High basal fractional cholesterol synthesis is associated with nonresponse of plasma LDL cholesterol to plant sterol therapy

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ABSTRACT

Background: The cholesterol-lowering effectiveness of plant sterol (PS) therapy is hindered by wide-ranging variability in LDL-cholesterol responsiveness across individuals. To capitalize on the LDL-cholesterol-lowering potential of PS in the clinical setting, it is paramount to characterize the metabolic factors that underlie this heterogeneity of responsiveness.

Objective: The objective was to investigate the relation between cholesterol synthesis and plasma LDL-cholesterol reductions in response to PS consumption.

Design: We evaluated previously conducted clinical PS interventions incorporating stable-isotope measures of cholesterol synthesis and conducted feeding studies in animal models of response (Syrian Golden hamsters) and nonresponse (C57BL/6J mice) to PS consumption.

Results: From our clinical study population (n = 113), we identified 47 nonresponders (3.73 ± 1.10% change in LDL cholesterol) and 66 responders (−15.16 ± 1.04% change in LDL cholesterol) to PS therapy. The basal cholesterol fractional synthesis rate (FSR) as measured by direct deuterium incorporation was 23% higher (P = 0.003) in the nonresponder subgroup than in responders to PS therapy. The basal cholesterol FSR correlated (r = 0.22, P = 0.02) with the percentage change in LDL cholesterol after PS intervention. In support of our clinical observations, nonresponding mice showed a 77% higher (P = 0.001) basal cholesterol FSR than that of responding hamsters. Compared with control mice, PS-fed mice showed an increase in hepatic nuclear sterol regulatory element binding protein 2 abundance (1.3-fold of control, P = 0.04) and β-hydroxy-β-methylglutaryl coenzyme A reductase–mRNA expression (2.4-fold of control, P = 0.00).

Conclusion: The results suggest that subjects with high basal cholesterol synthesis are less responsive to PS treatment than are subjects with low basal cholesterol synthesis. Am J Clin Nutr 2010; 92:41–6.

INTRODUCTION

Plant sterols (PSs) have a long-standing history as effective dietary cholesterol-lowering agents by interfering with intestinal cholesterol absorption. Recent analyses report a 5–15% reduction in plasma LDL cholesterol in response to PS therapy (1–3). However, clinical interventions show significant interindividual variability in the extent of cholesterol reductions, and in many cases a complete nonresponse of plasma cholesterol to PS therapy is evident (4, 5). Although compliance, food matrixing, dosage, and timing issues are thought to play a role in the lack of consistent response to PS across individuals within a population, it is clear that independent of such factors, some individuals respond with substantial reductions in circulating cholesterol, whereas other individuals are much more resistant or completely insensitive to PS challenges. Understanding the genetic and metabolic factors that underlie this heterogeneity of responsiveness is integral in establishing PSs as effective cholesterol-reducing agents in clinical practice.

Hepatic cholesterol synthesis is integral to the maintenance of whole-body cholesterol homeostasis and is regulated by multiple dietary factors (6–8). It has been suggested that high basal hepatic cholesterol synthesis confers protection against diet-induced hypercholesterolemia by creating a cholesterol-buffering capacity in which dietary cholesterol can maximally reduce cholesterol biosynthesis through negative feedback inhibition (9, 10). However, high basal cholesterol synthesis may also render pharmacologic inhibition of cholesterol absorption with Ezetimibe (Merck, Whitehouse Station, NJ) less effective than under conditions of low cholesterol synthesis (11). Therefore, we hypothesized that basal cholesterol synthesis capacity may underlie much of the variable cholesterol-lowering response to PS therapy.

To test this hypothesis, we conducted a retrospective evaluation of clinical data from several of our human studies to determine whether basal cholesterol synthesis as measured by direct stable-isotope methodology was different between hyper-and hyporesponders to PS intervention. In addition, to further investigate the effect of hepatic cholesterol biosynthesis and sterol trafficking on the hypocholesterolemic response to PS, we conducted studies in animal models of response (Syrian Golden hamsters) and nonresponse (C57BL/6J mouse) to PS consumption.

