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Original article

Dietary thylakoids suppress blood glucose and modulate appetite-regulating hormones in pigs exposed to oral glucose tolerance test☆

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SUMMARY

Background & aims: Dietary chloroplast thylakoids have previously been found to reduce food intake and body weight in animal models, and to change metabolic profiles in humans in mixed-food meal studies. The aim of this study was to investigate the modulatory effects of thylakoids on glucose metabolism and appetite-regulating hormones during an oral glucose tolerance test in pigs fed a high fat diet.
Methods: Six pigs were fed a high fat diet (36 energy% fat) for one month before oral glucose tolerance test (1 g/kg D-glucose) was performed. The experiment was designed as a cross-over study, either with or without addition of 0.5 g/kg body weight of thylakoid powder.
Results: The supplementation of thylakoids to the oral glucose tolerance test resulted in decreased blood glucose concentrations during the first hour, increased plasma cholecystokinin concentrations during the first two hours, and decreased late postprandial secretion of ghrelin.
Conclusion: Dietary thylakoids may be a novel agent in reducing the glycaemic responses to high carbohydrate and high glycaemic index foods. Thylakoids may in the future be promising for treatment and prevention of diabetes, overweight and obesity.

1. Introduction

The global obesity epidemic continuous to increase, with 65% of the world's population living in countries where obesity and overweight cause more death than underweight.[1a] The increased intake of palatable food rich in fat and refined carbohydrates, together with the decreased physical activity, are the main causes for obesity and overweight.[1a,1,2] Moreover, appetite control is easily deviated with palatable food.[3,4] Thus, to counteract the obesity epidemic, focus on endocrine appetite signals for cravings of palatable foods, as well as focus on physical activity, are essential. The body weight is fundamentally regulated by food intake and energy expenditure. Several hormones are responsible for the regulation of food intake, such as ghrelin, cholecystokinin (CCK) and leptin.[5,6] The energy expenditure on the other hand is regulated by physical activity, the basal metabolic rate and thermogenesis. Therefore, reduction of body weight requires control of food intake and a change in energy expenditure.[7,8]

The use of dietary thylakoids, extracted from chloroplasts in green leaves, as satiety-strengthening food components have previously been described.[1,3,5,9–12] Thylakoids have demonstrated an effect on body-weight gain, decreased food intake as well as decreased percentage of body fat in mice and rats.[9,10,12] *In vitro*, thylakoids prolong the uptake of glucose and the decrease the passage of macromolecules over the rat intestinal wall.[13,14] The addition of thylakoids for 10 days in rat resulted in an altered microflora and decreased plasma–insulin response to an oral glucose tolerance test of the 10th day, while levels of blood glucose were unchanged.[12,14] Moreover, thylakoids have been shown to decrease the lipase activity both *in vitro*[11,15] and *in vivo* in pig.[1] In humans, supplementation of a high dose of thylakoids to a high-fat

Abbrevations: CCK, cholecystokinin; OGTT, oral glucose tolerance test; AUC, area under the curve; E%, energy percent.

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meal was shown to decrease the plasma concentrations of insulin, free fatty acids and the hunger hormone ghrelin, and simultaneously elevate plasma concentrations of the satiety hormone CCK.[3] A high-carbohydrate meal supplemented of with a low dose of thylakoids resulted in an increased plasma concentration of glucose, insulin and CCK two hours after start of the meal.[5]

Since previous effects on blood glucose and insulin are not clear, the aim of the present study was to clarify the effect of thylakoid supplementation on glucose metabolism and to investigate the effect on the appetite-regulating hormones as CCK and ghrelin further in pigs, who had consumed a high fat diet for four weeks (to mimic the westernized food-habits).

2. Materials and methods

2.1. Animals

The experiments were performed on crossbred pigs ((Yorkshire × Swedish Landrace) × Hampshire) obtained from Odarslöv research farm, belonging to the Swedish University of Agricultural Sciences (Alnarp, Sweden). The pigs were 11 weeks old with an average body weight of 13.7 ± 1.1 kg at the start of the experiment. The pigs were transported to the departments' animal facilities one month before start of the experiment, at the age of 7 weeks, and were fed 4% of their body weight/day of a high fat diet. The diet consisted of 75% of a conventional pig chow (Växtill 320, Lantmännen, Malmö, Sweden), mixed with 17% fresh milk cream (40% fat, Skånemejerier, Malmö, Sweden) and 8% rapeseed oil (Eldorado, Axfood, Solna, Sweden). The energy distribution of the high fat diet was 36 E% fat, 14 E% protein and 50 E% carbohydrate. National guidelines for the care and use of pigs were followed and all experimental procedures were approved by the Ethical Committee of Animal Experiments at Lund University (M93-11).

