UNIVERSITY OF GLOUCESTERSHIRE

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Calcium Ingestion Suppresses Appetite and Produces Acute Overcompensation of Energy Intake Independent of Protein in Healthy Adults[1–3]

Javier T Gonzalez, Benjamin P Green, Meghan A Brown, Penny LS Rumbold, Louise A Turner, and Emma J Stevenson

Abstract

Background: Prior evidence suggests that high-calcium intake influences postprandial appetite and insulinemia, possibly due to elevated incretins. In vitro and ex vivo models demonstrate that extracellular calcium and protein synergistically enhance secretion of incretins. This is yet to be shown in humans.

Objective: This study was designed to assess energy intake compensation in response to protein and calcium ingestion.

Methods: Twenty healthy adults (13 men; 7 women) completed 4 trials in a randomized, double-blind crossover design separated by ≥48 h. During the trials, each participant consumed a low-calcium and low-protein control preload [(CON); 4 g and 104 mg, respectively], a high-protein preload (PRO; 29 g), a high-calcium preload (CAL; 1170 mg), or a high-protein and high-calcium preload (PROCAL). Blood samples were collected at baseline and 15, 30, 45, and 60 min after preload ingestion to determine insulin and incretin hormone concentrations. Energy intake was assessed by a homogenous test meal 60 min after the preload. Visual analog scales were completed immediately before blood sampling to assess subjective appetite sensations.

Results: Relative to the CON, the PRO produced 100% (95% CI: 85%, 115%) energy compensation, whereas the CAL produced significant overcompensation [118% (95% CI: 104%, 133%)], which was significantly more positive than with the PRO ($P < 0.05$). The PROCAL resulted in energy compensation of 109% (95% CI: 95%, 123%), which tended to be greater than with the PRO ($P = 0.06$). The mean difference in appetite sensations relative to the CON was not significantly different between the PRO (23 mm; 95% CI: 28, 3 mm), CAL (25 mm; 95% CI: 29, 0 mm), and PROCAL (25 mm; 95% CI: 210, 21 mm) ($P > 0.05$).

Conclusions: The addition of protein to a preload results in almost perfect energy compensation, whereas the addition of calcium, with or without protein, suppresses appetite and produces overcompensation of subsequent energy intake. The role of circulating insulin and incretin concentrations in these responses, however, remains unclear. This trial was registered at clinicaltrials.gov as NCT01986036.

Keywords: females, food intake, fullness, glucagon-like peptide-1, hunger, insulin, males, protein

Introduction

Habitual calcium intake is inversely associated with body fat percentage (1) and randomized controlled trials indicate that this may be a causal relation, i.e., calcium (plus vitamin D) supplementation augments fat loss under energy restriction (2). Although a decrease in dietary fat absorption is likely to partially account for this, fat excretion (typically increased by 2 g/d) cannot account for the effect size typically reported in energy-restriction studies (equivalent to an additional ~5 g/d) (2, 3). Thus, other mechanisms are likely to contribute. Some putative mechanisms include increased lipid utilization (4, 5) and reductions in ad libitum energy intake (6) and appetite sensations (7, 8).

Previous research has indicated that a single high-calcium (plus vitamin D) meal may decrease subsequent self-reported 24-h food intake (6). However, in this study, energy intake did not differ during the controlled (nonself-report) laboratory period.

This lack of an effect with nonself-report measures has been shown by others (9). It was only when participants provided self-reported food diaries for the subsequent 24 h that energy intake was lower with a high-calcium (plus vitamin D) breakfast (6). Therefore, whether calcium intake can influence acute food intake in humans with precise measurement of energy intake remains to be determined.

