Contribution of gastroenteropancreatic appetite hormones to protein-induced satiety1–3

Anita Belza, Christian Ritz, Mejse Q Sørensen, Jens J Holst, Jens F Rehfeld, and Arne Astrup

ABSTRACT

Background: Effects of protein intake on appetite-regulating hormones and their dynamics are unclear.

Objectives: We investigated the satiating effects of meals with varying protein contents and whether there was an effect of dose on appetite-regulating hormones and appetite ratings.

Design: Twenty-five men [mean ± SD age: 30.0 ± 8.7 y; body mass index (BMI; in kg/m²): 25.9 ± 4.7] participated in the 3-way, randomized, double-blind crossover study. Test meals were isocaloric with 30% of energy from fat and protein content adjusted at the expense of carbohydrate. Test meals were normal protein (NP; 14% of energy from protein), medium-high protein (MHP; 25% of energy from protein), and high protein (HP, 50% of energy from protein). Appetite ratings and blood samples were assessed every 0.5 h for 4 h. An ad libitum lunch was served 4 h after the meal.

Results: Protein increased dose-dependently glucagon-like peptide-1 (GLP-1), peptide YY (PYY) 3–36, and glucagon; MHP increased insulin and cholecystokinin and decreased ghrelin and glucose-dependent insulino-tropic polypeptide (P < 0.05). Satiety and fullness dose-dependently increased by 7% and 6% for MHP and 16% and 19% for HP compared with NP (P < 0.001). Hunger and prospective consumption dose-dependently decreased by 15% and 13% for MHP and by 25% and 26% for HP compared with NP (P < 0.0003). There was a combined effect of GLP-1 and PYY 3–36 (P = 0.03) next to the additive effect of GLP-1 (P = 0.006) on the composite appetite score. No difference was shown in ad libitum energy intake.

Conclusion: Protein dose-dependently increased satiety and GLP-1, PYY 3–36, and glucagon, which may, at least in part, be responsible for the satiety-stimulating effect of protein. This trial was registered at clinicaltrials.gov as NCT01561235. Am J Clin Nutr 2013;97:980–9.

INTRODUCTION

Several studies have shown that a high-protein, energy-restricted diet can lead to a reduction in body weight and fat mass (1–6) but can also preserve a larger proportion of the fat-free mass than an energy-restricted diet can with a normal protein content (4, 6).

Dietary protein appears to be the most satiating and thermogenic macronutrient (7–11). However, how protein exerts its effect on appetite is not fully understood, and the involvement of peripheral appetite-regulating hormones has not been adequately investigated (12). The effect of a high-protein diet compared with lower-protein diets has mainly been investigated with respect to glucagon-like peptide-1 (GLP-1)4, ghrelin, and cholecystokinin (5, 13–20). However, results are inconclusive, and the effect of protein on these appetite-regulating hormones is still not clear. Furthermore, we do not know whether increasing doses of protein induce corresponding increases in responses of these hormones. Existing studies have compared the effects of low- and high-protein preloads but have not investigated the effect of the dose of protein. Conflicting results of existing studies may be the consequence of an as yet unknown threshold of the effect of protein, which could lie between the 2 tested concentrations. There is a need to examine the effect of protein on appetite-regulating hormones in a dose-response manner and whether this effect corresponds to changes in subjective sensations of appetite and spontaneous energy intake (EI) (12). These relations can be examined by comparing the effects of >2 isocaloric meals in which the protein content and one other macronutrient are varied, and the content of the third macronutrient is fixed.

The objective of the current study was to investigate the mechanisms responsible for the satiating effects of protein in 3 isocaloric test meals with a protein content of 14%, 25%, or 50% of energy. The dose-response effect of protein on numbers of appetite-regulating hormones and peptides and changes in ad libitum EI were examined. Changes in subjective appetite sensations and sensory desires were also evaluated.

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4 Abbreviations used: EI, energy intake; GIP, glucose-dependent insulino-tropic polypeptide; GLP-1, glucagon-like peptide-1; HP, high-protein meal; iAUC, incremental AUC; LSM, least-square mean; MHP, medium-high–protein meal; NP, normal-protein meal; PYY, peptide YY; VAS, visual analog scale.

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SUBJECTS AND METHODS

Subjects

Thirteen normal-weight [BMI (in kg/m²) range: 19.1–24.8] and 12 overweight-to-obese (BMI range: 25.2–37), otherwise healthy, young Danish men participated in the study (Table 1). All subjects were weight stable (within ±3 kg) 2 mo before study inclusion, nonsmoking, and nonathletic and had no daily use of medication. Subjects were excluded if they had blood pressures >150/90 mm Hg or suffered from metabolic or psychiatric diseases. All subjects underwent assessments of height, body weight, blood pressure, and heart rate before the start of the study.

