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# **Supplementation by thylakoids to a high carbohydrate meal decreases feelings of hunger, elevates CCK levels and prevents postprandial hypoglycaemia in overweight women**

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## **Abstract**

Thylakoids are chlorophyll-containing membranes in chloroplasts that have been isolated from green leaves. It has been previously shown that thylakoids supplemented with a high-fat meal can affect cholecystokinin (CCK), ghrelin, insulin and blood lipids in humans, and can act to suppress food intake and prevent body weight gain in rodents. This study investigates the addition of thylakoids to a high carbohydrate meal and its effects upon hunger motivation and fullness, and the levels of glucose, insulin, CCK, ghrelin and tumour necrosis factor (TNF)-alpha in overweight women.

Twenty moderately overweight female subjects received test meals on three different occasions; two thylakoid enriched and one control, separated by one week. The test meals consisted of a high carbohydrate Swedish breakfast, with or without addition of thylakoids. Blood samples and VAS-questionnaires were evaluated over a four-hour period.

Addition of thylakoids suppressed hunger motivation and increased secretion of CCK from 180 minutes, and prevented postprandial hypoglycaemia from 90 minutes following food intake. These effects indicate that thylakoids may intensify signals of satiety. This study therefore suggests that the dietary addition of thylakoids could aid efforts to reduce food intake and prevent compensational eating later in the day, which may help to reduce body weight over time.

**Keywords:**

hunger, satiety, meal supplement, glucose, insulin, cholecystokinin

## **Introduction**

Obesity is a multifactorial disease developing from an interaction of the genotype and environment, which involves social, behavioural, cultural, physiological and metabolic factors. Under contemporary circumstances of modern society, our inherent appetite regulation may often be considered too weak to control energy homeostasis in that satiety signals may not always be strong enough to inhibit the effect of hunger signals (Erlanson-Albertsson, 2005). New ways to strengthen the appetite signalling system may be achievable by the enhancement of satiety hormones and the decreasing of hunger hormones. Even though by the most energydense nutrient of fat also generates potent satiety signals via cholecystokinin (CCK), it is not able to inhibit energy intake fast enough to prevent overconsumption (Holt, Miller, Petocz, & Farmakalidis, 1995). In Sweden, intake of carbohydrates and proteins increased during the last decades, while the intake of fat has remained stable (Swedish Board of Agriculture, 2009). The increased intake of refined carbohydrates, such as corn syrup, and a decreased consumption of fibers have appeared to evolve in parallel with the increased prevalence of type 2 diabetes during the 20th century (Gross, Li, Ford, & Liu, 2004; Holt et al., 1995). This increased ingestion of refined carbohydrates has also been shown to correlate with elevated levels of triacylglycerides and low density lipoprotein-cholesterol, and decreased high density lipoprotein-cholesterol, all of which are risk factors for cardiovascular disease (Siri-Tarino, Sun, Hu, & Krauss, 2010).

Today, popular diets rich in fat or in wholegrain carbohydrates are advertised as good ways to lose weight, by resulting in a lower over-all consumption of energy, and in many cases, high-fat diets result in short term weight reduction and improved glycemic control. However, in the long-term this type of diet may have detrimental affects on cardiovascular health, and

cognitive function, and could be associated with inflammation (Frigolet, Ramos Barragán, & Tamez González, 2011). On the other hand, diets rich in wholegrain carbohydrates are generally less tolerated by patients with various bowel diseases and syndromes (Smith, Humes, & Spiller, 2010; Unlü, Daniels, Vrouenraets, & Boermeester, 2012). There is therefore a need to find alternative and healthier satiety-promoting food supplements and additives, which are not associated with drastic changes in the macromolecular energy distribution of the meal.

Thylakoid membranes, part of the chloroplast extracted from green leaves, have been shown to prolong *in vitro* lipolysis by binding to both dietary fat, lipase and colipase (Albertsson et al., 2007). Thylakoids have also been shown *in vitro* to prolong the passage of glucose through the intestinal wall due to steric hindrance, and by the binding of macromolecules present on the mucosal side (Montelius et al., 2011). In both short-term and long-term studies with rats, thylakoids were shown to be associated with decreased food intake and body weight (Emek et al., 2010; Köhnke, Lindqvist, Göransson, Emek, Albertsson, Rehfeld, Hultgårdh- Nilsson, & Erlanson-Albertsson, 2009b). In humans, thylakoids have been suggested to increase satiety signals such as CCK, and decrease hunger signals such as ghrelin, in a single meal study consisting of 45% fat (Köhnke, Lindbo, Larsson, Lindqvist, Rayner, Emek, Albertsson, Rehfeld, et al., 2009a). A reduction of the insulin secretion by thylakoids has also been found in porcine and human studies (Köhnke, Lindbo, Larsson, Lindqvist, Rayner, Emek, Albertsson, Rehfeld, et al., 2009a; Köhnke et al., 2010).

The aim of the present study is to investigate the effects of thylakoid supplementation in a high carbohydrate breakfast on ratings of hunger, fullness, urge to eat and thoughts of food, as

well as serum levels of glucose, insulin, CCK, ghrelin and tumour necrosis factor (TNF)-alpha in overweight women.

## **Materials and methods**

### **Subjects**

The study was conducted on twenty healthy women aged 39-69 years with BMI 24-30 (non-vegetarian and non-smoking). The baseline characteristics of the participants are listed in Table 1. Participants were recruited through advertisement in the local community. Exclusion criteria included diabetes, inflammatory bowel disease, thyroid disease, food allergies, food intolerance and recent use of antibiotics. Procedures, objectives and requirements of the study were explained in detail to the participants, and a written consent was signed before the study started. All participants received a compensation of 1100 SEK (taxable) after completing the study. The Ethical Committee of Lund University approved the study protocol, and the research adheres to the tenants of the Declaration of Helsinki.

