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Changes in intestinal and liver global gene expression in response to a phytosterol-enriched diet

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Abstract

Background: Dietary phytosterols are a recommended therapeutic option for decreasing plasma cholesterol. The increased activity of ATP-binding cassette (ABC) transporters ABCA1, ABCG5 and ABCG8, or, alternatively, a decrease in Niemann–Pick C1 Like 1 (NPC1L1) could mediate the reduction in intestinal cholesterol absorption caused by phytosterols. Other biological properties such as a direct immune modulatory activity have recently been ascribed to these plant compounds.
Methods: To gain insight into the molecular effects of phytosterols, global genome-wide gene profiling and real-time RT-PCR studies were conducted in small intestines and livers of phytosterol-treated apolipoprotein E-deficient (apoE$^{-/-}$) mice. Re-testing of the main results was performed in C57BL/6J and LDL receptor-deficient (LDLR$^{-/-}$) mice.
Results: Intestinal cholesterol absorption was decreased in all mouse models but plasma cholesterol was only decreased in apoE$^{-/-}$ and LDLR$^{-/-}$ mice. ABCA1, ABCG5, ABCG8 and NPC1L1 mRNA levels were slightly reduced in the intestine of phytosterol-treated apoE$^{-/-}$ and LDLR$^{-/-}$ mice, but increased in C57BL/6J-treated mice. Phytosterols changed genes involved in immune regulation in apoE$^{-/-}$ mice. However, these changes were less extensive in LDLR$^{-/-}$ mice and were not found in C57BL/6J mice.
Conclusions: Inhibition of intestinal cholesterol absorption by phytosterols is not mediated via transcriptional changes in ABCA1, ABCG5, ABCG8 or NPC1L1. Changes suggestive of immunomodulation are associated with the hypocholesterolemic effect of phytosterols and with apoE deficiency.

Keywords: Phytosterols; Cholesterol; ATP-binding cassette transporter; Atherosclerosis; Immune system; Cancer

Abbreviations: ABCA1, ATP-binding cassette transporter A1; ABCG5, ATP-binding cassette transporter G5; ABCG8, ATP-binding cassette transporter G8; ALT, alanine aminotransferase; apoE, apolipoprotein E; apoE$^{-/-}$, apoE-deficient mice; ETV6, ETS variant gene 6; FDPS, farnesyl diphosphate synthase; FoxQ1, forkhead box Q1; FPLC, fast protein liquid chromatography; GC–MS, gas–liquid chromatography–mass spectrometry; HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein; IgK-V, immunoglobulin kappa chain variable; LDL, low-density lipoprotein; LDLR$^{-/-}$, LDL receptor-deficient mice; LFC, limit fold change; LXR, liver X receptor; MARCO, macrophage receptor with collagenous structure; NCEP, National Cholesterol Education Program; NPC1L1, Niemann–Pick C1 Like 1; Reg, regenerating islet-derived; RT-PCR, reverse-transcriptase polymerase chain reaction; SREBP, sterol regulatory element binding protein; SAA3, serum amyloid A3; VLDL, very-low-density lipoprotein

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1. Introduction

Phytosterols, and their saturated forms known as stanols, are the most abundant plant sterols. The cholesterol-lowering effect of phytosterols has been demonstrated in both humans and animals [1,2] and the most recent guidelines for cholesterol management of the National Cholesterol Education Program (NCEP) encouraged plant sterol/stanol consumption as a therapeutic dietary option to decrease LDL cholesterol [3]. Further, phytosterols can now be acquired in many countries without medical prescription. Thus, full understanding of their effects is highly desirable.

Competition between plant sterols and intestinal cholesterol for incorporation into mixed micelles has been proposed as the mechanism underlying the hypocholesterolemic effect of plant sterols [4]. However, one of the potential explanations for this competition, the cocrystallization of cholesterol and phytosterols or phystostanols in the intestinal lumen has been recently shown to be unlikely [5]. Further, recent studies have suggested that plant sterols may exert an unknown molecular action inside enterocytes and hepatocytes, especially considering that plant sterols/stanols do not need to be present in the intestinal lumen simultaneously with cholesterol to inhibit its absorption [6]. Recently, several adenosine triphosphate binding cassette (ABC) transporters have been proposed as carriers exchanging cholesterol and phytosterols in intestine and liver and sitostanol treatment of Caco-2 cells has been shown to increase ABCA1 expression [7]. On the other hand, mutations in ABCG5 and ABCG8 genes cause sitosterolemia, a rare autosomal recessive disorder characterized by elevated plasma levels and tissue accumulation of both plant and animal sterols [8]. As ABCA1, ABCG5 and ABCG8 genes are targets of liver X receptor (LXR) [9,10] and the overexpression of ABCG5 and ABCG8 in mice promotes biliary cholesterol secretion and reduces dietary cholesterol absorption [11], phytosterols or their derivatives could act as LXR ligands [12]. Also recently, Niemann–Pick C1 Like 1 (NPC1L1) protein has been shown to play a critical role in the absorption of intestinal cholesterol [13,14]. Ezetimibe, a drug that inhibits cholesterol absorption acting through the NPC1L1 pathway decreases the levels of plant sterols in patients with sitosterolemia [15]. Thus, dietary phytosterols could act also reducing the intestinal expression of NPC1L1.

Although most studies have focused on the cholesterol-lowering activity of phytosterols, other biological properties such as immunomodulation have been ascribed to these compounds [16,17]. β-Sitosterol and its glucoside act as potentially positive immune modulators in humans by increasing cytokines derived from T_{H1} helper cells [16–18]. Interestingly, LXR-dependent gene expression has been shown to play a role in the innate immune response [19]. Moreover, experimental studies suggest that phytosterols could protect against common cancers [20–23]. Possible mechanisms of protection include effects on membrane structure/function, signal transduction and immune function [21,24].

Apolipoprotein E-deficient ($apoE^{-/-}$) mice have been used as an animal model to show the antihypercholesterolemic and antiatherogenic effects of phytosterols [25,26]. In the present study, microarray-based technology and quantitative real-time RT-PCR were used to identify phytosterol-regulated genes in intestine and liver of $apoE^{-/-}$ mice and the main findings were re-tested in C57BL/6J and in LDL receptor-deficient ($LDLR^{-/-}$) mice.