All subjects gave their written consent to participate in the study after they received verbal and written information about the study. The study protocol was approved by The Danish National Committee on Health Research Ethics as being in accordance with the Helsinki II Declaration. This trial was registered at clinicaltrials.gov as NCT01561235.

Methods

The study was a 3-way, crossover, randomized, double-blind controlled study with each meal test separated by a 4-wk washout period. The 3 isocaloric test meals consisted of 1) a normal-protein meal (NP) with 14% of energy from protein, 2) a medium-high–protein meal (MHP) with 25% of energy from protein, and 3) a high-protein meal (HP) with 50% of energy from protein.

Participants were instructed to fast from 2000, except for intake of 0.5 L H2O on the evening before test days. Subjects were asked to refrain from hard physical activity for 36 h and from taking nonprescription drugs or alcohol for 48 h before test days. Subjects were given an identical standard supper, which was instructed to avoid taking nonprescription drugs or alcohol for 48 h before test days. Subjects were instructed to otherwise maintain their dietary and beverage habits, use of spices, and level of physical activity throughout the study period. Subjects were instructed to otherwise maintain their normal dietary and beverage habits, use of spices, and level of physical activity throughout the study. Subjects were instructed to otherwise maintain their normal dietary and beverage habits, use of spices, and level of physical activity throughout the study.

On each test day, subjects arrived at the department at 0800. After voiding of the bladder, body weight was measured to the nearest 0.05 kg on a decimal scale, and height was measured to the nearest 0.5 cm. Subsequently, participants rested in a supine position for 30 min before the other measurements were conducted. Body composition was assessed with electronic biocomposition monitoring unit (Animer, Quadscan 4000; Bodystat) and dual measures of blood pressure and heart rate were assessed by using a digital blood pressure meter (UA-743; A&D Co Ltd), and one blood sample was taken. Appetite sensations were assessed by using visual analog scales (VASs) before the test meal (see VASs for a description). One of the 3 test meals described was served. Subjects were instructed to consume the test meal within 15 min. After completion of the meal, subjects rated the organoleptic quality of the meal (ie, appearance, smell, taste, aftertaste, and general palatability) by using VASs.

Fifteen minutes after the initiation of the test meal, a second blood sample was collected, which was repeated at times 30, 45, 60, 90, 120, 150, 180, 240 min postintake, together with a VAS appetite-score assessment. The test lasted for 4 h after initiation of the meal.

Subjects were allowed to perform only sedentary physical activities, and they refrained from the consumption of food and beverages, except for 100 mL H2O, after breakfast and throughout the test until measurements of VAS scores and the last blood collection (4 h postmeal). Fifteen minutes after the conclusion of the 4-h postmeal period, subjects completed a VAS score assessment and were served an ad libitum lunch. The ad libitum lunch consisted of 960-g pizza slices with ham and cheese (1060 KJ/100 g; macronutrient composition: 17.6% of energy from protein, 56.1% of energy from carbohydrate, and 27.6% of energy from fat). Subjects were instructed to eat at a constant pace and to stop eating when they felt satiated. EI was assessed from the amount of the meal consumed. Immediately after completion of the meal, subjects rated their sensations of appetite and sensory-specific desires and palatability of the meal by using VASs.

To limit the diurnal variation and intersubject and intrasubject variations, all measurements were carried out according to a standardized time schedule at the same time on each of the test days.

Test meals

A standardized meal (pasta Bolognese; 4.5 MJ; macronutrient content: 18% of energy from protein, 22% of energy from fat, and 60% of energy from carbohydrate) was consumed at dinner on the evening before each test day.

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Test meals

All 3 test meals were isocaloric [which covered approximately one-third of daily energy requirements; ~3 or 4 MJ/meal depending on the subject’s body weight (21, 22)] with a fat content of ~30% of energy, and protein and carbohydrate contents that varied between test meals. The protein content was 14% of energy, 25% of energy, or 50% of energy. The fiber content was similar in all 3 test meals (5–6 g/3 MJ or 7–8 g/4 MJ). Ingredients and macronutrient contents of the 3 test meals are shown in Table 2. Test meals differed in volume (in the 3-MJ meal: NP, 559 g; MHP, 631 g; and HP, 700 g). Test meals were not typical breakfast meals but were served as pork, rice, and

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<sup>1</sup>FFM, fat-free mass; FM, fat mass.

<sup>2</sup>Mean ± SD (all such values).
mushroom pâtés flavored with thyme to blind differences in appearance and taste. A total of 300 mL H₂O was served together with the meal.

