

Plant sterols and stanols: effects on mixed micellar composition and LXR (target gene) activation

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Abstract Plant stanols and sterols of the 4-desmethyl family (e.g., sitostanol and sitosterol) effectively decrease LDL cholesterol concentrations, whereas 4,4-dimethylsterols (α -amyrin and lupeol) do not. Serum carotenoid concentrations, however, are decreased by both plant sterol families. The exact mechanisms underlying these effects are not known, although effects on micellar composition have been suggested. With a liver X receptor (LXR) coactivator peptide recruitment assay, we showed that plant sterols and stanols from the 4-desmethylsterol family activated both LXR α and LXR β , whereas 4,4-dimethyl plant sterols did not. In fully differentiated Caco-2 cells, the functionality of this effect was shown by the increased expression of ABCA1, one of the known LXR target genes expressed by Caco-2 cells in measurable amounts. The LXR-activating potential of the various plant sterols/stanols correlated positively with ABCA1 mRNA expression. Reductions in serum hydrocarbon carotenoids could be explained by the effects of the 4-desmethyl family and 4,4-dimethylsterols on micellar carotenoid incorporation. **Our findings indicate that the decreased intestinal absorption of cholesterol and carotenoids by plant sterols and stanols is caused by two distinct mechanisms.**—Plat, J., J. A. Nichols, and R. P. Mensink. Plant sterols and stanols: effects on mixed micellar composition and LXR (target gene) activation. *J. Lipid Res.* 2005. 46: 2468–2476.

Supplementary key words cholesterol • antioxidant • ATP binding cassette transporter • liver X receptor

Foods enriched with plant stanol or sterol esters (so-called functional foods) have gained a prominent position in decreasing cardiovascular risk by dietary means (1). Indeed, numerous intervention trials have demonstrated that plant stanol/sterol esters consistently and dose-dependently decrease serum LDL cholesterol concentrations in various populations and patient groups (2). The mechanism underlying this hypocholesterolemic effect is a reduction in cholesterol absorption from the intestinal

lumen into the circulation, as explained through a competition between plant stanols/sterols and intestinal cholesterol for incorporation into mixed micelles (3). Whether this is indeed the main or the only mechanism is still unknown. In this respect, we earlier suggested that sitostanol may affect cholesterol metabolism within intestinal cells (4). Cellular cholesterol concentrations within enterocytes are determined by several routes, such as cholesterol uptake from the lumen as mediated by Niemann-Pick C1-like 1 and/or annexin 2/caveolin 1 complexes (5, 6); however, the role of caveolin 1 in this process has recently been questioned (7). Besides by changing uptake, cellular cholesterol levels can also be regulated by cholesterol secretion back into the intestinal lumen by ATP binding cassette (ABC) transporters. In a series of elegant experiments, Yu and coworkers (8–10) have shown that ABCG5 and ABCG8 are involved in the liver X receptor (LXR) agonist-induced reduction of intestinal cholesterol absorption. Thus, it is possible that cholesterol metabolism within the enterocyte may change as a result of LXR agonist activity of plant stanols or sterols or one of their (oxidized) metabolites. To have any effects within the enterocyte, however, it is necessary that these components are taken up by enterocytes, as has been demonstrated (11).

Plant stanols/sterols decrease not only serum LDL cholesterol concentrations but also serum hydrocarbon carotenoid (i.e., α - + β -carotene and lycopene) concentrations. This reduction persisted after standardization for serum lipid concentrations, which indicated that this decrease is not attributable simply to a decreased number of LDL particles, a main carrier of the plasma carotenoids (12). Indeed, we have already shown that this reduction is significantly associated with the reduction of cholesterol absorption, as caused by the consumption of plant stanol esters (13). Therefore, it can be speculated that plant

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Abbreviations: ABC, ATP binding cassette; LXR, liver X receptor; SREBP-2, sterol-regulatory element binding protein-2.

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stanols/sterols affect not only micellar cholesterol incorporation but also that of the carotenoids.

Interestingly, not all plant sterols decrease both serum LDL cholesterol and carotenoid concentrations. In contrast to the 4-desmethylsterols such as sitosterol and sitosterol, 4,4-dimethylsterols such as lupeol and α -amyrin (Fig. 1) decrease only serum carotenoid concentrations (14). This suggests that the mechanisms underlying the reductions in serum LDL cholesterol and carotenoid concentrations are different. The aim of the present study, therefore, was to evaluate the effects of individual plant stanols/sterols on the incorporation of cholesterol as well as of carotenoids into in vitro-prepared mixed micelles. In addition, we have examined with an LXR coactivator peptide recruitment assay the potential LXR agonist activity of the same individual plant sterols to determine whether LXR target genes such as the ABC transporters are involved in the reduced cholesterol absorption found after consumption of plant stanol and sterol esters. To determine whether plant sterol- or stanol-induced LXR activation indeed increased gene expression, plant sterol- or stanol-enriched mixed micelles were added to the apical surface of polarized intestinal Caco-2 cells to measure the expression of the LXR target gene ABCA1. Although ABCA1 is not related to intestinal cholesterol absorption, this gene was chosen because the Caco-2 cells did not express ABCG5 and ABCG8, which are involved in cholesterol absorption, at detectable levels. It is known, however, that LXR activation by synthetic LXR ligands (15–17) increases intestinal ABCA1 as well as ABCG5 and ABCG8 expression and decreases intestinal cholesterol absorption (10, 15, 17).

