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Journal:	*Phytotherapy Research*
Manuscript ID:	PTR-08-1094.R1
Wiley - Manuscript type:	Full Paper
Date Submitted by the Author:	05-Mar-2009

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Keyword:	body fat, serum triglycerides, free fatty acids, leptin, colipase, lipase

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Running title: Long-term effect of thylakoids

Keywords: colipase, lipase, leptin, body fat, serum triglycerides, free fatty acids, satiety

Figures: 3 Tables: 1

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Abstract

Thylakoids are membranes isolated from plant chloroplasts which have previously been shown to inhibit pancreatic lipase/colipase catalysed hydrolysis of fat *in vitro* and induce short-term satiety *in vivo*. The purpose of the present study was to examine if dietary supplementation of thylakoids could affect food intake and body weight during long-term feeding in mice. Female apolipoprotein E-deficient mice were fed high-fat diet containing 44 % of fat by energy with and without thylakoids for 100 days. Mice fed thylakoid-enriched diet had suppressed food intake, body weight gain and body fat compared to high-fat fed control mice. We also found reduced serum glucose, serum triglyceride and serum free fatty acid levels in the thylakoid-treated animals. The satiety hormone cholecystokinin was elevated, suggesting this hormone to mediate satiety. Leptin levels were reduced, reflecting a decreased fat mass. There was no sign of desentization in the animals treated with thylakoids. The results suggest that thylakoids are useful to suppress appetite and body weight gain when supplemented to high-fat food during long-term feeding.

Introduction

Obesity is a major health problem. The reason for obesity is a dysregulated energy balance, energy intake and energy expenditure being unequal. In understanding the role of genes and environment it was found that the major impact was the environment (Fernandez *et al.,*2008; Papas *et al.,*2007). A relevant environmental factor is the ready availability of palatable energy-dense food containing fat and/or sucrose (Blundell *et al.,*2005; Lindqvist *et al.,*2005). Such food causes a disruption of the appetite regulating system (Erlanson-Albertsson2005). Thus, it has been described that hunger signals are still active after exposure to palatable food and that satiety signals postprandially are not strong enough to prevent over eating (Gaysinskaya *et al.,*2007).

Appetite regulation is governed by hunger and satiety signals, being produced both centrally and peripherally (Drazen & Woods2003; Woods2004; Woods *et al.,*2000). It has been shown that fat induces satiety as long as it stays in the intestine (Beglinger & Degen2004; Goedecke *et al.,*2003). The satiety signals are released from the gastrointestinal tract triggered by the entry of food products. One such important signal is cholecystokinin (CCK), being released from the intestine by the entry of fat and protein (Ritter2004). Another potential satiety signal for fat intake is enterostatin, released from pancreatic procolipase (Berger *et al.,*2004; Erlanson-Albertsson & York1997).

We have found that thylakoids isolated from green leaves, cause a retarded fat digestion by adhering to fat globules in the intestine thereby reducing the rate of the

lipase/colipase induced hydrolysis of intraluminal fat (Albertsson *et al.,*2007). When added to high-fat food and given to Sprague-Dawley rats for 12 days, thylakoids were found to reduce food intake, body weight gain and promote satiety by elevated CCK-release compared to control (Albertsson *et al.,*2007). Furthermore a significant reduction in serum triglyceride levels was observed (Albertsson *et al.,*2007). The main explanation for this retarding effect is the binding of thylakoid membranes to the lipid droplets, thereby decreasing the activity of lipase and colipase (Albertsson *et al.,*2007). The binding of the thylakoids to the lipid droplets occurs through the hydrophobic properties of the membrane spanning regions of the integral membrane proteins. In addition, lipase/colipase can bind directly to the thylakoid membranes thereby hindering the binding to the lipid droplet (Albertsson *et al.,*2007). The thylakoids are eventually digested in the intestine; their inhibitory effect is therefore temporary and results in a retardation of fat digestion as well as fat absorption rather than a total inhibition of fat digestion and absorption. One commercially available inhibitor of pancreatic lipase is Xenical® which produces weight loss in man. The side effect of Xenical is a total inhibition of all gastrointestinal lipases by an irreversible blockage of the active site of these enzymes. This does not occur during the action of thylakoids, whose action rather is a temporary blockage at the interface of the fat droplets and pancreatic lipase/colipase. Hence Xenical yields steatorrhea, which is not the case with thylakoids.