One week after adaptation to the animal facilities, the pigs were implanted with a jugular vein catheter, as described earlier.[7]

2.2. Thylakoids and Oral Glucose Tolerance Test (OGTT)

Thylakoids used in the present study were prepared from dry spinach leaves using the pH-method, as described by.[9] The thylakoid-slurry was drum dried to obtain a thylakoid powder, named *Appethyl*, donated by Green Leaf Medicals AB (Stockholm, Sweden).

Prior to the OGTT, pigs had been fasted overnight for approximately 18 h. Feeding was carried out via a syringe directly in the mouth during approximately 1 min. The oral glucose load consisted of 1 g/kg body weight of a 20% D-glucose solution, with or without supplementation of 0.5 g/kg body weight of thylakoids. Pigs had free access to tap water at all times. The test was a cross-over design, i.e. all pigs received one day of control OGTT and one day of thylakoid supplemented OGTT, separated by a wash-out of two days.

Baseline blood samples were taken from the jugular catheter before oral glucose loads were administered (time 0 min), and thereafter repeatedly during the day at 15, 30, 45, 60, 90, 120, 180, 240 and 360 min. In between blood sampling, the jugular catheter was flushed with saline (0.9% sodium chloride). Blood were collected into EDTA-tubes (6 mL), that were gently inverted several times after collection and stored on ice before being centrifuged 3000 × g for 15 min at 4 °C. Plasma samples were stored in cryogenic tubes at −20 before the insulin analysis (performed one week after the end of the experiment), and then at −80 °C.

2.3. Biochemical analysis

Blood glucose was analysed directly using a glucose-meter (ACCU-CHECK *Aviva*, Roche Diagnostics A/S, Hvidovre, Denmark).

Plasma insulin was analysed using an ELISA-kit for Porcine Insulin (10-1200-01, detection limit 0.01 μg/L, Mercodia AB, Uppsala, Sweden). Plasma CCK were measured using a radioimmunoassay (RIA) with a highly specific antiserum (no. 92128, detection limit 0.1 pmol/L).[16] Plasma ghrelin were measured using an RIA kit that recognises both the acylated and desacylated forms of the porcine hormone (RK-031-52, detection limit 10 pg/mL, Phoenix Pharmaceuticals GmbH, Karlsruhe, Germany).

2.4. Statistics

Statistical data analyses were done using Prism, version 6 (GraphPad Software, Inc, San Diego, CA, USA). Bonferroni corrected two-way Anova was used to calculate statistical differences between time points. For total comparison between thylakoid and control OGTT, both two-way Anova and numerical calculations of area under the curve (AUC) were used. Data were treated as not being normally distributed. All data are expressed as mean ± SEM. *P*-values <0.05 were considered to be statistically significant, and *p*-values <0.1 to be of interest to mention.

3. Results

The thylakoid supplemented OGTT, given orally to pigs, affected the outcome of blood glucose, plasma insulin and the hunger and satiety-regulating hormones ghrelin and CCK (Figs. 1–4).

Following the control OGTT, blood glucose concentrations were rapidly elevated and reached the highest level after 30 min (Fig. 1). Thereafter, a steep decrease was observed, and glucose concentrations below fasting levels were observed from 120 min. After the thylakoid supplemented OGTT, glucose concentrations initially increased rapid, but significantly lower peak concentrations were found at 30 min ($p < 0.01$) and 45 min ($p < 0.001$) compared to the control. The thylakoid supplemented OGTT in the later phase, measured with AUC between 120 and 360 min, resulted in blood glucose concentrations not below fasting levels, compared to after the control OGTT (control: 980 mmol/L vs. thylakoid: 1225 mmol/L; $p < 0.05$).

