formation of OPS after absorption of non-oxidized dietary plant sterols.

In contrast to OPS, besides oxidation in the ring structure, OS can also be generated by enzymatic side-chain hydroxylation [12]. While oxysterols oxidized in the ring are postulated to have toxic effects, sterols oxidized enzymatically in the sidechain play important biological roles [13]. Increased plasma 7-ketocholesterol and 7β-OH-cholesterol concentrations are found in cardiovascular disease (CVD) patients [14] and in atherosclerotic plaques [15]. Moreover, elevated plasma cholesterol concentrations are associated with increased 7-ketocholesterol concentration in hypercholesterolemic [16] and diabetic patients [16,17], demonstrating increased cholesterol concentrations as substrate for oxidative stress and higher absolute levels of oxysterols. In addition, a diet high in cholesterol has been shown to increase hepatic oxysterol concentrations [18]. In the present study, we evaluated the endogenous formation of OPS in apoE(-/-) (KO) mice and in apoExABCG8 double KO (dKO) mice that all consumed the same standard chow diets low in OPS by measuring cholesterol, oxysterol, plant sterol and OPS concentrations in plasma and in tissues. These mice were chosen, since apoE deficiency results in elevated plasma cholesterol concentrations whereas the deficiency in the half transporter ABCG8 severely elevates plasma plant sterol concentrations. The aim of this study was to assess OPS concentrations in plasma and in aortic, liver and brain tissue in an experimental mice model characterized by elevated plant sterol concentrations.

2. Methods

2.1. Animals, housing, and diets

This study was approved by the Institutional Animal Care and Use Committee (Dierexperimenten commissie, DEC-RUG). For this experiment, female apoE KO mice (N = 16) and female apoExABCG8 dKO (N = 16) were used. All mice had ad libitum access to their standard commercially pelleted laboratory chow – low in OPS concentrations (8 ng/mg) – (RMH-B, diets Hope Farms BV, Woerden, The Netherlands), and were housed individually during the whole experiment. At the age of 36 weeks, all animals were euthanized and flushed with ice-cold EDTA in PBS (2 μM). Next, the liver and brain were excised and rinsed with ice-cold BHT in PBS (5 mg BHT/100 ml). The aortic roots were isolated and immediately frozen in liquid nitrogen. All samples were kept at −80 °C until analysis. Plasma was obtained by low-speed centrifugation at 3000 rpm for 10 min at 4 °C and 10 μL Butylated hydroxytoluene (BHT, 25 mg/mL ethanol) was added per 1 mL of plasma, immediately after centrifugation. Plasma samples were snap frozen in liquid nitrogen and stored at −80 °C until analysis.

2.2. Cholesterol, plant sterol and oxy(phyto)sterol concentrations

Cholesterol, plant sterol, oxysterol and OPS concentrations were analyzed in plasma samples and in liver, brain and aortic root tissues. Cholesterol, plant sterols and oxy(phyto)sterols (7α-hydroxy(OH)-, 7β-OH- and 7keto-(phyto)sterol were extracted from tissue aliquots (dry weight) with 10 mL Folch-reagent (chloroform/methanol; 3:1; v:v) per mg tissue as described previously [19]. BHT (0.25 mg added per mL solvent) was used as antioxidant and added prior to extraction. Extraction was performed for 48 h at 4 °C in a dark cold room. The extracts were kept at 20 °C until analysis. Fifty microliters of mouse plasma and two mL of the Folch extract of liver, brain and aortic tissue was used for sterol and oxy(phyto)sterol determination. Cholesterol concentrations in plasma and tissue were analyzed by gas chromatography–flame ionization detection (GC–FID) and plant sterol concentrations were analyzed by highly specific and sensitive mass spectrometry in the selected ion monitoring mode (MS-SIM) [20]. Oxysterol and OPS concentrations were analyzed by gas chromatography-mass spectrometry (GC–MS) as described by Husche et al. [21]. In plasma, cholesterol and plant sterol concentrations are expressed as mg/dL and additionally plant sterols are also shown, standardized for total cholesterol. Cholesterol and plant sterol concentrations in tissues are expressed as μg/mg or ng/mg dry tissue. OPS concentrations in plasma are expressed as ng/mL and plasma OS concentrations are expressed as mg/dL and as 10^2 mg/mg when standardized for total cholesterol concentrations. In tissues, OPS and OS concentrations are expressed as ng/mg dry tissue. The isoforms of 7α-OH-, 7β-OH- and 7keto-(phyto)sterol concentrations were determined, which will be referred to as OPS or OS troughout this manuscript.

