Available online at www.sciencedirect.com

SciVerse ScienceDirect

Journal of Nutritional Biochemistry 24 (2013) 343–352

Journal of Nutritional Biochemistry

Plant stanols induce intestinal tumor formation by up-regulating Wnt and EGFR signaling in *Apc*^{Min} mice☆

Maija Marttinen[a,*], Essi Päivärinta[a], Markus Storvik[b], Laura Huikko[c], Heli Luoma-Halkola[a], Vieno Piironen[c], Anne-Maria Pajari[a], Marja Mutanen[a]

[a]Department of Food and Environmental Sciences, Division of Nutrition, P.O. Box 66 (Agnes Sjöbergin katu 2), FIN-00014 University of Helsinki, Finland
[b]School of Pharmacy, University of Eastern Finland, P.O. Box 1627, FIN-70211 Kuopio, Finland
[c]Department of Food and Environmental Sciences, Division of Food Chemistry, P.O. Box 27 (Latokartanonkaari 11), FIN-00014 University of Helsinki, Finland

Received 16 January 2012; received in revised form 20 June 2012; accepted 4 July 2012

Abstract

The rate of *APC* mutations in the intestine increases in middle-age. At the same period of life, plant sterol and stanol enriched functional foods are introduced to diet to lower blood cholesterol. This study examined the effect of plant stanol enriched diet on intestinal adenoma formation in the *Apc*^{Min} mouse. *Apc*^{Min} mice were fed 0.8% plant stanol diet or control diet for nine weeks. Cholesterol, plant sterols and plant stanols were analyzed from the caecum content and the intestinal mucosa. Levels of β-catenin, cyclin D1, epidermal growth factor receptor (EGFR) and extracellular signal-regulated kinase 1/2 (ERK1/2) were measured from the intestinal mucosa by Western blotting. Gene expression was determined from the intestinal mucosa using Affymetrix and the data were analyzed for enriched categories and pathways. Plant stanols induced adenoma formation in the small intestine, however, the adenoma size was not affected. We saw increased levels of nuclear β-catenin, phosphorylated β-catenin (Ser675 and Ser552), nuclear cyclin D1, total and phosphorylated EGFR and phosphorylated ERK1/2 in the intestinal mucosa after plant stanol feeding. The Affymetrix data demonstrate that several enzymes of cholesterol synthesis pathway were up-regulated, although the cholesterol level in the intestinal mucosa was not altered. We show that plant stanols induce adenoma formation by activating Wnt and EGFR signaling. EGFR signaling seems to have promoted β-catenin phosphorylation and its translocation into the nucleus, where the expression of cyclin D1 was increased. Up-regulated cholesterol synthesis may partly explain the increased EGFR signaling in the plant stanol-fed mice.

Keywords: Plant sterols; ApcMin mouse; β-catenin; EGFR; Cholesterol synthesis

1. Introduction

Plant sterol- and stanol-enriched functional foods are designed to lower blood cholesterol, a well-established risk factor of heart diseases. Plant sterols (phytosterols) are naturally existing compounds of plant origin, and they are present in small quantities in an average human diet. Plant stanols are saturated forms of sterols and they are less abundant in nature than plant sterols. Both plant sterols and stanols are poorly absorbed from the intestine and they interfere with cholesterol absorption, leading to increased concentrations of cholesterol and other sterol metabolites in the gut lumen [1]. The elevated level of cholesterol derivatives in the colon may lead to a modified gut environment. High intraluminal cholesterol concentration, from dietary and/or endogenous origin, has been associated with enhanced cell proliferation, aberrant crypt formation and tumor formation in the murine colon [2,3].

In some studies, plant sterols have been demonstrated to inhibit colon tumorigenesis in carcinogen-treated rats [4-6]. However, Quilliot et al. [7] found no protective effect of plant sterol supplement in their study with methylnitrosourea (MNU) rats. They concluded that plant sterols modified gut microflora negatively, which was seen as increased level of feacal coprostanol, a bacterial metabolite of cholesterol associated with colon carcinogenesis [8,9]. The Netherlands Cohort Study on Diet and Cancer did not find association with high dietary intake of plant sterols and lowered risk of colorectal cancer; however, high intake of β-sitostanol was positively associated with cancer of distal colon in men [10].

The risk of developing colorectal cancer increases with age. Mutation in the *adenomatous polyposis coli* (*APC*) tumor suppressor gene is required in developing hereditary colon cancer (familial

Abbreviations: Akt, protein kinase B; Apc, adenomatous polyposis coli; EGFR, epidermal growth factor; ERK1/2, extracellular signal-regulated kinase 1/2; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; LDL, low density lipoprotein; Min, multiple intestinal neoplasia; MNU, methylnitrosourea; PI3K, phosphatidylinostol 3-kinase; SREBP, sterol regulatory element binding protein.