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METHODS

Human clinical studies

Three previously published PS clinical studies from our group were chosen on the basis of similarity in study design and availability of data concerning cholesterol fractional synthesis rates (FSRs) directly measured by deuterium incorporation (5, 12, 13). Study 1 was conducted at the Clinical Nutrition Research Unit at the Richardson Centre for Functional Foods and Nutraceuticals (RCFFN), University of Manitoba, whereas studies 2 and 3 were conducted at the Mary Emily Clinical Nutrition Research Unit of McGill University, Montreal, Canada. Clinical studies were compliance-controlled feeding, randomized crossover investigations consisting of 4-wk control and PS-intervention phases separated by a washout period of ≥4 wk. The pooled study population included 135 hypercholesterolemic subjects (LDL cholesterol >3.5 mmol/L) with a body mass index (BMI; in kg/m²) between 22 and 32 and an age range of 20–80 y (Table 1). All studies excluded subjects who were smokers; had a history of diabetes, hypertension, or hypothyroidism; or were taking cholesterol-lowering medication in the 6 mo before the start of the study.

Study diets were designed to contain 35% of energy as fat, 50% as carbohydrate, and 15% as protein and to meet the energy requirements of each subject according to the Mifflin equation (14). Subjects were instructed to consume only the foods and beverages provided by the clinical research staff and to refrain from coffee and alcohol consumption. PSs were supplied in soy beverage, yogurt, or margarine food vehicles at ≈2 g/d. To monitor compliance, subjects consumed one meal per day with PS treatment or placebo under direct supervision. The remaining meals and treatments were packed for take-out. Each study protocol was approved by the Bioethical Research Ethics Board at the University of Manitoba or the Faculty of Medicine Institutional Review Board of McGill University, Montreal, Canada. All subjects signed informed consent to participate in the studies.

Fasting blood samples were taken at the beginning and at the end of each experimental period for plasma lipid analyses.

Table 1

| Study characteristics from clinical plant sterol interventions (1) |
|--------------------------|--------------------------|--------------------------|--------------------------|
|                         | Clinical studies (reference) |
| Study 1 (5)              | Study 2 (12)               | Study 3 (13)             |
| Study characteristics    | Crossover | Controlled | Crossover | Controlled | Crossover | Controlled |
| Diet                     |            |            |            |            |            |            |
| Plant sterol intake (g/d)| 1.95       | 2          | 1.6        |
| No. of subjects          | 23         | 82         | 30         |
| Male/female              | 10/13      | 82/0       | 17/13      |
| Duration (d)             | 28         | 30         | 30         |
| Subject characteristics (baseline) | 43 | 51 | 60 |
| Mean age (y)             | 29–30      | 28–29      | 27–30      |
| BMI (kg/m²)              | 6.46 ± 0.30^a | 5.82 ± 0.10^a | 5.74 ± 0.17^a |
| Total cholesterol (mmol/L) | 4.23 ± 0.21^a | 3.71 ± 0.10^a | 3.61 ± 0.15^a |
| LDL cholesterol (mmol/L) | 1.27 ± 0.07 | 1.21 ± 0.03 | 1.37 ± 0.09 |
| Triglycerides (mmol/L)   | 2.33 ± 0.39 | 2.03 ± 0.10 | 1.87 ± 0.21 |

Values with a different superscript letter are significantly different from each other (P < 0.05).

Twenty-four hours before the end of each experimental phase, subjects were given an oral dose of deuterium oxide (0.7 g/kg estimated body water) before breakfast as a tracer for measuring cholesterol FSRs according to previously established procedures (15). Cholesterol FSR measured at the end of the control period was used as an estimate of basal cholesterol synthesis. Specific details on experimental design and sample collection and analyses for the 3 studies have been published previously (5, 12, 13).

