Biochemical and Molecular Actions of Nutrients

Phytosterol Oxidation Products Are Absorbed in the Intestinal Lymphatics in Rats but Do Not Accelerate Atherosclerosis in Apolipoprotein E–Deficient Mice[1]

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ABSTRACT Phytosterol oxidation products (oxyphytosterols) are formed during the processing and storage of foods. However, it is unknown whether oxyphytosterols affect human health. To address these issues, we prepared β-sitosterol and campesterol oxides, evaluated their lymphatic absorption in rats, and examined the effect of an oxyphytosterol diet on atherosclerosis in apolipoprotein (apo) E–deficient mice. The lymphatic absorption of cholesterol and 6 oxyphytosterols (7α-hydroxy, 7β-hydroxy, β-epoxy, α-epoxy, dihydroxy, and 7-keto) of β-sitosterol or campesterol was assessed in thoracic duct–cannulated rats fed an AIN-93G–based diet containing 2.5 g of cholesterol, oxyphytosterols, or intact phytosterols per kg. Lymphatic recoveries (on a mass basis) of oxycampesterols (15.9 ± 2.8%, $n = 10$) and oxysitosterols (9.12 ± 1.77%, $n = 10$) were higher than for campesterol (5.47 ± 1.02%, $n = 12$, $P < 0.05$) and β-sitosterol (2.16 ± 0.37%, $n = 12$, $P < 0.05$), but lower than for cholesterol (37.3 ± 8.3%, $n = 6$, $P < 0.05$). Apo E–deficient mice were fed an AIN-93G–based diet containing 0.2 g oxyphytosterols or intact phytosterols per kg for 9 wk. Diet-derived oxyphytosterols accumulated in the serum, liver, and aorta. Furthermore, the oxyphytosterol diet increased oxycholesterol in the serum compared to the phytosterol diet. However, there was no significant difference between the 2 groups in the serum and aortic cholesterol concentration, the lesion area in the aortic root, or 8-iso-prostaglandin F2α concentration in the urine. These results indicate that exogenous oxyphytosterols are well-absorbed and accumulate in the body, but do not promote the development of atherosclerosis in apo E–deficient mice. J. Nutr. 134: 1690–1696, 2004.

KEY WORDS: • *phytosterol* • *oxyphytosterol* • *absorption* • *atherosclerosis*

Cholesterol oxidation products (oxycholesterols) are formed during the processing and storage of foods (1). Oxycholesterols are found in human plasma chylomicrons (2), indicating that they are absorbed from the digestive tract. Furthermore, Staprans et al. (3) showed that when a meal containing 400 mg cholestan-5α, 6α-epoxy-3β-ol (α-epoxycholesterol) is consumed by humans, α-epoxycholesterol in the serum is present in chylomicrons, chylomicron remnants, and endogenous lipoproteins (VLDL, LDL, and HDL). There are conflicting reports on the role of exogenous oxycholesterols in the development of atherosclerosis in experimental animals; although atherosclerosis was observed in some studies, it was not in others (4,5).

Some investigators found plant sterol oxidation products (oxyphytosterols) in foods (6–9) and in the serum from phytosterolemic patients (10), although there are fewer reports than for oxycholesterols. The oxides of β-sitosterol and campesterol cause cellular damage in cultured mouse macrophages similar to that caused by oxycholesterols (11). However, it is unknown whether oxyphytosterols affect human health.

To address these issues, we prepared β-sitosterol and campesterol oxides and evaluated their lymphatic absorption in rats with permanent cannulation of the thoracic duct (12). Then we examined the effect of an oxyphytosterol diet on atherosclerosis lesion size in apolipoprotein (apo)[3] E–deficient mice, as well as the level of oxyphytosterols in the serum, liver, and aorta and oxycholesterols in the serum. The level of 8-iso-prostaglandin F2α (8-iso-PGF2α) in the urine was also measured as an index of in vivo peroxidative stress.

MATERIALS AND METHODS

Materials. Highly purified campesterol (97.8% on a weight basis) and β-sitosterol (98.6% on a weight basis) were obtained from Tama Seikagaku and used as a starting material for the synthesis of [I] epimeric 7-hydroxyphytosterols and 7-ketophytosterols (13), [II] 5α,6α- and 5β,6β-epoxyphytosterols (14), [III] 5,6-dihydroxyphytosterols (15), and [IV] β-epoxyphytosterols (16). They were also used as

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[3] Abbreviations used: ACAT, acyl-coenzyme A:cholesterol acyltransferase; apo, apolipoprotein; GC/NICI-MS, GC negative ion chemical ionization mass spectrometry; 8-iso-PGF2α, 8-iso-prostaglandin F2α; LXR, liver X receptor.

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standards for quantifying oxysterols by GC/MS in diets and animal tissues. Alternatively, a phytosterol mixture (Merck-Japan) composed (on a weight basis) of 37.8% campesterol, 54.3% β-sitosterol, 3.4% stigmasterol, 0.5% campestanol, 1.3% sitostanol, and 2.7% unknown compounds was used as a starting material for the preparation of oxyphytosterols as described above (13–16), an equal amount of the synthesized oxyphytosterols was mixed, and the mixture was employed in Study 1 (**Table 1**). Because the methods used (13–16) for the synthesis of oxyphytosterols produced unknown compounds other than the target compound to different extents, the amount of individual target oxyphytosterols shown in Table 1 differed. In another experiment, the phytosterol mixture was heated at 150°C for 12 h. Then the heated products were applied to a silicic acid column and the resultant oxyphytosterols were eluted with acetone after being washed with diethyl ether as previously described (17). The heat-prepared oxysterols were then used in Study 2 (Table 1).

Animals and diets. Animals were individually housed in a temperature-controlled room at 22–25°C with a 12-h light-dark cycle (lights on 0800–2000 h). All diets were based on the AIN-93G diet formulation (18), as previously described (19). Experiments were carried out under the Guidelines for Animal Experiments at the Faculty of Agriculture and the Graduate Course, Kyushu University, Fukuoka, Japan, and Law No. 105 and Notification (No. 6) of the Government of Japan.