2. Materials and methods

2.1. Mice and diets

All animal procedures were in accordance with published recommendations for the use of laboratory animals [27]. Use of $apoE^{-/-}$ mice with a C57BL/6J background has been described previously [28]. Mice were maintained in a temperature-controlled (20 °C) room with a 12-h light:12-h dark cycle and food and water were provided ad libitum. Eight to ten-week-old female mice were randomized in four groups and fed either a control Western-type diet (200 g/kg fat, polyunsaturated/saturated = 0.07, 0.8 g/kg cholesterol, 170 g/kg protein, 105 g/kg fiber; Mucedola srl, Settimo Milanese, Italy) or a 0.5, 1 or 2% phytosterol-enriched Western-type diet (w/w) for 4 weeks. Homozygous $LDLR^{-/-}$ in C57BL/6J background and wild-type C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbour, ME) and were fed either a control Western-type diet or a 2% phytosterol-enriched Western-type diet (w/w) for 4 weeks. The phytosterol product was composed of 20% campesterol, 22% stigmasterol and 41% β-sitosterol (Lipofoods, S.L., Barcelona, Spain).

2.2. Lipid analyses of plasma, liver, small intestine, bile and stools

The methods used for plasma and liver lipid analyses have been described in detail elsewhere [28,29]. α-Tocopherol, α-carotene, β-carotene and retinol plasma content were quantified by reverse-phase HPLC [30]. Plasma and tissue phytosterols were analyzed by gas–liquid chromatography–mass spectrometry (GC–MS) [31]. Liver and intestine mixtures were extracted with isopropyl alcohol–hexane (2:3, v/v) and quantitative results were obtained by GC–MS and single ion monitoring; the m/z^+ values used were those described elsewhere [32].

Bile was removed from the gallbladders of anesthetized mice using a 30.5-gauge needle. Concentrations of cholesterol and phospholipids were determined enzymatically using commercial kits adapted to a BM/HITACHI 911 autoanalyzer (Roche Diagnostics GmbH, Mannheim, Germany). Bile acids were measured by the 3α-hydroxysteroid dehydrogenase method (Sigma Diagnostics, St. Louis, MO, USA). Gallbladder, liver and small intestine bile acids were measured and the bile acid pool size was calculated as the sum of all bile acids.

Stools from individually housed mice collected over 3 days were dried, weighed, and ground to a fine powder; bile acids of 0.5 g of feces were extracted in ethanol and used to determine total bile acid content as mentioned above.

2.3. Intestinal cholesterol and lipid absorption

Cholesterol absorption was measured by a fecal dual-isotope ratio method [33]. Lipid content of the diet and stools was determined using commercial kits as mentioned above [28,29]. These data, together with the amount of diet consumed and stools excreted (both expressed as g/(day 100 g body weight)), were used to calculate the fraction of consumed lipid absorbed.

2.4. Atherosclerosis analysis

At the end of the study, heart and proximal aorta were removed and atherosclerotic lesions quantified blindly as previously described [28].

2.5. Microarray intestine and liver gene expression analyses

Intestinal tissues were rinsed well of food/fecal materials using sterile phosphate buffered saline. Total liver and small intestine (an equivalent segment of duodenum, jejunum and ileum) RNA was isolated from four animals per group using the trizol RNA isolation method (Gibco/BRL, Grand Island, NY, USA). Total RNA samples were repurified (RNeasy mini kit; QIAGEN, Valencia, CA, USA) and checked for integrity by agarose gel electrophoresis. Probe synthesis from total RNA pooled samples, hybridization, detection and scanning were performed according to standard protocols from Affymetrix Inc. (Santa Clara, CA) at Progenika Biopharma S.L. (Barakaldo, Spain). Seven micrograms of labeled cRNA were hybridized to one mouse U74Av2 genechip (Affymetrix) and scanned after biotin amplification. These arrays allowed screening of 6000 functionally characterized sequences in the mouse Unigene database and 6000 EST clusters. To assess gene expression presence or absence and to compare changes in a given gene between 2% phytosterols and control treatment, the Microarray Suite (MAS) 5.0 software uses information from all probe pairs (15–20 per gene). This software allows classifying present mRNA under comparison as decreased, marginally decreased, not changed, marginally increased or increased. Information regarding this bioinformatic assessment is available at http://www.affymetrix.com/. The limit fold change (LFC) approach was then used to select differentially expressed genes [34]. The LFC model relates the expression level and the fold change (independently of being overexpressed or repressed) of every gene across the entire range of observed expressions. The model is developed by first binning gene expression data into tight classes across the entire range of expression values. Then, the 95th percentile of fold changes is selected for each bin, which is the higher fold change (HFC). The relationship between absolute expression, defined as min ADI (average difference intensity), and HFC, is plotted in order to set the LFC function. The equation $LFC = a + (b/\text{min ADI})$, which is fitted to the 95th percentile of each bin, produces the LFC curve that best models the expression data. The LFC curve in our experiments were 95th LFC model = 1.38 + (3.01/min ADI) in intestinal microarray and 95th LFC model = 1.44 + (2.54/min ADI) in liver microarray.

2.6. Quantitative real-time RT-PCR analyses

To confirm the results of the microarray study, a selection of genes that appeared to be highly regulated were subjected to real time RT-PCR.

Total RNA was reverse-transcribed with $Oligo(dT)_{15}$ using M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant (Promega Corporation, Madison, USA) to generate cDNA. Primer Express Software (Applied Biosystems, CA, USA) was used to design the primers (Table 1). PCR assays were performed on an Applied Biosystems Prism 7000 sequence detection system (Applied Biosystems). The PCR reaction contained (final volume 25 μL): 40 ng of reverse-transcribed RNA, 12.5 μL of 2× SYBR Green PCR Master Mix (Applied Biosystems) and a 400 nM concentration for each ABCA1 and ABCG8 primer, 900 nM concentration for each ABCG5 primer, and 800 nM for each Immunoglobulin kappa chain variable 28 (IgK-V28),