### **Experimental study design**

The study was conducted at the Overweight and Diabetes Unit, Skåne University Hospital, Lund, Sweden, in 2011, as a single-blinded, randomized, single centre meal supplementation study. Participants were given a high carbohydrate breakfast on three occasions, with one acting as a control and two being thylakoid-enriched. The washout period was of at least one week in duration.

The subjects were instructed not to consume high-fibre foods and alcohol, and avoid excessive physical activity on the day prior the experiment. After 8.00 pm the evening before each test day, no further intake of food or liquid was allowed, and subjects arrived fasting the

next morning. Subjects were weighed and measured the first day. The first blood sample was taken before breakfast was served. The addition of thylakoids to the thylakoid breakfasts was 3.7 g and 7.4 g respectively. All subjects were told to finish their breakfasts in under 15 minutes, after which no food or fluid was allowed for the next four hours. The subjects stayed on the premises during experimental days, and were asked to keep relatively still in a quiet, non-stressful environment, though sleeping was not allowed. Blood samples were taken and questionnaires filled out at 0, 15, 30, 45, 60, 90, 120, 180 and 240 minutes after the start of the meal. Subjects received the diet of low dose thylakoids, high dose thylakoids and control in a randomised order. All reported adverse events during and after trial days were registered.

### **Biochemical analyses**

Blood samples were taken through a venous catheter in the arm continuously during trial days. Plasma concentrations of glucose, insulin and CCK were measured at all time points, ghrelin was measured at 0, 30, 60, 90, 120, 180 and 240 minutes, and TNF-alpha was measured at 0 and 240 minutes after the start of the meal.

Plasma glucose was measured with an direct apparatus, HemoCue Glucose 201 (HemoCue AB, Ängelholm, Sweden). Plasma insulin and TNF-alpha were analysed by standard methods in the Department of Clinical Chemistry at Skåne University Hospital, Lund, Sweden. Plasma CCK was measured with a radio immunoassay using a highly specific antiserum (no. 92128) (Rehfeld, 1998). Plasma immunoreactive ghrelin was measured with a RIA human kit that recognises the acylated and desacyl forms of the hormone (Phoenix Pharmaceuticals, Belmont, CA, USA).



## **Test Breakfasts**

A common Swedish breakfast with high carbohydrate content (Table 2) was served at three separate occasions. The energy content of the breakfast was calculated upon the needs of a moderately active woman aged 31-60 years. The thylakoid breakfasts were adjusted (Table 2) for caloric and nutritional values, as the energy content of 100 g of thylakoid powder is 1470 kJ (351 kcal) (20.6 g carbohydrates, 45.8 g protein and 9.2 g fat). Total energy content and distribution of the breakfasts are presented in Table 3. The thylakoid powder was mixed with blackcurrant jam. Before serving, the blackcurrant jam was blended with yoghurt and muesli was placed on top. The jam contained no thylakoids in the control breakfast. Thylakoids have a taste of green tea and are a dark green colour. However, subjects were unable to distinguish any difference between the thylakoid and control breakfasts, since taste, colour and texture were concealed in the blackcurrant jam.

The thylakoids used in the present study were prepared by SwePharm AB (Södra Sandby, Sweden), using previously described methods (Albertsson et al., 2007). The particle size of the thylakoids was < 315 µm. The thylakoid powder contained 36.4 mg chlorophyll per gram.

## **Questionnaire**

At regular intervals throughout the experiment, participants answered questions about their state of appetite (Table 4). The questionnaires were designed as Visual Analogue Scales (VAS) (Flint, Raben, Blundell, & Astrup, 2000). Written instructions were given on the front page of the questionnaire. In addition, each subject was individually instructed on how to complete the questionnaire so as to avoid misinterpretation. Each question was followed by a 100 mm line anchored at each end by a descriptor (Table 4). Subjects were instructed to place a vertical line across the scale, thus rating objective sensations at every specific timepoint.

Ratings were scored as mm between “not at all” and the rater’s mark. Three of the parameters (hunger, urge to eat and thoughts of food) were analysed together, with the mean of all three parameters presented as one single graph termed “hunger motivation”.

## **Statistics**

Energy and macronutrient composition of the test meals were measured using the programme Dietist XP (Kostdata, Bromma, Sweden). All data were normally distributed, and were analysed for statistical significances using GraphPad Prism, version 4 (GraphPad Software, Inc, San Diego, CA, USA). Numerical calculations of area under the curve (AUC), mean score across time, were used to compare the outcome of control versus thylakoid diets. Wilcoxon signed rank test was used for all comparisons between thylakoid and control. Data are expressed as mean +/- standard error of the mean (SEM). Baseline characteristics (table 1) are expressed as standard deviation (SD). P-values  $\leq 0.05$  were considered to be statistically significant.

## **Results**

There were no statistically significant differences between the two concentrations of thylakoids (3.7g and 7.4g), using either objective or subjective measurements ( $p > 0.5$  for all parameters). The material was therefore analysed and presented as thylakoid vs control.

The supplementation of a high carbohydrate breakfast by thylakoids resulted in decreased ratings of hunger, thoughts of food and the urge to eat. These graphs had identical shape and the same score, and were therefore analysed and presented as a single graph, termed hunger motivation (Fig 1). As seen in this graph, hunger motivation was suppressed following

feeding in a similar way between thylakoids and control up to 120 minutes. From 180 minutes there was a significant difference in hunger motivation between thylakoid and control ( $p = 0.05$ ), with the thylakoid diet being associated with the suppression of hunger motivation (Fig 1). Numerical differences in rated fullness were not statistically reliable.

The secretion of CCK was increased from 180 minutes ( $p = 0.05$ ) following the thylakoid breakfast, compared to control (Fig 2). The concentrations of TNF- $\alpha$  decreased significantly from 0 to 240 minutes after both the thylakoid ( $p < 0.001$ ) and the control breakfasts ( $p < 0.05$ ), but no difference between the two meals was observed (Fig 3).