In Study 1, 8-wk-old male Sprague-Dawley rats were obtained from Seiwa Experimental Animals. They were trained to consume a basal diet containing 100 g/kg lard as dietary fat twice per day from 1000–1100 and 1600–1700 h, respectively, for 1 wk. Lard was chosen to avoid an effect of phytosterols derived from dietary oils on the lymphatic transport of the phytosterols in rats because it contains only a small amount of phytosterols (7.2 mg campesterol and 8.1 mg β-sitosterol per kg lard). Deionized water was freely available throughout the feeding periods. All rats were anesthetized with Nembutal prior to permanent cannulation of the thoracic duct according to an original method (12) modified by us (20,21). Briefly, a cannula (Silicon tube SH, i.d. 0.5 mm and o.d. 1.0 mm, product of Kaneka Medics) filled with heparinized saline was inserted into the thoracic duct and secured within the abdominal cavity. The rats were returned to their cages and provided with the basal diet twice per day. On d 3, the rats were attached to a long PE-cannula (i.d. 0.58 mm and o.d. 0.97 mm, Becton-Dickinson) to collect the lymph. The end of the cannula was 5–10 cm below the bottom of the cage to provide sufficient underside pressure to allow the lymph to enter the cannula. The lymph was collected for 15 min in a tube containing 50 μg BHT and the rats were then given free access to the diet (g/kg diet) supplemented with 2.5 cholesterol (control diet), 2.5 cholesterol plus 2.5 phytosterol mixture from Merck-Japan (phytosterol diet), or 2.5 cholesterol plus 2.5 oxyphytosterol (oxyphytosterol diet) (Table 1) for 30 min. This amount of oxycholesterols was used to evaluate the absorption (21), because we wanted to compare the absorption rate of phytosterols and oxyphytosterols with that of cholesterol. At this time, the lymph was collected every hour for 7 h. The rats freely consumed deionized water during lymph collection. After fibrin was removed, 268 nmol/L EDTA was added to the lymph solutions. The solution was flushed with argon (purity 99.9% on a volume, Hakata Kyoudou Sannso) and stored at −30°C for up to 12 h. Apparent lymphatic recovery (% on a mass) for 7 h worth of sterols (oxyphytosterols, phytosterols, or cholesterol) was calculated according to the formula [(lymphatic transport of sterols for 7 h after meal) − 4 × 7 × (lymphatic transport of sterols for 15 min before meal)]/(amount of sterols consumed for 30 min). Transport of sterols in the lymph collected for 7 h after the meal was first deducted from the values that were acquired by converting the transport of sterols in the basal lymph collected for 15 min to that of 7 h and then divided by the amount of sterols consumed for 30 min. The transport of sterols for 7 h in the basal lymph was estimated by multiplying the transport of sterols for 15 min by 28 (4 × 7).

TABLE 1

Composition of oxyphytosterols in Studies 1 and 2[1]

	Study 1	Study 2
	g/kg	
Oxycampesterols		
7α-Hydroxycampesterol	58 ± 5	10 ± 0
7β-Hydroxycampesterol	58 ± 3	28 ± 3
β-Epoxycampesterol	51 ± 4	62 ± 1
α-Epoxycampesterol	74 ± 6	76 ± 2
Dihydroxycampesterol	40 ± 4	18 ± 0
7-Ketocampesterol	40 ± 6	125 ± 3
Oxysitosterols		
7α-Hydroxysitosterol	85 ± 8	16 ± 1
7β-Hydroxysitosterol	78 ± 4	33 ± 1
β-Epoxycampesterol	57 ± 7	69 ± 1
α-Epoxycampesterol	104 ± 8	53 ± 1
Dihydroxycampesterol	54 ± 5	15 ± 1
7-Ketocampesterol	57 ± 8	130 ± 1
Unknown	243 ± 72	316 ± 11
Campesterol	0	23 ± 1
β-Sitosterol	0	27 ± 0

[1] Values are means ± SEM of triplicate analyses.

In Study 2, apo E–deficient mice purchased from Jackson Laboratories in 1994 were used (19). Male apo E–deficient mice (7–11 wk old) with an initial weight of 25.2 ± 0.6 g were divided into 2 groups, and the mice were fed a basal diet containing 100 g/kg palm oil as the dietary fat supplemented with 0.2 g/kg phytosterol mixture from Merck-Japan (phytosterol diet) or 0.2 g/kg heat-prepared oxyphytosterols (oxyphytosterol diet) (Table 1). This amount of oxycholesterols was used previously to evaluate their effect on atherosclerosis in mice (17). Palm oil was chosen to avoid an effect of cholesterol derived from dietary oils on the development of atherosclerosis in apo E–deficient mice because it is a dietary fat which contains negligible amounts of cholesterol. Experimental diets were packed in a pouch containing an O_2 absorbent (Ageless S-200; Mitsubishi Gas Chemical), flushed with N_2 and stored at 4°C. The diet was freshly prepared every week and changed every 2 d. Any remaining diet was discarded. At the end of the 9-wk feeding period, the mice were deprived of food for 4 h prior to killing. During the week before killing, mice were put in a metabolic cage (Shinano Seisakusho) where they freely consumed the diet and water, followed by urine collection in a container containing BHT (final concentration of 453 nmol/L) for 24 h. The urine was frozen in liquid nitrogen after Ar was blown through it and kept at −85°C. At the end of the experiment, the mice were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg body wt) and killed by withdrawing blood from the left ventricle. The blood was transferred into 1-mL microcentrifuge tubes containing 50 μg BHT. The serum was separated, bubbled with Ar, and stored at −85°C after being frozen in liquid nitrogen. Livers and aortas were immediately removed from the carcasses, frozen in liquid nitrogen, and stored at −85°C. Prior to freezing, adipose tissue around the aorta was dissected away, rinsed in fresh PBS, and blotted dry between filter paper.