2.3. Statistics

An unpaired two-tailed Student's *T*-test was used to examine differences between cholesterol, plant sterol, OS and OPS concentrations in apoE KO and apoExABCG8 dKO mice. Results were considered to be statistically significant if $P < 0.05$. Data are presented as means ± SD. All statistical analyses were performed using SPSS 20.0 for Mac Os X or higher (SPSS Inc., Chicago, IL, USA).

3. Results

Plasma campesterol (24.1 ± 4.3 vs. 11.8 ± 3.0 mg/dL) and sitosterol concentrations (67.4 ± 12.7 vs. 4.9 ± 1.1 mg/dL) were elevated in dKO mice as compared to apoE KO mice ($p < 0.001$). Results were comparable when standardized for total cholesterol concentrations (Table 1). Despite severely elevated plasma non-oxidized plant sterol concentrations, plasma OPS concentrations were comparable (sum oxysitosterol: 627.8 ± 102.5 vs. 589.9 ± 115.3 ng/mL) or even lower (sum oxycampesterol: 368.3 ± 57.3 vs. 531.5 ± 130.7 ng/mL) in dKO mice as compared to apoE KO mice (Table 2). Also in aortic root tissue and in brain tissue, campesterol (1172.6 ± 241.8 vs. 270.6 ± 124.8 and 406.3 ± 40.5 vs. 51.5 ± 6.3 ng/mg, respectively) and sitosterol concentrations (2607.0 ± 547.9 vs. 64.8 ± 41.0 and 655.3 ± 81.6 vs. 9.1 ± 2.1 ng/mg,

Table 1
Plasma plant sterol and cholesterol concentrations in apoE KO mice (N = 16) and apoExABCG8 dKO mice (N = 16) after 36 weeks low-OPS chow-diet.

	ApoE(-/-) mice	ApoE(-/-)xABCG8(-/-) mice	*P*-value[1]
Campesterol (mg/dL)	11.8 ± 3.0	24.1 ± 4.3	<0.001
Sitosterol (mg/dL)	4.9 ± 1.1	67.4 ± 12.7	<0.001
Campesterol[2]	22.4 ± 2.9	201.1 ± 45.6	<0.001
Sitosterol[2]	9.4 ± 1.2	563.8 ± 137.5	<0.001
Cholesterol (mg/dL)	520.8 ± 79.1	128.4 ± 50.5	<0.001

Values are means ± SD.
[1] P-values were calculated by an unpaired Student's *T*-test.
[2] Non-cholesterol sterols expressed as $*10^3$ dL/dL cholesterol.

Table 2
Plasma oxyphytosterol and oxycholesterol concentrations in apoE KO mice (N = 16) and apoExABCG8 dKO mice (N = 16) after 36 weeks chow-diet.

	ApoE(-/-) mice	ApoE(-/-)xABCG8(-/-) mice	*P*-value[1]
7α-OH-campesterol (ng/mL)	56.2 ± 19.1	15.3 ± 7.7	<0.001
7α-OH-sitosterol (ng/mL)	27.8 ± 10.1	15.6 ± 5.8	<0.001
7β-OH-campesterol (ng/mL)	218.2 ± 72.0	70.5 ± 27.4	<0.001
7β-OH-sitosterol (ng/mL)	212.9 ± 67.2	100.4 ± 34.9	<0.001
7-keto-campesterol (ng/mL)	257.1 ± 44.7	282.5 ± 31.2	NS
7-keto-sitosterol (ng/mL)	349.2 ± 60.8	511.9 ± 72.6	<0.001
Sum oxycampesterol (ng/mL)	531.5 ± 130.7	368.3 ± 57.3	<0.001
Sum oxysitosterol (ng/mL)	589.9 ± 115.3	627.8 ± 102.5	NS
7α-OH-cholesterol (mg/dL)	580.2 ± 154.9	137.8 ± 47.3	<0.001
7β-OH-cholesterol (mg/dL)	1361.9 ± 393.3	331.8 ± 139.1	<0.001
7-keto-cholesterol (mg/dL)	7335.2 ± 1817.3	1281.7 ± 519.5	<0.001
Sum oxysterol (mg/dL)	9277.2 ± 1911.9	1751.3 ± 663.1	<0.001