MATERIALS AND METHODS

Reagents and chemical compounds

Cholesterol (99% pure), sitosterol (96% pure), sitosterol (97% pure), campesterol (65% pure; remainder dihydrobrassicasterol), α -amyrin (purity unknown), lupeol (98% pure), fucosterol (95% pure), taurocholate, oleic acid, mono-olein, β -carotene, lutein, α -tocopherol, trimethylchlorosilane 5 α -cholestane, 5 β -cholestan-3 α -ol, retinylacetate, 22(*R*)-hydroxy cholesterol, and 9-*cis*-retinoic acid were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Campestanol [67% campestanol [i.e., C24(*R*)]; the remainder is the C24(*S*) epimer of campestanol derived from hydrogenation of 22,23-dihydrobrassicasterol] was prepared by RAISIO (Raisio, Finland) by hydrogenation of vegetable oil base sterols, mainly soy sterols, followed by preparative HPLC to isolate campestanol. Bis-(trimethylsilyl)-trifluoroacetamide and pyridine were obtained from Merck (Darmstadt, Germany). The sterols and stanols were always stored and handled under nitrogen to avoid autoxidation.

terol), α -amyrin (purity unknown), lupeol (98% pure), fucosterol (95% pure), taurocholate, oleic acid, mono-olein, β -carotene, lutein, α -tocopherol, trimethylchlorosilane 5 α -cholestane, 5 β -cholestan-3 α -ol, retinylacetate, 22(*R*)-hydroxy cholesterol, and 9-*cis*-retinoic acid were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Campestanol [67% campestanol [i.e., C24(*R*)]; the remainder is the C24(*S*) epimer of campestanol derived from hydrogenation of 22,23-dihydrobrassicasterol] was prepared by RAISIO (Raisio, Finland) by hydrogenation of vegetable oil base sterols, mainly soy sterols, followed by preparative HPLC to isolate campestanol. Bis-(trimethylsilyl)-trifluoroacetamide and pyridine were obtained from Merck (Darmstadt, Germany). The sterols and stanols were always stored and handled under nitrogen to avoid autoxidation.

In vitro mixed micelle synthesis

To evaluate the effects of the individual plant stanols/sterols on micellar cholesterol incorporation, mixed micelles were prepared in triplicate by adding 250 μ M of a particular sterol (i.e., cholesterol, sitosterol, sitosterol, campesterol, campestanol, fucosterol, α -amyrin, or lupeol) alone or in combination with cholesterol to 5 mM taurocholate, 390 μ M oleic acid, and 110 μ M mono-olein. After evaporation, DMEM (Gibco-BRL, Life Technologies, Breda, The Netherlands), supplemented with 1% nonessential amino acids, penicillin (100 U/ml), and streptomycin (100 mg/ml), was added to the residual to restore the concentrations as indicated. This mixture was sonicated for 30 min at 37°C. We have shown previously that under these conditions up to 500 μ M total sterols can easily be incorporated into the mixed micelles (4).

In a second series of experiments, similar procedures were used to examine the effects of the sterols and stanols on the incorporation of fat-soluble antioxidants into the mixed micelles. This experiment was carried out using 125 or 250 μ M of a particular sterol. Experiments were carried out in the dark to avoid possible breakdown of light-sensitive antioxidant structures. Three antioxidants were examined, in decreasing order of lipophilicity: β -carotene (a hydrocarbon carotenoid), lutein (an oxygenated carotenoid), and α -tocopherol. In a series of pilot experiments in which the concentrations of the antioxidants used were based on previous studies (18, 19), the maximum incorporation of the different antioxidants was evaluated (data not

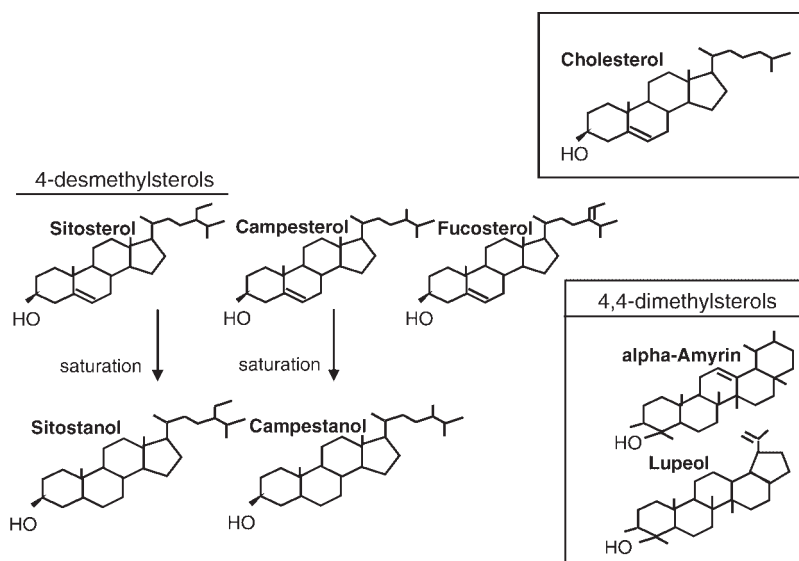


Fig. 1. Structures of 4-desmethylsterols (sitosterol, campesterol, fucosterol, sitostanol, and campestanol) and 4,4-dimethylsterols (α -amyrin and lupeol).

shown). Based on the outcomes of these experiments, it was decided to use 11 μ M lutein, 150 μ M α -tocopherol, and 25 μ M β -carotene in all further experiments; of these concentrations, both 11 μ M lutein and 150 μ M α -tocopherol were completely incorporated, whereas of the 25 μ M β -carotene, maximally 65% could be incorporated into the mixed micelles (data not shown). These micellar characteristics were used as a starting point to evaluate the influence of the different plant sterols on micellar antioxidant incorporation.