### VASs

VASs used consisted of a line, 100 mm in length, with words that expressed the most-positive and most-negative ratings of subject sensations of hunger, satiety, prospective consumption, fullness, and sensory-specific desires to eat something sweet, salty, rich in fat, or meat and fish anchored at each end (23, 24). On each test day, subjects received thorough instructions on how to rate their appetite sensations by using a VAS. The meaning of each appetite sensation was explained to subjects. A composite appetite score was calculated at each time of measurement by using the following formula:

\[
\text{Composite Appetite Score} = \frac{\text{Satiety} + \text{Hunger} + (100 - \text{Fullness}) - (100 - \text{Prospective Food Consumption})}{4}
\]

The composite appetite score indicated the overall appetite suppression, and it is increasingly used in the literature because it integrates appetite sensations into one index (25, 26).

### Blood samples

Blood samples were analyzed for insulin (intra-CV: 2.7%; inter-CV: 7.4%; Immulite 1000 insulin; Siemens Medical Solutions Diagnostics), glucose (intra-CV: 0.8%; inter-CV: 2.5%; ABX Pentra Glucose KH CP), and appetite-regulating hormones GLP-1 (intra-CV: <6%; inter-CV: <15%; tracer from Novo Nordisk A/S), peptide YY 3–36 (PYY 3–36) (intra-CV: <11%; inter-CV: <15%; PYY-67HK; Merck Millipore), total ghrelin (intra-CV: <10%; inter-CV: <17%; GHRT-89HK; Merck Millipore), and cholecystokinin (intra-CV: 8.5%; inter-CV: 11.3%; see reference 29 for an additional description). On each test day, samples of 235 mL full blood were collected from each subject.

### Statistical analysis

The sample size in the current study was based on Flint et al. (23). According to Flint et al. (23), 24 subjects must be studied in a paired design to detect a 10-mm difference in subjective appetite ratings of satiety by using a VAS with a statistical power of 0.90 and a significance level of 0.05. With regard to EI, Gregersen et al. (30) concluded that 26 subjects must be studied in a paired design to detect a 500-kJ difference in ad libitum EI with a statistical power of 0.8 and a significance level of 0.05. Therefore, the 25 subjects in the current study should have been sufficient to detect potential differences in subjective appetite ratings but not quite enough to detect differences in EI.

A mixed-model ANCOVA was used for VAS scores to compare meals, respecting the paired design. The fixed effects, including interactions of subgroup (normal weight compared with overweight/obese), time, and treatment, were included together with age, period, and quantitative, anthropometric measurements. No significant effect was shown on subgroups (normal compared with overweight/obese) with regard to responses of appetite hormones, EI, subjective appetite ratings, and sensory desires. Thus, the 2 groups were analyzed as one group. Adjustment of the general palatability, taste, and after-taste of test meals were also included. Subject-specific random effects were included to account for the intersubject variability introduced by the paired design to adjust for any nonspecific differences that were not captured by explanatory variables included. Repeated measurements within subjects, including subjective appetite ratings and plasma concentrations of glucose and appetite-regulating hormones/peptides, were additionally adjusted for baseline levels, and a serial correlation was modeled by using a spatial Gaussian correlation structure with an exponentially decaying correlation with an increasing time gap.

Model checking was based on graphical assessment by using a normal-probability plot and a residual plot. Approximate F tests were used for backward stepwise elimination of nonsignificant terms. For the resulting model, P values were reported, and model-based least-square means (LSMs) were used to test for differences between test meals (by using post hoc t tests).

Similarly, a mixed model was used to assess the combined effect of appetite-regulating hormones on the composite appetite score after adjustment for differences in baseline, between periods, subjects, and test meals.

To detect linear relations between the incremental AUC (iAUC) of responses of appetite-regulating hormones and glucose, and iAUCs of repeated measurements of subjective appetite ratings, repeated measures were analyzed by using a linear mixed model within subjects. Subjective appetite ratings and EI were included as explanatory variables. Period and subject effects were
also included. Approximate F tests were used for backward stepwise elimination of nonsignificant terms. P values were reported, and slopes were used to describe the relation between variables.

Statistical analyses were performed with SAS 9.2 software (SAS Institute) by using the SAS procedure MIXED. A significance level of 0.05 was used.

RESULTS

Appetite hormones

No differences in baseline between test meals were observed for any blood variables.

**GLP-1**

Protein dose-dependently increased the 4-h response of GLP-1 (Figure 1A). Compared with intake of the NP, intake of the MHP induced a 10% higher response [3.4 ± 2.4 pmol/L (LSM); \( P = 0.02 \)], and intake of the HP induced a 20% higher response [6.9 ± 2.4 pmol/L (LSM); \( P < 0.0001 \)]. The GLP-1 response to the HP was 8% higher [3.5 ± 2.4 pmol/L (LSM); \( P = 0.001 \)] than to the MHP. No interaction between time and treatments was shown. No difference in the peak concentration and time to peak was observed.