The plasma (*p*) insulin concentrations increased rapidly following the control OGTT, reaching the highest level after 30 min (Fig. 2). Thereafter, the concentrations decreased and returned to fasting concentrations at 120 min. The supplementation of thylakoids resulted in similar *p*-insulin concentrations during the first 60 min, with slightly lower concentrations found at 15 min and 30 min ($p < 0.1$), compared to control. Thereafter, the

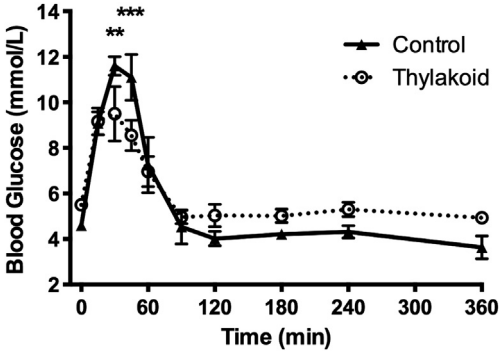

Fig. 1. Blood glucose following an oral glucose tolerance test (OGTT) in pigs, with or without supplementation of 0.5 g/kg body weight of thylakoids, resulted in significantly lower blood glucose levels the first hour, and no late postprandial hypoglycaemia (120–360 min) following the thylakoid OGTT (○), compared to the control OGTT (▲) ($p < 0.05$, calculated with AUC). Data are mean ± SEM, in a cross-over design in six pigs. Statistical differences between thylakoid and control OGTT is indicated by $^{**}p < 0.01$ and $^{***}p < 0.001$ (calculated with Bonferroni corrected 2-way Anova).

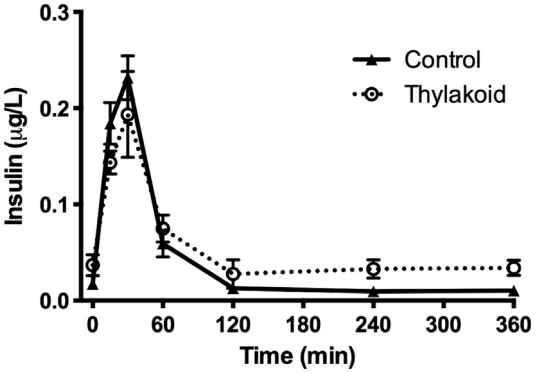

Fig. 2. Plasma insulin following an oral glucose tolerance test (OGTT) in pigs, with or without supplementation of 0.5 g/kg body weight of thylakoids, resulted in lower levels of *p*-insulin during the first hour, and elevated levels from 120 to 360 min after the thylakoid OGTT (○) compared to the control OGTT (▲) ($p < 0.1$, calculated with AUC). Data are mean ± SEM, in a cross-over design in six pigs.

AUC between 120 and 360 min resulted in elevated, but not significantly different, *p*-insulin concentrations for the thylakoid supplemented OGTT compared to the control OGTT (control: 2.54 µg/L vs. thylakoid: 7.64 µg/L; $p < 0.1$).

The concentrations of *p*-CCK increased rapidly after the control OGTT, peaking at 15 min, subsequently, the concentrations decreased immediately to fasting levels (Fig. 3). The thylakoid supplemented OGTT resulted in a significantly higher *p*-CCK concentration than control, being optimal at 30 min ($p < 0.05$), and continued to be increased during the following three hours. An increased CCK secretion during the entire experimental time, 0–360 min, was found following the thylakoid OGTT compared to the control OGTT, measured with AUC (control: 1415 pmol/L vs. thylakoid: 1852 pmol/L; $p < 0.1$).

The concentrations of *p*-ghrelin after the control OGTT decreased during the first hour, and thereafter increase above fasting levels in the 120–240 min interval (Fig. 4). With the addition of thylakoids, *p*-ghrelin concentrations decreased during the first hour, and stayed significantly lower at 120 min ($p < 0.001$), 180 min ($p < 0.001$) and 240 min ($p < 0.001$), compared to the control OGTT. The AUC between 0 and 360 min was lower, but not significantly different, after the thylakoid supplemented OGTT, compared to the control OGTT (control: 51,108 pg/mL vs. thylakoid: 40,971 pg/mL; $p < 0.1$).