Animal experiments

In 2 separate studies, 16 male C57BL/6J mice and Syrian Golden hamsters were acquired from Charles River (Wilmington, MA) and brought to the Animal Model Research Facility at the RCFFN. Mice were pair-housed in plastic cages with shavings in a temperature-controlled room (20°C) in a 12-h light/dark cycle. Hamsters were housed individually under similar conditions. Animals had free access to water and were acclimatized to the facility and research staff for 1 wk on a standard rodent nonpurified diet (Prolab RMH 3000; LabDiet, South Vistas Leduc, Canada) before commencement of the experiment. At the initiation of the experiment, animals were randomized to a hypercholesterolemic (0.25%) rodent diet with and without 2% PS (Reducol; Forbes Meditech, Vancouver, Canada) for 4–6 wk. Each study was designed as a randomized complete block. All procedures were approved by the Animal Care Committee at the University of Manitoba, and the animals were cared for in accordance with the guidelines established by Canadian Council of Animal Care, Ottawa, Canada.

Sample collection and processing

At the end of each study, animals were anesthetized with isoflurane for blood and tissue collection. Fasting blood was collected by cardiac puncture into EDTA-coated tubes. Plasma was separated from whole blood by centrifugation at 1000 × g for 10 min and stored in aliquots at −20°C. Livers from mice were collected and processed according to previously reported procedures (16).

Plasma lipid analyses

Plasma total cholesterol, HDL cholesterol, non-HDL cholesterol, and triglycerides were determined by automated enzymatic methods on a Vitros 350 chemistry analyzer (Ortho-Clinical Diagnostics, Markham, Canada).

RNA preparation and real-time reverse transcriptase–polymerase chain reaction

Total RNA was isolated from mouse whole-liver tissue by using TRIzol reagent (Invitrogen Canada Inc, Burlington, Canada). The amount of RNA and its integrity were determined by using spectrophotometry (260 nm) and agarose gel electrophoresis, respectively. RNA preparation and real-time reverse transcriptase–polymerase chain reaction (RT-PCR) were conducted with a one-step Quantitect SYBR Green RT-PCR kit (Qiagen Inc, Mississauga, Canada) on an Applied Biosystems 7500 system (Streetsville, Canada) according to previously established protocols (16). Sense and antisense primers were obtained from previously published sequences for β-hydroxy-β-methylglutaryl...
cholesterol (HMG-CoAr), sterol regulatory element binding protein 2 (SREBP2), β-actin (17), and ATP binding cassette transporters G5 and G8 (ABCG5 and ABCG8, respectively) (18).

Immunoblot analyses

Immunoblots were prepared as previously described (16). Nuclear and cytoplasmic extracts for immunoblot analyses of SREBP2 (SC-5603; Santa Cruz Biotechnology, Santa Cruz, CA) and β-actin (Ab8229; Abcam, Cambridge, MA) were prepared based on the previously published procedures (19). β-Actin was used as the housekeeping protein for all target proteins (Ab8229; Abcam).

Cholesterol synthesis and absorption

Cholesterol FSR (% pool/d) was quantified by using the uptake rate of deuterium from body water into the newly synthesized red blood cell–free cholesterol pool extracts over 2 h at the end of the feeding experiment (20, 21). Cholesterol absorption was measured with the single-stable-isotope-tracer approach with 48-h [3,4]-13C cholesterol–red blood cell enrichment reflecting cholesterol absorption efficiency (22).

Statistical analyses

Subjects in the control phase averaged a reduction in LDL cholesterol of 4% compared with baseline measurements, presumably due to the strict diet provided to the subjects. Therefore, subjects who did not show a reduction in LDL cholesterol beyond that observed on the control phase in response to PS intervention (<5%) were identified as nonresponders. Accordingly, clinical study subjects with an LDL-cholesterol reduction >5% in response to PS intervention were identified as responders. Basal cholesterol responses were analyzed as the percentage difference between end of treatment compared with control phases. Basal cholesterol synthesis rates (control phase FSR, % pool/d) between responder and nonresponder subgroups were analyzed by using the general linear model with trial as a fixed factor. The association between LDL-cholesterol response and basal cholesterol synthesis rates was assessed by using Pearson’s product–moment correlation coefficients. Plasma LDL-cholesterol response stratified by low, medium, and high basal cholesterol synthesis rates was compared with Tukey’s post hoc test.