**PYY 3–36**

Protein dose-dependently increased the 4-h response of PYY 3–36 (Figure 1B). Compared with intake of the NP, intake of the MHP induced a 7% higher response [5.6 ± 4.7 pmol/L (LSM); \( P = 0.0002 \)], and intake of the HP induced a 14% higher response [11.8 ± 4.7 pmol/L (LSM); \( P < 0.0001 \)]. The PYY 3–36 response was 7% [6.2 ± 4.7 pmol/L (LSM); \( P < 0.0001 \)] higher after intake of the HP than of the MHP. No interaction between time and treatments was shown. No difference in the peak concentration and time to peak was observed.

**GIP**

No dose-dependent difference was observed between meals. An interaction between time and treatment was detected in the 4-h repeated measures of GIP. When we tested for individual times of measurement, we showed that GIP concentrations were lower after intake of the HP than after the NP and MHP (\( P < 0.05 \)), except for the time 240 min postintake. Difference between the HP and NP ranged between 19% and 51% and between the HP and MHP by 25–45%. No difference between meals was observed at time 240 min (Figure 2B). The peak concentration was 35% (−30.1 ± 6.2 pmol/L; \( P < 0.0001 \)) and 36% (−31.4 ± 8.9 pmol/L; \( P < 0.0001 \)) lower after intake of the HP than after the NP and MHP, whereas the time to peak was 40% (39.2 ± 17.2; \( P = 0.04 \)) and 31% (32.4 ± 17.7; \( P = 0.04 \)) higher.

**Cholecystokinin**

No dose-dependent difference was observed between meals. An interaction between time and treatment was detected in the 4-h repeated measures of cholecystokinin. When we tested for individual times of measurement, 96% and 65% higher concentrations of cholecystokinin were shown at times 180 and

![FIGURE 1.](https://academic.oup.com/ajcn/article-abstract/97/5/980/4577230/B20) Mean ± SE 4-h concentrations of GLP-1 (A) and PYY (B) (presented as repeated measurements) \( n = 25 \); mean ± SD age: 29.7 ± 8.7 y; BMI (in kg/m²): 25.8 ± 4.7] after intake of an NP, MHP, or HP. Data were analyzed as repeated measurements by using a mixed-model ANCOVA. Post hoc comparisons were made between test meals with Tukey-Kramer adjustment of significance levels for the pairwise comparison. Significant 4-h dose-dependent differences (on the basis of least-square means of the 4-h repeated measures) in GLP-1 concentrations were shown between the NP and MHP (\( P = 0.02 \)), NP and HP (\( P < 0.0001 \)), and MHP and HP (\( P = 0.001 \)). Significant 4-h dose-dependent differences (least-square means) in PYY 3–36 concentrations were shown between the NP and MHP (\( P < 0.0001 \)), NP and HP (\( P < 0.0001 \)), and MHP and HP (\( P < 0.0001 \)). GLP-1, glucagon-like peptide-1; HP, high-protein meal; MHP, medium-high–protein meal; NP, normal-protein meal; PYY, peptide YY.

**Glucagon**

Protein dose-dependently increased the 4-h response of glucagon (Figure 2A). Compared with intake of the NP, intake of the MHP induced a 47% higher response [2.9 ± 0.6 pmol/L (LSM); \( P < 0.0001 \)], and intake of the HP induced a 116% higher response [7.2 ± 0.8 pmol/L (LSM); \( P < 0.0001 \)]. The HP increased the glucagon response by 44% compared with that of the MHP [4.1 ± 0.9 pmol/L (LSM); \( P < 0.0001 \)]. No interaction between time and treatments was shown. The peak concentration was protein dose-dependently higher after intakes of the MHP (47%; 4.1 ± 1.1 pmol/L; \( P = 0.0002 \)) and HP (119%; 10.3 ± 1.7 pmol/L; \( P < 0.0001 \)) than the NP. A difference in peak concentration was observed between the MHP and HP (49%, 6.3 ± 1.8 pmol/L; \( P < 0.0001 \)). The time to peak was increased by intakes of the MHP (39%; 31.3 ± 15.5 min; \( P < 0.0001 \)) and HP (134%; 107.5 ± 17.2 min; \( P < 0.0001 \)) than the NP.
240 min, respectively, for the HP compared with NP (P = 0.03), and a similar tendency was observed between meals at time 150 min (P = 0.06). Cholecystokinin was also increased by 31% at time 240 min after the HP than MHP (P = 0.0002) (Figure 3A). No differences in peak concentrations and the time to peak were observed.

Total ghrelin

No dose-dependent difference was observed between meals. An interaction between time and treatment was detected in the 4-h repeated measures of total ghrelin. Ghrelin concentrations were lower after intake of the HP than the NP and MHP at times 30–150 min (P < 0.03) expect for time 60 min, at which only a difference between the HP and MHP was observed (P = 0.01). Difference between the HP and NP ranged between 7% and 12% and between the HP and MHP by 5–8%. No difference was shown at times 180 and 240 min (Figure 3B). The peak concentration was 12% (62.0 ± 69.0 pmol/L; P = 0.004) higher after intake of the HP than NP. No difference in the time to peak was observed.