Determination of sterols and oxysterols. Oxysterols were measured using the method used for oxycholesterol (17,21). To 200 μL of serum and 0.25 g of liver was added 100 μg of 5α-cholestane (Sigma) and 1 μg of 19-hydroxycholesterol (Steraloids) as an internal standard. Then 200 μL of lymph was added to 50 μg of 5α-cholestane and 1 μg of 19-hydroxycholesterol. The total aorta was combined with 10 μg of 5α-cholestane and 1 μg of 19-hydroxycholesterol. Lipids in the samples were extracted with 20 vol of chloroform/methanol (2:1, v/v) (22) containing 453 nmol/L BHT. The extracts were dried under N_2 and the residue was dissolved in 1 mL of toluene (guaranteed reagent; Nacalai tesque) and applied to a Sep-Pak Vac silica cartridge (Waters Japan) to separate oxysterols from sterols (23). The cartridge was sequentially eluted with 1 mL of hexane, 8 mL of 2-propanol (5 mL 2-propanol/L hexane), and 5 mL of 2-propanol (300 mL 2-propanol/L hexane), which allowed for the sequential elution of 5α-cholestane, cholesterol plus phytosterol, and 19-hydroxycholesterol plus oxysterols, respectively. The recovery of individual oxycholesterols and oxyphytosterols from the cartridge was confirmed to be almost 100%. The samples were allowed to saponify at room temperature overnight

in the dark (24), and unsaponified lipids were converted into trimethylsilylethers in a mixture of trimethylchlorosilane, 1,1,1,3,3,3-hexamethyldisilazane, and dried pyridine (1:3:9, v:v:v) for 30 min at room temperature, as previously described (17,21). GC of sterols was performed as described (25). GC-MS was performed on a Shimadzu GC-17A ver. 3 coupled with the SPB-1 fused silica capillary column connected to a Shimadzu QP5050A series mass-selective detector. The following variables—ions monitored, relative retention time, correlation coefficient for calibration curves, response factors for the monitored ions, detection limit, and the CV for repeated injection—were determined as previously described (17,21).

Determination of 8-iso-PGF2α. Purification and measurement of urinary 8-iso-PGF2α were carried out by combining 4 methods (26–29). The 8-iso-PGF2α was analyzed by GC negative ion chemical ionization mass spectrometry (GC/NICI-MS). GC/NICI-MS was performed using a Shimadzu QP5050A GC/MS (Shimadzu). GC was performed using a 30-m, 0.25-mm-diameter, 0.25-μm-film-thick, SPB-20 fused silica capillary column (Supelco). The column temperature was initially maintained at 100°C for 2 min. The column was then heated to 250°C in 7 min and then to 290°C at 2°C/min and maintained at this temperature (29). Isobutane was used as a reactant gas for negative chemical ionization and helium as a carrier gas at 1.1 mL/min. The ion source temperature was 290°C and the electron energy was 70 ev. The ion monitor for endogenous 8-iso-PGF2α was set at m/z 569 (M-181). We used 8-iso-PGF2α-d4 (Cayman Chemicals) as an internal standard and ions at m/z 573 were monitored. Quantification of endogenous 8-iso-PGF2α was accomplished by selected ion monitoring analysis of the ratio of m/z 569 to m/z 573. The lower limit of detection (signal-to-noise ratio of 4:1) of 8-isoPGF2α was within the range for 50 μL urine. A standard curve was constructed by adding varying amounts of unlabeled 8-iso-PGF2α to 1 ng of 8-isoPGF2α-d4, and the measured ratio of m/z 569 to m/z 573 and the expected ratio were compared. The standard curve was linear over a 30-fold range in concentration. A concentration of urine 8-iso-PGF2α was expressed as a function of urinary creatine (Wako Pure Chemicals).

Analyses of serum and lymph lipids. Serum lipid levels were measured using commercially available kits (cholesterol C test, triglyceride G test, and phospholipid B test, Wako Pure Chemicals). Lymph lipids were chemically determined as previously described (21).

Morphometric determination of atherosclerosis. Apo E–deficient mice were perfused with 50 mL PBS (pH 7.4) via a cannula inserted into the left ventricle, which allowed unrestricted efflux from an incision in the vena cava. After the aorta and its main branches were dissected from the aortic valve to the iliac bifurcation, perfusion of the heart was immediately continued with 50 mL 10% (v/v) neutral formalin buffer solution (pH 7.4). The heart was removed and fixed in 10% (v/v) neutral formalin buffered solution (19). Hearts containing aortic roots were processed for quantitative atherosclerosis assay to determine the cross-sectional lesion volume as previously described (17,30).

Statistics. Results are expressed as means ± SE. Statistical analysis was carried out with Statcel (Excell 2000). A paired t test was used for comparisons within groups. Student's t test was used to compare 2 groups after Bartlett's test was used to check that variances were homogenous. Statistical comparison of 3 or more groups was done by Fisher's PLSD method, following detection of an effect by one-way ANOVA. Differences were considered significant at $P < 0.05$.

RESULTS

Study 1. Food consumption by the rats within 30 min did not differ among the groups (7.43 ± 0.57 g, $n = 7$; 6.43 ± 0.49 g, $n = 12$; 7.70 ± 0.33 g, $n = 10$, for the control, phytosterol, and oxyphytosterol groups, respectively). Lymph flow also did not differ among the groups (**Fig. 1**). Rats fed the phytosterol diet transported less cholesterol, triacylglycerols, and phospholipids for 3–7 h after the meal than controls. Rats fed the oxyphytosterol diet also transported less triacylglycerols and phospholipds than did control diet-fed rats. Rats fed the oxyphytosterol diet transported less cholesterol at 5 h after the meal than controls. Lipid transport did not differ between the phytosterol- and oxyphytosterol-fed groups.