Endpoint variables within and between animal studies were compared by using general linear model univariate analyses with block and study included as fixed factors. All data were analyzed with SPSS 16 (SPSS Inc, Chicago, IL) for Macintosh. Differences for clinical and animal studies were considered significant at \( P < 0.05 \).

RESULTS

Human studies

From a study population of 135 subjects, 113 subjects had complete data sets for plasma lipid and cholesterol synthesis. Out of the 113 subjects included in our analyses, 47 nonresponders and 66 responders to PS therapy were identified by using the threshold of 5% reduction in plasma LDL cholesterol. The pooled mean (±SE) LDL-cholesterol reduction (percentage change from control phase) for all study subjects in response to PS therapy was \(-7.3 \pm 1.2\%\). Plasma LDL cholesterol increased \((P = 0.01)\) in the nonresponder subgroup \((3.7 \pm 1.1\%\) and decreased \((P = 0.01)\) in the responder subgroup \((-15.2 \pm 1.0\%\) after PS intervention compared with the control phase.

Basal cholesterol FSR was \(23\%\) higher \((P = 0.003)\) in the nonresponder subgroup than in responders to PS (Figure 1). After the 4-wk PS intervention, nonresponders had a 14% higher \((P = 0.04)\) FSR compared with the responder subgroup (Figure 1). Basal FSR was correlated \((r = 0.22, P = 0.02)\) with percentage change in LDL cholesterol. Subjects with the lowest basal FSR showed greater reductions in plasma LDL cholesterol compared with subjects with higher FSR (Figure 2). In addition, when subjects were stratified by FSR (top and bottom 25%), those with the lowest basal FSR responded with a higher \((P = 0.03)\) percentage reduction in LDL cholesterol \((-12.31 \pm 2.22\%\) compared with \(-3.17 \pm 0.66\%\) compared with subjects with the highest FSR (Figure 3).

Animal studies

Compared with hamsters, mice showed a nonresponse to PS consumption with little reduction \((P < 0.05)\) of plasma total cholesterol \((-4.5 \pm 4.6\%\) compared with \(-55.3 \pm 4.8\%), non-HDL cholesterol \((-19 \pm 11.0\%\) compared with \(-85.5 \pm 9.6\%), and HDL cholesterol \((-1.3 \pm 1.4\%\) compared with \(-24.0 \pm 6.4\%)\) after PS consumption. Furthermore, compared with hamsters, nonrespiring mice showed a higher \((P < 0.01)\) cholesterol FSR in the control \((4.35 \pm 0.35\%)\) compared with \(1.92 \pm 0.42\%\) pool/d) and PS-supplemented phase \((6.96 \pm 0.78\%\) compared with \(3.92 \pm 0.33\%\) pool/d). PS-fed mice had lower \((P < 0.05)\) intestinal cholesterol absorption \((4.61 \pm 0.42\%)\) compared with \(6.82 \pm 0.83\%)\) and hepatic total cholesterol concentration \((1.81 \pm 0.37\%)\) compared with \(8.62 \pm 0.46 \mu mol/g tissue) compared with control mice.

mRNA and protein expression patterns of hepatic sterol regulatory targets are presented in Table 2. PS-fed mice had higher hepatic HMG-CoAr–mRNA expression (2.4-fold of control, Figure 1.

Mean (±SE) cholesterol fractional synthesis rates (% pool/d) in responder and nonresponder subgroups during control and plant sterol supplemented phases. *Significantly different from nonresponder subgroups, \(P < 0.05\) (general linear model with trial as a fixed factor). \(n = 113\) human subjects (47 nonresponders, 66 responders).

FIGURE 1.