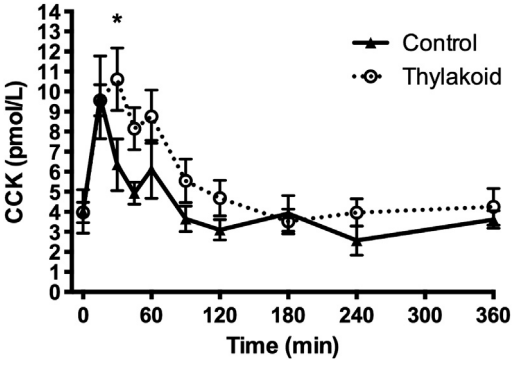

Fig. 3. Secretion of plasma cholecystokinin (CCK) after an oral glucose tolerance test (OGTT) in pigs with or without supplementation of 0.5 g/kg body weight of thylakoids, resulted in significantly increased levels of *p*-CCK 30 min after the thylakoid OGTT (○), compared to the control OGTT (▲). The secretion of *p*-CCK was also increased during the complete time period of 0–360 min for the thylakoid OGTT, compared to control OGTT ($p < 0.1$, calculated with AUC). Data are mean ± SEM, in a cross-over design in six pigs. Statistical differences between thylakoid and control OGTT is indicated by $^{*}p < 0.05$ (calculated with Bonferroni corrected 2-way Anova).

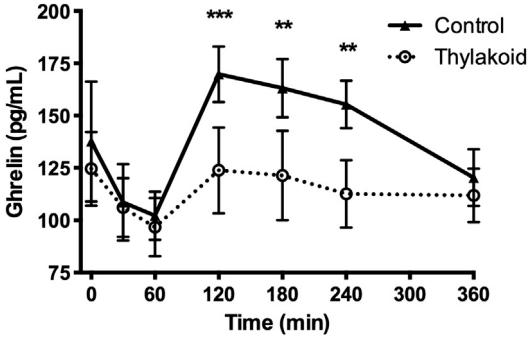

Fig. 4. Secretion of plasma ghrelin after an oral glucose tolerance test (OGTT) in pigs with or without supplementation of 0.5 g/kg body weight of thylakoids, resulted in significantly decreased levels of *p*-ghrelin from 120 to 240 min following the thylakoid OGTT (○), compared to control OGTT (▲). The secretion of *p*-ghrelin was also suppressed during the complete time period of 0–360 min for the thylakoid OGTT, compared to the control OGTT ($p < 0.1$, calculated with AUC). Data are mean ± SEM, in a cross-over design in six pigs. Statistical differences between thylakoid and control OGTT is indicated by $^{**}p < 0.01$ and $^{***}p < 0.001$ (calculated with Bonferroni corrected 2-way Anova).

4. Discussion

In this study we have found that the addition of thylakoids to an OGTT given to pigs, fed a high fat diet for four weeks, modify the postprandial concentrations of blood glucose, *p*-CCK and *p*-ghrelin in a significant manner, so as to promote glucose and energy balance.

The blood glucose-response after an OGTT is normally increased rapidly and then decreased below the concentrations found in the fasting state, as was also the case for the control OGTT in the present study. However, in the presence of thylakoids, the blood glucose concentrations did not increase to the same magnitude, and there was no postprandial hypoglycaemia. The supplementation of thylakoids thereby resulted in a similar blood glucose-response as can be seen after the consumption of a low GI-food.[13] The present study have thus shown that thylakoids have an effect on the blood glucose metabolism. We have previously shown in an *in vitro* model using rat small intestine in Ussing chambers, that the addition of thylakoids prolong the uptake of glucose significantly.[14] This was explained by the formation of an extra "membrane" seen with electron micrograph. This membrane can be formed since thylakoids expose both hydrophilic and hydrophobic surface groups, and these surface properties allow strong interaction with the intestinal surface. As thylakoids cover the intestinal mucosa, a prolongation of both the active and passive passage of glucose over the intestinal wall can be explained.[14] In addition, thylakoids was found to have an affinity of 17% to glucose, which also could, in part, explain a prolonged uptake since glucose would stay in the GI-tract for a longer time period. The similar phenomenon with a formation of a "thylakoid barrier" and binding to glucose would explain the present *in vivo* results.

The changed *p*-insulin levels after the addition of thylakoids reflect the lower but prolonged blood glucose concentrations. Taken together, the changed glucose and insulin curves after addition of thylakoids, compared to the control OGTT, indicate that dietary supplementation of thylakoids could be beneficial for suppressing the incidence of diabetes.[13]