Table 1
Primers used in quantitative real-time RT-PCR analyses

Gene	Forward primer 5′–3′	Reverse primer 5′–3′
Ig kappa chain variable 28	ACTCTCCAATCCTGTCACTTCTGG	GAAACCAATTCAAGTATGTCTTCCC
Regenerating islet-derived 2	TAATTGAAGACCGTTTGACCTGG	AAAGTTGCTCTCAGCCTGGC
Forkhead box Q1	CGAGATCAACGAGTACCTCATGG	GCATCCAGTAGTTGTCCTTGCC
Ig heavy chain V region fragment	GATTGCTGCAAGTAGAAACAAAGC	ACAGTGTCCTCAGCTCTCAGGG
ABCA1	CTTCCCACATTTTTGCCTGG	AAGGTTCCGTCCTACCAAGTCC
ABCG5	TGTCCTACAGCGTCAGCAACC	GGCCACTCTCGATGTACAAGG
ABCG8	AGAGTTGCATCCCCCTAGCC	TCCTTGACACAGGCATGAAGC
Niemann–Pick C1 Like 1	ATCCTCATCCTGGGCTTTGC	GCAAGGTGATCAGGAGGTTGA
β-Actin	CAGATCATGTTTGAGACCTTCAAC	TCGAAGTCTAGAGCAACATAGCAC

Regenerating islet derived 2 (Reg2), Forkhead box Q1 (FoxQ1), Immunoglobulin heavy chain V region fragment and Niemann–Pick C1 Like 1 (NPC1L1). Optimal primer amplification efficiency for each primer set was assessed and a dissociation protocol was carried out to assure a single PCR product. All analyses were performed in duplicate and relative RNA levels were determined using β-actin as internal control.

2.7. Statistical analysis

All values are expressed as mean ± S.E.M. Comparison of data for two groups was performed by Student's *t*-test or Mann–Whitney *U*-test, depending on whether the distribution of data was Gaussian or not. Correlations among variables were determined by Pearson coefficient of correlation (r_P). Statistical tests were performed using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA). A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Plasma biochemical analyses in apoE$^{-/-}$ mice

Mice seemed healthy during the study and tolerated well the Western diets with or without phytosterols. Final body weights, weight gain and food consumption per cage during the study were similar in all groups (data not shown). Total cholesterol levels were determined at baseline and at weeks 2 and 4 (Fig. 1A). Plasma total cholesterol was markedly increased after consumption of Western diet in control apoE$^{-/-}$ mice. Mice given phytosterols showed no significant dose-related increase in plasma cholesterol after baseline relative to control. ApoE$^{-/-}$ mice given 2% phytosterols for 4 weeks exhibited a marked decrease in plasma total cholesterol (from 18.6 to 9.5 mmol/L), VLDL and IDL fractions and, to a lesser degree, in LDL when isolated by FPLC (Fig. 1B). The mean atherosclerotic lesion areas of apoE$^{-/-}$ mice fed a control Western-type diet were 2.2-fold larger compared with those treated with 2% phytosterols (Fig. 1C).

In order to ascertain the major pathophysiological and molecular mechanisms implicated in the hypocholesterolemic effects of phytosterols, a series of experiments was conducted in mice given 2% phytosterols for 4 weeks. Plasma phytosterols were measured in 2% phytosterol-treated and control mice (Fig. 2). Only a slight amount of campesterol was detected in control mice, whereas plasma levels of campesterol and β-sitosterol increased markedly and slightly, respectively, in mice fed the phytosterol-enriched diet. Stigmasterol was not detected in any group (Fig. 2A). In addition, plasma carotenoids were not found at a detectable level in treated or control mice, and no differences were observed in plasma α-tocopherol levels (16.0 μM versus 15.8 μM) and

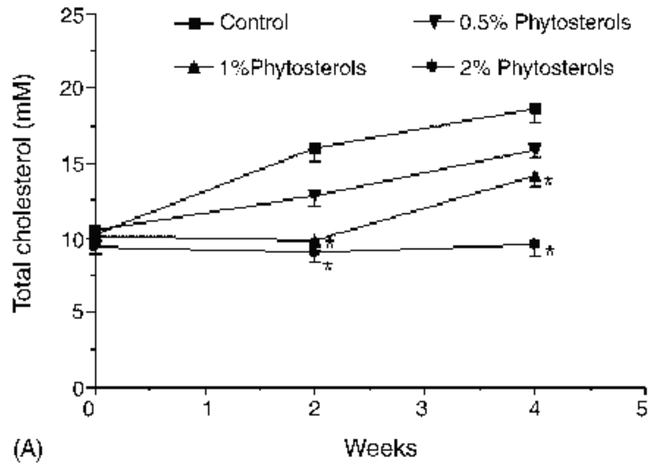

Fig. 1. Effects of phytosterols in plasma lipoproteins and atherosclerosis susceptibility in apoE$^{-/-}$ mice. (A) Line graph shows plasma total cholesterol from mice fed either a 0.5, 1 or 2% phytosterol-enriched Western diet at 0, 2 and 4 weeks of treatment. Values represent the mean ± S.E.M. of data ($n = 13$ for each group). (B) FPLC was performed on 0.2 mL of pooled plasma samples obtained from mice feeding each diet for 4 weeks. The position of elution of the VLDL, IDL/LDL and HDL are represented by horizontal lines. (C) Mean area of atherosclerotic lesion of phytosterol-treated and control mice. Results are expressed as mean ± S.E.M. of the average area of lesion of four proximal aortic sections from each mouse. * Significantly different ($P < 0.05$) from control mice.