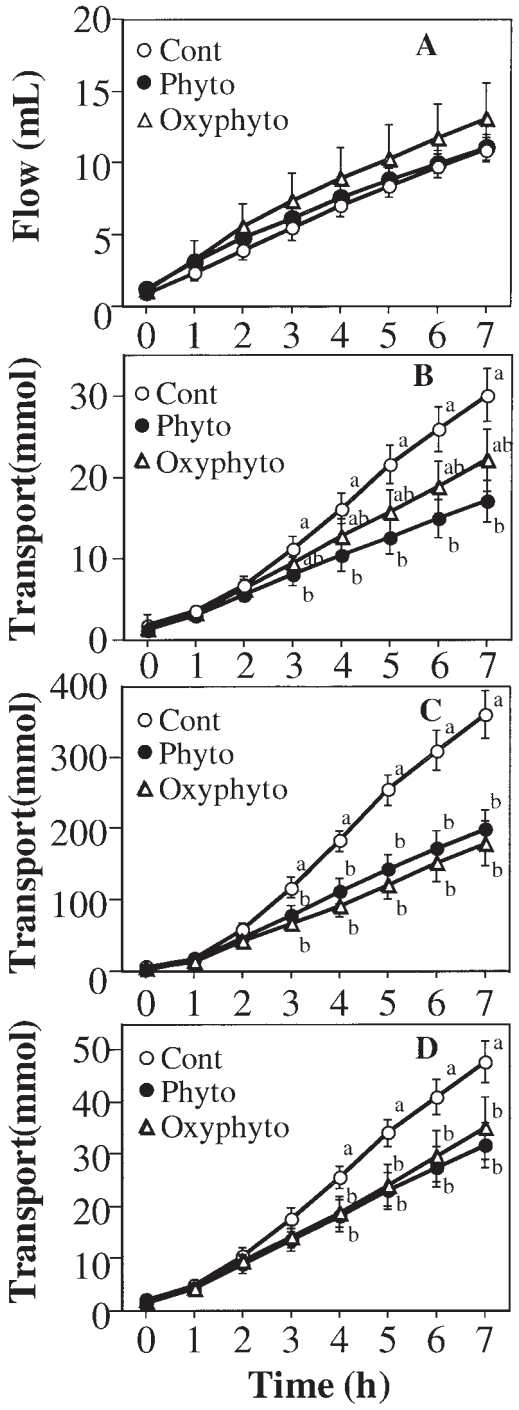

FIGURE 1 Lymph flow (*A*) and the cumulative transport of cholesterol (*B*), triacylglycerols (*C*), and phospholipids (*D*) in rats fed a control (Cont, $n = 7$), phytosterol (Phyto, $n = 12$), or oxyphytosterol (Oxyphyto, $n = 10$) diet. Values are means ± SEM. Means without a common letter differ, $P < 0.05$.

In the basal lymph collected prior to the consumption of oxyphytosterols, the following oxidized campesterols and sitosterols were detected (in nmol/h, $n = 10$): 7α-hydroxycampesterol (1.36 ± 0.60), 7β-hydroxycampesterol (0.81 ± 0.24), β-epoxycampesterol (0.50 ± 0.19), α-epoxycampesterol (0.74

± 0.31), dihydroxycampesterol (0.69 ± 0.28), 7-ketocampesterol (0.70 ± 0.27), 7α-hydroxysitosterol (2.07 ± 1.02), 7β-hydroxysitosterol (1.15 ± 0.50), β-epoxysitosterol (0.49 ± 0.16), α-epoxysitosterol (0.79 ± 0.34), dihydroxysitosterol (0.83 ± 0.28), and 7-ketositosterol (1.10 ± 0.60). After the meal, the lymphatic recovery of oxycampesterols was greater than that of the corresponding oxysitosterols, except for the dihydroxy oxysterols (**Fig. 2***A*). Among the oxycampesterols, the lymphatic recoveries of 7β- and 7α-hydroxy were greater than those of the other oxyphytosterols, as was also the case for oxysitosterols. The lymphatic recoveries (on a mass basis) of the 6 oxycampesterols (15.9 ± 2.8%, $n = 10$) and 6 oxysitosterols (9.12 ± 1.77%, $n = 10$) were greater than those of the corresponding unoxidized campesterol (5.47 ± 1.02%, $n = 12$) or β-sitosterol (2.16 ± 0.37%, $n = 12$) (Fig. 2*A*, *B*). Among the lymphatic sterols, the recovery (on a mass basis) of campesterol was greater than that of β-sitosterol or stigmasterol (1.39 ± 0.33%, $n = 12$), but lower than for cholesterol (37.3 ± 8.3%, $n = 6$) (Fig. 2*B*).

Study 2. The phytosterol and oxyphytosterol diets did not affect the body weight gain, food intake, or liver weight of apo E–deficient mice (results not shown). Serum cholesterol concentrations did not differ between mice fed phytosterol (13.7 ± 1.1 mmol/L) and mice fed oxyphytosterol (16.0 ± 1.9 mmol/L) diets. The liver cholesterol concentration in the mice fed the oxyphytosterol diet (12.7 ± 0.5 mmol/kg, $n = 7$) was greater than that in phytosterol diet–fed mice (14.9 ± 0.9 mmol/kg, $n = 7$) ($P < 0.05$).

Even in mice fed the phytosterol diet, there was a small but measurable amount of oxyphytosterols in the serum, liver, and aorta (**Table 2**). Mice fed oxyphytosterols had significantly more oxyphytosterols in these tissues than mice fed phytosterols. However, the concentrations of oxysitosterols in the aorta did not differ between the 2 groups, except that the oxysterol fed–mice had a higher concentration of 7β-hydroxysitosterol than the phytosterol fed–mice. In mice fed the oxyphytosterol diet, 7β-hydroxycampesterol and 7β-hydroxysitosterol were the most abundant species of oxyphytosterol in the serum, liver, and aorta, although they were not the most abundant species in the diet (Table 1). Mice fed phytosterols had significantly more campesterol and β-sitosterol in the serum, liver, and aorta than mice fed oxyphytosterols. The concentrations of campesterol in these tissues were higher than that of β-sitosterol ($P < 0.05$). Unlike phytosterols, the concentrations of total oxycampesterols in the serum and liver of the 2 groups were almost the same as that of the total oxysitosterols.

Mice fed oxyphytosterols had significantly higher serum concentrations of 7α-hydroxycholesterol, 7β-hydroxycholesterol, dihydroxycholesterol, 7-ketocholesterol, and 25-hydroxycholesterol in the serum than the phytosterol-fed mice (**Table 3**). In particular, the elevation of 25-hydroxycholesterol (~600%) was greater than that of other oxycholesterols. However, serum concentrations of α-epoxycholesterol, β-epoxycholesterol, and 27-hydroxycholesterol did not differ between the groups.

Cholesterol concentrations in the aorta and the lesion area in the aortic root did not differ between the phytosterol and oxyphytosterol groups (data not shown). Furthermore, dietary oxyphytosterol did not affect the amount of urinary 8-iso-PGF2α excreted (data not shown).