Thylakoids are membranes inside the chloroplast and constitute the site of the light-dependent reactions of photosynthesis with the photosynthetic pigments chlorophyll and carotenoids embedded in the membrane proteins. Thylakoids in general have a favourable nutritional composition. They contain membrane proteins which are mostly

found in major protein complexes (Albertsson2001; Nelson & Ben.Sham2004; Nelson & Yocum2006). These membrane proteins together with bound pigments account for approximately 70% of the thylakoids mass. Galactolipids with a dominance of ω-3 polyunsaturated fatty acids (Duchene & Siegenthaler2000) account for the remaining 30%. Hence, thylakoids membranes have a great potential as food additive for preparation of so called "functional food" with health promoting properties.

In the present study, we were interested to know if thylakoids have any long-term effect on food intake, blood lipid levels and body weight regulation. In the choice of mouse strain, we chose the apolipoprotein (apo) E-deficient mouse, a strain of mice that is highly sensitive to high-fat diet with the induction of atherosclerotic plaques as a result of such a regimen (van Ree *et al.,*1994). We found that thylakoids reduced food intake, serum triglycerides and body weight gain compared to control animals after long-term treatment. There was however no effect of thylakoids on the high-fat diet induced atherosclerosis.

Materials and methods

Preparation of thylakoids

The preparation of thylakoids was made as follows: 1000 g spinach leaves were homogenised in a blender with 1250 ml water and filtered through four layers of Monodur polyester mesh (20 µm). The filtrate was diluted 10 times with distilled water and pH adjusted to 4.7 with HCl as maximum precipitation occurs at pH 4.7 which is the isoelectric point of the thylakoids. The thylakoids flocculate and after standing in the cold (+4^{o}C) for 4 h, a green sediment was obtained with a clear, slightly yellowish supernatant. The supernatant was discarded and the sediment washed with water and sedimentation repeated at the same pH 4.7 and then the sediment was collected. Sediment was adjusted with NaOH to pH 7.0. The final sediment collected is the thylakoids that we used in the high-fat diet enriched with thylakoids.

Determination of chlorophyll

The chlorophyll content of crude thylakoids was determined according to Porra et al. (Porra *et al.,*1989). Chlorophyll content is a method to determine the amount of thylakoid membrane (concentration of chlorophyll is proportional to the amount of thylakoids). We used a dose of 6 mg chlorophyll/g diet (approximately the amount of thylakoids taken from 10 g of spinach), which is three times higher than the effective dose used in our previous rat study (Albertsson *et al.,*2007). Data from a pilot study showed that the higher dose was more effective (data not shown).

Measurements of food intake, body weight and body fat

Thirty female apoE-deficient mice were obtained from Taconic (Taconic Europe, Lille Skensved, Denmark) and divided into two groups (n=15 per group); one group receiving high-fat diet containing 44% fat by energy (control group; R638, Lactamin, Kimsta, Sweden) and the other group receiving high-fat diet (R638) enriched with thylakoids. The control diet and the thylakoid enriched diet were isocaloric with approximately the same macronutrient energy-composition (Table 1). The mice were housed three per cage in a temperature-controlled environment (21ºC), with a 12 h light/dark cycle (lights on at 6 a.m.). The mice had free access to food and tap water. Food intake was measured every other day. The cages were carefully monitored for evidence of spillage. Body weight was measured every fourth day. The study lasted for 100 days. After four hours fasting the mice were sacrificed by cervical dislocation. The amount of body fat was measured using dual energy X-ray analysis (DEXA; PIXImus, Lunar Corporation, Madison, WI, USA). The experiments described were approved by the Local Animal Welfare Committee, Lund, Sweden.