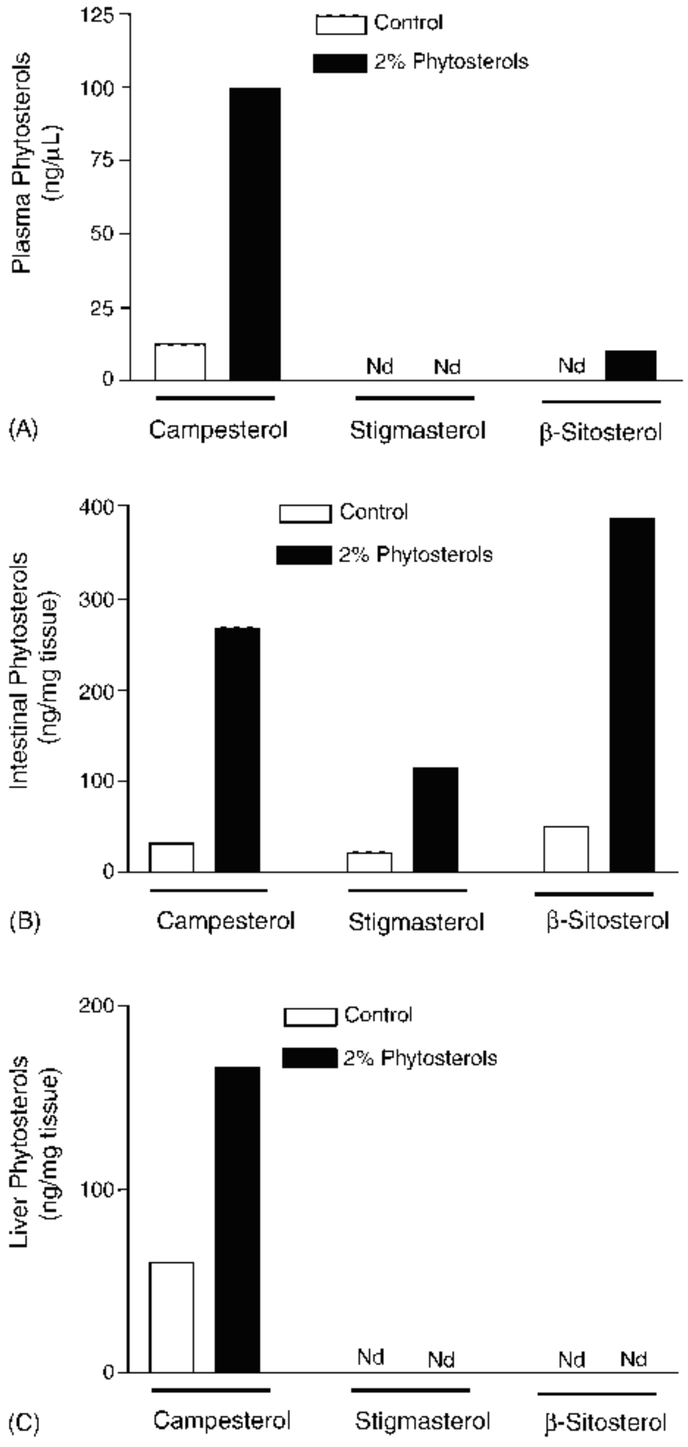

Fig. 2. Phytosterol levels of 4-week-phytosterol-treated and control apoE$^{-/-}$ mice. Lipids from pooled plasma (A), intestine (B) and liver (C) of each group of mice. Nd, not detected.

retinol (0.6 μM versus 0.7 μM) between phytosterol-treated and control mice.

3.2. Intestinal, liver and bile biochemical parameters in apoE$^{-/-}$ mice

Intestinal phytosterols, especially campesterol and β-sitosterol, were markedly increased in phytosterol-treated mice (Fig. 2B). Intestinal cholesterol absorption was reduced by 45% in 2% phytosterol-treated mice compared with control mice. In contrast, no significant differences in total lipid absorption and fecal bile acid excretion were found in these mice (Table 2).

Table 2
Liver, biliary and intestinal parameters in 4-week-phytosterol-treated and control apoE$^{-/-}$ mice

	Control	2% Phytosterols
Number of mice	13	13
Liver weight (g)	1.1 ± 0.08	1.1 ± 0.06
Liver cholesterol (mg/g tissue)	19.6 ± 2.0	7.5 ± 0.7*
Liver phospholipids (mg/g tissue)	24.3 ± 2.7	19.1 ± 1.1
Liver triglyceride (mg/g tissue)	115.5 ± 19.0	95.3 ± 11.3
Plasma ALT (U/L)	36.1 ± 2.4	36.9 ± 4.4
Biliary cholesterol (μmol/mL)	4.0 ± 0.5	2.6 ± 0.3*
Biliary bile acids (μmol/mL)	91.4 ± 25.3	86.7 ± 1.4
Biliary phospholipids (μmol/mL)	29.0 ± 5.7	20.9 ± 1.0*
Bile acid pool size (μmol/100 bw)	48.8 ± 14.2	44.5 ± 1.9
Intestinal cholesterol absorption (%)[a]	71.5 ± 6.9	39.1 ± 4.0*
Intestinal lipid absorption (%)[a]	96.3 ± 0.8	93.8 ± 1.5
Fecal bile acid excretion (μmol/(day 100 g bw))[a]	1.9 ± 0.6	2.7 ± 1.1

Values represent mean ± S.E.M. of data. Bw, body weight; ALT, alanine aminotransferase.
[a] Six animals per group.
* Significantly different ($P < 0.05$) from control mice.

Liver campesterol in phytosterol-treated mice was higher than that of control mice (Fig. 2C). Although the phytosterol-treated liver weight, liver phospholipids and triglyceride levels and alanine aminotransferase (ALT) activities did not differ significantly from those of control mice, liver cholesterol content was significantly decreased in 2% phytosterol-treated mice compared with that of control mice (Table 2).

Significant decreases were observed in cholesterol and phospholipid concentrations of bile from 2% phytosterol-treated mice, but no significant differences were found in biliary bile acid content and bile acid pool size between phytosterol-treated and control mice (Table 2).

3.3. Gene expression profiles in apoE$^{-/-}$ mice

2864 and 2608 transcripts were defined as present in small intestines and livers of control mice, respectively. The small intestines and livers of 2% phytosterol-treated apoE$^{-/-}$ mice expressed 2953 and 2816 transcripts, respectively. Differentially expressed genes were determined using the Microarray Suite and the LFC model, considering hybridization intensity >48 in at least one of the samples (which is the 95th percentile of gene transcripts determined as absent), as explained in Section 2.