Values are means ± SD.
[1] P-values were calculated by an unpaired Student's *T*-test. NS: non-significant.

respectively) were significantly elevated in dKO mice (Tables 3 and 4 p < 0.001), while OPS concentrations in aortic root and brain tissue were comparable between dKO and apoE KO mice (Tables 3 and 4). In contrast to plasma, aortic and brain tissue, in liver tissue, both plant sterol concentrations (campesterol: 824.3 ± 138.1 vs. 156.6 ± 40.0, sitosterol: 1963.6 ± 329.7 vs. 39.9 ± 10.1 ng/mg, p < 0.001) and also OPS were significantly elevated in dKO mice compared to apoE KO mice (sum oxycampesterol: 2.74 ± 0.58 vs. 1.57 ± 0.31, sum oxysitosterol: 4.68 ± 1.04 vs. 2.52 ± 0.48, p < 0.001) (Table 5).

Looking in more detail to the different OPS isoforms, the highest concentrations in liver tissue were seen for 7-keto-sitosterol / campesterol, followed by 7β-sitosterol / campesterol and finally 7α-sitosterol / campesterol, in both dKO mice and ApoE KO mice. All hepatic OPS concentrations were higher in dKO compared with apoE KO mice with the largest increases seen for the 7α-isoforms (∼500 % higher), followed by the 7β-isoforms (∼200 % higher) and the 7-keto-isoforms (∼50 % higher) (Fig. 1). In addition, a comparable pattern for 7-keto-isoforms was seen in liver and in plasma. In liver tissue, the highest concentration for the 7-keto-isoforms was observed for 7-keto-

Table 3
Sterol and oxyphytosterol and oxycholesterol concentrations in brain tissue from apoE KO mice (N = 16) and apoExABCG8 dKO mice (N = 16) after 36 weeks chow-diet.

	ApoE(-/-) mice	ApoE(-/-)xABCG8(-/-) mice	*P*-value[1]
Brain			
Campesterol (ng/mg)	51.5 ± 6.3	406.3 ± 40.5	<0.001
Sitosterol (ng/mg)	9.1 ± 2.1	655.3 ± 81.6	<0.001
7α-OH-campesterol (ng/mg)	0.08 ± 0.24	0.05 ± 0.05	NS
7α-OH-sitosterol (ng/mg)	0.04 ± 0.12	0.02 ± 0.01	NS
7β-OH-campesterol (ng/mg)	0.08 ± 0.15	0.09 ± 0.06	NS
7β-OH-sitosterol (ng/mg)	0.05 ± 0.09	0.08 ± 0.03	NS
7-keto-campesterol (ng/mg)	1.57 ± 1.00	2.35 ± 0.33	<0.01
7-keto-sitosterol (ng/mg)	2.88 ± 2.09	3.61 ± 0.48	NS
Sum oxycampesterol (ng/mg)	1.74 ± 1.39	2.49 ± 0.34	NS
Sum oxysitosterol (ng/mg)	2.97 ± 2.29	3.72 ± 0.49	NS
Cholesterol (ug/mg)	65.9 ± 1.8	64.2 ± 3.4	NS
7α-OH-cholesterol (ng/mg)	0.8 ± 0.1	0.7 ± 0.1	NS
7β-OH-cholesterol (ng/mg)	2.4 ± 0.3	2.6 ± 0.3	<0.05
7-keto-cholesterol (ng/mg)	77.7 ± 19.5	77.9 ± 23.6	NS
Sum oxysterol (ng/mg)	80.8 ± 19.7	81.2 ± 23.7	NS

Values are means ± SD.
[1] P-values were calculated by an unpaired Student's *T*-test. NS: non-significant.