☆ Grant support and funding: The work was funded by Yrjö Jahnsson Foundation, Juho Vainio Foundation and the Finnish Graduate School on Applied Bioscience.

* Corresponding author. Tel.: +358 9 19158276.
E-mail address: maija.marttinen@helsinki.fi (M. Marttinen).

0955-2863/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jnutbio.2012.07.002

adenomatous polyposis, FAP) and is found in the majority of sporadic colorectal tumors [11]. The incidence of *APC* mutations in the colon increases in middle age [12] and often at the same time consumption of plant sterol enriched products starts. Therefore, we studied the effect of plant stanol enriched semisynthetic diet on intestinal adenoma formation in the *Apc*^{Min} mouse (*Adenomatous polyposis coli*, Multiple intestinal neoplasia), a well-characterized model of colon cancer. No previous studies have reported the effect of plant sterols or stanols in the tumor prone *Apc*^{Min} mouse. The *Apc*^{Min} mouse carries a heterozygous mutation in the *Apc* gene [13], which leads to spontaneous development of intestinal tumors. Disturbed function of Apc protein results in the activation of the Wnt pathway with concomitant down-regulation of β-catenin degradation and its accumulation in the nucleus [14]. In the nucleus, β-catenin interacts with the Tcf/Lef transcription factor and regulates transcription of genes, e.g., c-*myc* and *cyclin D1* [15,16] that enhance cell proliferation and growth and mediate cellular program for ongogenic transformation in colorectal cancer [17,18]. In addition to Wnt activation, mitogen-activated protein kinase (MAPK) signaling occurs in response to almost any change in the extracellular or intracellular milieu. One subfamily of MAPKs is extracellular signal-regulated kinases (ERK1/2) that are activated through, e.g., growth factor receptor signaling, including the epidermal growth factor receptor (EGFR). The other pathway activated by EGFR phosphorylation is the PI3K/Akt pathway, and through these two pathways EGFR regulates the homeostasis between cell proliferation and maturation in the gut [19,20]. On the membrane EGFRs locate in lipid rafts that are rich in cholesterol and sphingolipids [21-23]. Plant stanols and sterols may be incorporated to cellular membranes, which may result in disturbed membrane properties and receptor function [24-26]. In this study, levels of cholesterol, plant sterols and plant stanols were measured from caecum content and intestinal mucosa in order to detect changes in the extracellular and intracellular milieu of enterocytes after plant stanol feeding. To determine how these changes in the sterol homeostasis affect cell signaling in intestinal tumorigenesis, protein levels of β-catenin and its phosphorylated forms (Ser552 and Ser675), cyclin D1, EGFR, ERK1/2 and Akt were measured from the normal appearing mucosa of small intestine. Finally, microarray analysis was performed to discover the impact of plant stanol feeding on gene expression in the *Apc*^{Min} mouse.

2. Materials and methods

2.1. Animals

The study protocol was approved by the Laboratory Animal Ethics Committee, University of Helsinki. Male and female C57BL/6J *Apc*$^{Min/+}$ mice were bred at the Laboratory Animal Centre in Viikki, Helsinki, from inbred mice originally obtained from the Jackson Laboratory (Bar Harbor, ME, USA). After weaning, the mice were screened for the *Min* genotype using a PCR assay described by Dietrich et al. [27]. The 5-week old *Apc*^{Min} mice were stratified by weight and litter background and assigned into experimental groups of 15 mice each group, eight male and seven female mice into both groups. The mice were housed in plastic cages in humidity- and temperature-controlled facilities with 12-h light–dark cycle. They were fed ad libitum and had free access to tap water. The mice were weighed and monitored weekly and record on their growth was kept throughout the experiment. Rapid loss of weight and a relapse of the rectum were indications of impaired health. One mouse from both experimental groups was sacrificed before the end of experiment due to a considerable weight-loss within 1 week. These mice were excluded from the analysis. The total number of mice included in the data is 28 (14 mice per group).