Insulin

No dose-dependent difference was observed between meals. There was an interaction between time and treatment when repeated measures were analyzed. The insulin response was lower after intake of the HP than NP and MHP at times 30–150 min (P < 0.05) (Figure 4A). Difference between the HP and NP ranged between 38–47% and between the HP and MHP by 33–45%. A higher concentration of insulin with the MHP than NP was also observed at time 180 min. Peak concentrations were 40% (196.1 ± 81.0 pmol/L; P < 0.0001) and 39% (188.4 ± 75.6 pmol/L; P < 0.0001) lower after intake of the HP than NP and MHP. No difference in the time to peak was observed.

Glucose

No dose-dependent difference was observed between meals. An interaction between time and treatment was observed in 4-h repeated measures of glucose. The HP induced lower concentrations of glucose at times 30–90 min (P < 0.004) compared with those with the NP. The difference between the HP and NP

**FIGURE 2.** Mean ± SE 4-h concentrations of glucagon (A) and GIP (B) (presented as repeated measurements) [n = 25; mean ± SD age: 29.7 ± 8.7 y; BMI (in kg/m²): 25.8 ± 4.7] after intake of an NP, MHP, or HP. Data were analyzed by using a mixed-model ANCOVA. Post hoc comparisons were made between test meals with Tukey-Kramer adjustment of significance levels for the pairwise comparison. Significant 4-h dose-dependent differences (on the basis of least-square means of the 4-h repeated measures) in glucagon concentrations were shown between the NP and MHP (P < 0.0001), NP and HP (P < 0.0001), and MHP and HP (P < 0.0001). A time-treatment interaction was shown on GIP data, and individual time points of assessment were analyzed. bSignificant difference between the NP and HP, P < 0.05; csignificant difference between the MHP and HP, P < 0.05. GIP, glucose-dependent insulinotropic polypeptide; HP, high-protein meal; MHP, medium-high–protein meal; NP, normal-protein meal.

**FIGURE 3.** Mean ± SE 4-h concentrations of CCK (A) and ghrelin (B) (presented as repeated measurements) [n = 25; mean ± SD age: 29.7 ± 8.7 y; BMI (in kg/m²): 25.8 ± 4.7] after intake of an NP, MHP, or HP. Data were analyzed by using a mixed-model ANCOVA. A time-treatment interaction was shown on CCK and ghrelin data, which entailed that individual time points of assessment were analyzed instead of repeated measurements. Post hoc comparisonswere made between test meals, with Tukey-Kramer adjustment of significance levels for the pairwise comparison. bDifference between the NP and HP, P < 0.05; cdifference between the MHP and HP, P < 0.05. CCK, cholecystokinin; HP, high-protein meal; MHP, medium-high–protein meal; NP, normal-protein meal.
ranged between 14% and 17%. The MHP induced a 10% lower concentration at time 90 min ($P = 0.01$) (Figure 4B) compared with that with the NP. The concentration was decreased after intake of the HP than MHP at times 30 and 45 min ($P < 0.03$) with a difference of 13–14%. The peak concentration was 15% (−1.1 ± 0.3 mmol/L; $P < 0.0001$) and 13% (−0.9 ± 0.3 mmol/L; $P < 0.0001$) lower after intake of the HP than NP and MHP. No difference in the time to peak was observed.

**Ratings of subjective appetite sensations**

No differences between test meals were observed in any appetite ratings at baseline, and no interaction between time and treatments was shown.

Four-hour ratings of satiety and fullness (LSM) were dose-dependently increased after intakes of the MHP [satiety: 7%; 4.4 ± 1.2 mm (LSM); $P = 0.001$; fullness: 6%, 3.7 ± 1.3 mm (LSM); $P < 0.0001$] and HP [satiety: 16%; 10.1 ± 1.3 mm (LSM); $P < 0.0001$; fullness: 19%; 11.1 ± 1.3 mm (LSM); $P < 0.0001$] (Figure 5A). Four-hour ratings of satiety and fullness were 9% [5.8 ± 1.3 mm (LSM); $P < 0.0001$] and 12% [7.4 ± 1.3 mm (LSM); $P = 0.01$] higher after intake of the HP than after the MHP, respectively. The peak level of sensation of satiety was 12% (9.8 ± 2.6 mm) and 7% (5.8 ± 2.4 mm) higher after intake of the HP than after the NP and MHP ($P < 0.0001$). No difference in the time to peak was observed.