For analysis of the composition of the mixed micelles, the micellar phase was first isolated by ultracentrifugation (3). Next, micellar stanol/sterol and antioxidant concentrations were quantified by gas-liquid chromatography and reversed-phase HPLC analysis, respectively. Incorporation of cholesterol or a specific antioxidant in the presence of a particular plant stanol/sterol was calculated compared with the mixed micelles prepared with 250 μ M cholesterol only, which was set at 100%.

Mixed micellar sterol, stanol, and antioxidant concentrations

Micellar cholesterol and plant sterol and stanol concentrations were analyzed by gas-liquid chromatography as described previously (13). Briefly, 1.0 ml of the micellar fraction was saponified for 1.5 h at 70°C with 2 ml of a mixture consisting of a 10 M KOH solution in water and ethanol (1:9, v/v). After cooling down, sterols were extracted twice with hexane. Next, the hexane extracts were evaporated to dryness at 37°C under a moderate nitrogen flow. The nonsaponifiable lipids were silylated for 15 min after addition of 200 μ l of bis-(trimethylsilyl)-trifluoroacetamide containing 1% trimethylchlorosilane and 100 μ l of pyridine. After evaporation under a moderate nitrogen flow at 50°C, the samples were dissolved in 500 μ l of pentane. Samples were then analyzed with a GC8000 Top gas chromatograph (Carlo Erba, Milan, Italy) fitted with a 25 m AT1701 capillary column with an inner diameter of 0.32 mm and 0.30 mm film thickness (Alltech, Breda, The Netherlands) using cold on-column injection. One hundred micrograms of 5 α -cholestan-3 α -ol were added to all samples before extraction and used as internal standards for cholesterol and plant sterols/stanols, respectively.

The micellar concentrations of lipophylic antioxidants were determined as described by Hess, Keller, and Oberlin (20) and modified by Oostenbrug, Mensink, and Hardeman (21). Briefly, 50 μ l of the micellar fractions was combined with 950 μ l of water and 1,000 μ l of ethanol-methanol (1:1, v/v) with retinylacetate as an internal standard. This mixture was extracted twice with hexane and evaporated, and the remaining material was resuspended in 200 μ l of ethanol-dioxan (1:1, v/v). The amounts of lipid-soluble antioxidants were determined by reversed-phase HPLC using a Inertsil ODS-2 C-18 reversed-phase column with simultaneous fluorescence detection for α -tocopherol and absorbance measurement for β -carotene and lutein.

In vitro preparation of oxysterol and oxyphytosterol mixtures

Oxysterol and oxyphytosterol mixtures were prepared by heating cholesterol or soy sterols (46% sitosterol, 28% campesterol, and 16% stigmasterol) for 3 h at 180°C. Oxidized sterols were fractionated by SPE silica gel and eluted with acetone. The compositions of the oxysterol and oxyphytosterol fractions were analyzed by GC-flame ionization detection (HP6890, column HP-5; Agilent Technologies) and GC-MS (HP6890-HP5973 electron ionization, column HP-5MS; Agilent Technologies), both as trimethylsilyl derivatives. The mixtures were composed mainly of ring oxidation products such as 7 α - and 7 β -hydroxy sterols, 7-keto sterols, and 5 α ,6 α - and 5 β ,6 β -epoxy sterols (Table 1). The composi-

TABLE 1. Composition of the in vitro-prepared oxysterol and oxyphytosterol mixtures analyzed in the liver X receptor coactivator peptide assay

Component	Cholesterol	Plant Sterols
7-Keto (% of total)	40.3	28.2
Epoxides (% of total)	35.7	20.0
5 α ,6 α -epoxy	30.3	7.2
5 β ,6 β -epoxy	5.4	12.7
Hydroxides (% of total)	24.0	51.8
7 α -hydroxy	4.0	19.8
7 β -hydroxy	13.8	32.0
Others	6.1	0.0

Oxysterols and oxyphytosterol mixtures were prepared by in vitro oxidation of cholesterol and a plant sterol mixture (46% sitosterol, 28% campesterol, and 16% stigmasterol) as described in Materials and Methods. In the end, 95.9% of cholesterol used in the oxidation reaction was present in the mixture in one of its oxidized forms. For the plant sterols, this was 93.6%. Concentrations were determined by GC-MS. Ketones are 7-keto cholesterol or the sum of 7-keto sitosterol/campesterol/stigmasterol. Identified epoxides were 5 α ,6 α -epoxy cholesterol + 5 β ,6 β -epoxy cholesterol or 5 α ,6 α -epoxy sitosterol/campesterol/stigmasterol + 5 β ,6 β -epoxy sitosterol/campesterol/stigmasterol. Hydroxides were identified as 7 α -hydroxy + 7 β -hydroxy cholesterol or 7 α -hydroxy sitosterol/campesterol/stigmasterol + 7 β -hydroxy sitosterol/campesterol/stigmasterol.

tions of the oxysterol and oxyphytosterol mixtures were comparable in terms of the proportions of keto, hydroxy, and epoxy sterols. No side chain oxidation products, such as 22(R)-hydroxy or 24(S),25-epoxy sterols, which are known natural LXR ligands, could be detected.