The supplementation of thylakoids resulted in a tendency towards higher plasma glucose levels from 90 minutes ( $p = 0.09$ ), compared to control (Fig 4). However, calculation of total AUC for control versus thylakoid breakfasts was statistically not significant ( $p > 0.05$ ). The concentration of insulin was also significantly higher than control from 90 minutes after supplementation with thylakoids ( $p < 0.05$ ) (Fig 5).

All subjects completed the entire study. No adverse events were reported during or after the trial.

## **Discussion**

Supplementation by thylakoids resulted in a greater increase in CCK levels from 180 minutes than control. In an earlier single meal study with humans, a high fat diet and higher concentrations of thylakoids (10, 25 and 50 g respectively) were used, resulting in increased CCK levels with thylakoid supplementation (Albertsson et al., 2007; Köhnke, Lindbo, Larsson, Lindqvist, Rayner, Emek, Albertsson, Rehfeld, et al., 2009a). In the present study,

we demonstrate that supplementation of a high carbohydrate / low fat breakfast by thylakoids at lower doses results in increased CCK levels three hours postprandially. The release of CCK is known to be dependent on the hydrolysis of triglycerides into fatty acids in humans (Beglinger et al., 2010) and an irreversible lipase inhibitor such as orlistat attenuates the release of CCK (Ellrichmann et al., 2008). The observed augmented release of CCK by thylakoids in our experiments suggests that fat hydrolysis does occur, though a longer time is needed since the augmented CCK response is observed only at late time points, i.e. after 180 minutes, which is in agreement with previous observations (Köhnke, Lindbo, Larsson, Lindqvist, Rayner, Emek, Albertsson, Rehfeld, et al., 2009a). Elevated levels of CCK are consistent with the observed reduced sensation of appetite. Furthermore, thylakoids, unlike orlistat (Baretić, 2012), do not cause steatorrhea.

Plasma glucose levels increased rapidly after intake of both control and thylakoid breakfasts, which was to be expected following a high carbohydrate breakfast. The first peaks in glucose levels are identical after the control and thylakoid breakfasts, suggesting that easily digested carbohydrates, with high concentration of sugars, were not affected by the supplementation of thylakoids. However, supplementation resulted in higher glucose levels from 90 minutes after the start of the breakfast, and a second peak in blood glucose was observed at 120 minutes. This did not occur after the control breakfast, where glucose levels dropped below fasting level at 60 minutes and continued to decrease. An explanation of the second blood glucose peak at 120 minutes may be a prolongation of carbohydrate digestion and absorption, caused by thylakoids. Such a hypothesis is supported by calculations of the total AUC of glucose in the present study, where there was no statistically significant difference between thylakoid and control breakfast. Furthermore, findings in a previous *in vitro* study support the result of the present study, in that thylakoid membranes appear to prolong the uptake of free glucose

through the intestinal wall of the small intestine (Montelius et al., 2011). The mechanism of action for thylakoids is not clear, but may involve binding to disaccharidases in the brush border of the mucosa, which are responsible for the hydrolysis of oligosaccharides to glucose. We propose that preventing postprandial hypoglycaemia is important for weight control in the long run. Such a hypothesis is supported by a recent report showing that a reactive hypoglycaemia was predictive of a more pronounced weight gain in humans (Tremblay & Chaput, 2012). Increased hypothalamic glucose sensing has also been shown to be important for the suppression of hunger hormones, such as Agouti related peptide and neuropeptide Y (Jordan, Könnér, & Brüning, 2010).

The secretion of insulin was identical after the thylakoid and control breakfasts during the first 90 minutes following the start of the meal. Thereafter, the supplementation of thylakoids resulted in stabilised levels of insulin compared to control. The slightly elevated insulin concentrations at 120 and 180 minutes may indicate that thylakoids have an incretin effect (Hardikar, 2004; Holst & Gromada, 2004). Also, these increased insulin levels postprandially may be an effect of CCK and glucagon-like peptide-1 (GLP-1), since both peptides act to promote satiety.

Different foods and meal compositions have various effects on inflammatory markers (Egger & Dixon, 2010), such as TNF-alpha. Meals high in refined carbohydrates with a high GI-value have been regarded as pro-inflammatory, while low-GI foods have been regarded as anti-inflammatory. The present test-meals, although carbohydrate-rich with high GI-values, resulted in significantly decreased TNF-alpha concentrations between 0 and 240 minutes. We speculate that this could arise from the high content of dietary fibre and antioxidants in the

blackcurrant jam, orange and red pepper. Indeed, no difference in TNF-alpha concentration between the thylakoid and control breakfasts was found.

The effects found after supplementation of thylakoid in the present study indicate a prolongation of the phase satiation seen after the control meal. The increased levels of CCK at later timepoints might explain, at least in part, the decreased ratings of hunger motivation described. Subjects experienced less hunger motivation from 180 minutes after the thylakoid breakfast compared to control. The higher blood glucose levels from 90 minutes, preventing postprandial hypoglycaemia, may also explain this observation. These findings agree with Jean Mayers' proposal 50 years ago that blood glucose concentrations act to regulate energy intake and that a trend towards hypoglycaemia and/or glucose instability might induce excess energy intake and overweight in humans (Chaput & Tremblay, 2009).

A possible limitation of this study was the limited number of time points that the subjects were followed after intake of the test meals. In future studies, subjects may be best followed during the entire day, with all meals eaten under observation to monitor second meal effects.

In summary, we have found that supplementation of a single, carbohydrate rich breakfast with thylakoids suppress feelings of hunger. We also show that thylakoid supplementation increases secretion of CCK three hours postprandially, and prevents postprandial hypoglycaemia from 90 minutes after the commencement of the meal. If the supplementation of thylakoids can result in a consistent reduction in food intake and prevent compensatory eating later in the day, we suggest that thylakoids may help in a program of weight reduction over time.