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responsive to PS therapy and showed no reduction in LDL cholesterol with relatively high basal cholesterol synthesis were nonresponders, 66 responders). $P < 0.01$; however, no difference ($P = 0.29$) in HMG-CoAr protein abundance was observed between control and PS-fed mice. Although PS consumption did not affect ($P = 0.41$) hepatic protein abundance of cytoplasmic precursor SREBP2, the nuclear active form was higher (1.3-fold of control, $P = 0.04$) in the PS-fed mice compared with control mice. PS-fed mice had lower mRNA expression of hepatic ABCG5 (2.6-fold of control, $P = 0.01$) and ABCG8 (1.9-fold of control, $P = 0.01$) compared with control mice.

**FIGURE 2.** Pearson’s product-moment correlation ($r$) between basal cholesterol fractional synthesis rate (% pool/d) and percentage of LDL cholesterol (LDL-C) change from control phase in response to plant sterol consumption. Values are means ± SEs; $n = 113$ human subjects (47 nonresponders, 66 responders).

**DISCUSSION**

The present study shows in multiple species that the degree of plasma LDL-cholesterol reduction in response to dietary PS therapy is influenced by the basal cholesterol synthesis rate. In the human studies presented, individuals with low basal cholesterol synthesis responded to PS therapy with clinically significant reductions in LDL cholesterol ($-15.2 ± 1.0\%$), whereas those with relatively high basal cholesterol synthesis were unresponsive to PS therapy and showed no reduction in LDL cholesterol after PS consumption ($3.7 ± 1.1\%$). Results from our studies in animal models of response (hamster) and nonresponse (mouse) to PS consumption support the association between basal cholesterol synthesis and LDL-cholesterol-lowering response to PS therapy in humans.

LDL-cholesterol reductions in subjects from the current retrospective analysis ranged from $-40\%$ to $-0.19\%$, confirming recent reports on the variable cholesterol-lowering response to PS consumption (4, 23). As the hypocholesterolemic effects of PS are achieved through direct interference with intestinal cholesterol absorption, the substantial range of responsiveness is most often examined in the context of genetic factors that underscore the wide variability in intestinal cholesterol absorption across populations (13, 23–25). Basal serum ratios of campesterol to cholesterol, an indirect measure of intestinal cholesterol absorption, have been reported to predict the LDL-cholesterol-lowering response to PS (26, 27). Overall, results from these studies suggest that subjects with high intestinal cholesterol absorptive efficiency respond with greater reductions in plasma LDL cholesterol to PS therapy than individuals with low absorption efficiency. Similarly, several studies have observed a lower LDL-cholesterol-lowering response to PS therapy in subjects with high basal sterol synthesis marker concentrations that provides an indirect reflection of whole-body cholesterol synthesis (28–30). After prescreening for high and low plasma lathosterol:campesterol ratios, greater LDL-cholesterol reductions have been observed in a small group of subjects ($n = 8$) with plasma marker ratios indicative of low basal cholesterol synthesis compared with subjects with marker ratios reflecting high basal synthesis in response to plant stanol consumption ($-13.8\%$ compared with $+4.2\%$) (31). Our study confirms these results and is the first to show nonresponse to PS therapy in subjects with high basal cholesterol synthesis evaluated with direct kinetic stable-isotope measures of endogenous cholesterol synthesis (%/d).

Through an elegant feedback mechanism and precise molecular regulation of enterohepatic sterol homeostasis, cholesterol absorption and synthesis maintain a reciprocal relation to sustain body pools (32–36). Because PS therapy has been shown to work most effectively in subjects with high intestinal cholesterol absorption efficiency (13), it is reasonable that PS-responsive subjects would possess low cholesterol synthetic capacities. Alternatively, nonresponders to PS therapy may compensate for PS-induced reductions in intestinal cholesterol absorption by increasing whole-body cholesterol synthesis, thereby maintaining plasma cholesterol concentrations. Although measures of intestinal cholesterol absorption were not available for all study subjects included in this analysis, results from our animal studies suggest that mice show a nonresponse to PS consumption even in the face of significant reductions ($-39\%$) in intestinal cholesterol absorption.