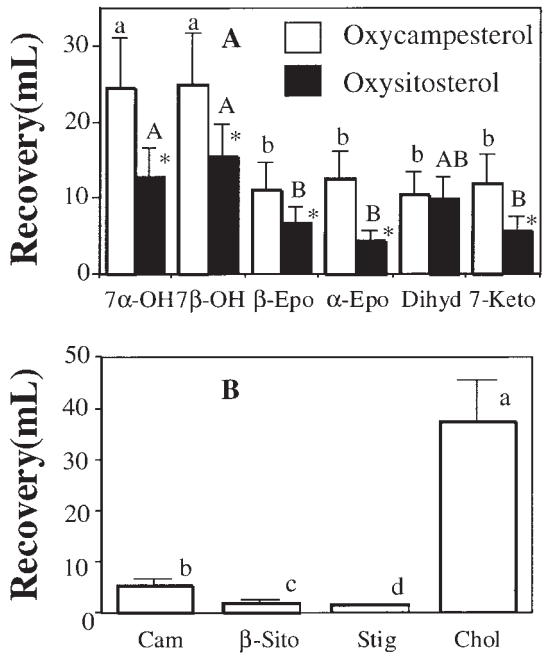

FIGURE 2 Lymphatic recovery of oxyphytosterols (*A*) in rats fed an oxyphytosterol ($n = 10$) and sterols (*B*) in rats fed a phytosterol ($n = 12$) or control ($n = 7$) diet. Values are means ± SEM. Means without a common letter differ, $P < 0.05$. *Different from the corresponding oxycampesterols, $P < 0.05$. Abbreviations: β-Epo, β-epoxy; α-Epo, α-epoxy; β-Sito, β-sitosterol; Cam, campesterol; Chol, cholesterol; Dihyd, dihydro; Stig, stigmasterol

DISCUSSION

The present study on thoracic duct lymph from rats confirmed the discriminatory capacity of the small intestine between cholesterol and plant sterols, in that cholesterol is preferentially absorbed and transported into the lymph over plant sterols (31–33). The pattern of discrimination depends on the side chain; the addition of 1 methyl group at the C-24 position (campesterol), 1 ethyl group at the C-24 position (β-sitisterol), or 1 ethyl group at the C-24 position, and the Δ22 double bond (stigma sterol). Because the uptake of cholesterol and plant sterols from the intestinal lumen into enterocytes is a rapid process in mice (34), the efflux rather than uptake of sterols may be the main process that discriminates between the lymphatic transport of sterols (35,36). Cholestery ester synthesis in enterocytes via acyl-coenzyme A:cholesterol acyltransferase (ACAT) might be a crucial step for discrimination in that cholesterol is preferentially esterified. Therefore, less free cholesterol than free plant sterols is available for transport out of the cells and back into the intestinal lumen (25,33). β-Sitosterol is a poorer ACAT1 substrate than cholesterol in mixed micelles and reconstituted vesicles (37).

For the lymphatic absorption of oxidation products of campesterol and β-sitosterol, the pattern of discrimination depended on the side chain: lymphatic recovery of oxycampesterols was higher than that of oxysitosterols, except for their trihydroxy types. Of note, the lymphatic recovery of oxyphytosterols was higher than that of unoxidized plant sterols. In particular, the addition of a hydroxyl group (7α-OH and 7β-OH) to the 7 position of campesterol and β-sitosterol resulted in the greatest recovery in the lymph. These results contradicted our previous results for cholesterol with a second oxygen atom, present as a carbonyl, hydroxyl, or epoxide group in the cyclopentanoperhydrophenanthrene nucleus, in that it was transported at a reduced rate into the lymph compared with unoxidized cholesterol (21). In that study, recoveries of α-epoxycholesterol in the lymph were the highest. Further-

TABLE 2

Oxyphytosterol and phytosterol levels in serum, liver, and aorta of apo E–deficient mice fed a phytosterol or oxyphytosterol diet[1]

	Serum		Liver		Aorta	
Sterols	Phytosterol[2]	Oxyphytosterol[3]	Phytosterol	Oxyphytosterol	Phytosterol	Oxyphytosterol
	μmol/L		*nmol/g*			
Plant sterols						
Campesterol	354.4 ± 42.4	80.1 ± 42.2*	227.9 ± 14.7	51.7 ± 3.2*	117.5 ± 19.2	25.7 ± 2.0*
β-Sitosterol	43.4 ± 5.1	14.2 ± 5.1*	21.6 ± 1.3	8.1 ± 0.7*	48.0 ± 10.4	16.9 ± 1.4*
Oxidized campesterols						
7α-Hydroxy	0.08 ± 0.01	1.50 ± 0.01*	0.07 ± 0.00	0.55 ± 0.14*	0.31 ± 0.05	0.31 ± 0.07
7β-Hydroxy	0.48 ± 0.05	23.04 ± 0.05*	0.14 ± 0.00	13.20 ± 2.04*	1.15 ± 0.38	6.05 ± 1.06*
β-Epoxy	0.28 ± 0.04	4.03 ± 0.04*	0.22 ± 0.05	1.75 ± 0.41*	0.31 ± 0.05	0.94 ± 0.14*
α-Epoxy	0.32 ± 0.06	6.07 ± 0.06*	0.24 ± 0.05	4.70 ± 0.53*	0.46 ± 0.07	1.42 ± 0.34*
Dihydroxy	0.81 ± 0.05	2.42 ± 0.05*	0.87 ± 0.14	2.09 ± 0.21*	0.83 ± 0.14	1.06 ± 0.23
7-Keto	0.16 ± 0.02	7.02 ± 0.02*	0.19 ± 0.02	2.44 ± 0.65*	0.51 ± 0.10	1.01 ± 0.15*
Total	2.14 ± 0.18	43.85 ± 0.18*	1.77 ± 0.17	24.56 ± 3.58*	3.51 ± 0.38	10.75 ± 1.86*
Oxidized β-sitosterols						
7α-Hydroxy	0.21 ± 0.03	1.87 ± 0.03*	0.14 ± 0.02	0.70 ± 0.14*	3.07 ± 0.19	2.51 ± 0.58
7β-Hydroxy	0.68 ± 0.07	24.29 ± 0.07*	0.23 ± 0.02	13.30 ± 1.81*	1.32 ± 0.30	3.99 ± 0.93*
β-Epoxy	0.19 ± 0.02	3.04 ± 0.02*	0.37 ± 0.09	1.56 ± 0.37*	0.95 ± 0.14	1.81 ± 0.44
α-Epoxy	0.09 ± 0.02	3.37 ± 0.02*	0.21 ± 0.07	3.30 ± 0.37*	0.77 ± 0.23	1.11 ± 0.12
Dihydroxy	0.28 ± 0.04	1.41 ± 0.04*	0.11 ± 0.02	1.18 ± 0.18*	2.23 ± 0.51	2.39 ± 0.40
7-Keto	0.34 ± 0.08	1.89 ± 0.08*	0.54 ± 0.09	1.21 ± 0.19*	1.72 ± 0.33	1.42 ± 0.28
Total	1.78 ± 0.21	35.72 ± 0.21*	1.57 ± 0.21	21.18 ± 2.42*	10.06 ± 0.72	13.25 ± 1.92

[1] Values are means ± SEM, $n = 7$; * different from the phytosterol group, $P < 0.05$.
[2] The phytosterol diet contained 0.2 g/kg phytosterol mixture.
[3] The oxyphytosterol diet contained 0.2 g/kg heat-prepared oxyphytosterols.

more, the present study also revealed that the addition of a second oxygen atom to the cyclopentanoperhydrophenanthrene nucleus of phytosterols lowered their ability to reduce the absorption of cholesterol into the lymph (Fig. 1). These data suggest that the intestine has a complex pattern for discriminating between the cyclopentanoperhydrophenanthrene nucleus and the side chain of sterols for their absorption and lymphatic transport.