Tables 3 and 4 show gene expression changes (≥1.5-fold) in small intestines and livers of phytosterol-treated apoE$^{-/-}$ mice compared with control mice. Significant upregulation of 17 sequences and downregulation of 12 sequences were found in small intestines of phytosterol-treated apoE$^{-/-}$ mice. In liver, 26 sequences were significantly upregulated and 17 were downregulated in mice given phytosterols. The rest of comparisons were not classified as increased or

Table 3
Differentially expressed genes in small intestines of 4-week-phytosterol-treated apoE$^{-/-}$ mice

Gene symbol	Full name	Phyt/Ctrl ratio	Gene Bank ID	Affymetrix ID	Process
Igk-V28	Ig kappa chain variable 28	39.4	V00779	96969_at	Humoral immune response
Reg2	Regenerating islet-derived 2	19.7	D14011	95786_at	Cell proliferation
Foxq1	Forkhead box Q1	6.9	AF010405	92658_at	Cell immune response
Ig heavy chain V region fragment	Ig heavy chain V region fragment	6.4	AF000913	97720_at	Unknown
Reg3g	Regenerating islet-derived 3 gamma	2.0	D63362	96064_at	Immune response/Cell proliferation
Ig K light chain	Ig K light chain	1.8	AF044198	97570_at	Unknown
9030624C24Rik		1.7	AF045953	93162_f_at	Unknown
Pap	Pancreatitis-associated protein	1.7	AV371861	161890_f_at	Inflammatory response
Mail-pending	Molecule possessing ankyrin repeats-induced by LPS	1.6	AA614971	98988_at	Inflammatory response
Prom	Prominin	1.6	AF039663	93390_g_at	Phototransduction
2010309G21Rik		1.6	J00592	92316_f_at	Antigen binding
Calb3	Calbindin-D9K	1.6	AF028071	160918_at	Calcium ion binding
Slc11a2	Solute carrier family 11	1.6	AI852578	104451_at	Iron transport
Akp3	Alkaline phosphatase 3	1.6	M61705	102147_at	Hydrolase activity
Asml3a-pending	Acid sphingomyelinase-like phosphodiesterase 3a	1.5	Y08135	94872_at	Carbohydrate metabolism
ESTs		1.5	AI594427	93471_at	Unknown
ABCB1a	ATP-binding cassette, sub-family B (MDR/TAP), member 1A	1.5	M24417	102910_at	Transport
Rfxank	Regulatory factor X-associated ankyrin-containing protein	−1.5	AF094761	103801_at	Transcription regulation
ESTs		−1.5	AA881307	99825_at	Unknown
0610039N19Rik		−1.5	AW047688	95026_at	Unknown
Ace	Angiotensin converting enzyme	−1.5	AV258262	161224_f_at	Proteolysis and peptidolysis
Igtp	Interferon gamma induced GTPase	−1.5	U53219	160933_at	GTPase activity
Isg15	Interferon-stimulated protein (15 kDa)	−1.6	X56602	98822_at	Immune response
Cyp2e1	Cytochrome P450, 2e1, ethanol inducible	−1.7	X010226	93996_at	Electron transport
H2-Q10	Histocompatibility 2, Q region locus 10	−1.7	X16426	101898_s_at	Defense response
Spi1-3	Serine protease inhibitor 1–3	−1.7	M75720	101565_f_at	Serine proteases inhibition
Ifit1	Interferon-induced protein with tetratricopeptide repeats 1	−1.7	U43084	100981_at	Immune response
Adn	Adipsin	−1.8	X04673	99671_at	Complement activation
Alb 1	Albumin 1	−2.5	X13060	94777_at	Transport

For each group, total RNA from small intestines of four animals were prepared for Affymetrix oligonucleotide hybridization as described in Section 2. Relative expression profile of each phytosterol (Phyt)/control (Ctrl) gene shows whether a gene is significantly upregulated or downregulated.

decreased by the Microarray Suite software or did not reach the cut-off level set by the LFC (see Section 2 for details).

3.4. Real-time RT-PCR analyses in apoE$^{-/-}$ mice

Gene expression absolute changes >3-fold in microarray analyses were also studied by real-time RT-PCR (Fig. 3). In all cases, these genes were confirmed to be overexpressed. The level of concordance of the two methods was very good with the exception of the Ig heavy chain V region fragment that was found to be overexpressed an average of 21.6-fold in the real-time RT-PCR assay and 6.4-fold in the microarray.

ABCA1 was expressed at low levels in small intestine and ABCG5, ABCG8 and NPC1L1 were not represented on the above-mentioned microarray. Thus, real-time RT-PCR was also used to measure the intestinal and hepatic expression of these genes (Fig. 4). Ingestion of phytosterols was associated with a small reduction in the intestinal expression of the three mRNA ATP-binding cassette transporters analyzed and NPC1L1 (Fig. 4). However, no significant correlation was

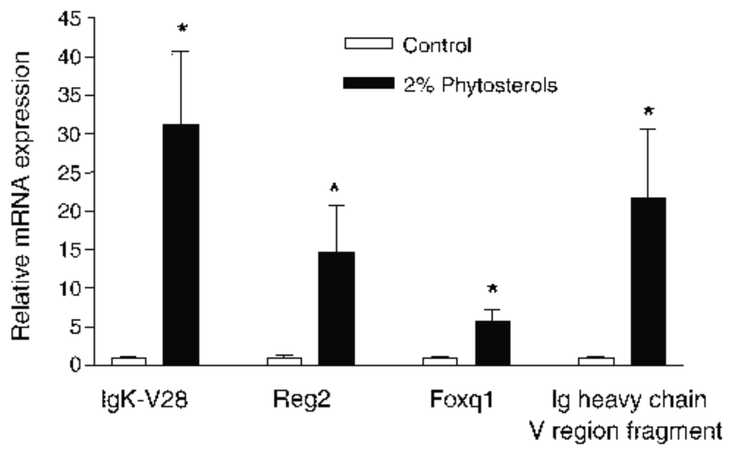

Fig. 3. Gene expression changes confirmed by real-time RT-PCR. Gene expression changes >3-fold in microarray analyses were confirmed by real-time RT-PCR. β-Actin was used as an internal control for these studies, and values represent the amount relative to the amount in the apoE$^{-/-}$ control mice, which was arbitrarily standardized to 1. Results are expressed as mean ± S.E.M. of real-time RT-PCR analyses performed on samples from individual animals ($n = 4$, same animals of microarray analyses plus 3 additional samples for each group). * $P < 0.05$ compared with the control.