Table 4
Sterol and oxyphytosterol and oxycholesterol concentrations in aortic root tissue from apoE KO mice (N = 16) and apoExABCG8 dKO mice (N = 16) after 36 weeks chow-diet.

	ApoE(-/-) mice	ApoE(-/-)xABCG8(-/-) mice	*P*-value[1]
Aortic root			
Campesterol (ng/mg)	270.6 ± 124.8	1172.6 ± 241.8	<0.001
Sitosterol (ng/mg)	64.8 ± 41.0	2607.0 ± 547.9	<0.001
7α-OH-campesterol (ng/mg)	0.52 ± 0.08	0.48 ± 0.12	NS
7α-OH-sitosterol (ng/mg)	0.17 ± 0.02	0.26 ± 0.08	<0.01
7β-OH-campesterol (ng/mg)	0.34 ± 0.09	0.45 ± 0.09	<0.01
7β-OH-sitosterol (ng/mg)	0.22 ± 0.05	0.54 ± 0.07	<0.001
7-keto-campesterol (ng/mg)	5.20 ± 0.82	4.60 ± 0.94	NS
7-keto-sitosterol (ng/mg)	8.11 ± 1.17	7.50 ± 1.57	NS
Sum oxycampesterol (ng/mg)	6.06 ± 0.92	5.53 ± 1.05	NS
Sum oxysitosterol (ng/mg)	8.51 ± 1.18	8.30 ± 1.65	NS
Cholesterol (ug/mg)	11.7 ± 3.5	4.3 ± 1.0	<0.001
7α-OH-cholesterol (ng/mg)	2.4 ± 1.0	1.0 ± 0.2	<0.001
7β-OH-cholesterol (ng/mg)	6.5 ± 2.7	2.6 ± 0.4	<0.001
7-keto-cholesterol (ng/mg)	28.0 ± 17.5	8.5 ± 4.1	<0.001
Sum oxysterol (ng/mg)	36.9 ± 19.9	12.1 ± 4.0	<0.001

Values are means ± SD.
[1] P-values were calculated by an unpaired Student's *T*-test. NS: non-significant.

Table 5
Sterol and oxyphytosterol and oxycholesterol concentrations in liver tissue from apoE KO mice (N = 16) and apoExABCG8 dKO mice (N = 16) after 36 weeks chow-diet.

	ApoE(-/-) mice	ApoE(-/-)xABCG8(-/-) mice	*P*-value[1]
Liver			
Campesterol (ng/mg)	156.6 ± 40.0	824.3 ± 138.1	<0.001
Sitosterol (ng/mg)	39.9 ± 10.1	1963.6 ± 329.7	<0.001
7α-OH-campesterol (ng/mg)	0.06 ± 0.02	0.37 ± 0.17	<0.001
7α-OH-sitosterol (ng/mg)	0.03 ± 0.01	0.18 ± 0.09	<0.001
7β-OH-campesterol (ng/mg)	0.18 ± 0.06	0.42 ± 0.08	<0.001
7β-OH-sitosterol (ng/mg)	0.15 ± 0.04	0.55 ± 0.21	<0.001
7-keto-campesterol (ng/mg)	1.33 ± 0.26	1.95 ± 0.44	<0.001
7-keto-sitosterol (ng/mg)	2.34 ± 0.47	3.95 ± 0.89	<0.001
Sum oxycampesterol (ng/mg)	1.57 ± 0.31	2.74 ± 0.58	<0.001
Sum oxysitosterol (ng/mg)	2.52 ± 0.48	4.68 ± 1.04	<0.001
Cholesterol (ug/mg)	10.5 ± 1.5	3.3 ± 0.6	<0.001
7α-OH-cholesterol (ng/mg)	1.6 ± 0.4	0.5 ± 0.1	<0.001
7β-OH-cholesterol (ng/mg)	1.8 ± 0.5	1.2 ± 0.4	<0.001
7-keto-cholesterol (ng/mg)	23.6 ± 10.0	6.8 ± 2.6	<0.001
Sum oxysterol (ng/mg)	26.9 ± 10.3	8.4 ± 2.5	<0.001

Values are means ± SD.
[1] P-values were calculated by an unpaired Student's *T*-test. NS: non-significant.

