Phytosterol-mediated inhibition of intestinal cholesterol absorption is independent of ATP-binding cassette transporter A1

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An increased activity of ATP-binding cassette transporter (ABC) A1 has been proposed as a mechanism underlying the hypcholesterolaemic effect of phytosterols. In the present study, ABCA1-deficient mice (ABCA1–/– mice) were used to examine the involvement of the ABCA1 in the reduction of intestinal cholesterol absorption in response to a phytosterol-enriched diet. A decrease in intestinal cholesterol absorption of 39 and 35 % was observed after phytosterol treatment in ABCA1+/+ mice and in ABCA1–/– mice, respectively. No statistically significant changes in plasma lipoprotein profile or in intestinal ABCG5, ABCG8 and Niemann-Pick C1-Like 1 gene expression levels were found when phytosterol-treated ABCA1–/– mice and untreated ABCA1–/– mice were compared. We conclude that phytosterol inhibition of cholesterol absorption in mice is independent of ABCA1.

Phytosterols: Intestinal cholesterol absorption: ATP-binding cassette transporter A1: Mice

Dietary consumption of phytosterols, or their saturated forms known as stanols, is a recommended therapeutic option to decrease LDL-cholesterol in the most recent guidelines of the National Cholesterol Education Program (Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults, 2001). Although it has been clearly demonstrated that phytosterols decrease intestinal cholesterol absorption, the mechanisms involved in this action remain unclear (de Jong et al. 2003). One mechanism could be physical competition between phytosterols and cholesterol for incorporation into micelles (de Jong et al. 2003). However, phytosterols do not need to be present in the intestinal lumen simultaneously with cholesterol to inhibit its absorption (Plat et al. 2000). In recent years, important advances in the understanding of intestinal sterol absorption (Sudhop et al. 2005) have provided potential molecular targets of phytosterols. One of these is ATP-binding cassette transporter (ABC) A1 (Brousseau, 2003), especially considering that ABCA1 gene expression increased when Caco-2 cells were incubated with sitostanol added in a micellar solution (Plat & Mensink, 2002), particularly considering that ABCA1 expression is independent of ATP-binding cassette transporter A1 (Sudhop & Mensink, 2002; Brousseau, 2003). One mechanism could be competitive inhibition between phytosterols and cholesterol for incorporation into micelles (de Jong et al. 2003). However, phytosterols do not need to be present in the intestinal lumen simultaneously with cholesterol to inhibit its absorption (Plat et al. 2000). In recent years, important advances in the understanding of intestinal sterol absorption (Sudhop et al. 2005) have provided potential molecular targets of phytosterols. One of these is ATP-binding cassette transporter (ABC) A1 (Brousseau, 2003), especially considering that ABCA1 gene expression increased when Caco-2 cells were incubated with sitostanol added in a micellar solution (Plat & Mensink, 2002). This transcriptional increase, if maintained at protein level, could decrease ABCA1-mediated intestinal cholesterol absorption (Plat & Mensink, 2002; Brousseau, 2003). We have used ABCA1-deficient mice to study whether this protein is needed by phytosterols to decrease intestinal cholesterol absorption.

Materials and methods

Mice and diets

ABCA1 heterozygous mice (ABCA1+/–) in the DBA/1 background were obtained from Jackson Laboratories (no. 003897; Bar Harbor, ME, USA) and were crossed to produce wild-type ABCA1+/+ and ABCA1-deficient (ABCA1–/–) mice (McNeill et al. 2000). Genotype of the offspring was confirmed by PCR using the wild-type and the targeted allele-specific primers recommended by Jackson Laboratories (http://jaxmice.jax.org/pub-cgi/protocols/protocols.sh). Sex- and age-matched ABCA1+/+ and –/– were used in our experiments. Eight-week-old ABCA1+/+ and –/– mice were randomised into two groups and fed either a control Western-type diet (fat, 200 g/kg; PUFA:saturated fatty acids, 0·07; cholesterol, 0·8 g/kg; protein, 170 g/kg; fibre, 105 g/kg; Mucedola srl, Settimo Milanese, Milan, Italy) or a 2 % phytosterol-enriched Western-type diet (w/w) for 2 weeks. The phytosterol product was composed of 20 % campesterol, 22 % stigmasterol and 41 % β-sitosterol (Lipofoods S.L., Barcelona, Spain) (Calpe-Berdiel et al. 2005). All the procedures described were approved by the ethical committee of the Ministry of Agriculture, Livestock and Fishing of the Generalitat de Catalunya.