That the palatability of the thylakoid diet was not important for the suppressed appetite was proven by studies where mice were given low-fat diet with thylakoids. These had no effect on food intake or body weight (data not shown).

Serum analysis

Blood was collected and serum was stored at -20ºC until analysis. Free fatty acids (FFA) were measured using a NEFA C kit from Wako (Wako Chemicals, Neuss, Germany). Triglycerides (TG) were measured with a GPO-Trinder kit from Sigma (Sigma Diagnostics, Steinheim, Germany). Cholesterol was measured using a kit from Wako Chemicals (Chol-H L-type). CCK was measured using a radioimmunoassay (RIA) with a highly specific antibody (no. 92128), which does not cross-react with any of the homologous gastrin peptides (Rehfeld1998). Glucose was measured using a colorimetric kit from ThermoElectron (Melbourne, Australia). Peptide YY (PYY) was measured using an enzyme immunoassay (EIA) kit from Phoenix Pharmaceuticals (Burlingame, CA, USA). Leptin was measured using an enzyme-linked immunosorbent assay (ELISA) from CrystalChem (Downers Grove, IL, USA).

Measurements of pancreatic lipase activity

Pancreas was dissected out from the mice and homogenised in buffer (10 mM sodium phosphate, pH 6.0, 0.5% digitonin). Lipase activity was determined with pH-stat titration (TitraLab 854, Radiometer Analytical SAS, Lyon, France) using 0.1 M NaOH. The substrate was prepared in a vial by adding 500 µl tributyrine to 15 ml colipase buffer (2 mM Tris-maleate, pH 7.0; 4 mM NaTDC; 1 mM $CaCl_2$ and 0.15 M NaCl) with addition of pure colipase (0.1 mg/ml; 10 µl) (Borgstrom & Erlanson1973). The analysis was performed at 25ºC and stirring was maintained using a magnetic rod under standardised conditions. Protein concentration was measured in the homogenates according to Bradford (Bradford1976). Activity was expressed as units per mg protein, where one unit corresponds to one micromole of fatty acids released per minute. An

aliquot of the solution of homogenised pancreas was added to the vial and the lipase/colipase activity recorded.

Statistical analysis.

All data are presented as mean±SEM. Differences were analysed statistically by two-way repeated-measures analysis of variance (ANOVA) (Statview version 5.0) followed by Bonferroni´s post-hoc test, where appropriate, or by Student´s t-test. A p-value<0.05 was considered statistically significant.

Results

Effect of long-term treatment with thylakoids on food intake, body weight and adiposity.

Feeding apoE-deficient mice a high-fat diet enriched with thylakoids resulted in decreased food intake (Figure 1A). The food intake of the mice fed the thylakoid-enriched diet remained lower than the control mice throughout the study. There was no dramatic change in food intake, hence suggesting that the palatability of the diet was not affected. A subsequent reduction in body weight gain was evident from approximately 20 days of feeding (Figure 1B). The mice fed the thylakoid-enriched diet weighed approximately 82 % of the control mice at the end of the study (35±1.5 g for control mice vs. 29±0.6 g for thylakoid-mice). Using dual energy X-ray analysis (DEXA), the thylakoid-diet reduced the amount of body fat mass by approximately 33 % (Figure 1C).

Effect of long-term treatment with thylakoids on blood lipids and glucose.

Feeding the mice thylakoid-enriched diet for 100 days resulted in a decrease of serum free fatty acids (FFA) levels compared to control feeding (Figure 2A). Triglycerides were also found to be lower (Figure 2B), whereas serum cholesterol was not affected (Figure 2C). Serum glucose concentrations were also decreased by the thylakoid-enriched diet (Figure 2D).

Effect of long-term treatment of thylakoids on serum lipase, CCK, PYY and leptin.

After 100 days of feeding pancreatic lipase activity was increased 1.7 fold in the thylakoid-fed animals compared to control (Figure 3A). CCK was increased approximately 1.5-fold (Figure 3B), whereas serum PYY was unaffected (Figure 3C). Serum leptin was decreased in the mice receiving the thylakoid-enriched diet (Figure 3D).