LXR coactivator peptide assay

A cell-free ligand-sensing assay, initially developed to analyze the structural requirements of activating LXR α and LXR β , was used to evaluate the potential LXR-activating capacity of various plant stanols/sterols. In this assay, the ligand-dependent recruitment of the peptide steroid receptor coactivator 1 to the nuclear receptor was measured by fluorescence energy transfer, as described (22). Moreover, the LXR proteins used were histidine-tagged and biotinylated "human" ligand binding domains. The synthetic LXR agonist GW683965A (GlaxoSmithKline) and DMSO were used as positive and vehicle controls, respectively. All compounds were tested in duplicate in a range from 10 nM to 200 μ M. Data were then normalized using the following equation: $B_{\max} = (\text{test compound signal} - \text{average basal signal}) / (\text{average GW683965A signal} - \text{average basal signal}) \times 100\%$. This B_{\max} (maximal signal) can be regarded as the relative efficacy (percentage maximal increase in fluorescence compared with the maximal signal obtained with GW683965A) value for each compound. Next, EC₅₀ values were calculated using a least-squares fit to a standard three-parameter saturation binding equation.

Caco-2 cell culture and ABCA1 mRNA expression

Caco-2 cells were cultured on collagen-coated polytetrafluoroethylene membrane transwell filter inserts with a 0.4 mm membrane pore size (Costar Co., Cambridge, MA) in DMEM (Gibco, Life Technologies) supplemented with heat-inactivated fetal calf serum (20%), nonessential amino acids (1%), sodium pyruvate (1%), penicillin (100 U/ml), and streptomycin (100 mg/ml). When cultured on the transwell filter system, Caco-2 cells behave as if they have an apical (luminal) and a basolateral (lymphatic) side, which resembles the in vivo situation. Mixed micelles, enriched with the various sterols (prepared as described above), were presented for 6 h to the apical surface of fully differentiated

Caco-2 cells. A combination of the natural LXR ligands 22(*R*)-hydroxy cholesterol (10 μ M) plus 9-*cis*-retinoic acid (10 μ M) was used as a positive control for LXR activation. The effect of a particular sterol on ABCA1 expression was expressed as the percentage change compared with the effect of mixed micelles prepared without sterols. ABCA1 expression was evaluated as a model gene for LXR activation, because Caco-2 cells do not express ABCG5 and ABCG8 mRNAs, which are probably the LXR target genes responsible for decreased cholesterol absorption, at detectable levels. All experiments were conducted in triplicate, under serum-free conditions, \sim 14 days after confluence was reached. Total RNA from the fully differentiated Caco-2 cells was isolated using Trizol (Gibco, Life Technologies). After removing possible traces of contaminating DNA (DNase Rnase-free; Promega), ABCA1 and β -actin (housekeeping gene) expression were measured by RT-PCR using ABCA1-specific primers (sense, TGACAAGTCTGTGCAATGGATCAA; antisense, GATACGAGACACAGCCTGGTAGAT) and β -actin-specific primers (sense, ACCTGACTGACTACCTCATGAAGATC; antisense, CGTCATACTCCTGCTTGCTGAT). The sense primers were 5'-Cy-5 labeled (Sigma-Genosys, Cambridge, UK), enabling detection of the subsequently formed fluorescently labeled PCR fragments on an ALF-express DNA sequencer (Pharmacia Biotech, Roosendaal, The Netherlands) as described previously (4).

Statistics

Data are presented as means \pm SD. Differences between mean values were tested for statistical significance by ANOVA, in which $P < 0.05$ was considered statistically significant. In case a significant treatment effect was found ($P < 0.05$), the treatments were compared pairwise using the Bonferroni multiple comparison test.

RESULTS

Sterol incorporation into mixed micelles

Figure 2 shows that addition of 250 μ M sitostanol, but also of sitosterol, to mixtures containing 250 μ M cholesterol decreased the incorporation of cholesterol into mixed micelles by \sim 25%. Although less pronounced than the effects of these two 4-desmethylsterols, α -amyrin and lupeol, two 4,4-dimethylsterols, also decreased micellar cholesterol incorporation by 14.8% and 9.8%, respectively, whereas fucosterol reduced micellar cholesterol incorporation by only 3.3%.

Antioxidant incorporation into mixed micelles

Compared with cholesterol, the addition of all plant stanols/sterols evaluated decreased the incorporation of β -carotene into mixed micelles (Fig. 3). However, large differences between the various sterols were observed. The effects of the three 4-desmethylsterols (sitostanol, sitosterol, and fucosterol) on β -carotene incorporation were modest (-28.3 , -38.4 , and -37.2% , respectively) compared with that of the 4,4-dimethylsterol α -amyrin (-91.3%). The effects of lupeol, another 4,4-dimethylsterol, were less striking (-41.9%). In contrast to the highly lipophilic hydrocarbon carotenoid β -carotene, lutein (Fig. 3) and α -tocopherol (100% incorporation for all plant stanols/sterols; data not shown) were well incorporated into the micelles, irrespective of the stanol/sterol added to the