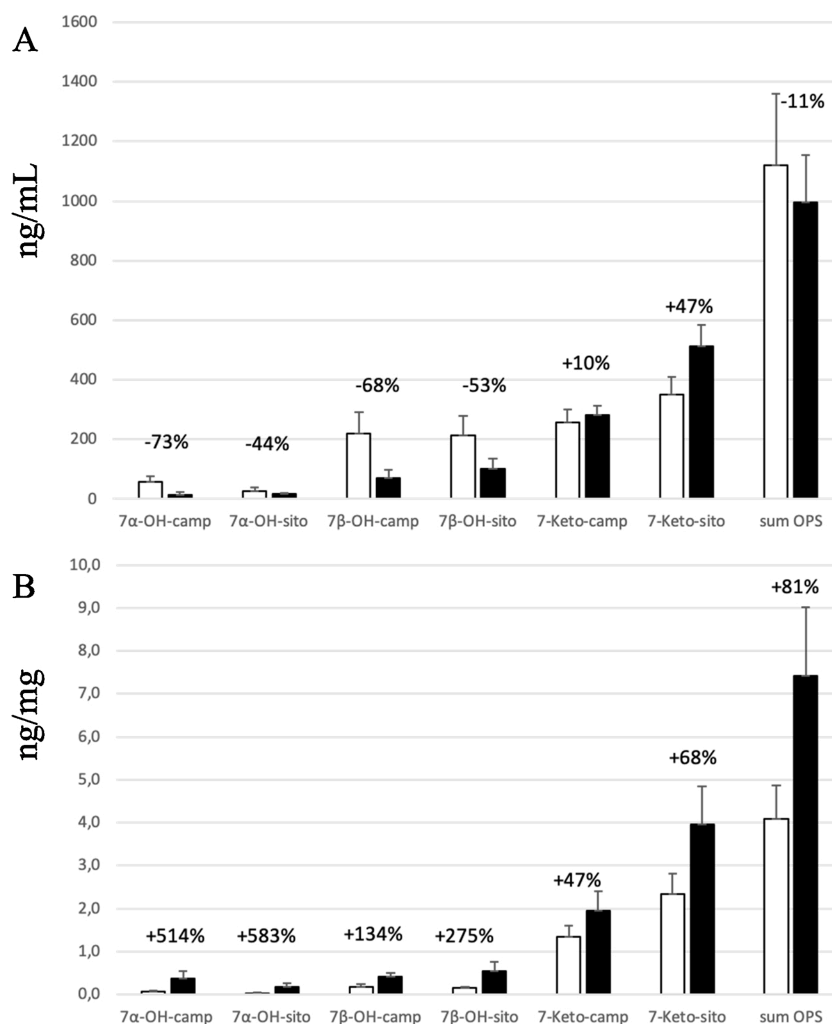

Fig. 1. Oxyphytosterol isoform concentrations in plasma (panel A) and in liver tissue (panel B) in apoE KO mice (N = 16, white bars) and apoExABCG8 dKO mice (N = 16, black bars) after 36 weeks low-OPS chow-diet.

sitosterol in dKO mice, followed by 7-keto-sitosterol in apoE KO mice, 7-keto-campesterol in dKO mice and 7-keto-campesterol in apoE KO mice (i.e. 3.95−2.34-1.95−1.33 ng/mg). In plasma, this order of magnitude was comparable for 7-keto-isoforms (i.e. 512-349-282−257 ng/mL). As displayed in Fig. 1, such a pattern could not be identified for 7α-OH-isoforms and 7β-OH-isoforms in liver tissue and in plasma. In contrast, all oxycholesterol isoforms followed a comparable pattern in the same order of magnitude (range: 36 %–83 %) in plasma and liver tissue in ApoE KO and dKO mice (data not shown).