Discussion

This study shows that thylakoids significantly reduce food intake, body weight gain and body fat when added to a high-fat diet in mice. One significant finding in this long term study is that the effects on food intake and body weight as well as metabolites sustain throughout the study period.

The effect of thylakoids on satiety and body weight is believed to occur through the inhibition of pancreatic lipase/colipase activity during the hydrolysis of intraluminal fat. The retarded fat digestion in turn was shown to stimulate cholecystokinin (CCK) secretion in a previous study (Albertsson *et al.,*2007). Here we show that CCK is released also after long term treatment with thylakoids (Figure 3B).

CCK is a gut peptide promoting satiety and inhibiting food intake (Coll *et al.,*2007). It is released into the circulation from enteroendocrine CCK-cells in the upper small intestine by fatty acids and protein (French *et al.,*2000). Thylakoids, consisting of hydrophobic proteins and galactolipids, are able to accumulate at the surface of lipid droplets, thereby reducing the rate of fat hydrolysis catalysed by pancreatic lipase/colipase (Albertsson *et al.,*2007). CCK is released by free fatty acids rather than by unhydrolysed fat (Beglinger & Degen2004). We observed no sign of steatorrhea in the mice receiving the thylakoid diet. This supports the hypothesis that there is a complete absorption of dietary fat. Compared to the short-term treatment with thylakoid-enriched diet (Albertsson *et al.,*2007), long-term treatment with thylakoids resulted in a further increase in serum CCK levels (27% increase in the short-term (Albertsson *et al.,*2007) vs. 48% increase in the long-term). Thus the CCK release is a robust effect, which occurs both in short-term studies and long-term studies. Further

experiments are needed to understand whether CCK alone is responsible for the satiety effect or other gastro-intestinal peptides are involved.

The elevated level of CCK may explain why an increased lipase activity in the thylakoid treated animals was observed (Figure 3A). CCK has been reported to stimulate the release of exocrine pancreatic enzymes (Evilevitch *et al.,*2003). This is in agreement with previous studies where an elevated level of pancreatic lipase was observed in the thylakoid treated animals compared to control (Albertsson *et al.,*2007).

PYY is known to be released by dietary fat and hence could be a candidate for a fat specific satiety signal (Batterham *et al.,*2003; Degen *et al.,*2007). We found no statistical effect of thylakoids on the concentrations of PYY compared to the control animals. PYY is released from the distal gut (ileum and colon) (Batterham *et al.,*2002) and may not be relevant for the thylakoid effect, which starts already in the stomach or upper small intestine.

Serum leptin (Figure 3D) was significantly reduced by treatment with thylakoids, which is a consequence of the reduced adipose tissue mass (Figure 1C). This is in agreement with previous studies (Frederich *et al.,*1995). Reduced levels of leptin may suggest enhanced leptin sensitivity (Yamada *et al.,*2006), which may be positive to stimulate fatty acid oxidation.

At the end of the study serum glucose levels were lower in the thylakoid-treated mice compared to the control. This was true also for the triglyceride levels as well as the levels of free fatty acids. Both hyperglycemia and hyperlipidemia are major contributors to the development of type 2 diabetes (Prentki *et al.,*1998; Reaven1988). We have seen in our laboratory that sugar and fat at high concentrations have a inhibitory effect on ATP synthesis in insulinoma β-cells, leading to impaired insulin production (Kohnke *et*

*al.,*2007). The reducing effect of thylakoids on serum glucose, triglycerides and free fatty acids concentration are hence beneficial to prevent the development of type 2 diabetes (Winzell *et al.,*2003).

In spite of the reduction in free fatty acid and triglyceride levels there was no effect of the thylakoid diet on the development of atherosclerotic plaques in the apoE-deficient mice. ApoE is essential for the clearance of dietary cholesterol from the blood (Coleman *et al.,*2006). Genetic deletion of the apoE-gene generates mice with severe hypercholesterolemia. We saw no effect on serum cholesterol and probably for that reason we were unable to detect any difference between plaque size and plaque number in the aorta between the control mice and the mice receiving thylakoids (data not shown). Normally apoE-deficient mice develop plaques spontaneously on high-fat diet, starting after 10 weeks (Coleman *et al.,*2006). It might be that a longer time period is required to see significant differences on plaque size since the procedure of obtaining plaque in apoE-deficient mice is a rather slow process (Coleman *et al.,*2006).