Four-hour ratings of hunger and prospective consumption (LSM) were dose-dependently decreased after intake of the MHP [hunger: 15%; −5.6 ± 1.4 mm (LSM); $P = 0.0002$; prospective consumption: 13%; −5.3 ± 1.3 mm (LSM); $P = 0.0001$] and HP [hunger: 25%; −9.6 ± 1.4 mm (LSM); $P < 0.0001$; prospective consumption: 26%; −10.9 ± 1.3 mm (LSM); $P < 0.0001$]. Ratings of hunger and prospective consumption were 13% [−4.0 ± 1.4 mm (LSM); $P = 0.01$] and 16% [−5.6 ± 1.3 mm (LSM); $P < 0.0001$] decreased after intake of the HP than after the MHP. The peak level of sensation of hunger was 58% (−9 ± 2.7 mm) and 40% (−4.3 ± 2.2 mm) lower after intake of the HP than after the NP and MHP ($P < 0.004$). No difference in the time to peak was observed.

The combination of the 4 appetite ratings to a composite appetite score also resulted in a protein dose-dependent effect. The 4-h composite appetite score (LSM) was 8% and 17%
increased after intakes of the MHP and HP than NP [MHP: 4.9 ± 1.1 mm (LSM); \( P < 0.0001 \); HP: 10.4 ± 1.2 mm (LSM); \( P < 0.0001 \)] (Figure 5B). The score was 7% increased with the HP in comparison with the MHP [5.5 ± 1.2 mm (LSM); \( P < 0.0001 \)]. The peak level of composite appetite score was 11% (9.2 ± 2.7 mm) and 7% (6.1 ± 2.6 mm) higher after intake of the HP than NP and MHP (\( P < 0.0001 \)). No difference in the time to peak was observed.

Four-hour ratings of subjective sensory-specific desires

No differences between test meals were observed in any sensory desires at baseline, and no interaction between time and treatment was shown. Compared with the NP, the HP induced 4-h decreased desires for something sweet, fat, salty, or meat (sweet: 8%; 4.3 ± 1.6 mm (LSM); \( P = 0.03 \); fat: 11%; 8.8 ± 1.2 mm (LSM); \( P < 0.0001 \); salt: 10%; 7.5 ± 1.3 mm (LSM); \( P < 0.0001 \); meat: 9%; 6.9 ± 1.3 mm (LSM); \( P < 0.0001 \)). Similar differences were observed for the HP compared with MHP [sweet: 7%; 4.0 ± 1.6 mm (LSM); \( P = 0.04 \); fat: 8%; 6.4 ± 1.2 mm (LSM); \( P < 0.0001 \); salt: 8%; 5.9 ± 1.3 mm (LSM); \( P < 0.0001 \); meat: 12%; 9.4 ± 1.3 mm (LSM); \( P < 0.0001 \)]. No difference between the NP and MHP was observed.

The peak level of desires for something sweet, salty, or fat was higher (decreased desire) after the HP than after the NP and MHP (\( P < 0.004 \)). No difference in the time to peak was observed. The peak level of the desire for meat was higher (decreased desire) after the HP than after the NP (\( P = 0.004 \)). The time to peak was lower after the HP than after the MHP (\( P = 0.002 \)) but not HP.

Ad libitum EI

No difference in ad libitum EI was observed between test meals (NP: 4167 ± 322 kJ; MHP: 4122 ± 304 kJ; HP: 3471 ± 332 kJ; \( P = 0.3 \)). Subjects rated the palatability of the ad libitum meal as a little above medium, and there was no significant difference between meals. There were no differences between test meals in VAS ratings of appetite sensations after intake of the ad libitum meal.

Putative contribution of appetite hormones to appetite ratings and EI

Four-hour iAUCs of PYY 3–36 response were associated with the 4-h iAUC of satiety (slope: –0.31; \( P = 0.02 \)), 4-h iAUC of prospective consumption (slope: –0.49; \( P = 0.004 \)), and 4-h iAUC desire for something sweet (slope: –0.37; \( P = 0.0005 \)), whereas the iAUC of 4-h glucagon response was associated with the 4-h iAUC of fullness (slope: 0.051; \( P = 0.04 \)), 4-h iAUC of desire for something salty (slope: 0.052; \( P = 0.02 \)), and EI (slope: 0.17; \( P = 0.02 \)). The 4-h iAUC of cholecystokinin response was associated with 4-h iAUC prospective consumption (slope: 0.025; \( P = 0.0009 \)) and 4-h iAUC of desire for something salty (slope: 0.017; \( P = 0.007 \)). The 4-h iAUC of GIP was associated with the 4-h iAUC of fullness (slope: 0.56; \( P = 0.01 \)) and 4-h iAUC of composite appetite score (slope: –0.51; \( P = 0.04 \)). The 4-h iAUC of ghrelin response was associated with the 4-h iAUC of prospective consumption (slope: 1.04; \( P = 0.04 \)) and EI (slope: –2.96; \( P = 0.04 \)). No associations between either 4-h iAUCs of GLP-1 or insulin responses and subjective appetite ratings and EI were shown.

