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Short Communication

Effects of high-calcium diets with different whey proteins on weight loss and weight regain in high-fat-fed C57BL/6J mice

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The aim of the study was to compare the effect of different whey protein-containing high-Ca diets on weight loss and weight regain in a model of diet-induced obesity. Obesity was induced in C57BL/6J mice with a high-fat (60 % of energy) diet. Weight loss by energy restriction was performed on four different high-Ca diets (1·8 % $CaCO_3$) containing different whey proteins (18 % of energy): α-lactalbumin (ALA), β-lactoglobulin (BLG), lactoferrin (LF) and whey protein isolate (WPI). After 7 weeks of energy restriction some of the mice were killed and the rest were fed with the same diets *ad libitum* for 7 weeks. The mice on the LF diet lost significantly more weight than mice on the WPI diet. The body fat content in the ALA and LF groups was significantly lower than in the WPI group ($P<0·05$) and the LF group differed significantly even from the BLG group ($P<0·05$). *Ad libitum* feeding after weight loss resulted in weight regain in all groups and only the ALA diet significantly reduced fat accumulation during weight regain. The weight regain was most pronounced in the LF group, but the adipocyte size was still significantly smaller than in the other groups. There were no differences in food intake or apparent fat digestibility between the groups. It can be concluded that a high-Ca diet with ALA significantly improves the outcome of weight loss and subsequent weight regain during the feeding of a high-fat diet in C57BL/6J mice, in comparison with WPI.

Whey protein: Diet-induced obesity: Dietary calcium: High-fat diet

The increased intake of dairy products has been shown to augment weight loss[1–4]. This has been primarily explained by Ca. Ca binds fatty acids in the intestine and thereby reduces the absorption of dietary fat[5–9] and may also regulate adipose tissue metabolism via 1,25-dihydroxyvitamin D_3 [10,11]. However, the effect of dairy products has been found to be comparable or superior to the effect of supplemental Ca[3,12–14] and especially whey proteins have been proposed to be the source of this additional effect on body weight[15,16].

We have previously shown that a high-Ca diet with whey protein isolate (WPI) inhibits weight and fat tissue gain in high-fat diet-fed C57BL/6J mice, a well-established model of diet-induced obesity[17]. A similar effect of WPI on body-weight gain has also been observed in rats[18] and a recent clinical intervention study demonstrated that whey protein increased fat loss during a weight-loss diet[19]. However, the mechanisms of action remain unknown.

Weight loss through lifestyle changes is the primary treatment for obesity. Therefore it is crucial to find nutritional approaches that maximise the effect of weight loss and inhibit weight regain thereafter. In order to develop more effective nutritional approaches against obesity and to understand the mechanism of action of whey proteins, the active components of whey protein should be identified. In the present study our aim was to characterise the effect of different whey protein fractions of WPI, α-lactalbumin (ALA), β-lactoglobulin (BLG) and lactoferrin (LF) on weight loss and weight regain in a model of diet-induced obesity.

Methods

Animals and diets

Male C57BL/6J mice (8 weeks old) were purchased from Harlan (Horst, The Netherlands). The mice were housed

Abbreviations: ALA, α-lactalbumin; BLG, β-lactoglobulin; LF, lactoferrin; WPI, whey protein isolate.

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five per cage in a standard experimental animal laboratory, illuminated from 06.30 to 18.30 hours (temperature 22 ± 1°C). The protocols were approved by the Animal Experimentation Committee of the University of Helsinki, Finland. After a 1-week acclimatisation period the mice (25·7 (SEM 0·2) g; *n* 66) were put on a low-Ca (0·4 %) high-fat diet (60 % of energy from fat; D05031101M; Research Diets Inc., New Brunswick, NJ, USA) for 100 d. After the weight-gain period ten mice were killed and the remaining mice were divided into four groups: (1) WPI group (45·1 (SEM 1·4) g), diet D05031104M[17], protein source whey protein isolate (Alacen™ 895; NZMP, Auckland, New Zealand); (2) ALA group (45·9 (SEM 1·4) g), diet D07041804M, protein source α-lactalbumin (Davisco BioPURE; Davisco Foods International, LeSueur, MN, USA); (3) BLG group (45·1 (SEM 1·2) g), diet D07041803M, protein source β-lactoglobulin (Davisco BioPURE; Davisco Foods International); (4) LF group (46·2 (SEM 1·1) g), diet D07041805M, protein source lactoferrin (Glanbia Bioferrin; Glanbia, Kilkenny, Republic of Ireland) (ANOVA $P=0{\cdot}904$). All groups were put on an energy-restriction diet (70 % of *ad libitum* energy intake) for 50 d. After the energy restriction five mice per group were killed and the remaining mice were fed with the same diets *ad libitum* for 50 d.