Table 4
Differentially expressed genes in livers of 4-week-phytosterol-treated apoE$^{-/-}$ mice

Gene symbol	Full name	Phyt/Ctrl ratio	Gene Bank ID	Affymetrix ID	Process
Etv6	ETS variant gene 6 (TEL oncogene)	2.5	AI845538	160119_at	Transcription regulation
Fdps	Farnesyl diphosphate synthetase	2	AI846851	160424_f_at	Sterol synthesis
Gas5	Growth arrest specific 5	1.9	AI849615	98531_g_at	Cell cycle arrest
LOC207933		1.9	AA716963	96269_at	Sterol biosynthesis
3110041O18Rik		1.9	AW047445	92437_at	Unknown
Pbx1	Pre B-cell leukemia transcription factor 1	1.7	AW124932	94325_at	Sex differentiation
Sc4mol	Sterol-C4-methyl oxidase-like	1.7	AI848668	160388_at	Sterol biosynthesis
Vnn1	Vanin 1	1.7	AJ132098	104165_at	Nitrogen metabolism
Tieg	TGFB inducible early growth response	1.6	AF064088	99603_g_at	Transcription regulation
5730469M10Rik		1.6	AI850090	96634_at	Unknown
Rgs16	Regulator of G-protein signaling 16	1.6	U94828	94378_at	G-protein regulation
Ech1	Enoyl coenzyme A hydratase 1, peroxisomal	1.6	AF030343	93754_at	Fatty acid metabolism
Cct6a	Chaperonin subunit 6a (zeta)	1.6	AV370410	162279_f_at	Protein folding
4933432H23Rik		1.6	AA597220	160959_at	Unknown
Bhlhb2	Basic helix–loop–helix domain containing, class B2	1.6	Y07836	104701_at	Transcription regulation
Nsdhl	NAD(P) dependent steroid dehydrogenase-like	1.5	AW106745	98631_g_at	Cholesterol metabolism
Cappa2	Capping protein alpha 2	1.5	U16741	98127_at	Actin cytoskeleton organization
Fdft1	Farnesyl diphosphate farnesyl transferase 1	1.5	D29016	97518_at	Isoprenoid biosynthesis
3110001A13Rik		1.5	AI644158	96640_at	Unknown
2610207I16Rik		1.5	AI648018	96095_i_at	Sterol carrier activity
Slc16a7	Solute carrier family 16, member 7	1.5	AF058054	95060_at	Transport
1190008F14Rik		1.5	AI848671	93806_at	Unknown
Clk	CDC-like kinase	1.5	M38381	93274_at	Protein amino acid phosphorylation
Decr1	2,4-Dienoyl CoA reductase 1, mitochondrial	1.5	AI844846	160711_at	Reductase activity
Rad51l1	RAD51-like 1 (*S. cerevisiae*)	1.5	U92068	103944_at	DNA repair
Npm1	Nucleophosmin 1	1.5	M33212	101634_at	Nucleic acid, RNA and protein binding
Bcl2l11	BCL2-like 11 (apoptosis facilitator)	−1.5	AF032459	99418_at	Apoptosis
Gtpbp1	GTP binding protein 1	−1.5	AV239949	161683_r_at	Protein synthesis elongation
Abcg1	ATP-binding cassette, sub-family G (WHITE), member 1	−1.5	Z48745	160612_at	Transport
1110032A03Rik		−1.5	AI851206	104314_r_at	Unknown
Egfr	Epidermal growth factor receptor	−1.5	AW049716	101841_at	Protein amino acid phosphorylation
Mt2	Metallothionein 2	−1.5	K02236	101561_at	Metallothionein
Srebf1	Sterol regulatory element binding factor 1	−1.6	AI843895	93264_at	Lipid metabolism
Emr1	EGF-like module containing, mucin-like, hormone receptor-like 1	−1.6	X93328	103507_at	G-protein regulation
Pfc	Properdin factor, complement	−1.6	X12905	101468_at	Complement activation
AI326478	Ig heavy chain 6	−1.7	V00817	93583_s_at	Humoral immune response
Hamp	Hepcidin antimicrobial peptide	−1.7	*AI*255961	104588_at	Antibacterial peptide activity
Lgals3	Lectin, galactose binding, soluble 3	−1.8	X16834	95706_at	Sugar binding
Rpo1–2	RNA polymerase 1–2 (128 kDa subunit)	−1.8	U58280	92225_f_at	Transcription
Basp1	Brain abundant, membrane attached signal protein 1	−2.0	AW124113	95673_s_at	Unknown
Saa3	Serum amyloid A 3	−2.0	X03505	102712_at	Oxidative stress response
Marco	Macrophage receptor with collagenous structure	−2.3	U18424	102974_at	Immune response
Ldh2	Lactate dehydrogenase 2, B chain	−2.6	X51905	101990_at	Anaerobic glycolysis pathway

For each group, total RNA from livers of four animals were prepared for Affymetrix oligonucleotide hybridization as described in Section 2. See legend to Table 3.

found between these intestinal lipid transporters expression and non-HDL cholesterol levels (data not shown). A significant increase in ABCG8 and NPC1L1 mRNA expression was observed in livers of mice fed with phytosterols. Again, no significant correlation was found between liver ABCA1, ABCG5, ABCG8 and NPC1L1 mRNA expression and non-HDL cholesterol levels (data not shown).

3.5. Re-testing main results obtained in apoE$^{-/-}$ in C57BL/6J and LDLR$^{-/-}$ mice

To ascertain whether the changes found in apoE$^{-/-}$ mice were due to the hypocholesterolemic effect of phytosterols or to the lack of apoE, two other strains (C57BL/6J and LDLR$^{-/-}$) of mice were also analyzed after being fed with