Notwithstanding this, we previously reported that the addition of calcium to a mixed-macronutrient meal suppresses postprandial appetite sensations while concomitantly elevating insulinemia (7, 8). These responses may be in part due to the gastrointestinal peptides glucose-dependent insulinotropic polypeptide$_{1-42}$ (GIP$_{1-42}$[6]; formerly known as gastric inhibitory peptide) and glucagon-like peptide-1$_{7-36}$ (GLP-1$_{7-36}$) (8). GIP$_{1-42}$ and GLP-1$_{7-36}$ are secreted by enteroendocrine cells in the gastrointestinal tract and are degraded by the enzyme dipeptidyl peptidase-IV (DPP-IV) (10). Evidence from both human embryonic kidney cells (11) and an isolated rodent intestinal model (12) suggest that the secretion of these peptides is elevated by stimulation of the extracellular calcium sensing receptor [present in the human gastrointestinal tract (13)] by an elevated extracellular/luminal calcium concentration. Moreover, this effect is potentiated by the presence of amino acids (11, 12). Taken in concert with the observation that milk peptides display DPP-IV inhibitory activity (14), the presence of protein and calcium in a meal may act synergistically to enhance plasma glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1 concentrations. This, in turn, may make a contribution to a reduction in appetite and improve energy intake compensation.

Therefore, the primary aim of this study was to assess the effects of the protein and calcium in a preload on subsequent compensation of energy intake. Secondary aims were to assess the subjective appetite, and plasma insulin, GIP$_{1-42}$, and GLP- 1$_{7-36}$ responses to the preloads.

Methods

Study design. This study was a randomized, double-blind (both investigators and participants were blinded to the intervention) cross-over study consisting of 4 main trials composed of a low-calcium and low-protein control preload (CON) trial, a high-calcium preload (CAL) trial, a high-protein preload (PRO) trial, and high-protein and high-calcium preload (PROCAL) trial. Each trial was separated by ≥2 d and ≤7 d. Trials were conducted in the nutrition and metabolism laboratories of Northumbria University (Newcastle-upon-Tyne, United Kingdom) in accordance with the Second Declaration of Helsinki after approval from the Northumbria University Faculty of Health and Life Sciences Ethics Committee. Random assignment with the use of www.randomization.com, blinding, and the preparation of preload meals was performed by PLS Rumbold, who had no further involvement in data acquisition.

Participants. A sample size estimation was conducted based on the reported 9.3% difference in ad libitum energy intake after a single high- calcium meal vs. a low-calcium meal (6). Given that the day-to-day variation in this measure is 8.9% (15), it was estimated that 16 participants would provide > 80% chance of statistically detecting a difference with $P < 0.05$. In order to account for potential dropouts, after informed written consent, 20 participants (12 men and 8 women) were recruited from the Northumbria University student and staff population (characteristics displayed in **Table 1**) between October 2013 and January 2014. Inclusion criteria included a BMI between 18.5 and 29.9 kg/m^2 and aged 18–40 y. Participants were excluded if they smoked, had any history of food allergies or metabolic disorders such as type 2 diabetes, or displayed dietary restraint [defined as a score of >13 on the Three-Factor Eating Questionnaire (16)]. No direct male-female comparisons were made because of the difference in group sizes; however, for information on the homogeneity of the participants, their characteristics are provided as men alone, women alone, and the total group.

Main trials. Participants arrived in the laboratory at 0800 ± 1 h after an overnight fast (10–14 h) and 24 h of physical activity standardization. Participants were asked to refrain from alcohol and caffeine for 24 h and to record and replicate their evening meal before trials. For all female participants, all main trials were carried out during the early follicular phase of the menstrual cycle (3–6 d after the day 1 of menses). An intravenous catheter was inserted into an antecubital vein and, after a baseline blood sample and visual analog scale (VAS), participants consumed one of 4 preloads (CON, PRO, CAL, or PROCAL). A timer was started when participants consumed the first mouthful of the preload, after which blood samples and a VAS were taken at 15, 30, 45, and 60 min post-preload. Food intake was then assessed (60 min after preload ingestion) by providing participants with a homogenous pasta meal [as previously described (17)], which they were asked to consume until ''comfortably full.'' The mass of food consumed was then converted into energy intake taking into account water losses from reheating. The time frame after the preload was based on our previous findings in which appetite sensations after a high-calcium breakfast were divergent within the first 60 min of the postprandial period (7, 8). Participants were initially served a subserving of the whole portion, which was augmented at regular intervals. This method prevents participants from feeling overwhelmed by a whole, large portion of pasta while never allowing the serving bowl to be empty, thus preventing participants from stopping eating because they reached the end of a ''portion.''