The diet of all the groups was a high-fat diet (60 % of energy from fat) and the only difference between the diets was the protein source. The diets were manufactured by Research Diets Inc. (New Brunswick, NJ, USA). The powdered diets were moistened with tap water (200 ml/kg in WPI and BLG, 205 ml/kg in ALA, 190 ml/kg in LF diets) using an industrial dough mixer, packed in 1 d portions and stored at −20°C.

Body weight was monitored once per week and the consumption of feed was monitored daily using a standard table scale (Ohaus Scout™ Pro SP4001; Ohaus Europe, Nänikon, Switzerland). Mean energy intake was calculated from the food intake data. Body fat content was analysed by dual-energy X-ray absorptiometry (Lunar PIXImus; GE Healthcare, Chalfont St Giles, Bucks, UK) before and after energy restriction and at the end of the study.

Faecal fat and calcium excretion

For the collection of faeces, the mice (*n* 7–9 per group) were housed individually in metabolism cages for 72 h at the end of the weight-gain, energy-restriction and weight-regain periods. The intake of feed and drink was monitored daily. All faeces excreted during the 72 h period were collected at the end of the 72 h period. The faeces were weighed and stored at −70°C until assayed. The fat content of the faecal samples was determined by the Schmid–Bondzynski–Ratzlaff (SBR) method[20] and the Ca content was determined using an inductively coupled plasma mass spectrometer (Elan 6100; Perkin Elmer, Boston, MA, USA). The apparent fat digestibility was calculated from the amount of feed consumed and the amount of fat excreted during the housing in metabolism cages. Apparent fat digestibility (%) was determined as 100 × ((fat intake − faecal fat)/(fat intake)). To estimate how much the increased fat excretion actually decreased the amount of digestible energy from fat during the whole study period, we calculated 'the apparent cumulative energy digestibility from fat'. For this we used the cumulative energy intake data and apparent fat digestibility percentage (apparent fat digestibility % × cumulative energy intake from fat) as described previously[7].

Blood glucose and serum lipids

Blood glucose was analysed from the blood samples taken when the animals were killed. Blood glucose was determined using a glucometer (Super Glucocard™ II, GT-1630; Arkray Factory Inc., Shiga, Japan).

Sample preparation

At the end of the treatment period the mice were rendered unconscious with CO_2–O_2 (95:5, v/v) (AGA, Riihimäki, Finland) after a 4 h fast, and decapitated. The subcutaneous, epididymal, abdominal and perirenal fat pads were removed, washed with saline, blotted dry and weighed.

The distal end of the perirenal fat pad was fixed in 10 % formalin, and embedded in paraffin with routine techniques. Sections (5 μm) of paraffin-embedded adipose tissue samples were cut with a microtome and mounted on charged glass slides, deparaffinised in xylene, and stained. Cross-sectional area was determined for each adipocyte in six fields per sample (*n* 5 per group) using Leica QWin Standard software (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK).

Statistical analysis

Data are presented as mean values with their standard errors. Statistically significant difference in mean values were tested by ANOVA followed by Tukey's test. ANOVA for repeated measurements was applied for data consisting of repeated observations at successive time points. The difference was considered significant when $P<0{\cdot}05$. The data were analysed using GraphPad Prism (version 4.02; GraphPad Software, Inc., San Diego, CA, USA) and SPSS (version 10.1; SPSS Inc., Chicago, IL, USA).

Results

Changes in body weight and body fat during energy restriction

Weight loss was most pronounced in the LF group (Fig. 1 (a)). The fat percentages of the LF and ALA groups were significantly lower than the fat percentage of the WPI group (Fig. 1 (b)). Weight loss effectively reduced the fat pad weights in all groups except of the epididymal fat, which was reduced significantly only in the ALA and LF groups (Table 1).

Changes in body weight and body fat during weight regain

In the LF group the mice gained significantly more weight than in the ALA and BLG groups ($P<0{\cdot}05$), but there were no differences in the body fat percentage between any of the groups at the end of the weight-regain period. The total fat pad weight of the ALA group was smaller than in the LF group (Fig. 1 (c)) and visceral fat pad weight was significantly smaller in the ALA group in comparison with the WPI and LF groups (Fig. 1 (d)). Also the epididymal fat pads were smaller in the ALA than in the WPI group (1·7 (SEM 0·1) *v.* 2·5 (SEM 0·2) g; $P<0{\cdot}05$).