Net intestinal cholesterol absorption

Net cholesterol absorption was measured in treated and untreated ABCA1+/+ and –/– mice by a faecal dual-isotope ratio method as previously described (Calpe-Berdiel et al. 2005). Briefly, five mice from each group were intragastrically administered a mixture of 2 μCi [5,6-3H]sitostanol (American Radiolabeled Chemicals Inc., St Louis, MO, USA) and 1 μCi [4-14C]cholesterol (NEN Life Science Products, Boston, MA, USA). They were then individually housed and stools were collected over the next 3 d. Lipids were extracted from stools with
isopropyl alcohol–hexane (2:3, v/v) and the $^{14}$C:$^3$H ratio in each sample was determined. The percentage of intestinal cholesterol absorption was calculated from these data (Calpe-Berdiel et al. 2005). At the end of the present study, plasma was also taken when mice were killed and $^{14}$C counts per min determined.

Plasma and liver lipid analyses

The methods used for plasma lipid and liver analyses have been described in detail elsewhere (Escola-Gil et al. 2000). Size fractionation of plasma lipoproteins was performed by fast performance liquid chromatography of pooled plasma samples and total cholesterol content on each fraction was then assayed (Escola-Gil et al. 2001).

Quantitative real-time RT PCR analyses

The small intestine of four animals in each experimental group was removed, flushed with ice-cold saline solution, and cut into three segments with length ratios of 1:3:2 (duodenum–jejenum–ileum). From the middle of each intestinal segment, 1·5 cm of the duodenal, jejunal, and ileal tissues were cut out and pooled. Small-intestine RNA was isolated using the trizol RNA isolation method (Gibco/BRL, Grand Island, NY, USA). Total RNA samples were repurified, checked for integrity by agarose gel electrophoresis and reverse-transcribed with oligo(dT)15 using M-MLV RT, RNase H Minus, Point Mutant to generate cDNA (Calpe-Berdiel et al. 2005). Primer sequences for ABCA1, ABCG5, ABCG8, Niemann-Pick C1-Like 1 protein (NPC1L1) and $\beta$-actin have been published elsewhere (Calpe-Berdiel et al. 2005). PCR assays were performed on an Applied Biosystems Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) as described (Calpe-Berdiel et al. 2005). All analyses were performed in duplicate and relative RNA levels were determined using $\beta$-actin as the internal control.

Statistical analysis

Results are expressed as mean values and standard deviations. Two-way ANOVA with Bonferroni post hoc tests was 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.

Table 1. Effects of phytosterols on plasma lipoproteins and liver cholesterol content in ATP-binding cassette transporter A1 (ABCA1) $+/+$ and ABCA1 $−/−$ mice after 2 weeks on each diet (eight animals per group)

<table>
<thead>
<tr>
<th></th>
<th>ABCA1 $+/+$ mice</th>
<th></th>
<th>ABCA1 $−/−$ mice</th>
<th></th>
<th>Effect of genotype (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>2 % Phytosterols</td>
<td>Control</td>
<td>2 % Phytosterols</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>sd</td>
<td>Mean</td>
<td>sd</td>
<td>Mean</td>
</tr>
<tr>
<td>Plasma total cholesterol (mmol/l)</td>
<td>3·5</td>
<td>1·5</td>
<td>3·3</td>
<td>0·9</td>
<td>2·0</td>
</tr>
<tr>
<td>Plasma non-HDL-cholesterol (mmol/l)</td>
<td>0·9</td>
<td>0·6</td>
<td>0·9</td>
<td>0·3</td>
<td>1·9</td>
</tr>
<tr>
<td>Plasma HDL-cholesterol (mmol/l)</td>
<td>2·5</td>
<td>0·8</td>
<td>2·5</td>
<td>0·8</td>
<td>0·1</td>
</tr>
<tr>
<td>Liver cholesterol (mg/g tissue)</td>
<td>0·8</td>
<td>0·6</td>
<td>0·6</td>
<td>0·2</td>
<td>0·8</td>
</tr>
</tbody>
</table>

*Two-way ANOVA.
Furthermore, phytosterols or their derivatives could act as liver receptor ligands and increase ABCA1 expression at a transcriptional level (Kaneko et al. 2003; Plat et al. 2005). However, the importance of ABCA1 in net intestinal cholesterol absorption is unclear (Brousseau, 2003). Several reports in animal models, although not all (Groen et al. 2001), have provided substantial in vivo evidence that ABCA1 influences intestinal net cholesterol absorption (McNeish et al. 2000; Drobnik et al. 2001; Mulligan et al. 2003; Temel et al. 2005). However, ABCA1 is dominantly expressed on the basolateral surface of intestinal cells (Ohama et al. 2002) and liver X receptor activation increases intestinal cholesterol excretion independently of ABCA1, probably by increasing the intestinal transcription of ABCG5 and ABCG8 (Plow et al. 2002). ABCG5/G8 heterodimers may exchange phytosterols and cholesterol in the intestinal lumen (Plow et al. 2002; Sehayek, 2003). NPC1L1 is a critical mediator of cholesterol absorption as revealed in mice lacking a functional NPC1L1 (Altmann et al. 2004). Thus, activation of these ABC transporters and the reduction in NPC1L1 could also explain the phytosterol-mediated inhibition of intestinal net cholesterol absorption (Duan et al. 2004; Davies et al. 2005).