In comparison to differences in plant sterol and OPS concentrations, cholesterol concentrations in plasma, liver and aortic tissue were lower in dKO mice as compared to apoE KO mice (Tables 2, 4 and 5, $p < 0.001$) and the same pattern, i.e. reduced concentrations were seen for oxidized cholesterol concentrations ((Tables 2, 4 and 5, $p < 0.001$) at all three locations. Cholesterol and oxysterol concentrations in brain tissue were comparable between dKO and apoE KO mice.

4. Discussion

A still unanswered question is whether OPS in plasma originate from intestinal dietary uptake or from endogenous synthesis. An additional question is the possible site of oxidation of dietary plant sterols. Therefore, we evaluated OPS concentrations in apoE(-/-) (KO) mice and in apoE x ABCG8 double KO (dKO) mice that consumed the same low-OPS chow diets for 36 weeks. Plant sterol and OPS concentrations were measured in plasma and tissues, and compared to cholesterol and oxycholesterol concentrations. The ROS-driven oxidation of cholesterol was used as reference for sterol oxidation in all compartments. We demonstrated that despite severely elevated plant sterol concentrations in plasma, brain, aorta and liver tissue in the dKO mice, OPS levels were only consistently elevated in liver tissue **(**Fig. 2**)**. Based on this data we hypothesize that 1) OPS are primarily formed in the liver and plasma OPS concentrations originate from hepatic spill-over into the circulation. Alternatively, it is possible that 2) OPS are formed in plasma and preferentially cleared from the circulation by the liver explaining the higher hepatic concentrations or 3) OPS are absorbed from the diet and preferentially taken up by the liver. In contrast, oxysterol concentrations followed non-oxidized cholesterol concentrations in all tissues in these mice models.

All mice consumed the same chow diets and – due to the deletion of ABCG8 – plant sterol concentrations, especially those of sitosterol, were severely elevated in plasma, liver, aortic and brain tissue in the dKO as compared with the apoE KO mice. However, OPS concentrations were only elevated in liver tissue in the dKO mice, suggesting that the liver is the major site of endogenous oxidation. Even though OPS intake from the chow was extremely low (~28 μg/day), it can also be postulated that OPS are absorbed from the diet and not excreted back into the interstitial lumen, resulting in higher hepatic concentrations. Plant sterols are used as functional food ingredients that effectively lower plasma LDL-C concentrations. Analogue to cholesterol, plant sterols possess a double

	Plant sterol concentrations in dKO compared to apoE KO mice	**Oxyphytosterol concentrations in dKO compared to apoE KO mice**	**Cholesterol concentrations in dKO compared to apoE KO mice**	**Oxysterol concentrations in dKO compared to apoE KO mice**
Circulation	↑	↑	↓	↓
Brain tissue	↑	↔	↔	↔
Aortic tissue	↑	↔	↓	↓
Liver tissue	↑	↑	↓	↓

Fig. 2. Non-oxidized plant sterol, cholesterol and oxy(phytosterol concentrations in the circulation and in brain, aortic and liver tissue in apoExABCG8 double knock-out (dKO) mice compared to apoE KO mice.
Values are means ± SD. [1]P-values were calculated by an unpaired Student's *T*-test. NS: non-significant.

bond in their ring structure and are therefore susceptible to oxidation in allylic position by non-enzymatic processes, such as reactions with reactive oxygen species and can be oxidized into OPS [9]. It is relevant to know the origin of plasma OPS, since we have for example previously shown that plasma OPS concentrations are elevated in (pre)diabetic patients compared with healthy controls [22] and increased postprandially after the intake of a plant sterol-enriched meal [10]. In addition, in some studies [23,24], but not all [25], lesion formation increased in various rodent models after the addition of OPS to the diet or after intraperitoneal application of OPS in apoE KO mice [26]. Since non-oxidized plant sterols cannot be synthesized in the human body, the origin of circulating plant sterol concentrations is evident: they are taken up in the intestine, transported into the circulation by chylomicrons and distributed via LDL and other lipoproteins into the various tissues, such as aortic valve cusps [27]. For circulating OPS concentrations, this question is more difficult to answer since OPS can theoretically either be absorbed in the intestine – alongside the plant sterols – or be endogenously formed. Earlier, we have demonstrated in a double-blind randomized study that consumption of plant sterol enriched margarines for four weeks increased fasting serum plant sterol concentrations without affecting fasting OPS concentrations [28]. Furthermore, plasma OPS and non-oxidized plasma plant sterols do not correlate as strongly with each other as oxysterol and non-oxidized cholesterol [21,28,29], suggesting that substrate oxidation in the circulation is not the most important origin of plasma OPS. It is also unlikely that plasma OPS are solely dependent from intestinal absorption, because dietary levels as well as the intestinal bioavailability are very low [30,31]. Recently, Lin et al. [11] have demonstrated that even a high intake of OPS (9–37 mg/d) every day for a period of 6 weeks resulted in new steady state concentrations already after 1 week without any further increase, suggesting that dietary absorption of OPS occurs but only to a limited extent.