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**Table 1.** Baseline characteristics of the twenty subjects of the study.

<b>Variable</b>	<b>Range</b>	<b>Mean</b>	<b>SD</b>
Age (years)	39-69	53.3	7.49
Body weight (kg)	64.6-85.7	74.7	6.42
BMI (kg/m <sup>2</sup> )	24.6-31.8	27.0	1.65
Waist circumference (cm)	78-102	88.9	6.25
Waist/hip ratio	0.73-0.98	0.86	0.06

**Table 2:** The composition of the control and thylakoid supplemented breakfasts.

<b>Ingredients</b>	<b>Control meal (g)</b>	<b>Low-dose</b>	<b>High-dose</b>
		<b>thylakoid meal (g)</b>	<b>thylakoid meal (g)</b>
Muesli - fruits and nuts, homemade	55	60	60
White bread (Skogaholmslimpan, Pågen AB, Malmö, Sweden)	40	40	40
Vanilla yogurt 2.5 % fat (Skånemejerier, Malmö, Sweden)	180	175	175
Cheese 17 % fat (Herrgårdsost, Skånemejerier, Malmö, Sweden)	20	20	18
Ham 6 % fat	31	20	18
Butter 60 % fat	2.0	2.8	3.5
Black currant jam	50	50	50
Orange juice	230	220	220
Red pepper	25	20	20
Banana	10	25	20
Orange	50	0	0

**Table 3:** Nutritional composition of the control and thylakoid supplemented breakfasts.

<b>Nutritional value</b>	<b>Control meal</b>		<b>Low-dose thylakoid meal</b>		<b>High-dose thylakoid meal</b>	
<sup>1</sup> E% = Energy %						
Energy content	554 kcal	2319 kJ	546 kcal	2282 kJ	537 kcal	2245 kJ
Carbohydrates	112.6 g	71 E% <sup>1</sup>	113.1 g	71 E% <sup>1</sup>	113.0 g	71 E% <sup>1</sup>
- Sucrose	11.0 g		11.0		11.0 g	
- Fibres	8.5 g		8.0		8.0 g	
Fat	16.9 g	11 E% <sup>1</sup>	17.5 g	11 E% <sup>1</sup>	17.9 g	11 E% <sup>1</sup>
Protein	28.6 g	18 E% <sup>1</sup>	28.4 g	18 E% <sup>1</sup>	29.6 g	18 E% <sup>1</sup>

**Table 4:** Questions and anchors for line ratings of strength of appetite.

<b>Questions</b>	<b>Anchor phrases</b>	
	<b>Score 0</b>	<b>Score 100</b>
How hungry are you right now?	Not at all hungry	Hungrier than I ever felt before
How full are you right now?	Not at all full	More full than I ever felt before
How strong is your urge to eat right now?	I have no urge to eat at all	Very strong urge to eat right now
How preoccupied are you with thoughts of food right now?	Not thinking of food at all	I can hardly think of anything but food

## Legends

Fig 1. Hunger motivation (mean of feelings of hunger, the urge to eat and thoughts of food), rated with questionnaires over four hours, after intake of either a thylakoid (▲) or control meal (■). Rating of 100 at Y-axis means very hungry, very strong urge to eat and I can't think of anything but food (Table 4). Rating of 0 means not hungry, no urge to eat and not thinking of food. From 180 minutes after the start of the thylakoid-supplemented meal (Thyl), compared to the control (Ctr) meal, subjects rated their hunger motivation lower (AUC,  $p = 0.05$ ).

Fig 2. The secretion of cholecystokinin (CCK) measured over four hours, after intake of either a thylakoid (▲) or a control meal (■). Thylakoid supplementation (Thyl) resulted in increased secretion of CCK from 180 minutes (AUC,  $p=0.050$ ) compared to control (Ctr).

Fig 3. Levels of TNF-alpha measured in plasma before the start of the meal (0 min) and 240 minutes after intake of either a thylakoid or a control meal. Both meals resulted in significantly decreased levels;  $p < 0.05$  for the control meal and  $p < 0.001$  for the thylakoid meal (AUC). No difference between thylakoid and control meals was observed.

Fig 4. Plasma glucose levels measured over four hours after food intake. The supplementation of thylakoids (▲) resulted in a tendency of higher glucose from 90 minutes after the start of the meal (AUC,  $p < 0.1$ ) compared to control (■), thus

preventing hypoglycaemia. There were no significant differences in AUC between thylakoid (Thyl) and control (Ctr) breakfasts for the entire four-hour study ( $p > 0.05$ ).

Fig 5. Plasma insulin measured over four hours after the start of the meal. The supplementation of thylakoids (Thyl, ▲) resulted in stabilised insulin levels from 90 minutes (AUC,  $p < 0.01$ ) compared to control (Ctr, ■).

Figure 1.

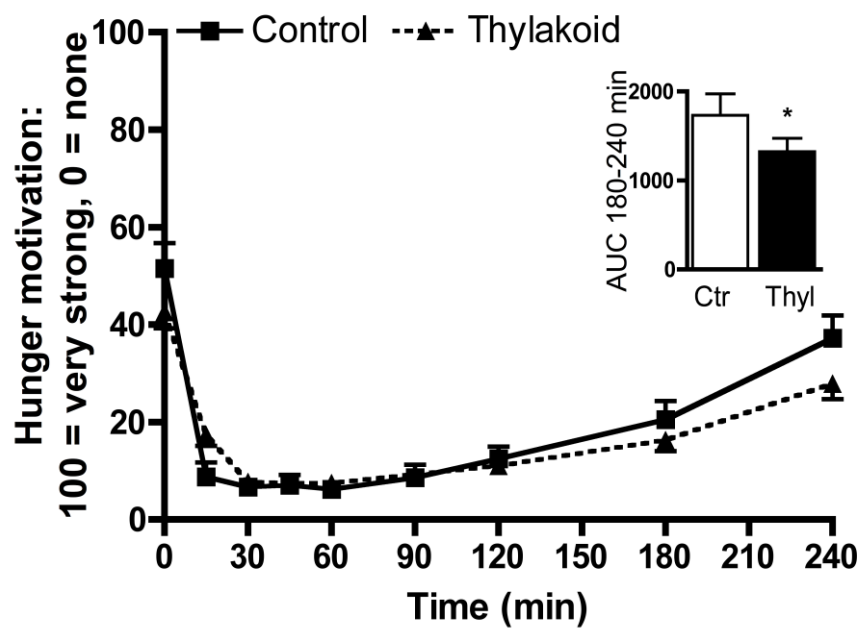




Figure 2.