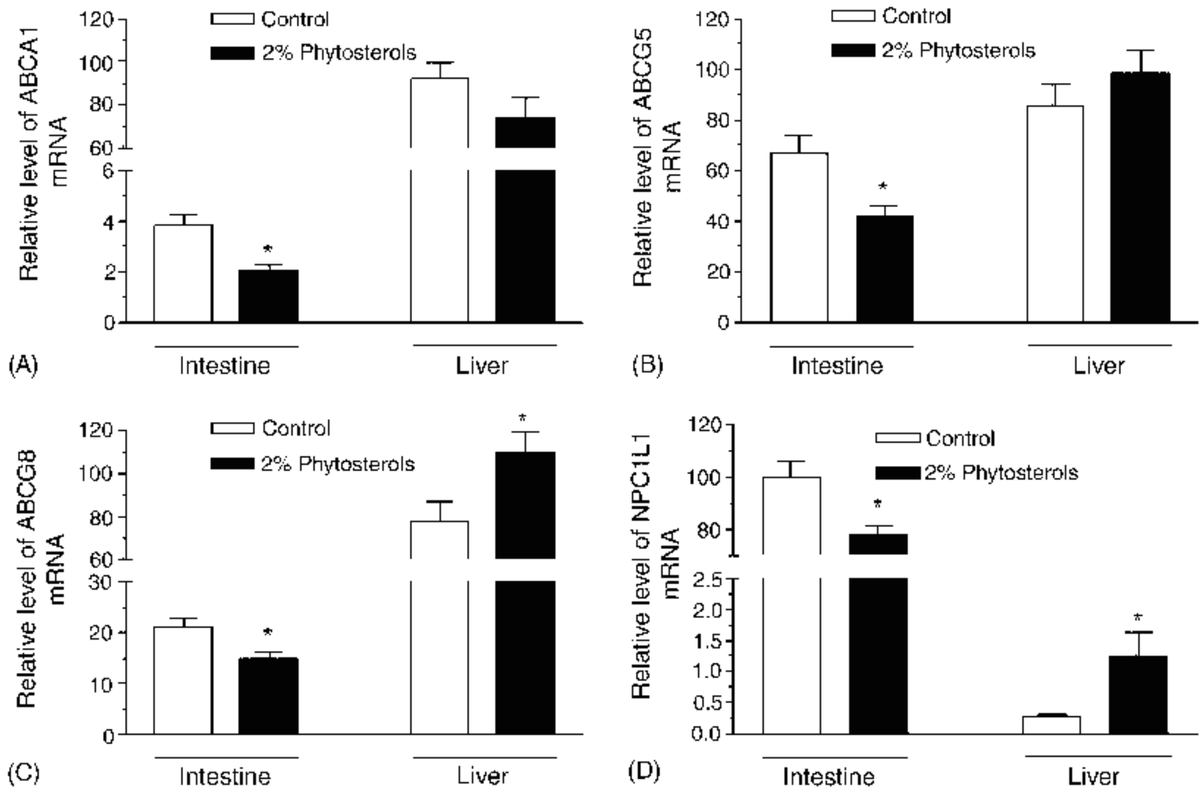

Fig. 4. Relative mRNA levels for ABCA1, ABCG5, ABCG8 and NPC1L1 of apoE$^{-/-}$ Western-fed mice treated with or without phytosterols for 4 weeks. mRNA levels for ABCA1 (A), ABCG5 (B) and ABCG8 (C) and NPC1L1 (D) were quantified by real-time RT-PCR and β-actin used as an internal control. Tissue with the most abundant signal was set to a normalized value of 100 arbitrary units. Results are expressed as mean ± S.E.M. of individual animals ($n = 4$, same animals of microarray analyses plus 3 additional samples for each group). * $P < 0.05$ compared with the control.

the same Western-type diet enriched or not with phytosterols. These strains were selected because their plasma cholesterol levels were very different before and after phytosterol treatment. The results of these analyses are presented in Table 5. Phytosterols inhibited intestinal cholesterol in both types of mice but only prevented increase in plasma cholesterol in LDLR$^{-/-}$ mice. Plasma total cholesterol in phytosterol-treated LDLR$^{-/-}$ mice was 9.8 mmol/L versus 15.8 mmol/L in non-treated animals, a reduction that corresponded to the non-HDL fraction. Phytosterol-treated LDLR$^{-/-}$ mice showed a small but statistically significant decrease in intestinal ABCA1, ABCG5, ABCG8, NPC1L1 and Ig heavy chain V region mRNA expression, whereas the relative level of IgK-V28 and Reg2 increased by almost fourfold (Table 5). In contrast, phytosterol-treated C57BL/6J mice presented a small increase in the expression of intestinal ABCA1, ABCG8 and NPC1L1 and decreased relative level of Reg2 mRNA compared with non-treated C57BL/6J mice (Table 5).

Table 5
Effects of phytosterols on plasma lipoproteins, intestinal cholesterol absorption and relative intestinal mRNA levels of selected genes in C57BL/6J and LDLR$^{-/-}$ mice

	C57BL/6J		LDLR$^{-/-}$	
	Control	2% Phytosterols	Control	2% Phytosterols
Plasma total cholesterol (mM)	2.9 ± 0.1	2.6 ± 0.2	15.8 ± 1.6	9.8 ± 1.2*
Plasma non-HDL cholesterol (mM)	0.6 ± 0.08	0.5 ± 0.2	14.1 ± 1.6	8.4 ± 1.1*
Intestinal cholesterol absorption (%)	57.9 ± 3.7	23.8 ± 7.9*	78.7 ± 2.2	36.0 ± 2.0*
Relative level of ABCA1 mRNA	100 ± 9.7	149 ± 12*	100 ± 9.4	44.7 ± 2.8*
Relative level of ABCG5 mRNA	100 ± 10.3	105.9 ± 9.0	100 ± 6.4	49.9 ± 5.3*
Relative level of ABCG8 mRNA	100 ± 8.3	153.6 ± 16.8*	100 ± 7.7	53.9 ± 3.2*
Relative level of NPC1L1 mRNA	100 ± 8.1	132.7 ± 13.1*	100 ± 7.0	76.7 ± 5.3*
Relative level of IgK-V28 mRNA	100 ± 8.4	167.9 ± 56.0	100 ± 14.9	370.4 ± 94.4*
Relative level of Reg2 mRNA	100 ± 14.3	56.4 ± 8.6*	100 ± 17.9	391.5 ± 99.2*
Relative level of FoxQ1 mRNA	100 ± 13.3	85.8 ± 14.2	100 ± 21.1	85.7 ± 8.7
Relative level of Ig heavy chain V region mRNA	100 ± 15.7	166.0 ± 45.5	100 ± 23.6	25.1 ± 5.0*

Results are expressed as mean ± S.E.M. of individual animals ($n = 5$ for each group). Tissue of control animals was set to a normalized value of 100 arbitrary units.
* $P < 0.05$ compared with the control.