CCK has been considered an important satiety hormone for years. The regulation of CCK secretion is similar in pigs and humans, and is stimulated both by carbohydrates, proteins and fat.[17,18] The effects of CCK include stimulation of pancreatic secretion of enzymes, pancreatic growth, gallbladder emptying, as well as control of gut motility and gastric emptying. The present results show a

steady increased secretion of *p*-CCK during the first two hours following the OGTT with addition of thylakoids, as compared to the control OGTT. The increased CCK secretion seen in our previous studies have been explained by the prolonged lipolysis caused by the accumulation of thylakoids at the surface of lipid droplets, as well as binding of thylakoids to lipase/colipase.[10] The secretion of CCK has previously been found to be increased as a response to low GI foods.[19] This was explained by a slower rate of gastric emptying and hence entry of carbohydrates into the proximal small intestine after intake of low GI foods compared to high GI foods, prolonging the stimulation of the CCK-secreting I cells.[19] The stimulation of I cells would further decrease gastric emptying and thereby prolong the CCK secretion. The same mechanistic explanation could theoretically explain how thylakoids may affect the secretion of CCK after an OGTT. The present results of thylakoids effect on CCK secretion does support our previous findings that thylakoids promote a continuous release of CCK for a prolonged time after nutrients have reached the stomach and intestines,[3,10] and that these effects are not only achieved by the fat content, but also by carbohydrates.

In recent years, the role of ghrelin to regulate pre-prandial hunger has been discussed. It has been reported that ghrelin administered systemically in doses mimicking basal plasma concentrations stimulates food intake in a broad range of animals.[20] Postprandially, when macronutrient composition and volume are kept constant, ghrelin is suppressed in proportion to the calories ingested.[21] Moreover, carbohydrates and proteins have been found to promote the ghrelin suppression post-prandially more potently than fat.[22,23] Present results of a postprandial suppression of the ghrelin secretion may be explained by the prolonged gastric emptying, as discussed regarding CCK above. Since ghrelin is primarily synthesised and secreted from endocrine cells in the gastric fundus,[20] a prolonged gastric emptying would theoretically blunt the synthesis and secretion of ghrelin as thylakoids was added to the OGTT. In long-term regulation of body weight and increased adiposity, ghrelin has also been found to play a crucial role, as rodents given chronic administration gain weight and have an increased stimulation of adipogenesis as long as the administration continuous.[20,24] The postprandial suppression of *p*-ghrelin found in the present study demonstrates that the supplementation of thylakoids may be important for suppression of hunger, and thereby might be useful for suppression of overweight and adiposity.

Control of the secretion of gut hormones, such as ghrelin and CCK, are of greatest interest for long-term approaches to control weight and counteract metabolic diseases, without deleterious side effects.[25,26] The present study demonstrates that the addition of thylakoids to glucose influences the release of relevant gut hormones, by increasing the secretion of *p*-CCK, and suppressing the late post-prandial secretion of *p*-ghrelin. Moreover, the addition of thylakoids lowered the blood glucose curve, similar to curves seen after ingesting a low GI-food. With lower blood glucose and insulin responses in daily life, the risk of cardiovascular disease and the prevalence of diabetes type 2 and of obesity is diminished.[13] If thylakoids have the same effects in overweight/obese/healthy humans, and in diabetic/pre-diabetic patients, as shown in the present study will be addressed in the future. However, several longer studies have been performed in mice and rats, showing that the addition of thylakoids to the diet, for a time period of 10–100 days, does affect the ad libitum food intake, body weight gain, body composition and several metabolic profiles such as glucose, insulin, free fatty acids, triglycerides, leptin and CCK.[9–12] The proposed mechanisms of action for thylakoids are 1) a prevention of postprandial hypoglycaemia by a prolonged uptake of glucose through both a direct binding of glucose to thylakoids, and a binding of thylakoids to the gastric and intestinal mucosa, 2) a possible interference by thylakoids to the small intestinal mucosa affecting the direct release of gut hormones, and 3) a delayed gastric emptying affecting secretion of CCK and ghrelin.

In summary, the present study shows that thylakoids affect intestinal glucose absorption and the secretion of major gut hormones involved in appetite regulation, such as CCK and ghrelin, after an OGTT in pigs. We suggest thylakoids as a promising novel food component for reducing the glycaemic index after a high-carbohydrate load and in high glycaemic foods. Thylakoids may therefore in the future be useful for prevention of diabetes, overweight and obesity.

Statement of authorship

C.M. is responsible for all parts of this work. K.S. and M.K. assisted in performing the experiments and in animal care. L.L. performed the insulin analysis and J.F.R performed the CCK analysis and assisted in writing the paper. S.P., C.E-A and B.W. assisted in planning the study, interpreting the results and in writing the paper. All authors have read and approved the final manuscript.

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Conflict of interest statement

CEA has an on-going collaboration with Green Leaf Medicals AB. The other authors have no interests to declare.

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