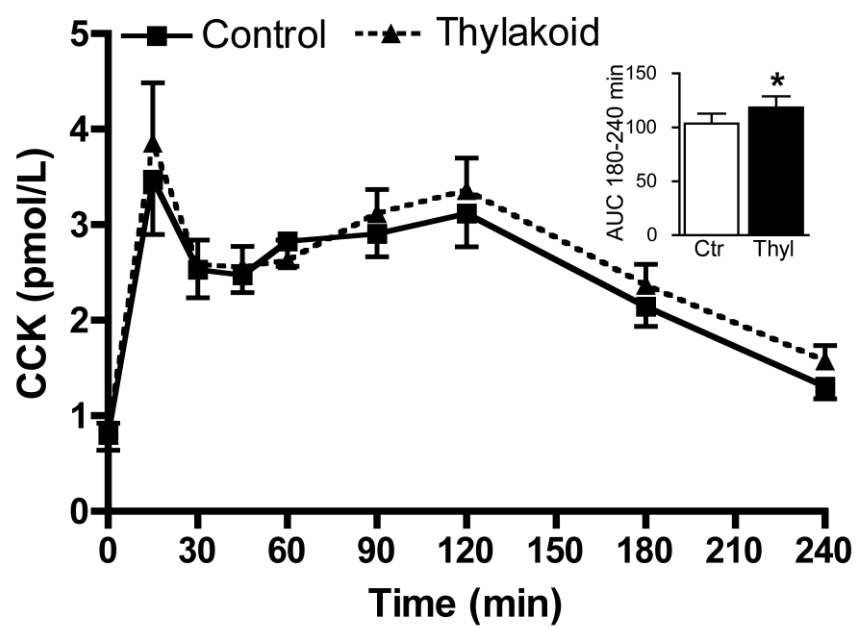


Figure 3.

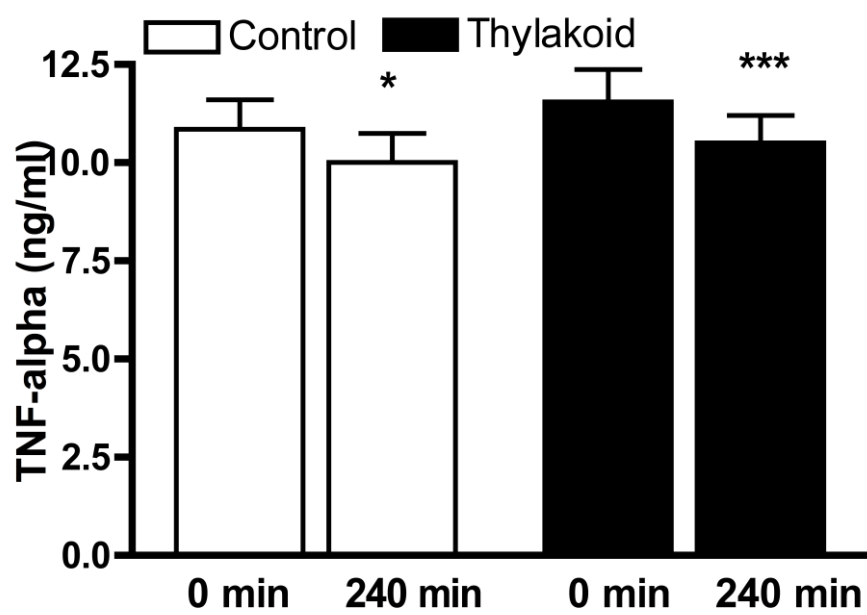


Figure 4.

