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New insight into the cholesterol-lowering effect of phytosterols in rat cardiomyocytes

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ABSTRACT

The exact mechanisms for cholesterol-lowering effect of phytosterols (PS) are not well defined yet. Dietary cholesterol minimally affects blood cholesterol, and regulation of cholesterolaemia could not exclusively rely on modification in intestinal absorption. Activation of sterol regulatory element-binding proteins (SREBPs), master regulators of lipid homeostasis, is tightly regulated through a sterol-sensing domain (SSD); in cholesterol-depleted cells, SREBP activation upregulates the expression of genes involved in cholesterol synthesis and trafficking. Since PS structure is very similar to cholesterol, they could be sensed by SSD, so preventing SREBP cleavage. To verify this hypothesis primary cultures of rat cardiomyocytes were supplemented with PS (sitosterol, campesterol, and brassicasterol, separately or in a mixture), cholesterol or mevastatin.
PS were actively incorporated by cardiac cells, and caused a significant decrease in cholesterol cellular content. Genes encoding for SREBP2, HMGCR and LDLR were upregulated in cells treated with mevastatin, while no increase in their transcription was detected in PS-supplemented cardiomyocytes, although the reduction of cholesterol was similar in PS- and mevastatin-treated cells. According to herein reported data, it is conceivable that PS are sensed by the SSD so preventing the activation of the nuclear receptors and consequent upregulation of genes connected with cholesterol metabolism.

1. Introduction

Phytosterols (PS) are plant-derived sterols that are structurally similar and functionally analogous to cholesterol in vertebrate animals. Sitosterol and campesterol are the most frequently occurring phytosterols in food, followed by stigmasterol. Plant oils, legumes, nuts, and seeds have relatively high concentrations of PS, whereas cereal grains, fruits, and vegetables contain only modest amounts (Ostlund, 2002; Weihrauch & Gardner, 1978).

Several clinical studies support the effectiveness of PS-rich diet in the reduction of hypercholesterolaemia, a major risk factor for cardiovascular diseases (AbuMweis, Barake, & Jones, 2008; Katan et al., 2003; Ras, Geleijnse, & Trautwein, 2014); added to a statin, PS supplementation is equivalent to doubling the statin dose with regard to LDL cholesterol-lowering (Scholle, Baker, Talati, & Coleman, 2009).

The exact mechanisms for PS cholesterol-lowering effect are not well defined yet. Different hypotheses have been proposed (De Smet, Mensink, & Plat, 2012; Trautwein et al., 2003), mainly the reduction of intestinal cholesterol absorption by competition of PS with cholesterol for micelle formation, reducing the transport of cholesterol to the enterocyte (Hui & Howles, 2005), and the *trans*-intestinal cholesterol excretion, increasing faecal loss of neutral sterols (De Smet et al., 2012). Since cholesterol in the body comes from two sources, *de novo* synthesis and dietary intake, and dietary cholesterol contributes a relatively minor proportion (about 10%) of the cholesterol in the system

Abbreviations: ACTB, β-actin; ANOVA, analysis of variance; AP, alkaline phosphatase; BA, bile acids; Brassica, brassicasterol; Campe, campesterol; Chole, cholesterol plus 25-OH-cholesterol; Ctrl, control; CYP7A1, cholesterol-7-α-hydroxylase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; FCS, foetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GC–MS, gas chromatography-mass spectrometry; gDNA, genomic DNA; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; HS, horse serum; HSD, honest significant difference; IS, internal standard; LDH, lactate dehydrogenase; LDL, low density lipoprotein; LDLR, low density lipoprotein receptor; LXR, liver X receptor; n.d., not detectable; *n.s.*, not significant; NIH, National Institutes of Health; NIST, National Institute of Standards and Technology; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; *p*NPP, *p*-nitrophenyl phosphate; PPAR, peroxisome proliferator-activated receptor; PS, phytosterols; qPCR, quantitative real-time PCR; SCAP, SREBP cleavage-activating protein; SE, standard error; Sito, sitosterol; *Srebf*, sterol regulatory element binding transcription factor; SREBP, sterol regulatory element-binding protein; SSD, sterol-sensing domain.

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Table 1
Sterols content in the PS mixture.
(Danesi et al., 2011).

Compound	Content (g/100 g)
Sitosterol	44.797 ± 2.464
Campesterol	34.494 ± 0.923
Stigmasterol	5.193 ± 0.177
Cholesterol	1.809 ± 0.317
Avenasterol	0.985 ± 0.052
Sitostanol	0.963 ± 0.083
Campestanol	0.652 ± 0.024
Clerosterol	0.212 ± 0.034
Δ7-Sitosterol	0.182 ± 0.022
Stigmastanol	0.169 ± 0.007
Fucosterol	0.168 ± 0.010
Brassicasterol	0.119 ± 0.021

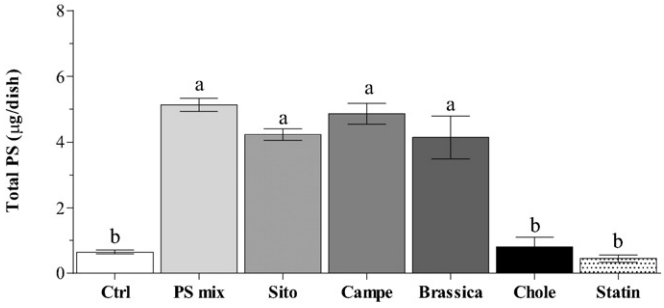

Fig. 1. Total PS content in unsupplemented (Ctrl), PS-supplemented, cholesterol- and statin-treated cardiomyocytes. Data are expressed as µg/dish. Statistical analysis was carried out by one-way ANOVA ($p < 0.001$) using Tukey's HSD test. Different letters indicate statistical significance (at least $p < 0.05$).

and minimally affects blood cholesterol (McNamara, 2000), regulation of cholesterolaemia cannot exclusively rely on modification in intestinal absorption.