In conclusion, thylakoids have a long-term suppressive effect on appetite and body weight gain. This effect is possibly mediated by CCK. Thylakoids might hence be suitable as potential treatment against obesity. One advantage is the ability of thylakoids to activate gastrointestinal satiety hormones, which may limit overeating. Present experiments have confirmed the ability of thylakoids to promote satiety signalling in healthy humans (Köhnke *et al.,*2009). Future studies will be performed where thylakoids are given as food supplement to obese individuals.

Acknowledgments

This work is supported by grants from the Swedish Medical Research Council (K2007-03X-007904-20A), the Färs and Frosta Foundation (Sparbankstiftelsen), Dr P. Håkanson Foundation and Ferrosan AB. The authors report no conflict of interest in the preparation of this paper. We thank Gunnel Roos for her technical expertise.

Figure legends

Figure 1. ApoE-deficient mice were offered either a high-fat diet or a high-fat diet with thylakoids for 100 days. The mice offered the thylakoid-enriched diet consumed less food than the control mice [$F_{(1,1372)}$=85,197, p<0.001] (food intake is presented as g/day per mice) (A). The decreased amount of food ingested by the mice offered the thylakoid-enriched diet resulted in a reduced body weight gain compared to the control mice [$F_{(1,700)}$=14,34, p<0.001] (B). The thylakoid diet gave a reduced amount of body fat (Figure 1C) as assessed using dual energy X-ray analysis (DEXA). ***, p<0.001.

Figure 2. After feeding the ApoE-deficient mice the thylakoid diet, serum levels of free fatty acids (FFA) were reduced compared to control (A). Thylakoid diet also resulted in decreased levels of serum triglycerides (TG) (B). Serum cholesterol was unaffected by the thylakoids (C), whereas serum glucose was decreased (D). *, p<0.05; n.s. (not significant).

Figure 3. Pancreatic lipase activity was increased by the thylakoid diet in mice compared to control (A). Serum levels of CCK were elevated in mice receiving thylakoid diet (B). Serum PYY was statistically unaffected by the thylakoid diet (C). Serum leptin was reduced in mice receiving thylakoid diet compared to control (D). *, p<0.05; **, p<0.01.

Table 1
Composition of diet

Content	**g/100g**
Casein	19,50
Cellulose	5,00
Glucose	7,50
Sucrose	7,50
Starch (corn)	34,35
Vitamin mix	1,00
Mineral mix	4,00
Cholesterol	0,15
Coconut butter	21,00
	100,00

	Control diet		Thylakoid diet	
Energy derived from:	g/100g	kcal%	g/100g	kcal%
Protein	17,2	15%	22,3	19%
Carbohydrate	50,4	44%	47,7	42%
Fat	20,8	41%	20,0	39%
Energy	457,6		460,0	