Although, to the best of our knowledge, this is the first study to specifically use wild-type C57BL/6j mice as a model of nonresponse to PS consumption, 3 previous reports have observed similar findings (37–39). Alternatively, hamsters show consistent reductions in intestinal cholesterol absorption and plasma cholesterol in response to dietary PS supplementation (40–42). In support of our clinical findings, basal cholesterol synthesis in nonresponsive mice was considerably higher ($77\%$) than in responsive hamsters. A previous analysis confirms that mice

**FIGURE 3.** Mean (±SE) percentage change in LDL cholesterol (LDL-C) in the top 25% and bottom 25% of subjects stratified by low ($n = 28$), medium ($n = 57$), and high ($n = 28$) basal cholesterol fractional synthesis rate (% pool/d). *Significantly different from the low fractional synthetic rate group, $P < 0.05$ (Tukey’s post hoc test). $n = 113$ human subjects (47 nonresponders, 66 responders).
TABLE 2
mRNA and protein expression patterns (in arbitrary units) of hepatic regulatory targets in C57BL/6J control and plant sterol–supplemented mice

<table>
<thead>
<tr>
<th>Target gene/protein</th>
<th>mRNA</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Plant sterol</td>
</tr>
<tr>
<td>HMG-CoAr</td>
<td>1.0 ± 0.1</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>ABCG5</td>
<td>1.0 ± 0.1</td>
<td>0.4 ± 0.03</td>
</tr>
<tr>
<td>ABCG8</td>
<td>1.0 ± 0.1</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>SREBP2 (cytoplasmic)</td>
<td>—</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>SREBP2 (nuclear)</td>
<td>—</td>
<td>1.0 ± 0.1</td>
</tr>
</tbody>
</table>

1 Values are means ± SEs; n = 8. All data were analyzed by general linear model univariate analyses with block as a fixed factor. HMG-CoAr, β-hydroxy-β-methylglutaryl coenzyme A reductase; ABCG5 and ABCG8, ATP binding cassette transporters G5 and G8, respectively; SREBP2, sterol regulatory element binding protein 2.

2 Significantly different from control, P < 0.05.

possess a higher cholesterol synthetic capacity than hamsters (160 compared with 40 mg · kg⁻¹ · d⁻¹, respectively) (43).

The inhibition of intestinal cholesterol absorption and associated reduction in hepatic cholesterol stores observed in the PS-fed mice were associated with modulation in the expression of hepatic sterol regulatory and trafficking machinery. As far as we are aware, this is the first report of enhanced SREBP2 nuclear protein abundance in response to PS consumption. SREBP2 is a master transcriptional regulator of genes involved in hepatic cholesterol synthesis (44) and was likely responsible for the compensatory increase in HMG-CoAr mRNA and cholesterol synthesis in PS-fed mice displaying reduced hepatic cholesterol concentrations. Alternatively, the observed reduction in hepatic ABCG5- and ABCG8-mRNA expression in the current study has been reported previously in the same mouse model after PS consumption (37) and may be a mechanism to reduce biliary sterol loss under conditions of cholesterol depletion.

In summary, with the wide degree of LDL-cholesterol-lowering responsiveness to PS therapy, it is important to characterize metabolic factors to explain this variability and assist in identifying patients for whom PS therapy would be an appropriate therapeutic strategy. Our results in human clinical studies and animal models of nonresponse (mouse) and response (hamster) to PS therapy suggest that patients with high basal cholesterol synthesis are unresponsive to PS treatment.

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The authors’ responsibilities were as follows—TCR: was the principal manuscript author and project lead on the animal model experiments and contributed to the preparation of the manuscript; DM: analyzed the human clinical data and contributed to the preparation of the manuscript; SSA: organized the data from the previously run clinical studies and revised the final manuscript; and PIJH: was the principal investigator on the human clinical studies and contributed to the preparation of the manuscript. The authors had no conflicts of interest to declare.

REFERENCES
5. Rideout TC, Chan YM, Harding SV, Jones PJ. Low and moderate-fat plant sterol fortified soy milk in modulation of plasma lipids and cholesterol kinetics in subjects with normal to high cholesterol concentrations: report on two randomized crossover studies. Lipids Health Dis 2009;8:45.