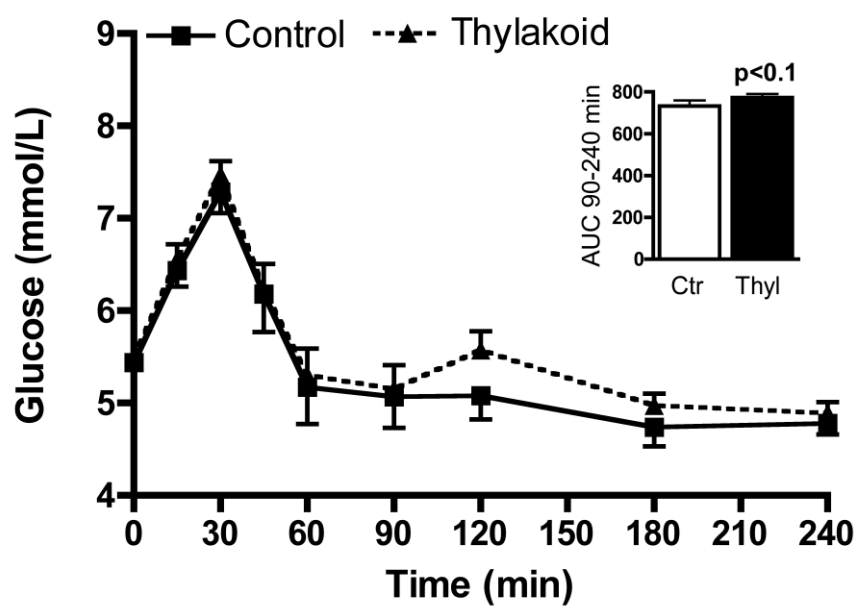


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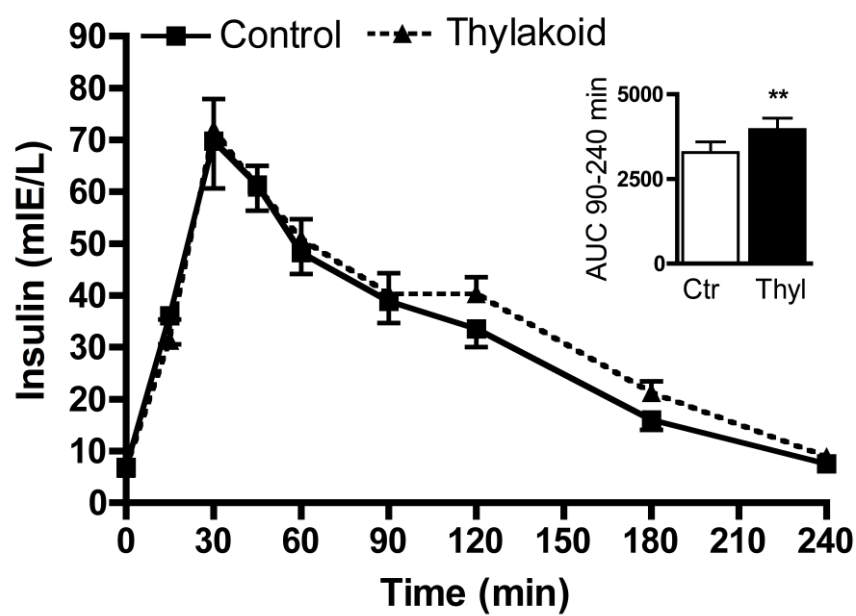


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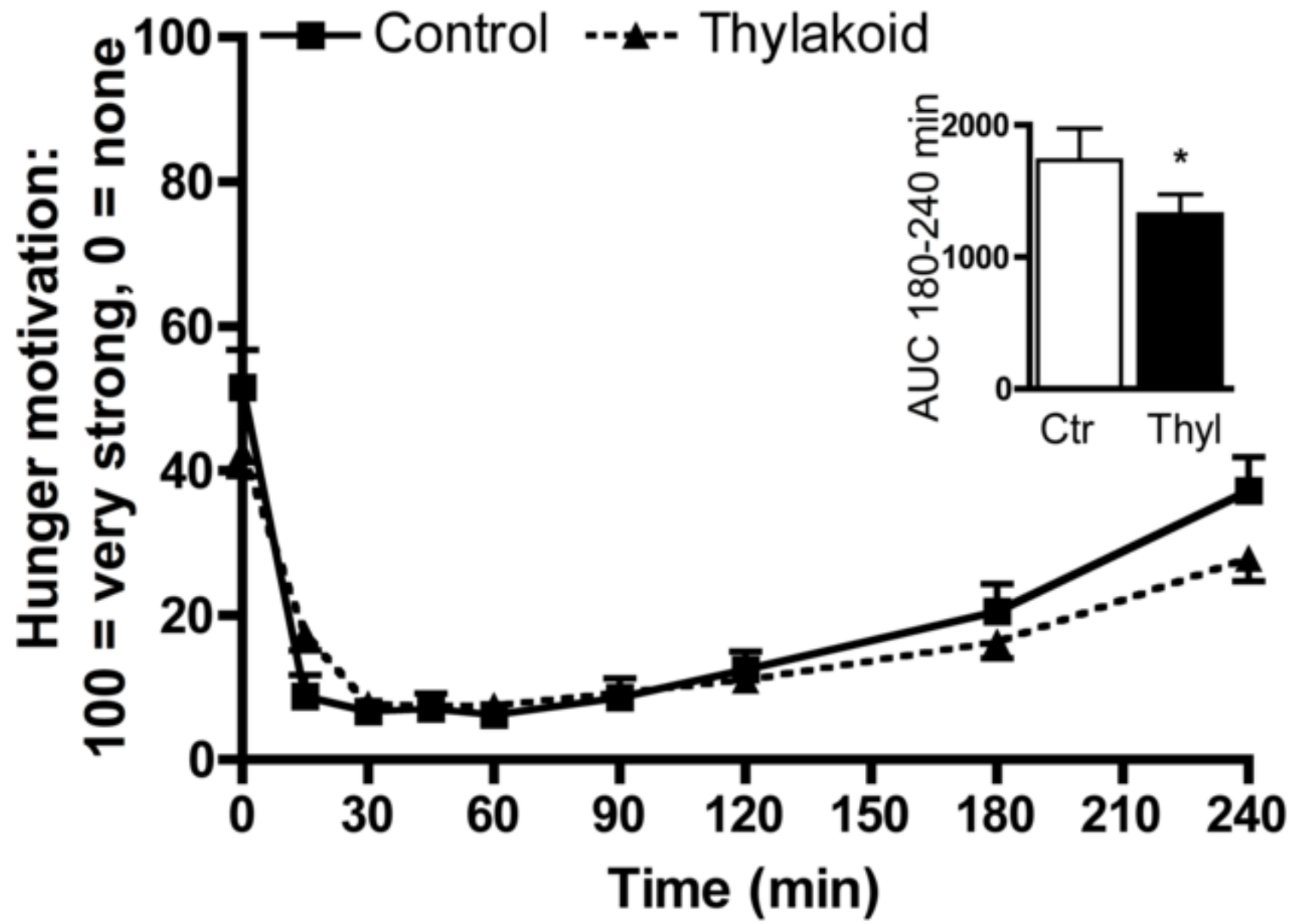