There was a combined effect of GLP-1 and PYY 3–36 (\( P = 0.03 \)) next to the additive effect of GLP-1 (\( P = 0.006 \)) on composite appetite scores, whereas cholecystokinin and PYY 3–36 showed no additive effects (\( P > 0.20 \)). The combined effect explained 1% of the variation in composite appetite scores compared with 4% explained by test meals.

Rating of palatability of test meals

The palatability of the NP was rated 23% higher (rated slightly above medium) than that of the HP (10.2 ± 6.1 mm; \( P < 0.05 \)) (rated slightly below medium). The aftertaste of the MHP was rated 13% more acceptable than that of the HP (8.6 ± 6.6 mm; \( P = 0.02 \)). No other differences were observed between test meals.

DISCUSSION

The current results indicated that protein has greater satiety stimulating properties than carbohydrate and emphasized previous findings of an effect of a high protein intake on appetite-regulating hormones (13, 16, 31), subjective appetite sensations, and subsequent EI (19, 20) compared with that of a lower protein intake. We showed clear-cut dose-response relations for protein’s effect on GLP-1, PYY 3–36, glucagon, and subjective appetite ratings. To our knowledge, only 2 studies have previously investigated the dose response to protein in a design with <2 preloads of different protein concentrations, and these studies only examined subjective appetite ratings and EI (32, 33). A few studies have examined effects of preloads with a rather low protein content (10% of energy) compared with a high protein content (25–50% of energy). However, it is difficult to identify a dose response by comparing only 2 concentrations of protein. The current inclusion of 3 preloads of 14%, 25%, and 50% of energy from protein strengthened the evidence of a dose-dependent response of subjective appetite sensations and appetite-regulating hormones GLP-1, PYY 3–36, and glucagon to protein. It was an additional strength that the lowest protein dose (14% of energy) was within the normal range of a habitual protein intake. The validity of the results was strengthened because a dose response was observed even though subjects were given 3 preloads of normal to high protein concentrations.

Several studies have reported that the GLP-1 concentration increases after protein intake, but only one study has shown a trend to a dose-dependent increase in GLP-1 after a high protein intake (30% of energy) compared with a low intake (10% of energy) (16). Other studies have shown no difference in the GLP-1 response after consumption of a high- compared with low-protein preload (13, 19, 20, 34, 35). In contrast to some other studies, Hochstenbach-Waelen et al (36) reported that the GLP-1 response was lower after intake of 3 successive HPs (25% of energy) compared with 3 successive low-protein meals (10% of energy) consumed over a 12-h test period. However, no significant difference was observed between high (25% of energy) and low protein (10%) when the total continuous12-h GLP-1 response was compared. The authors (36) suggested that the decreased GLP-1 response after the three 25%-of-energy preloads may have been caused by a delayed gastric emptying after each main meal. They observed a similar decrease in PYY response.
after a protein preload of 25% compared with 10% of energy (36). However, Leidy et al (31) showed an increased PYY response after meals with 25% of energy from protein compared with isocaloric meals with 14% of energy from protein. Furthermore, the PYY concentration fluctuated more if the protein was consumed on only 3 compared with 6 eating occasions per test day. This finding accords with the current results, which suggested that a preload with larger amount of protein seems to induce a higher PYY response. Other studies have not shown a dose response of PYY to protein (34, 35, 37). Coinfusions of lower doses of GLP-1 and PYY 3–36 have reduced EI in an additive manner in humans and rodents (Schmidt JB, Gregersen NT, Pedersen S, Holst JJ, Schwartz T, Astrup A, Sjödén A, unpublished data, 20 August 2012; 38, 39), which is an effect that may be explained by an inhibition of the vagal tone (40). We showed a combined effect of GLP-1 and PYY 3–36 on the composite appetite score. This result may indicate that the 2 hormones, in combination, affect appetite postintake of a protein-rich meal, probably in interaction with other hormones. However, the results must be interpreted with some reservations because the identified associations between appetite sensations and concentrations of appetite hormones were weak and, in some cases, inconsistent with the current literature (ie, PYY 3–36 was inversely associated with satiety, and cholecystokinin was associated with prospective consumption) (41). A comparison of the effects of protein on appetite sensations with appetite hormones was difficult because of the different timing and responses of individual variables (42). Associations between appetite sensations and appetite hormones need to be explored further. The dose response of glucagon to protein has been sparsely investigated. Blom et al (13) showed that glucagon increased dose-dependently by 380% after consumption of a 58%-of-energy protein meal compared with an isocaloric 19%-of-energy protein meal.