In contrast to our observations, Grandgirad et al. (38) reported that lymphatic recoveries of oxyphytosterols (7-keto and epoxides) in rats are lower than that of β-sitosterol and campesterol, respectively. However, their results in which oxycampesterols were better absorbed than oxysitosterols are in agreement with those of the present study. The discrepancies between the 2 studies might be due to the different methods used to administer oxyphytosterols, collect lymph, and analyze oxyphytosterols. They administered oxyphytosterols into the stomach of thoracic lymph duct cannulated rats, collected the lymph immediately after the operation, and analyzed sterols with GLC.

Reflecting the lymphatic transport of sterols in rats, apo E–deficient mice fed a diet of oxyphytosterols had elevated amounts of these compounds in the serum, liver, and aorta compared to mice fed the phytosterol diet. The concentrations of 7β-hydroxycampesterol and 7β-hydroxysitosterol in the serum, liver, and aorta were higher than that of other oxyphytosterols. Furthermore, the concentration of campesterol in the serum, liver, and aorta was consistently higher than the concentration of β-sitosterol. 7β-Hydroxysitosterol and 7β-hydroxycampesterol are present in the serum of phytosterolemic patients, who have highly elevated serum plant sterol concentrations (10). These results might be due to discrimination by the circulatory system for 7β-hydroxysterols and other sterols and between campesterol and β-sitosterol. Some investigators hypothesized that ABC monomers (ABCG5, ABCG8) expressed in the liver and intestine (39) are able to discriminate between cholesterol and other sterols (40,41). Therefore, the circulatory system appears to have a discrimination system that eliminates campesterol and 7β-hydroxyphytosterols less efficiently compared with β-sitosterol and other oxyphytosterols.

In the present study, apo E–deficient mice fed a diet containing oxyphytosterols had elevated levels of 5 kinds of oxycholesterols (7α-hydroxycholesterol, 7β-hydroxycholesterol, dihydroxycholesterol, 7-ketocholesterol, 25-hydroxycholesterol) in the serum compared to mice fed the phytosterol diet.

TABLE 3

Oxycholesterol levels of serum in apo E–deficient mice fed a phytosterol or oxyphytosterol diet[1]

Sterols	Phytosterol[2]	Oxyphytosterol[3]
	μmol/L	
7α-Hydroxy	0.454 ± 0.020	0.787 ± 0.107*
7β-Hydroxy	0.323 ± 0.010	1.110 ± 0.171*
β-Epoxy	3.775 ± 0.695	3.154 ± 0.720
α-Epoxy	4.246 ± 0.596	5.215 ± 0.571
Dihydroxy	0.047 ± 0.007	0.074 ± 0.006*
7-Keto	0.107 ± 0.010	0.252 ± 0.022*
25-Hydroxy	0.042 ± 0.004	0.298 ± 0.040*
27-Hydroxy	0.273 ± 0.025	0.315 ± 0.032
Total	9.267 ± 0.469	11.205 ± 1.480

[1] Values are means ± SEM, $n = 7$; * different from the phytosterol group, $P < 0.05$.
[2] The phytosterol diet contained 0.2 g/kg phytosterol mixture.
[3] The oxyphytosterol diet contained 0.2 g/kg heat-prepared oxyphytosterols.

The groups did not differ in the concentrations of β-epoxycholesterol, α-epoxycholesterol, and 27-hydroxycholesterol. Although a portion might be formed during the extraction and analyses, oxysterols formed in vivo include 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, and 24-, 25-, and 27-hydroxycholesterols, as shown by $^{18}O_2$ inhalation [reviewed by Björkhem (42)]. Because serum cholesterol did not differ between the groups, it is likely that elevated oxycholesterol in mice fed the oxyphytosterol-containing diet was due to the consumption of oxyphytosterols. Rosenblat and Aviram (43) showed that the enrichment of peritoneal macrophages from C57BL6 mice with 3 kinds of oxysterols (7-ketocholesterol, β-epoxycholesterol, and 7β-hydroxycholesterol) dose-dependently increased in superoxide anion release and cell-mediated oxidation of LDL.

In the present study, the adverse effects of oxyphytosterols on the development of atherosclerosis were evaluated in apo E–deficient mice by measuring aortic cholesterol levels and lesion size in the aortic root. Oxidative stress was also measured through the urinary excretion of 8-iso-PGF2α, one of the most reliable biomarkers of oxidative stress in vivo (44). Although comparisons to mice fed the AIN diet alone were not performed, our results showed for the first time that there was no significant difference in the size of lesions or aortic cholesterol concentration in apo E–deficient mice fed the phytosterol- and oxyphytosterol-supplemented diets. These results agree with our previous results, which showed no significant effect of dietary oxycholesterols on the development of atherosclerosis in apo E–deficient mice (17). Furthermore, the oxyphytosterol and phytosterol groups did not differ in creatine-indexed urinary isoprostanes. Accordingly, although apo E–deficient mice have a high antibody titer against oxidized lipids (45) and dietary oxyphytosterols might locally accelerate oxidative stress as reflected by the elevation of oxycholesterol in the serum, the endogenous antioxidant defense system and dietary antioxidants are apparently adequate to minimize the amounts of in vivo oxidant damage (5,17). Alternatively, some dietary oxysterols might exert a beneficial effect on the development of atherosclerosis. Oxycholesterols such as 22(R)-hydroxycholesterol, 20(S)-hydroxycholesterol, 24-hydroxycholesterol, 25-hydroxycholesterol, 7α-hydroxycholesterol, and 27-hydroxycholesterol are ligands for the liver X receptor (LXR). This receptor is a transcription factor for the ATP-binding cassette transporter A1, which is involved in cholesterol efflux from the arterial intima (46). Similarly, oxyphytosterols derived from ergosterol and brassicasterol are LXR agonists, and act as effectively as the ligands 22(R)-hydroxycholesterol and 24(S), 25-epoxycholesterol (47). Therefore, oxycholesterol and oxyphytosterol may exert their effects on the sterol regulatory machinery and thereby prevent the development of atherosclerosis. This hypothesis, however, will need to be tested in future studies.