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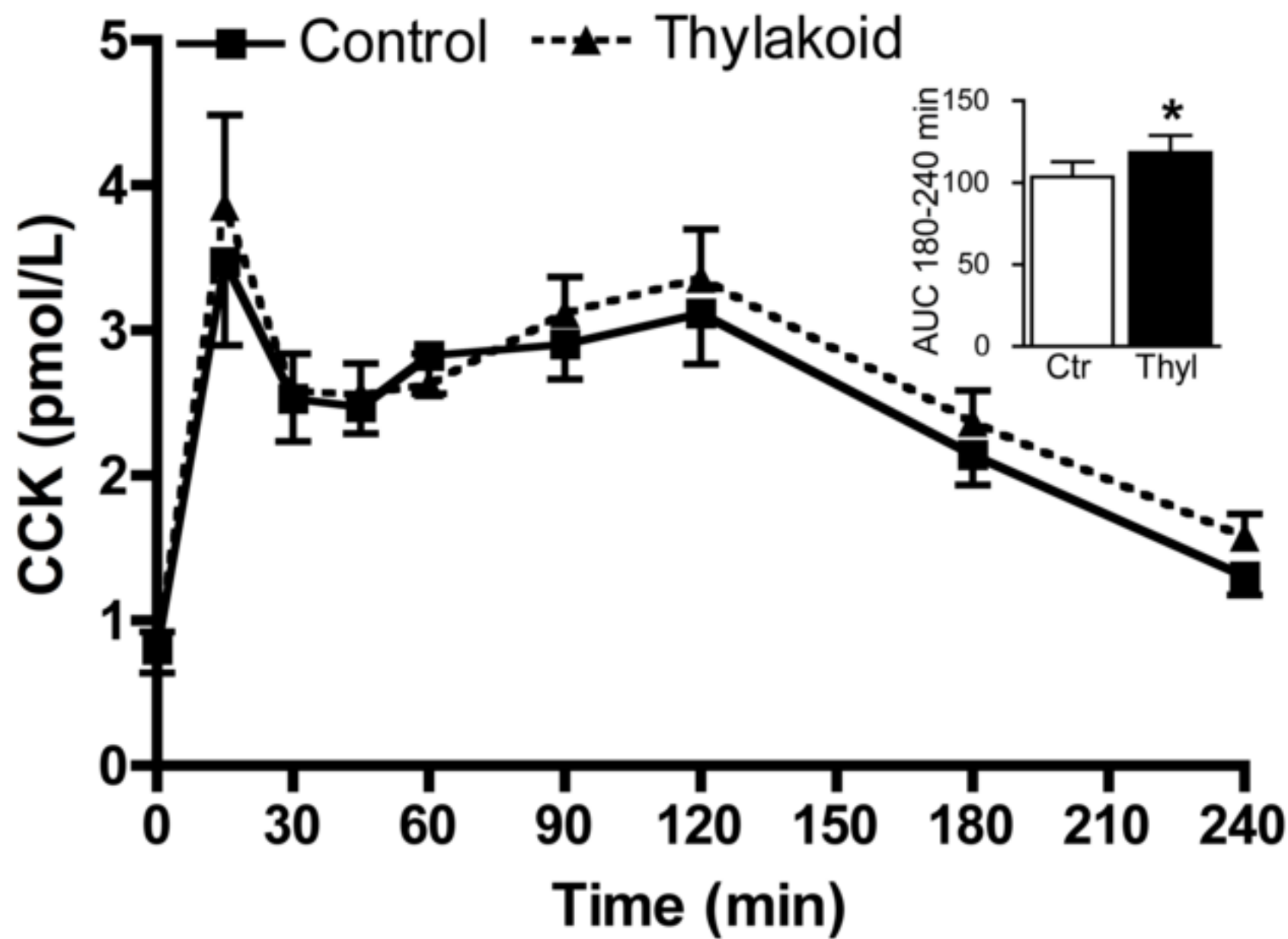