Preloads. All preloads contained instant porridge oats (Oatso Simple Golden Syrup, Quaker Oats UK) and water to provide 0.5 g carbohydrate/kg body mass. These were cooked in a microwave for 2 min at 1000 W and cooled for 5 min before being served. For CAL trials, a milk-extracted calcium powder [Capolac, Arla Foods Ingredients; from the same batch that was validated independently previously (18)] was added to the porridge to increase the calcium content by 15 mg/kg body mass. For PRO trials, milk protein concentrate (MyProtein.co.uk) was added to increase the protein content of the porridge by 0.35 g/kg body mass. To test the synergy of protein and calcium, the PROCAL was composed of the addition of protein and calcium in identical absolute quantities to the PRO and CAL

[6]Abbreviations used: CAL, high-calcium preload; CON, low-calcium and low-protein control preload; DPP-IV, dipeptidyl peptidase-IV; GIP_{1-42}, glucose-dependent insulinotropic polypeptide$_{1-42}$; $GLP-1_{7-36}$, glucagon-like peptide-1_{7-36}; PRO, high-protein preload; PROCAL, high-protein and high-calcium preload; VAS, visual analog scale; DCON, change from control.

Table 1 Participant characteristics and fasting plasma variables[1]

	Total ($n = 20$)	Men ($n = 13$)	Women ($n = 7$)	P [2]
Characteristics				
Age, y	23 ± 1	24 ± 1	22 ± 1	0.15
Body mass, kg	71.0 ± 2.4	77.4 ± 1.7	59.0 ± 2.4	<0.001
Height, cm	175 ± 2	180 ± 2	164 ± 2	<0.001
BMI, kg/m^2	23.2 ± 0.6	23.9 ± 0.7	21.9 ± 1.1	0.11
Habitual calcium intake, mg/d	1000 ± 126	1080 ± 169	855 ± 180	0.41
Fasting plasma variables[3]				
Insulin, pmol/L	91 ± 8	79 ± 9	112 ± 14	0.049
GIP_{1-42}, pmol/L	2.1 ± 0.3	2.4 ± 0.4	1.7 ± 0.3	0.25
$GLP-1_{7-36}$, pmol/L	0.41 ± 0.08	1.58 ± 0.40	0.99 ± 0.28	0.32

[1] Values are means ± SEMs. GIP_{1-42}, glucose-dependent insulinotropic polypeptide$_{1-42}$; $GLP-1_{7-36}$, glucagon-like peptide-1_{7-36}.

[2] Men vs. women, compared by independent Student's t test.

[3] Mean of 4 visits; $n = 12$ for men and $n = 7$ for women.

Table 2 Nutritional composition of preloads[1]

	CON	PRO	CAL	PROCAL
Energy, kJ	773 ± 27	1244 ± 43	783 ± 27	1253 ± 43
Energy, kcal	185 ± 6	297 ± 10	187 ± 6	299 ± 10
Carbohydrate, g	36 ± 1	37 ± 1	36 ± 1	38 ± 1
Fat, g	3 ± 0	4 ± 0	4 ± 0	4 ± 0
Protein, g	4 ± 0	29 ± 1	5 ± 0	29 ± 1
Calcium, mg	104 ± 4	104 ± 4	1170 ± 40	1170 ± 40
Energy density, kJ/g	2.1 ± 0.0	3.1 ± 0.0	2.1 ± 0.0	3.1 ± 0.0

[1] Values are means ± SEMs. CAL, high-calcium preload; CON, low-calcium and low- protein control preload; PRO, high-protein preload; PROCAL, high-protein and high- calcium preload.

trials (**Table 2**).The calcium concentration of the drinking water used to make the porridge was determined in duplicate with the use of a photometric technique (Modular P, Roche Diagnostics). This was determined as 0.82 6 0.01 mmol/L (given an atomic mass of 40.078 g/mol, this equates to 3.27 6 0.03 mg/dL) and was taken into account in the calcium content of the preloads (Table 2).

Anthropometric variables. Body mass was determined to the nearest 0.1 kg with the use of balance scales (Seca) when participants, wearing only light clothing, arrived at the laboratory. Height was measured to the nearest 0.1 cm with the use of a stadiometer (Seca).