In summary, the present study showed that the intestine and liver possess a discrimination system that eliminates less campesterol and 7β-hydroxyphytosterols. Dietary oxyphytosterols are absorbed and accumulate in the serum, liver, and aorta in apo E–deficient mice. However, dietary oxyphytosterols do not affect aortic cholesterol or the lesion volume in the aortic valve compared to phytosterols. The results indicate that exogenous oxysterols do not promote the develomment of atherosclerosis in apo E–deficient mice.

LITERATURE CITED

1. Jacobson, M. S. (1987) Cholesterol oxides in Indian ghee: possible cause of unexplained high risk of atherosclerosis in Indian immigrant populations. Lancet 2: 656–658.

2. Emanuel, H. A., Hassel, C. A., Addis, P. B., Bergmann, S. D. & Zavoral, J. H. (1991) Plasma cholesterol oxidation products in human subjects fed a meal rich in oxysterols. J. Food Sci. 56: 843–847.

3. Staprans, I., Pan, X.-M., Rapp, J. H. & Feingold, K. R. (2003) Oxidized cholesterol in the diet is a source of oxidized lipoproteins in human serum. J. Lipid Res. 44: 705–715.

4. Brown, A. J. & Jessup, W. (1999) Oxysterols and atherosclerosis. Atherosclerosis 142: 1–28.

5. Carpenter, K.L.H. (2002) Good COP, bad COP: an unsolved murder. Are dietary cholesterol oxidation products guilty of atherogenicity? Br. J. Nutr. 88: 335–338.

6. Dutta, P. C. & Appelqvist, L-Å. (1997) Studies on phytosterol oxides. I: Effect of storage on the content in potato chips prepared in different vegetable oils. J. Am. Oil Chem. Soc. 74: 647–657.

7. Dutta, P. C. (1997) Studies on phytosterol oxides. II: Content in some vegetable oils and in French fries prepared in these oils. J. Am. Oil Chem. Soc. 74: 659–666.

8. Lampi, A.-M., Juntunen, L., Toivo, J. & Piironen, V. (2002) Determination of thermo-oxidation products of plant sterols. J. Chrom. B 777: 83–92.

9. Bortolomeazzi, R., Cordaro, F., Pizzale, L. & Conte, L. S. (2003) Presence of phytosterol oxides in crude vegetable oils and their fate during refining. J. Agric. Food Chem. 51: 2394–2401.

10. Plat, J., Brzezinka, H., Lütjohann, D., Mensink, R. P. & von Bergmann, K. (2001) Oxidized plant sterols in human serum and lipid infusions as measured by combined gas-liquid chromatography-mass spectrometry. J. Lipid Res. 42: 2030–2038.

11. Adcox, C., Boyd, L., Oehrl, L., Allen, J. & Fenner, G. (2001) Comparative effects of phytosterol oxides and cholesterol oxides in cultured macrophage-derived cell lines. J. Agric. Food Chem. 49: 2090–2095.

12. Remie, R., van Dongen, J. J. & Rensema, J. W. (1990) Permanent cannulation of the thoracic duct. In: Mannual of Microsurgery on the Laboratory Rat (van Dongen, J. J., Remie, R., Rensema, J. W. & van Wunnik, G.H.J., eds.), pp. 243–254. Elsevier, Amsterdam.

13. Chicoye, E., Powrie, W. D. & Fennema, O. (1968) Isolation and characterization of cholesterol-5β,6β-oxide from an aerated aqueous dispersion of cholesterol. Lipids 3: 335–334.

14. Nourooz-Zadeh, J. & Appelqvist, L.-Å. (1992) Isolation and quantitative determination of sterol oxides in plant-based foods: soybean oil and wheat flour. J. Am. Oil Chem. Soc. 69: 288–293.

15. Fieser, L. F. & Rajagopalan, S. (1949) Selective oxidation with *N*-bromosuccinimide. II. Cholestane-3β,5α,6β-triol. J. Am. Chem. Soc. 71: 3938–3941.

16. Davis, M. & Petrow, V. (1949) Steroids and related compounds. Part VI. The stereochemical configuration and dehydration of the isomeric androstane-3:5:6-triol-ones. J. Chem. Soc. 4: 2536–2539.

17. Ando, M., Tomoyori, H. & Imaizumi, K. (2002) Dietary cholesterol-oxidation products accumulate in serum and liver in apolipoprotein E-deficient mice, but do not accelerate atherosclerosis. Br. J. Nutr. 88: 339–345.

18. Reeves, P. G., Nielsen, F. H. & Fahey, G. C.. Jr. (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition *ad hoc* Writing Committee on the recommendation of the AIN-76A rodent diet. J. Nutr. 123: 1939–1951.

19. Ni, W., Tsuda, Y., Sakono, M. & Imaizumi, K. (1998) Dietary soy protein isolate, compared with casein, reduces atherosclerotic lesion area in apolipoprotein E-deficient mice. J. Nutr. 128: 1884–1889.

20. Carvajal, O., Nakayama, M., Kishi, T., Sato, M., Ikeda, M., Sugano, M. & Imaizumi, K. (2000) Effect of medium-chain fatty acid positional distribution in dietary triacylglycerol on lymphatic lipid transport and chylomicron composition in rats. Lipids 35: 1345–1351.

21. Tomoyori, H., Carvajal, O., Nakayama, M., Kishi, T., Sato, M., Ikeda, I. & Imaizumi, K. (2002) Lympyhatic transport of dietary cholesterol oxidation products, cholesterol and triacylglycerol in rats. Biosci. Biotechnol. Biochem. 66: 828–834.

22. Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957) A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226: 497–509.

23. Dzeletovic, S., Breuer, O., Lund, E. & Diczfalusy, U. (1995) Determination of cholesterol oxidation products in human plasma by isotope dilution-mass spectrometry. Anal. Biochem. 225: 73–80.