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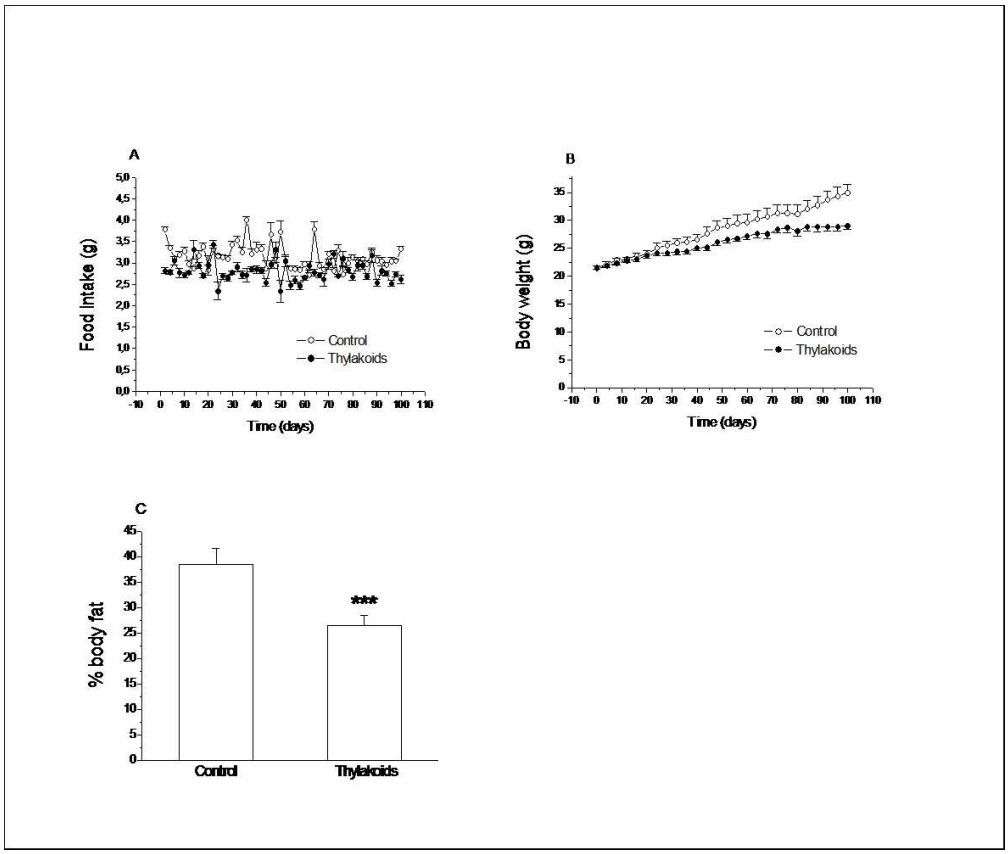

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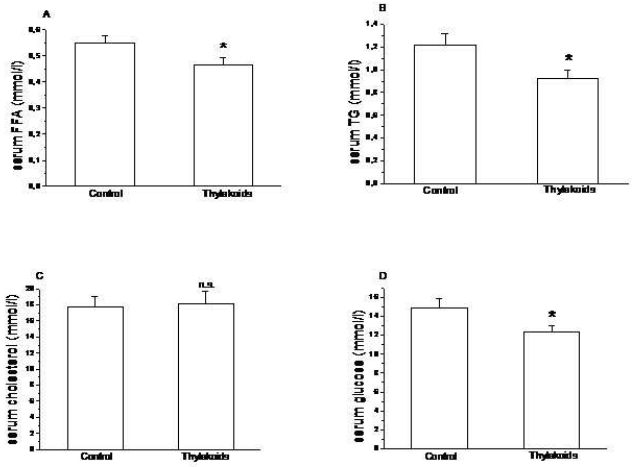

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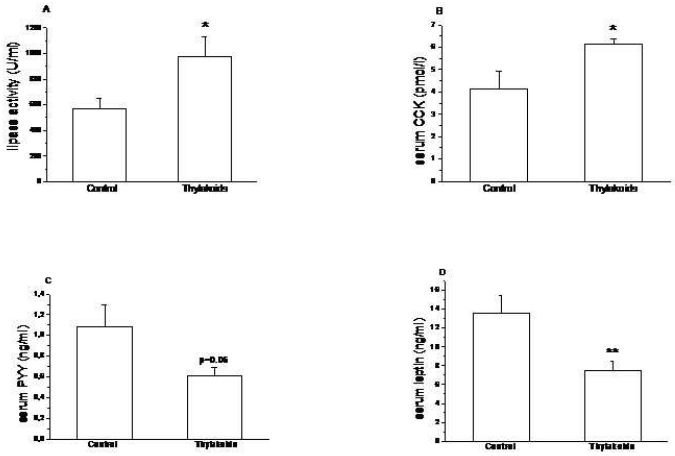

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