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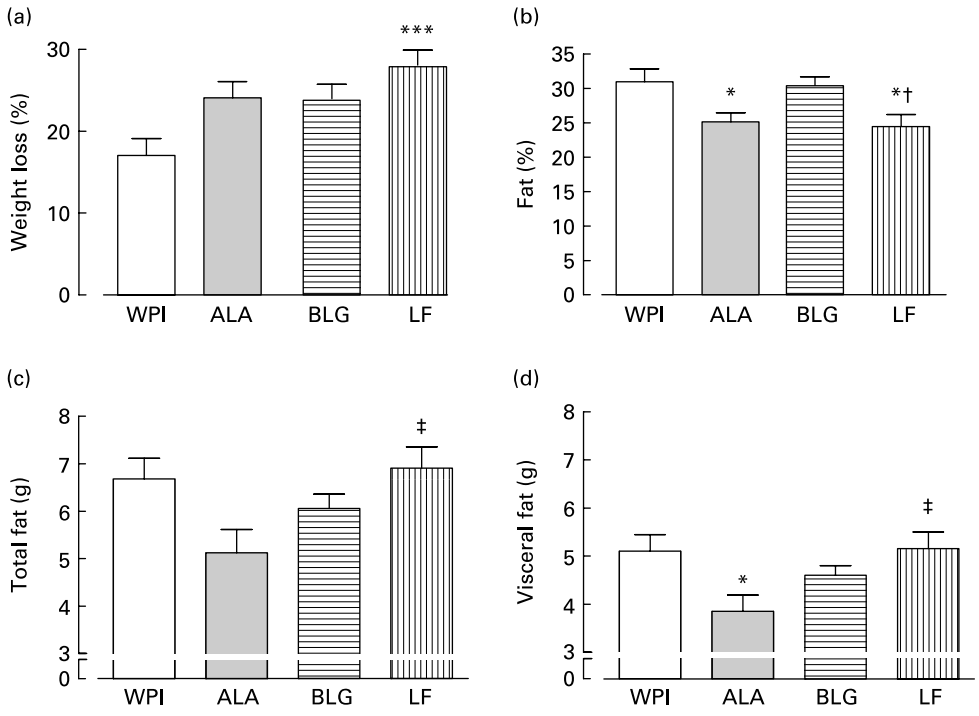

Fig. 1. (a) Weight loss during energy restriction as percentage of body weight at the beginning of energy restriction (*n* 14 per group). (b) Body fat content of high-fat-fed C57BL/6J mice after weight loss. (c) Total fat pad weight after weight regain. (d) Visceral fat pad weight after weight regain (*n* 8 in α-lactalbumin (ALA) group and *n* 9 in other groups). WPI, whey protein isolate; BLG, β-lactoglobulin; LF, lactoferrin. Values are means, with standard errors represented by vertical bars. Mean value was significantly different from that of the WPI group: * $P<0·05$, *** $P<0·001$. Mean value was significantly different from that of the BLG group: † $P<0·05$. Mean value was significantly different from that of the ALA group: ‡ $P<0·05$.

Adipocyte size

Adipocyte size after weight loss was significantly smaller in the ALA than in the WPI and BLG groups (Table 1). After weight regain there was no difference in adipocyte size between the groups (ANOVA $P=0·232$).

Blood glucose

Weight loss induced a significant decrease in blood glucose in the LF group (Table 1). Weight regain increased the blood glucose level back to what was seen in the obese state and there were no differences between the groups in blood glucose after weight regain (ANOVA $P=0·0566$).

Food and energy intake

The mean daily energy intake during energy restriction was 70 % of the intake during *ad libitum* feeding. During the weight-regain period the mice were fed *ad libitum* and the mean daily energy intake did not differ between the groups (ALA 58·8 (SEM 0·1) kJ/mouse per d, BLG 52·9 (SEM 1·7) kJ/mouse per d, LF 63·0 (SEM 4·6) kJ/mouse per d and WPI 63·8 (SEM 5·0) kJ/mouse per d; ANOVA $P=0·132$).

Table 1. Fad pad weights, blood glucose and adipocyte size after weight loss in C57BL/6J mice
(Mean values with their standard errors for five mice per group)

	Obese		WPI		ALA		BLG		LF		
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	ANOVA *P*
Fat pad weight (g)											
Subcutaneous	1·559	0·11	0·938**	0·13	0·563***	0·14	1·023*	0·07	0·497***	0·10	<0·0001
Abdominal	1·402	0·13	0·607***	0·05	0·370***	0·08	0·764**	0·04	0·297***	0·04	0·0012
Epididymal	2·019	0·19	1·416	0·23	0·984**	0·18	1·662†	0·13	0·737***	0·14	0·0003
Perirenal	1·339	0·07	0·694***	0·10	0·504*‡	0·10	0·876**††	0·03	0·418***	0·09	<0·0001
Total	6·319	0·35	3·656***	0·42	2·421***‡	0·49	4·326**†	0·18	1·949***	0·33	<0·0001
Visceral	4·760	0·26	2·718***	0·33	1·858***‡	0·36	3·302**††	0·16	1·452***	0·24	<0·0001
Blood glucose (mmol/l)	6·3	0·4	7·8††	1·0	5·4	0·3	7·2†	0·6	4·5	0·5	0·0059
Adipocyte size (μm^2)	5340	400	3814*	409	2381***	209	4045	220	3147**	284	0·0042