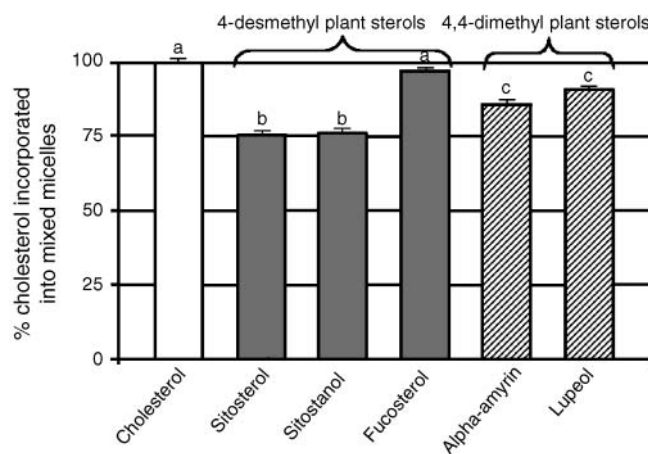


Fig. 2. Effects of individual plant stanols/sterols (250 μ M) on micellar cholesterol (250 μ M) incorporation. Mixed micelles were prepared as described in Materials and Methods. Data shown are means \pm SD. Different letters indicate statistically significant differences between the indicated conditions ($P < 0.001$).

mixtures. Only α -amyrin decreased micellar lutein concentrations (-41.5%), but reductions were not as dramatic as those for β -carotene. Only data for 125 μ M plant stanols/sterols are presented. When the concentration was increased to 250 μ M, the same pattern of micellar antioxidant incorporation was observed (data not shown).

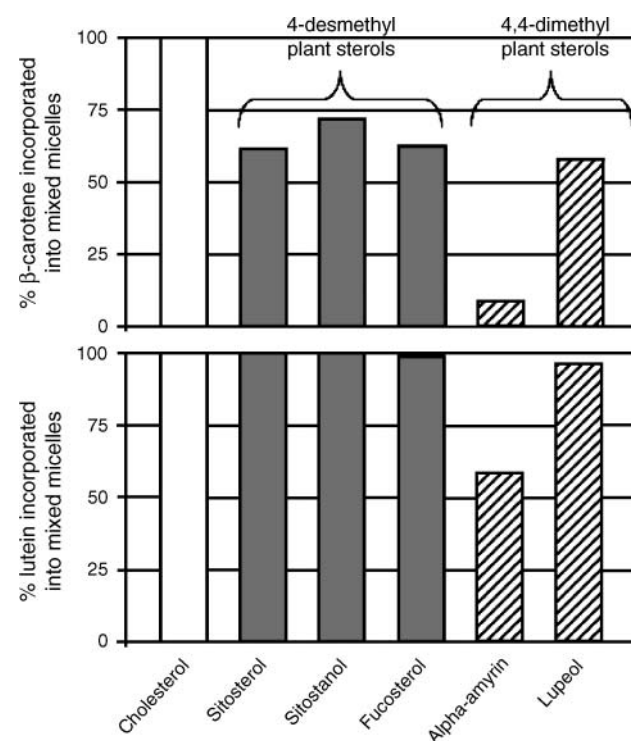


Fig. 3. Effects of individual plant stanols/sterols (125 μ M) on micellar β -carotene (25 μ M; upper panel) or micellar lutein (11 μ M; lower panel) incorporation in mixed micelles containing 250 μ M cholesterol. Mixed micelles were prepared as described in Materials and Methods.

LXR activity of individual plant sterols

Potential effects of the different stanols/sterols on LXR activity were evaluated in a coactivator peptide recruitment assay with GW683965A, a synthetic LXR agonist, as a positive control. EC₅₀ values for the 4-desmethylsterols (sitosterol, campesterol) clearly indicated that these plant sterols can activate LXR (Fig. 4). Oxysterols are known natural ligands of LXR. We previously identified oxidized plant sterols (oxyphytosterols) in serum from sitosterolemic patients. Circulating oxyphytosterols were 7-keto/7 α -hydroxy/7 β -hydroxy, 5 α ,6 α -epoxides, or 3 β ,5 α ,6 β -trianols of sitosterol and/or campesterol, and all nonenzymatically formed ring oxidation products (23). Because we previously suggested that metabolites rather than the plant sterols or stanols themselves might be responsible for the effects on gene expression (4), we also evaluated the effects of a mixture of these in vivo-circulating nonenzymatically formed oxidized plant sterols. This mixture was composed mainly of 7 α - and 7 β -hydroxy sitosterol/campesterol/stigmasterol, 7-keto sitosterol/campesterol/stigmasterol, and 5 α ,6 α - and 5 β ,6 β -epoxy sitosterol/campesterol/stigmasterol (Table 1). However, this mixture was ineffective as an LXR activator. Also, an oxysterol mixture made from cholesterol with a comparable composition in terms of the proportions of keto, hydroxy, and epoxy sterols (Table 1) did not activate LXR. Furthermore, both 4,4-dimethylsterols (α -amyrin and lupeol) showed no LXR activation activity (Fig. 4). Interestingly, fucosterol had the strongest effects, which were even more pronounced (LXR α) or similar (LXR β) compared with those of GW683965A (Fig. 4). Although slightly less powerful than their sterol equivalents, sitostanol and campestanol also activated LXR.