24. Mori, T. A., Croft, K. D., Puddey, I. B. & Beilin, L. J. (1996) Analysis of native and oxidized low-density lipoprotein oxysterols using gas chromatography-mass spectrometry with selective ion monitoring. Redox Rep. 2: 25–34.

25. Ikeda, I., Nakagiri, H., Sugano, M., Ohara, S., Hamada, T., Nonaka, M. & Imaizumi, K. (2001) Mechanism of phytosterolemia in stroke-prone spontaneously hypertensive and WKY rats. Metabolism 50: 1361–1368.

26. Morrow, J. D. & Roberts, L. J. (1994) Mass spectrometry of prostanoids: F2-isoprostanes produced by non-cyclooxygenase free radical-catalyzed mechanism. Methods Enzymol. 233: 163–175.

27. Zhao, Z., Hjelm, N. M., Lam, C.W.K. & Ho, C. (2001) One-step solid-phase extraction procedure for F2-isoprosanes. Clin. Chem. 47: 1306–1308.

28. Schwedhelm, E., Tsikas, D., Durand, T., Gutzki, F.-M., Guy, A., Rossi, J.-C. & Frölich, J. C. (2000) Tandem mass spectrometric quantification of 8-iso-prostaglandin F2α and its metabolite 2,3-dinor-5,6-dihydro-8-iso-prostaglandin F2α in human urine. J. Chrom. B 744: 99–112.

29. Wübert, J., Reder, E., Kaser, A., Weber, P. C. & Lorenz, R. L. (1997) Simultaneous solid phase extraction, derivatization, and gas chromatographic

mass spectrometric quantification of thromboxane and prostacyclin metabolites, prostaglandins, and isoprosanes in urine. Anal. Chem. 69: 2143–2146.

30. Ni, W., Tsuda, Y., Takashima, T., Sato, H., Sato, M. & Imaizumi, K. (2003) Anti-atherogenic effect of soya and rice-protein isolate, compared with casein, in apolipoprotein E-deficient mice. Br. J. Nutr. 90: 13–20.

31. Hassan, A. S. & Rampone, A. J. (1979) Intestinal absorption of and lymphtic transport of cholesterol and beta-sitosterol in the rat. J. Lipid Res. 20: 646–653.

32. Vahouny, G. W., Connor, W. E., Subramaniam, S., Li, D. S. & Gallo, L. L. (1983) Comparative lymphatic absorption of sitosterol, stigmasterol, and fucosterol and differential inhibition of cholesterol absorption. Am. J. Clin. Nutr. 37: 805–809.

33. Ikeda, I., Tanaka, M., Sugano, M., Vahouny, G. V. & Gallo, L. L. (1988) Discrimination between cholesterol and sitosterol for absorption in rats. J. Lipid Res. 29: 1583–1591.

34. Igel, M., Giesa, U., Lütjohann, D. & von Bergmann, K. (2003) Comparison of the intestinal uptake of cholesterol, plant sterols, and stanols in mice. J. Lipid Res. 44: 533–538.

35. Allayee, H., Laffitte, B. A. & Lusis, A. J. (2000) Biochemistry. An absorption study of cholesterol. Science 290: 1709–1711.

36. Chen, H. C. (2001) Molecular mechanisms of sterol absorption. J. Nutr. 131: 2603–2605.

37. Zhang, Y., Yu, C., Liu, J., Spencer, T. A., Chang, C.C.Y. & Chang, T.-Y. (2003) Cholesterol is superior to 7-ketocholesterol or 7α-hydroxycholesterol as an allosteric activator for acyl-coenzyme A:cholesterol acyltransferase 1. J. Biol. Chem. 278: 11642–11647.

38. Grandgirard, A., Sergiel, J.-P., Nour, M., Demaison-Meloche, J. & Ginies, C. (1999) Lymphatic absorption of phytosterol oxides in rats. Lipids 34: 563–570.

39. Graf, G. A., Li, W. P., Gerard, R. D., Gelissen, I., White, A., Cohen, J. C. & Hobbs, H. H. (2002) Coexpression of ATP-binding cassette proteins ABCG5 and ABCG8 permits their transport to the apical surface. J. Clin. Invest. 110: 659–669.

40. Berge, K. E., Tian, H., Graf, G. A., Yu, L., Grishin, N. V., Schultz, J., Kwiterovich, P., Shan, B., Barnes, R. & Hobbs, H. H. (2000) Accumulation of dietary cholesterol in sitosterolemia caused by mutations in adjacent ABC transporter. Science 290: 1771–1775.

41. Yu, L., Li-Hawkins, J., Hammer, R. E., Berge, K. E., Horton, J. D., Cohen, J. C. & Hobbs, H. H. (2002) Overexpression of ABCG5 and ABCG8 promotes biliary cholesterol secretion and reduces fractional absorption of dietary cholesterol. J. Clin. Invest. 110: 671–680.

42. Björkhem, I. (2002) Do oxystgerols control cholesterol homeostasis? J. Clin. Invest. 110: 725–730.

43. Rosenblat, M. & Aviram, M. (2002) Oxysterol-induced activation of macrophage NADPH-oxidase enhances cell-mediated oxidation of LDL in the atherosclerotic apolipoprotein E deficient mouse: inhibitory role for vitamin E. Atherosclerosis 160: 69–80.

44. Morrow, J. D. & Roberts, L. J. (1997) The isoprostanes: unique bioactive products of lipid peroxidation. Prog. Lipid Res. 36: 1–21.

45. Palinski, W., Hörkko, S., Miller, E., Steinbrecher, U. P., Powell, H. C., Curtiss, L. K. & Witztum, J. L. (1996) Cloning of monoclonal autoantibodies to epitopes of oxidized lipoproteins from apolipoprotein E-deficient mice: demonstration of epitopes of oxidized low density lipoprotein in human plasma. J. Clin. Invest. 98: 800–814.

46. Janowski, B. A., Willy, P. J., Devi, T. R., Falck, J. R. & Mangelsdorf, D. J. (1996) An oxysterol signaling pathway mediated by the nuclear receptor LXRα. Nature 383: 728–731.

47. Kaneko, E., Matsuda, M., Yamada, Y., Tachibana, Y., Shimomura, I. & Makishima, M. (2003) Induction of intestinal ATP-binding cassette transporters by a phytosterol-derived liver X receptor agonist. J. Biol. Chem. 278: 36091–36098.