WPI, whey protein isolate; ALA, α-lactalbumin; BLG, β-lactoglobulin; LF, lactoferrin.
Mean value was significantly different from that of the obese group: * $P<0·05$, ** $P<0·01$, *** $P<0·001$.
Mean value was significantly different from that of the LF group: † $P<0·05$, †† $P<0·01$.
Mean value was significantly different from that of the BLG group: ‡ $P<0·05$.

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Apparent fat digestibility and calcium excretion

During weight loss, the LF diet decreased apparent fat digestibility (93·2 (SEM 2·0) % in the LF group compared with 98·0 (SEM 0·3) % in the WPI, 97·6 (SEM 0·2) % in the ALA and 97·1 (SEM 0·4) % in the BLG groups; $P<0·05$). During the weight-regain phase, there were no differences between the groups (ANOVA $P=0·796$).

Discussion

Whey proteins have been postulated to contribute to the body weight-regulating properties of dairy products, but so far the active components have not been identified. These results suggest that ALA has beneficial effects on body fat during both weight loss and weight regain. The other major whey protein, BLG, also had a significant effect on body weight but not on the amount of fat tissue. LF, a minor whey protein, accelerated both weight loss and weight regain. In addition to these protein components, whey contains a substantial amount of other minor proteins and peptides[21], which may have an effect on body weight and can also explain the anti-obesity effect of dairy products.

In addition to distinguishing the potential active protein component in dairy products, the active component in the body should also be identified. Studies investigating what kind of peptides are formed during digestion of these proteins are scarce. *In vitro* digestion of caprine whey proteins with human gastric and duodenal juice has been shown to rapidly degrade LF while ALA and BLG are more resistant and part of these proteins stay in their native form through digestion[22]. Digestion of milk proteins with human gastric and duodenal juice produces different protein and peptide profile from digestion with porcine enzymes. Therefore it is crucial that the formation or stability of bioactive peptides is studied in the study model in question.

It is often stated that the health effects of whey proteins result from their optimal amino acid composition. An especially high amount of leucine or other branched-chain amino acids is suggested to explain the findings, since they have a role as energy substrates and in the regulation of muscle protein synthesis[16,23,24]. In the present study the amount of leucine is not in line with the effect of proteins on body weight and amount of fat tissue. The amount of leucine was highest in the BLG and WPI diets and lowest in the control and LF diets (10·7 g/100 g protein in ALA, 13·0 g/100 g protein in BLG, 9·8 g/100 g protein in LF and in 13·1 g/100 g protein in WPI) and thus it is unlikely that leucine would account for the effects seen in the present study.

The glycine content of LF and ALA is greater than in BLG or WPI and it may contribute to the effect on adipocyte size, since glycine intake has been shown to reduce adipocyte size[25]. Interestingly, adipocyte size after weight loss was significantly smaller only in the ALA group even though body fat percentage decreased significantly also in the LF group. ALA is a source of angiotensin-converting enzyme (ACE)-inhibiting peptides[26] and ACE inhibition has been shown to decrease adipocyte size[27]. However, it is known that ACE-inhibiting peptides are also formed during the hydrolysis of other whey proteins and hence this may not explain why the effect on adipocyte size was more pronounced in the ALA group.

In our previous study we saw a significant decrease in apparent fat digestibility in high-Ca whey protein-fed mice during high-fat feeding[17]. In the present study apparent fat digestibility was decreased only in the LF group during weight loss, but there were no differences between the groups during the weight-regain period. Ca from dairy sources has been shown to be more effective in increasing fat excretion than supplemental Ca, but the reason for that is unknown[9]. Even though our previous results suggested that the protein source of the diet could have an effect on fat excretion these results do not conclusively support the theory.

In conclusion, ALA was the most beneficial since it accelerated fat loss during weight loss and the amount of visceral fat was reduced after the weight-regain period. The differences in body weight are not explained by energy intake or fat excretion and the molecular mechanisms of these effects and actual functional compounds in these proteins remain to be studied further. Since all of the diets in the present experiment were high-Ca diets, the interaction between Ca and whey proteins should also be further explored in the future.

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