Effects on mRNA expression of ABCA1, a LXR target gene

Mixed micelles enriched with the different stanols/sterols were added for 6 h to the apical surface of fully differentiated Caco-2 cells. As shown in Fig. 5, sitostanol and sitosterol increased ABCA1 expression by 244% and 273%, respectively. These effects were greater than those obtained with micelles enriched with 250 μ M cholesterol (+163%). In addition, campesterol (213%) and fucosterol (166%) affected ABCA1 expression, whereas both 4,4-dimethylsterols, lupeol (–18%) and α -amyrin (67%), did not show an even pronounced effect as the 4-dimethylsterols did on ABCA1 expression. Moreover, the effects of campesterol and campestanol were lower compared with those of sitosterol and sitostanol. This suggests that sterols with an ethyl side chain at the carbon-24 position are more potent LXR agonists than sterols with a methyl side chain at this position. It should be noted, however, that the campesterol used in these experiments was only 65% pure (the remainder being dihydrobrassicasterol), which may have influenced the estimates for campesterol. The combination of the known natural LXR/retinoid X receptor heterodimer agonists 22R-OH cholesterol (10 μ M) plus 9-*cis*-retinoic acid (10 μ M) had by far the strongest effects (+649%) on ABCA1 expression. Interestingly, the change in ABCA1 mRNA expression upon sterol supplementation correlated positively with the maximum activating potential of that particular plant stanol/sterol in the ligand-sensing assays. This relation was found for both LXR α ($r = 0.684$, $P = 0.061$) and LXR β ($r = 0.711$, $P = 0.046$).

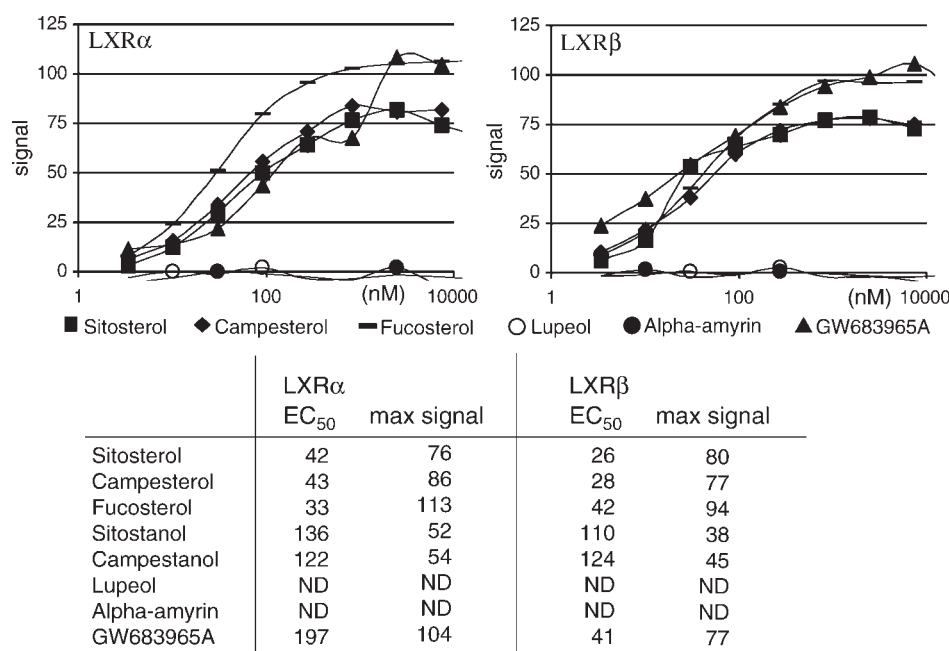


Fig. 4. Liver X receptor (LXR) agonist activity of individual sterols as evaluated in a coactivator peptide recruitment assay (22) compared with GW683965A or DMSO. In the table, EC₅₀ values as well as maximal values obtained at 10 mM incubations (indicated as relative efficacy to GW683965A) are shown. ND indicates not detectable. A typical curve example is shown, and the table presents averages of the duplicate analyses.

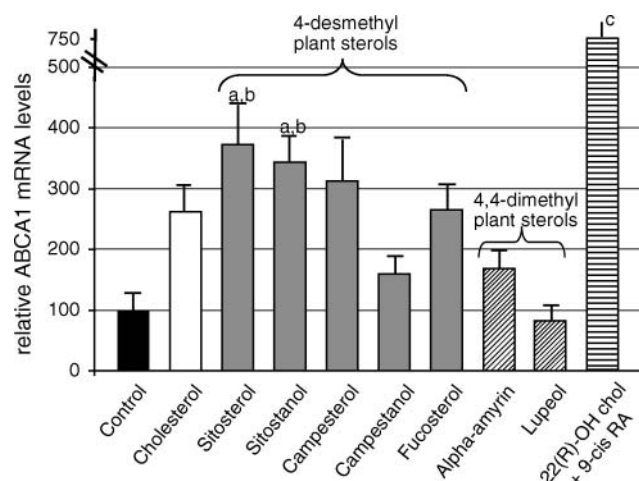


Fig. 5. Effects on ABCA1 mRNA expression of cholesterol or individual plant stanols/sterols (250 μ M) as part of in vitro-prepared mixed micelles added for 6 h to the apical surface of fully differentiated Caco-2 cells. A combination of 10 μ M 22(*R*)-hydroxy cholesterol [22(*R*)-OH chol] and 10 μ M 9-*cis*-retinoic acid (9-*cis* RA) was used as a positive control. Data shown are means \pm SD. Each value represents the mRNA level relative to the amount of transcript in the control condition, which was set arbitrarily to 100%. ^a $P < 0.05$ versus control; ^b $P < 0.05$ versus lupeol; ^c $P < 0.01$ versus all other conditions.