4. Discussion

As expected [25,26], phytosterols decreased serum cholesterol levels and atherosclerosis in apoE$^{-/-}$ mice. They also did so in LDLR$^{-/-}$ mice, but not in C57BL/6J mice, even though a reduction in intestinal cholesterol absorption was observed in all models. This may be due to the low level of plasma cholesterol in C57BL/6J mice that could lead cells to compensate lower absorption of cholesterol with increased synthesis. In line with previous observations, no effects of phytosterols were found on bile acid pool size, biliary bile acid levels and fecal excretion of bile acids [35]. In contrast, a phytosterol-mediated decrease in bile cholesterol and phospholipid content was found in apoE$^{-/-}$, the only animal model in which these parameters were analyzed. These data suggest that the reduced liver cholesterol levels were rate-limiting for the excretion of cholesterol into the bile, which is usually coupled to phospholipid excretion [36].

4.1. Small intestine

4.1.1. Lipid metabolism

The results of this study demonstrate that the inhibition of cholesterol absorption by phytosterols does not require increases in the mRNA expression of intestinal ABCA1, ABCG5 and ABCG8 transporters. Moreover, it reveals that the downregulation found in intestinal NPC1L1 does not correlate with the hypocholesterolemic effect of phytosterols. In fact, these transporters are slightly decreased in the cases of phytosterol-treated apoE$^{-/-}$ and LDLR$^{-/-}$ mice. This observation contrasts with phytosterol-treated C57BL/6J mice, which presented slightly increased gene expression of all these sterol transporters. Similar findings have recently been reported in hamsters in which the intestinal expression of these genes after a phytosterol-treatment changed in the same direction as plasma cholesterol after different interventions, such as cholesterol feeding, stanol ester feeding and cholestyramine/lovastatin treatment [37]. We speculate that although phystosterols may increase per se the intestinal gene expression of ABCA1, ABCG5, ABCG8 and NPC1L1, their hypocholesterolemic effect could decrease the intestinal expression of these transporters, perhaps through an associated decrease in oxysterol signaling to LXR [38]. Obviously, the possibility exists that activities of these intestinal proteins may be altered by phytosterols through postranscriptional mechanisms.

4.1.2. Immune regulation

Results of the small intestine microarray analyses in apoE$^{-/-}$ mice were indicative of activation of genes involved in immune response. This is the case of IgK-V28 [39], Ig heavy chain V region fragment, Reg2 [40–42], and FoxQ1 [43]. However, only IgK-V28 and Reg2 were found to be upregulated, at a lower level, in phytosterol-treated LDLR$^{-/-}$ mice and none of these changes in intestinal gene expression were present in phytosterol-treated C57BL/6J mice. This suggests that most changes found in intestinal gene expression are more related to the level of plasma cholesterol (or its consequences) than to a direct phytosterol effect. Previous evidence of a relationship between cholesterol levels and immunity status does exist [19]. Further, there is an apoE-dependence effect in some of these changes in intestinal gene expression whose origin is unknown. In this context, it should be pointed out that apoE-deficiency also modulates dietary cholesterol absorption and cholesterol bile excretion [44].

4.2. Liver

It is noteworthy that gene expression and detailed biochemical studies of the liver were only conducted in apoE$^{-/-}$.

4.2.1. Lipid metabolism

It is noticeable the fact that ABCG8 and NPC1L1 mRNA expression increased in liver of phytosterol-treated apoE$^{-/-}$ mice. However, liver ABCG8 overexpression has no effect on the plasma lipid profile [45]. The increase in liver NPC1L1 expression is unlikely to be of relevance in the hepatic clearance of phytosterols since the liver expression of this gene in mouse is very small relative to intestine. This contrasts with a high liver expression of NPC1L1 in humans [13].

The relatively lower levels of liver cholesterol were not compensated and this is consistent with the absence of transcriptional changes in 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase and LDL receptor genes. This fact has previously been observed in mice [35] but not in humans treated with stanols [46]. Taken together, most of the observed changes in liver gene expression may be the primary or the compensatory response to an increased campesterol liver concentration together with reduced liver cholesterol content that could lead, at least in part, to a decrease in LXR-mediated activation. This could be the case of the moderate downregulations of the sterol regulatory element binding factor 1 (SREBP-1) or the ABCG1 transporter [47,48].

4.2.2. Immune regulation

Changes in genes that operate in cell growth/differentiation and stress/inflammatory pathways were also identified in apoE$^{-/-}$ mice. The E-Twenty-Six (ETS) family member TEL (ETV6) [49] upregulation observed would be consistent with a potential role of phytosterols in cancer protection [20,21,24]. Further, mRNA expression of two stress-responsive genes, serum amyloid A3 (SAA3) and macrophage receptor with collagenous structure (MARCO) were downregulated. SAA3 is an acute-phase reactant protein specially induced by interleukin-1 through a mechanism that involves a nuclear factor kappa beta-mediated increase in transcription [50,51]. MARCO, an LXR-controlled gene, plays a role in host defense as well as in innate immunity against infectious agents and its capacity to scavenge acetylated LDL has also been described [19,52,53]. In line with the other results, we may hypothesize that a number of these

changes are related to the decreased liver cholesterol content and the lack of apoE.

4.2.3. Study limitations

It is noteworthy that some changes in gene expression could be even more pronounced given the dilution of immune cells in an enterocyte- or hepatocyte-enriched medium. Thus, gene expression profiles in specific cell cultures incubated with phytosterols may provide additional significant information. This approach has been followed in other recent studies [7,54]. Our experimental approach does not allow us to determine whether changes in gene expression are due to decreased cholesterol levels, increased plant sterols, or both. However, this question has also been addressed recently by another group [55].

5. Conclusions

The data presented here provide, to our knowledge, the first global assessment of gene expression patterns in response to phytosterol treatment. Our data show that transcriptional changes in ABCA1, ABCG5, ABCG8 and NPC1L1 do not play an essential role in the phytosterol-sensitive reduction in cholesterol absorption in mice. Rather, the intestinal expression of these transporters seems to be modified in relation to plasma cholesterol concentrations. Microarray analyses permitted identification of changes in the expression of four novel phytosterol-inducible genes involved in immune response of the gut, although these changes are dependent, first, on the hypocholesterolemic action of phytosterols and, second, at least in part to the lack of apoE.

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