Intake of PS-enriched foods and supplements increases plasma PS concentrations, and although total plasma PS concentration is very low (5–25 µM) (Chadwick et al., 2003; Chan et al., 2006; Ras et al., 2013), PS can be taken up by cells (Danesi et al., 2011), and could regulate genes and proteins related to cholesterol metabolism. Different molecular effects have been already evidenced for PS; they were shown to be potent liver X receptor (LXR) ligands (Hoang et al., 2012; Plat, Nichols, & Mensink, 2005; Yang et al., 2004), to activate peroxisome proliferator activated receptors (PPARs) (Nomaguchi et al., 2011), and to induced the expression of cholesterol-7-α-hydroxylase (*Cyp7a1*), which governs the synthesis of bile acids (BA) (Davis, Miyake, Hui, & Spann, 2002), thus indicating that the increased excretion of cholesterol via BA represents an additional mechanism of cholesterol reduction. PS from *Aloe vera* have been also shown to decrease the expression of sterol regulatory element binding protein 1 in Zucker diabetic fatty rats (Misawa et al., 2012). Notwithstanding the increasing evidence of a molecular mechanism of action of PS (De Smet, Mensink, Boekschoten, et al., 2015; De Smet, Mensink, Konings, et al., 2015), the physiological phenotypes induced by PS intake is not clearly established. Among the few studies supplementing animals with pure PS, Rideout et al. (2015) evidenced that pups of apoE(−/−) mice born to mothers supplemented with PS during gestation and lactation exhibit favourable liver and serum lipid responses. This could indicate that PS consumption induces a hypolipidaemic phenotype.

Sterol regulatory element binding proteins (SREBPs) are a family of transcription factors that regulate lipid homeostasis by controlling the expression of >30 genes involved in the biosynthesis of cholesterol, triacylglycerols, phospholipids, and fatty acids (Eberlé, Hegarty, Bossard, Ferré, & Foufelle, 2004; Ye & DeBose-Boyd, 2011). The regulation of the expression of genes encoding for SREBPs is still unclear (Guillou, Martin, & Pineau, 2008), and few data are reported in the literature about the possible modulatory effect of PS on their transcription (Gil-Ramirez et al., 2016; Misawa et al., 2012; Rideout, Harding, & Jones, 2010). The three SREBP isoforms (SREBP1a, 1c, and 2) are synthesized as inactive precursors bound to the endoplasmic reticulum (ER) membranes. When cells are deprived of cholesterol, NH_2-terminal fragments of SREBPs are proteolytically released from membranes and migrate to the nucleus to activate transcription of genes required for lipid synthesis and trafficking. On the contrary, a normal/high cholesterol cellular concentration keeps SREBPs in their inactive form, thus preventing additional synthesis and uptake. This cholesterol-mediated regulation relies on the presence of a sterol-sensing domain (SSD) in the cleavage-activating protein (SCAP). SCAP is an integral membrane protein located in the ER that is required for the proteolytic cleavage of SREBPs. When cholesterol is present, SCAP undergoes a conformational change that prevents it from activating SREBPs (Sun, Seemann, Goldstein, & Brown, 2007).

In the attempt to further unravel the molecular effects and mechanism of action of PS, in the present study we focused on three different PS (sitosterol, campesterol, and brassicasterol) which were supplemented, separately or in a mixture, to the growth media of primary rat neonatal cardiomyocytes. The PS concentration employed in the current study (13 µM) was within a physiological range, achievable by a moderate intake of PS-enriched foods (Ras et al., 2013). In parallel experiments cells were exposed to cholesterol or treated with statins. PS incorporation by cell and their cholesterol-lowering effect were evaluated, and the transcription of the SREBP encoding genes (*Srebf1* and *2*) and of two SREBP target genes (*Hmgcr* and *Ldlr*) was assessed. *Hmgcr* and *Ldlr* were selected among SREBP target genes since they encode for key proteins in cholesterol synthesis and uptake, i.e. HMGCR, the enzyme catalysing the rate-limiting step in cholesterol synthesis, and

Table 2
Primer sequences for qPCR.

Gene name	GenBank accession number	Primer sequence	Amplicon size
Reference genes			
Actb β-actin	NC_005111.4	F: GGGAAATCGTGCGTGACATT R: GCGGCAGTGGCCATCTC	76 bp
Gapdh glyceraldehyde-3-phosphate dehydrogenase	NC_005103.4	F: ATGACTCTACCCACGGCAAG R: GGAAGATGGTGATGGGTTTC	87 bp
Target genes			
Hmgcr 3-hydroxy-3-methylglutaryl-CoA reductase	NC_005101.4	F: TCACAGGATGAAGTAAGGGAGAA R: GGTGCCAACTCCAATCACAA	105 bp
Ldlr low density lipoprotein receptor	NC_005107.4	F: CAAGGAGTGCAAGACCAACG R: AAACCGCTGGGACATAGGC	102 bp
Srebf1 sterol regulatory element binding transcription factor 1	NC_005109.4	F: CCATGGGCAAGTACACAGGA R: GCCACGTAGATCTCTGCCAGT	121 bp
Srebf2 sterol regulatory element binding transcription factor 2	NC_005106.4	F: TGACTGAGAGTCCCTTGGTGA R: ACCACTGGCCCTCTCACAAT	119 bp

F: forward primer; R: reverse primer; bp: base pairs.