DISCUSSION

Foods enriched with plant stanols and sterols potently decrease serum LDL cholesterol concentrations as a result of effects in the intestinal lumen or within enterocytes. However, the exact cholesterol-lowering mechanism is not known. In this study, we have examined the effects of plant stanols and sterols on mixed micellar composition and on LXR activation.

LXR α and LXR β are nuclear receptors for oxysterols, in particular 22(*R*)-hydroxy cholesterol and 24(*S*),25-epoxy cholesterol (24, 25), and are of great interest as targets for the prevention and treatment of cardiovascular diseases, because several relevant genes (i.e., cholesteryl ester transfer protein, cytochrome P450 dependent 7 α -hydroxylase, ABCA1, ABCG5/G8) are LXR-regulated. With our coactivator peptide recruitment assay, we showed that 4-desmethylsterol plant stanols and sterols could activate both LXR α and LXR β . Because synthetic LXR agonists decrease intestinal cholesterol absorption in animals (10, 15, 17), it can be speculated that 4-desmethyl plant sterols have a comparable effect. This hypothesis was further supported by our finding that in Caco-2 cells, mixed micelles enriched with plant stanols and sterols increased the expression of ABCA1, one of the known LXR target genes. Cholesterol efflux from enterocytes back into the intestinal lumen, however, is not mediated by ABCA1, because ABCA1 is located at the basolateral side of enterocytes (26), but most probably by ABCG5 and ABCG8. Measurement of the expression of these latter two genes would have been preferred, but mRNA levels

of these genes were not detectable under our experimental conditions. Intestinal ABCA1 expression, however, correlates positively with that of ABCG5 or ABCG8 after LXR activation by synthetic LXR agonists (15–17). Interestingly, in our experiments, LXR activation and ABCA1 mRNA expression were positively related, although some apparent discrepancies were evident. For example, fucosterol and sitostanol showed the strongest and the weakest effects, respectively, in the coactivator recruitment assay, whereas the effects of sitostanol on ABCA1 expression in Caco-2 cells were stronger. Furthermore, the effects of plant stanols on LXR activity seemed less compared with those of plant sterols. In Caco-2 cells, however, sitosterol and sitostanol increased ABCA1 expression to the same extent, whereas human studies also showed comparable effects on intestinal cholesterol absorption and serum LDL cholesterol concentrations (14, 27, 28). This emphasizes the need to use different approaches to study the effects of nutrients at the molecular level.

In contrast to the 4-desmethylsterols, 4,4-dimethylsterols did not activate LXR. These differential effects on LXR activation agree with their effects on LDL cholesterol, as 4-desmethylstanols and desmethylsterols decrease LDL, but 4,4-dimethyl plant sterols do not (14). On the other hand, both types of sterols decreased the micellar incorporation of cholesterol. This suggests that effects on micellar cholesterol incorporation are not the main mechanism by which plant sterols decrease intestinal cholesterol absorption. In support of this idea, fucosterol did not decrease micellar cholesterol incorporation, but it did activate LXR and decrease cholesterol absorption in rats (3). Therefore, our results suggest that LXR-regulated genes are involved in the effects of plant stanols/sterols on intestinal cholesterol absorption. Interesting candidate genes are, of course, ABCG5 and ABCG8 (10). Unfortunately, in our study, the expression of ABCG5 and ABCG8 by Caco-2 cells was low and could not be measured reliably. Whether ABCG5 and ABCG8 are indeed involved in intestinal cholesterol absorption is also uncertain, because disruption of the ABCG5 and ABCG8 genes increased the fractional absorption of plant sterols but not of cholesterol (8, 10, 29). Therefore, other (LXR-regulated) candidate genes should not be ruled out. It is also possible, however, that in addition to effects on LXR-regulated genes, plant sterols and stanols interact with proteins involved in cholesterol uptake, such as Niemann-Pick C1-like 1 (5), resulting in decreased cholesterol absorption. The suggestion that not the micellar composition but LXR activation within the enterocyte is responsible for the effects of plant stanols/sterols on intestinal cholesterol absorption also explains why it is not necessary to consume products rich in plant stanol/sterol esters at each meal or simultaneously with dietary cholesterol to decrease LDL cholesterol (30). After intake, plant sterols/stanols enter the enterocyte and can exert their effects for a longer period. In fact, sitosterol, sitostanol, campesterol, and campestanol have been detected in intestinal cells from plant stanol- or sterol-


fed mice (11). Based on these data, it can be calculated that intracellular cholesterol, sitosterol, campesterol, sitostanol, and campestanol concentrations in the upper small intestine were all well above EC₅₀ values, as found in our LXR assay. If fucosterol enters the enterocyte, it is not known.