In studies that investigated the effect of varied contents of protein and carbohydrate in isocaloric meals (13, 15, 31, 43, 44), it has been observed that insulin increased correspondingly to an increased protein consumption. The opposite was shown in the current study when we compared high and normal protein intakes in that we showed the glucose response to be higher (at times 30–90 min) after intake of the NP. We speculate that the varying carbohydrate content may have masked the effect of protein on insulin. Results from HP studies with a fixed carbohydrate content are contradictory and do not further an understanding of the effect of protein on insulin (5, 19, 20, 36).

The effect of a protein dose on cholecystokinin (13, 43, 44) and GIP (13) responses has only been sparingly investigated, and results have been inconsistent. Blom et al (13) showed a significant increase of cholecystokinin after consumption of a 58%-of-energy protein meal compared with 19%-of-energy protein meal, but showed no difference in the GIP response. We showed no significant differences in cholecystokinin and GIP responses to the 3 protein meals, but we observed that the GIP response was numerically lower after the HP than after the 2 other meals. One explanation of the lower GIP response could be that GIP release may not be as effectively stimulated by protein as by carbohydrates (45).

In contrast to other studies (13, 15–17, 20, 31, 35–37, 43, 44), the current response of ghrelin was less suppressed after consumption of the HP compared with at lower doses. The decreased suppression of ghrelin secretion was mirrored by a correspondingly lower release of GIP. However, there is no indication that GIP is able to inhibit ghrelin secretion effectively (46, 47). The regulation of ghrelin in relation to stimulation by protein intake remains to be explored. The current findings on ghrelin and GLP-1 responses suggested that the effect of protein is more probably linked to satiety (related to the dose-response effect on GLP-1) than to hunger (related to the ghrelin response).

To our knowledge, only 2 studies have investigated the dose-dependent response of subjective appetite sensations to protein by including 3 preloads of different protein concentrations (33, 34). None of these studies showed a dose-dependent effect of protein. Results from studies that compared only 2 doses of protein have been conflicting. The majority of studies observed that high-protein preloads could suppress subjective appetite sensations more (5, 16, 17, 19, 20, 34–37, 44, 48), but other studies did not find difference in subjective appetite ratings (13, 15, 31, 43, 49). The current test meals were not of the same volume, which may especially have affected subjective appetite sensations and subsequent EI. However, no significant difference was observed in EI, which indicated that the difference in volume did not play a significant role.

The HP induced a 17% lower (NS) EI in comparison with the 2 lower doses. The lack of a significant difference was probably related to the lack of power. Gregersen et al (30) concluded that 26 subjects must be studied in a paired design (with an 80% study power) to detect a difference of 500 kJ in EI. This could possibly also explain similar results regarding EI from other protein-meal studies in which only 12–25 subjects were included (5, 13, 19, 20, 43). Unfortunately, the current lack of EI reduction made it difficult to determine the quantity of protein that could increase satiety to the point of either prolonging the time before a renewed drive to eat or reducing the subsequent EI. Ratliff et al (34) observed that intakes of energy, protein, and fat were decreased for 24 h after consumption of a high-protein diet. Similar findings were reported by Gosby et al (50) who investigated ad libitum EI and nutrient intake over 3 different 4-d periods of dietary manipulation with fixed protein contents of 10%, 15%, or 25% of energy. The low-protein diet led to higher intakes of energy, fat, carbohydrate, and fiber, of which the 3 latter intakes were dose-dependent of protein. Thus, and increased protein consumption seems to lead to appetite suppression and subsequent EI, whereas fat and carbohydrates may promote overconsumption and enhance risk of weight gain. Thus, it would have been an additional strength if we had included a 24-h follow-up on food intake in the current study.

The meat-based pâté meals were not typical Danish breakfast foods. Meat was chosen for its protein quality, ability to blind meals, and low fiber content because fiber may have a confounding effect. It may be a limitation of the study that no acclimation to the different eating pattern or protein intake of the test meals or screening of the habitual eating behaviors of subjects (breakfast skipping) (37) were included in the protocol. Therefore, it was not clear whether habituation to the meals might have caused some other responses than those shown in this study.

In conclusion, protein dose-dependently increases satiety. Results of the current study support the view that postprandial changes in circulating GLP-1, PYY 3–36, and glucagon are, at
least in part, responsible for the satiety-stimulating effect of protein. Although associations between postprandial responses in satiety hormones and satiety were shown, our findings show that the various satiety hormones are stimulated dose-dependently by protein and, hence, may be involved in the mediation of the greater satiety induced by protein that has been shown to be important in the control of body-weight regulation. The authors' responsibilities were as follows—AB and AA: designed the research and wrote the manuscript; AB, MQS, JJH, and JFR: conducted the research; AB and CR: analyzed data; CR, MQS, JJH, and JFR: coedited the manuscript; AB: had primary responsibility for the final content of the manuscript; and all authors: read and approved the final manuscript. None of the authors had a conflict of interest.

REFERENCES