2.2. Diets

The control diet was a modified high-fat (41% of energy from fat; 13% butter, 5.5% rapeseed oil, 1.1% sunflower oil), very low fiber AIN93-G diet. The plant stanol diet was similar to control diet but contained 0.8% (w/w) plant stanol (sum of campestanol and sitostanol). The protein, carbohydrate, fat, fatty acid, cholesterol, plant sterol, vitamin and mineral contents of both diets were otherwise similar (Table 1). Plant stanol was added to the diet in form of freeze-dried plant stanol ester enriched products found on market in Finland 2003. The control diet was prepared with the same freeze-dried products without plant stanol enrichment. The nutrient content of the products with or without added plant stanol was similar because they were purchased when possible from the same manufacturer in Finland (Supplementary Table 1). The origin of plant sterols in both diets was vegetable oils added to the diets. The approximate daily intake of plant stanol was calculated to be 20 mg per mouse in the plant stanol group.

2.3. Tumor scoring and tissue samples

After the 9-week feeding period, the mice were sacrificed by CO_2 asphyxiation. Blood was collected from abdominal aorta and after centrifugation the plasma was stored at −70°C. The small intestine, caecum and colon were removed and opened along the longitudinal axis and rinsed with ice-cold saline. The small intestine was divided into five sections of equal length. The caecum and colon were separated from the small intestine and kept together for analysis. The contents of caecum were collected and stored at −20°C. The intestinal sections were spread flat on microscope slides. Each section of the intestine and colon and caecum was analyzed under inverse light microscope attached to a monitor. The number and diameter of each adenoma was recorded for each section separately. The observers keeping record were blinded to the treatment. The intestinal adenomas were excised from the tissue and the normal-appearing mucosa that was left behind was scraped off from lamina propria. The tissue samples were snap frozen in liquid nitrogen and stored at −70°C. For microarray analysis, a 0.5-cm section was cut from middle of the distal part of small intestine and stored in RNAlater solution at -20 °C (Qiagen).

2.4. Sterol analysis by direct saponification and gas chromatography

Cholesterol, plant sterols and stanols were analyzed as total amount of each sterol instead of separating the esterified and free sterols. The sterol composition was analyzed from the faeces of caecum and duodenal/jejunal mucosa for each mouse by applying a method described by Soupas et al. [28]. A sample of rapeseed oil was used to control interassay variation. Amount of dehydrocholesterol in ethanol was added to each sample as an internal standard. Shortly, ethanol and saturated potassium hydroxide were added to each sample and the esterified sterols were hydrolyzed in a shaking incubator for 30 min in 85°C. After hydrolyzation, water and cyclo-hexane were added and then shaked vigourously to extract sterols into solvent phase. To obtain a detectable concentration of sterols, solvent was evaporated from the samples of the control mice. Silylation was carried out to obtain trimethylsilyl derivatives of sterols. Before silylation, the solvent was evaporated under stream of nitrogen in 50°C. Pyridine and silylation solution (BSTFA: TMCS, 99:1) were added (1:1) and the samples were silylated in an incubator for 30 min in 60°C. The silylation reagent was evaporated under nitrogen in 50°C and remaining sterol sample was dissolved into heptane. The sample was injected into a RTX-5 w/Integra Guard (crossbond 5% diphenyl-95% dimethyl polysiloxane) capillary column (60 m × 0.32 mm i.d., 0,10 μm film) (Restek, Bellefonte, PA, USA) by automated injector of a gas chromatograph equipped with a flame ionization detector (Agilent 6890N Network GC System, Agilent Technologies, USA). Sterols were quantified using an internal standard method. Identification was based on retention times and GC-mass spectrometric analysis.

2.5. Western blotting analysis

Proteins were isolated from normal-appearing mucosa of the distal small intestine which represents approximately 40% of total small intestine. Sample preparation has been described earlier in detail by Misikangas et al. [29]. Samples were fractionated to membranous, cytosolic and nuclear fractions. For proteinase inhibition a solution of 0.4 mM leupeptin, 3.0 μM pepstatin and 1.0 mM PMSF (in DMSO) was used. For whole mucosa lysate 10% Triton-X solution was added to the total homogenate, the sample was efficiently mixed for 20 min with 5 min intervals and centrifuged 15 000×*g* 10 min at 4°C. The supernatant was collected and protein concentration measured as described by Misikangas et al. [29]. All protein samples were stored at −70°C.

Table 1
Analyzed composition of experimental diets

Component	Control diet	Plant stanol diet
	/100 g of diet	
Energy (kJ)*	1959	1965
Protein (g)*	21.3	21.4
Carbohydrate (g)*	51.5	51.1
Crude fat (g)*	19.5	19.8
Water (g)*	4.28	4.37
Ash (g)*	3.41	3.36
Cholesterol (mg)*	50.3	49.0
Plant sterols (mg)**	56.1	68.4
Plant stanols (mg)**	0.8	764.2

* Analysis by Agrifood Research Finland.
** Analysis by Division of Food Chemistry, University of Helsinki.