From structure-activity relationships, it is evident that particularly side chain-modified sterols are effective LXR ligands (22, 24). In addition, the presence of a 3 β -hydroxyl group is required (24). This may explain our finding that a mixture of ring-oxidized plant sterols or cholesterol did not activate LXR. It is even possible that because of spatial hindrance, the 3 β -hydroxyl group of the oxidized plant sterols could not interact with the ligand binding domain of LXR, explaining why there was no activation at all. This does not rule out the possibility, however, that in vivo other metabolites, for example, side chain oxidation products, are formed that do have LXR activity. The known natural oxysterol ligands for LXR, 22(*R*)-hydroxy cholesterol and 24(*S*),25-epoxy cholesterol, (22) are side chain oxidation products, which were not present in our mixtures. Ring-oxidized sterols were examined, because these sterols have been identified in human plasma (23, 31). Except for the effects on structure, the effects also depend on cell type, possibly as a result of the presence of specific cofactors/repressors. Yang et al. (32), for example, recently demonstrated in LXR α -transfected CHO-7 cells that stigmaterol, but not sitosterol, activated LXR α . In LXR α -transfected human embryonic kidney 293 cells, however, stigmaterol did not activate LXR α (15). Effects of cell type have also been demonstrated for sterol-regulatory element binding protein-2 (SREBP-2)-mediated processes. In cultured adrenocortical (Y1-BS1) cells, both stigmasterol and campesterol decreased SREBP-2 processing and, consequently, HMG-CoA synthase protein levels, whereas sitosterol did not. In U937 monocyte cells, however, sitostanol increased the expression of LDL receptor protein, another SREBP-2-regulated gene (4). This again illustrates the need to study effects under different experimental conditions. Moreover, it has been shown that feeding plant sterol-enriched diets to apolipoprotein E-deficient or LDL receptor-deficient mice did not increase the intestinal mRNA expression of ABCA1, ABCG5, and ABCG8, whereas an increase was observed in C57BL/6J mice (33). There is currently no conclusive explanation for the observed differences between the different models. However, interestingly, Kaneko and coworkers (15) showed also in C57BL/6J mice the potential of enhanced intestinal ABC transporter expression by an oxidized plant sterol metabolite. Moreover, plant stanol esters did not increase mRNA levels of these proteins in hamsters (34). Together, these results could be interpreted as if plant sterols and stanols are inactive in vivo; however, it is also possible that the influence of plant sterols/stanols on the expression or activity of ABCA1 and/or ABCG5/G8 is regulated at another level.

LXR agonists have a regulatory role not only in cholesterol metabolism but also in the hepatic expression of genes

involved in fatty acid metabolism. The effects on the hepatic LXR target genes fatty acid synthase, stearyl-CoA desaturase-1, and acetyl-CoA carboxylase may explain the unwanted hypertriglyceridemic effects during LXR agonist treatment (35, 36). Therefore, LXR agonists that favorably affect cholesterol metabolism, without unfavorable effects on hepatic triglyceride metabolism, are of particular interest. Plant stanols/sterols meet these criteria, because the effects are limited mainly to the enterocytes as a result of their low absorption into the circulation. Indeed, plant stanol and sterol intervention trials have never demonstrated an increase in serum triglycerides. Furthermore, intestine-specific effects have been shown by Kaneko and coworkers (15). Feeding (22*E*)-ergost-22-ene-1 α ,3 β -diol, an ergosterol- and brassicasterol-related oxidized plant sterol, increased ABCA1, ABCG5, and ABCG8 expression in the intestine but not the liver of C57BL/6J mice. In that study, sitosterol, campesterol, and fucosterol did not show pronounced effects in vitro on LXR α activation. This discrepancy with our findings may be explained by the fact that a cell-based reporter assay was used (i.e., human embryonic kidney 293 cells cotransfected with a GAL4-hLXR α expression vector in combination with a GAL4-responsive luciferase reporter). In our experiments, we used a cell-free assay to measure LXR-activating capacity. Results of a cell-free assay are not affected by the ability of a potential ligand to cross the cell membranes. For plant sterols, this may have been a disturbing factor in the assay with human kidney cells.

Our present findings further suggest that plant stanols and sterols decrease the absorption of the hydrocarbon carotenoids through effects on micellar incorporation. Compared with the moderate effects of sitostanol or sitosterol, α -amyrin had dramatic effects on micellar β -carotene incorporation, which is in agreement with the results of controlled human intervention trials (14). Previously, we showed that reductions in serum hydrocarbon carotenoid concentrations after plant stanol ester consumption were associated with reduced cholesterol absorption, whereas the reductions in serum concentrations of the less lipophilic oxygenated carotenoids and the tocopherols were related to a reduced number of LDL particles, a main carrier of these antioxidants (13). The reason why in particular the micellar incorporation of hydrocarbon carotenoids is affected may be related to their polarity and the consequent surface-to-core distribution of these compounds in the mixed micelle. The apolar hydrocarbon carotenoids are almost exclusively solubilized in the core of a micelle, whereas the less polar oxygenated carotenoids and tocopherols are preferentially solubilized in the surface phospholipid layer (37). Because plant stanols/sterols can replace the core component cholesterol, it is very possible that other core components, such as the hydrocarbon carotenoids, are replaced. Because the mucosal uptake of lipophilic compounds requires incorporation into mixed micelles, our results may explain the differential effects of plant stanols/sterols on plasma carotenoid and tocopherol levels.

In conclusion, our results indicate that the serum LDL cholesterol-lowering plant stanols and sterols from the 4-desmethylsterol family can activate LXR target genes. In contrast, 4,4-dimethylsterols could not activate LXR, which is in agreement with their lack of effect on serum LDL cholesterol concentrations. Therefore, we postulate that the reduced intestinal cholesterol absorption in response to plant sterol or stanol consumption is attributable, at least partly, to local activation of LXR-regulated genes. Because plant sterols are easily taken into enterocytes but are poorly absorbed, systemic effects of synthetic LXR agonists, such as hypertriglyceridemia, do not occur. In addition, the reduction in serum hydrocarbon carotenoids is determined mainly by effects on micellar composition, showing that the decreased intestinal absorption of cholesterol and carotenoids are caused by two distinct mechanisms. 

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