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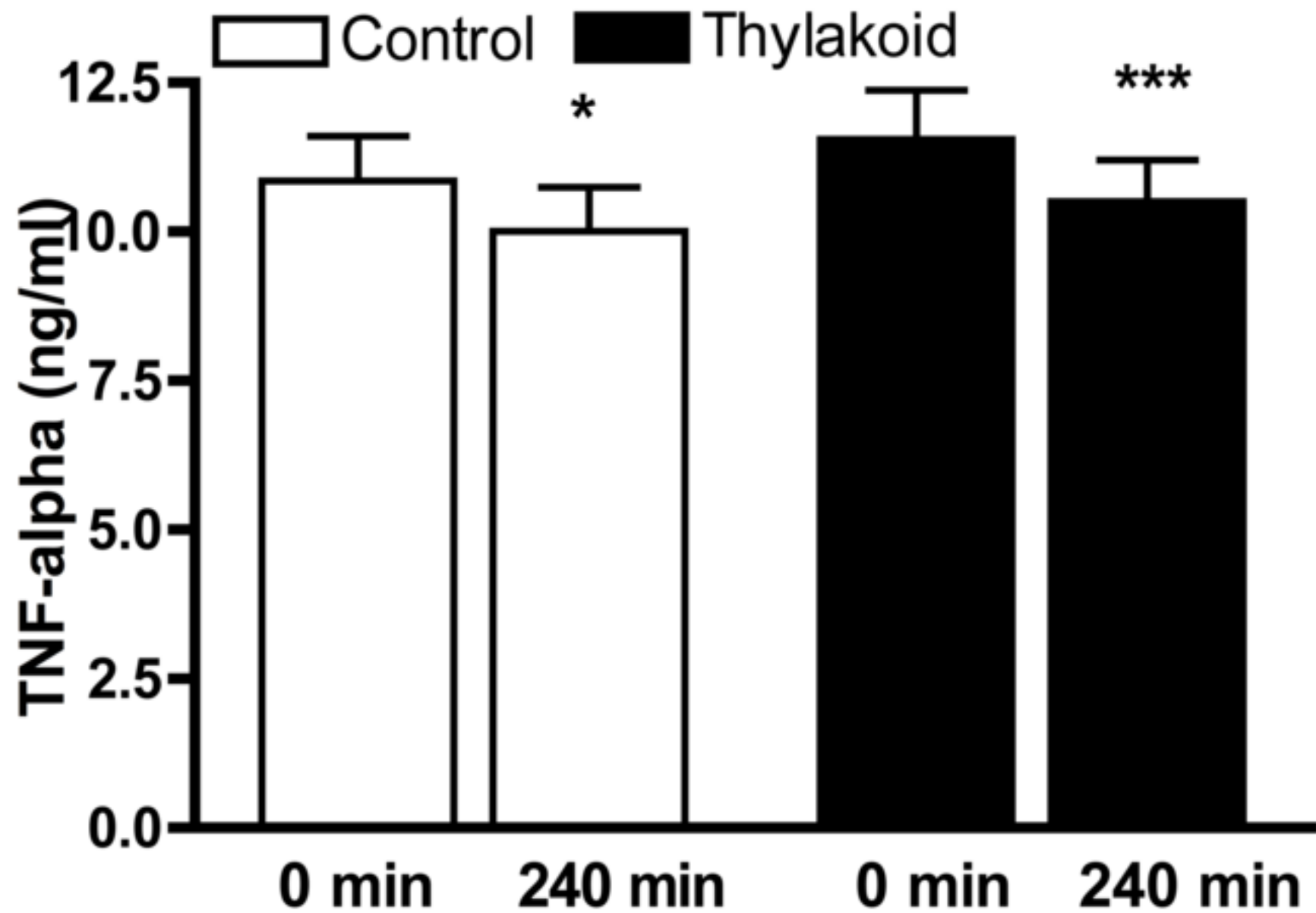


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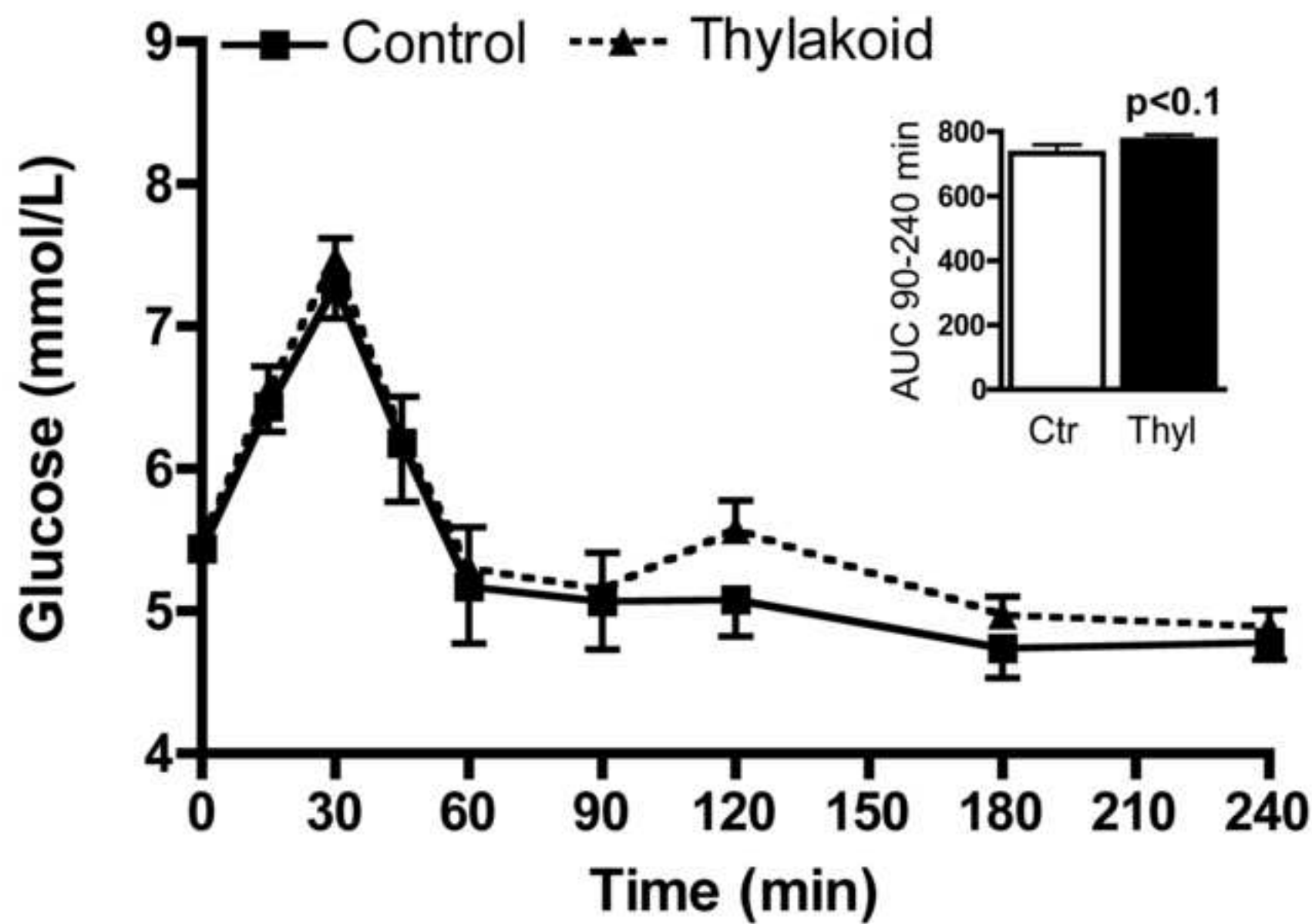




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