We have reported that dietary plant stanols and sterols decrease intestinal net cholesterol absorption regardless of increases or decreases in intestinal gene expression of ABCA1, ABCG5/G8 and in NPC1L1 in apoE−/− mice, LDL-receptor-deficient mice and C57BL/6 mice (Calpe-Berdiel et al. 2005). Other authors have reported similar results in hamsters (Field et al. 2004). An oxidised plant sterol metabolite has also been found to enhance intestinal ABC expression in C57BL/6 mice (Kaneko et al. 2003). Liver X receptor activation with increased ABCA1 gene expression has also been described in Caco-2 cells after the addition of plant sterols and stanols from the 4-desmethylsterol family (Plow et al. 2005). However, in our opinion, there is no conclusive explanation for the differences observed between the different models and thus it cannot be ruled out that phytosterols changed the activity of these transporters through post-transcriptional mechanisms. Furthermore, these results do not permit differentiation between primary and compensatory changes (Kaneko et al. 2003; Field et al. 2004; Calpe-Berdiel et al. 2005; Plat et al. 2005).

In the present study, we analysed the involvement of ABCA1 as a molecular target of phytosterols. The major finding was that ABCA1 do not play an essential role in the phytosterol-mediated reduction in net cholesterol absorption since the effect of phytosterols was similar in ABCA1−/− and ABCA1+/+ mice. Of note, we found very low, but not undetectable, intestinal mRNA ABCA1 in ABCA1−/− mice. This has also been found by other groups (Plosch et al. 2002; Timmins et al. 2005) and may be due to the fact that the PCR primers were located outside the disrupted exons (McNeish et al. 2000).

We did find a modest increase in net cholesterol absorption in untreated ABCA1−/− mice with regard to untreated ABCA1+/+ animals. Although the cause of this increase is unknown, this observation is consistent with previous observations made during the characterisation of these animals (McNeish et al. 2000) but in contrast to that of another group that studied intestinal net cholesterol absorption in ABCA1−/− mice fed a cholesterol-free diet (Drobnik et al. 2001). We found no major compensatory changes in the intestinal gene expression of other sterol transporters such as ABCG5, ABCG8 or NPC1L1.
The decreased plasma labelled \( ^{14} \text{C} \) cholesterol at 72 h in phytosterol-fed ABCA1+/+ mice compared with non-treated ABCA1+/+ mice may be due to the decreased intestinal net cholesterol absorption in the first group. In contrast, the increased plasma radioactivity in ABCA1−/− mice compared with ABCA1+/+ could be due only in part to their increased intestinal net cholesterol absorption. The increase in VLDL particles observed in ABCA1−/− animals fed a Western-type diet has also been described previously (McNeish et al. 2000). Thus, elevated radioactivity in ABCA1−/− mice may also be due to enhanced VLDL particle formation (Plosch et al. 2002; Sahoo et al. 2004) and/or reduced catabolism of apoB-containing lipoproteins (Joyce et al. 2003). In fact, the increase (2-fold) in plasma cholesterol radioactivity and non-HDL-cholesterol found in ABCA1−/− mice, compared with ABCA1+/+ mice, is consistent.

Interestingly, phytosterol treatment did not decrease plasma cholesterol in either of the two mouse genotypes, although a reduction in intestinal net cholesterol absorption was observed in both models. We and other authors have reported that wild-type mice and gerbils fed a low-cholesterol diet present unchanged low plasma cholesterol concentrations regardless of manipulations that alter intestinal net cholesterol absorption, such as treatment with ezetimibe or phytosterols (Repa et al. 2002; Calpe-Berdiel et al. 2005; Hayes et al. 2005). Thus, it is possible that with a non-expanded whole-body pool of cholesterol, as occurs in mice with low non-HDL-cholesterol, changes in cholesterol synthesis compensate for the decrease in net intestinal absorption. However, detailed studies will be required to prove this hypothesis. In preliminary experiments we found no change in either liver cholesterol (Table 1) or HMGCoA reductase gene expression (data not shown).

In conclusion, the present results clearly demonstrate that ABCA1 is not the primary transporter involved in the reduction in intestinal net cholesterol absorption induced by phytosterols in mice. The wide availability in the future of other GM mice, such as ABCG5-, ABCG8- and NPC1L1-deficient mice, should permit an in vivo evaluation of other potential targets of phytosterols.

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**References**