An interesting finding in this experiment are the relative differences in plasma and hepatic OPS isoforms in dKO compared with apoE KO mice. Since 7-keto-isoforms are the endproduct of plant sterol oxidation, concentrations may be higher than those of 7α-OH- and 7β-OH-isoforms, which we indeed observed both in plasma as well as in liver tissue. In liver tissue in dKO mice, all OPS isoforms were higher than in ApoE KO mice and relatively more 7α-OH- and 7β-OH-isoforms were present as compared to 7-keto-isoforms (increases ~200−500 % compared to ~50 %), which could indicate a preference to form 7α-OH- and 7β-OH-isoforms, probably as a first step in the oxidation cascade, in the liver. In addition, in both mice models the order of magnitude was comparable for 7-keto-isoforms in plasma and liver tissue, while such a pattern could not be identified for 7α-OH- and 7β-OH-isoforms. Although speculative, this could suggest that there might be some kind of selective or preferred spill-over of 7-keto-isoforms from the liver to the circulation.

We also observed that in plasma as well as in aortic and liver tissue, cholesterol and oxysterol concentrations concentrations were lower in the dKO mice. Even though lower cholesterol concentrations in the dKO mice were not expected, this data demonstrates that oxysterol concentrations followed non-oxidized cholesterol concentration in plasma and aortic and liver tissues in our mice models. This observation suggests that – in contrast to plant sterols – cholesterol was oxidized in all tissues. Dias et al. demonstrated that plasma oxysterol concentrations follow cholesterol concentrations in hypercholesterolemic men, where non-enzymatically oxysterols were elevated but normalized after 3 months simvastin treatment [32]. In addition, high-cholesterol feeding in rats resulted in increased hepatic oxysterols that are formed via non-enzymatic or ROS-driven oxidation [18]. In our data, cholesterol concentrations in brain tissue are an exception since cholesterol metabolism in the brain is independent from that in peripheral tissues. Due to the blood-barrier, cholesterol levels in the brain are kept constant [33] and cholesterol and oxysterol concentration were comparable in both mice models. It remains to be determined why in the different tissue oxysterol levels were more related to cholesterol levels than plant sterols to OPS levels. Although speculative, it could be related to cholesterol – in contrast to plant sterols – being produced endogenously, which may increase local tissue oxidation.

An important question is what the pathophysiological meaning is of our finding that elevated plant sterol concentrations in plasma, aortic and brain tissue do not necessarily translate to elevated OPS concentrations, except for liver tissue. We can only speculate on the pathophysiological meaning of these elevated OPS concentrations in the liver. However, if the analogy is made with OS concentrations, then increased OS levels would negatively impact liver health. Sasaki et al. have shown that hepatic function was worsened after short-term feeding of OS in rats [34] and Hur et al. [35] demonstrated the induction of pathological lesions in rabbit liver tissues after dietary intake of OS. Furthermore, the addition of dietary 7-ketocholesterol aggravated liver steatosis and inflammation in a diabetic/obese mouse model [36]. These data show the impact of elevated OS concentration on the liver and whether such deleterious effects on the liver can occur increased hepatic OPS concentrations warrants further research.

In conclusion, irrespective of whether OPS concentrations result from hepatic spill over or whether OPS are preferentially cleared by the liver, future research should determine whether increased hepatic OPS concentrations are functionally related to health or disease.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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