Subjective ratings. Subjective appetite ratings were assessed with the use of a previously validated 100 mm VAS (19) upon participants' arrival at the laboratory (in the fasted, resting state). Questions asked included the following: ''How hungry do you feel?,'' ''How full do you feel?,'' ''How satisfied do you feel?,'' and ''How much do you think you can eat?'' These were also converted into a composite appetite score, which combined hunger, fullness, satisfaction, and prospective consumption to provide a single value, as used previously (20).

Blood sampling and analysis. Blood samples were collected into EDTA tubes with 25 µL of aprotinin per mL of whole blood and were immediately centrifuged (10 min, 1509 X *g*, 4°C). Aliquots of plasma were stored at 280°C before analysis. Plasma was analyzed for insulin (IBL International), GIP_{1-42} (Immuno-Biological Laboratories), and $GLP-1_{7-36}$ (MesoScale Discovery) concentrations with the use of commercially available kits. Samples from all trials for each individual participant were always included on the same plate to minimize variation. Intra-assay CVs were below 10%.

Statistical analysis. Because of difficulties with blood sampling from one participant, data for all blood variables are based on $n = 19$. Where data for a single time point during an individual's trial was missing [11 points were missing out of a total of 380 (<3%) for each blood-based variable], the linear interpolation was used to complete the data set. For clarity and to account for the additional energy in the high protein trials (while the calcium contained negligible additional energy), energy intake is reported as both absolute values (intake at the test meal only in kilojoules) and energy compensation (percentage), calculated as follows:

$$\text{Energy compensation} = (\text{EI}_{\text{CON}}/\text{EI}_{\text{EXP}} + \Delta\text{EP}) \text{ X } 100 \tag{1}$$

where EI represents ad libitum energy intake after the control (EI_{CON}) or experimental (EI_{EXP}) preloads

and ΔEP represents the additional energy (above control) provided by the experimental preload. Energy compensation was calculated for the PRO, CAL, and PROCAL trials, with the CON as the reference. Data for energy compensation are reported as means (95% CIs); thus, if the 95% CIs do not overlap with 100, then there was significant under- or overcompensation.

Plasma variables and subjective ratings were converted into time-averaged postprandial AUC values. Data are expressed as means ± SEMs for absolute data, whereas 95% CIs are presented for mean differences relative to the CON (i.e., PRO-CON, CAL-CON, and PROCAL-CON) and were analyzed with the use of Prism v5 (GraphPad Software). Data were checked for normal distribution with the use of the Shapiro-Wilk normality test and were log-transformed if appropriate before statistical analysis. Male vs. female participant characteristics were compared by independent Student's t tests. A 2-factor (trial X time) repeated-measures ANOVA was used to detect differences between plasma and appetite variables over time. A 1-factor ANOVA was used to detect differences between all trials (CON vs. PRO vs. CAL vs. PROCAL) in energy intake, energy compensation, and AUC data and to compare the mean differences of each trial with the control trial (PRO-CON vs. CAL-CON vs. PROCAL-CON). If there was a significant effect, post-hoc tests adjusted for multiple comparisons (Holm-Sidak) were used to determine the location of variance. Differences were considered significant at $P < 0.05$. Associations between variables [expressed as the change relative to the CON trial (ΔCON)] were assessed by Pearson product-moment correlation coefficients. Raw data are available as a **Supplemental Data** file.


Results

Energy intake. Repeated measures ANOVA detected a significant effect for energy intake at the test meal ($P < 0.05$). After adjustment for multiple comparisons, energy intake after the PROCAL (3419 ± 345 kJ; $P < 0.05$) was significantly less than after the CON (4126 ± 395 kJ), but not after the PRO (3699 ± 304 kJ; $P > 0.05$) or CAL (3501 ± 253 kJ; $P > 0.05$).

Energy compensation was significantly greater (overcompensation) with the CAL vs. the PRO ($P < 0.01$) (**Figure 1**) and tended to be greater with the PROCAL vs. the PRO ($P = 0.06$). The PRO produced almost perfect compensation (perfect compensation = 100%), whereas participants overcompensated after the CAL (Figure 1).

Subjective appetite sensations. A 2-factor repeated measures ANOVA revealed a significant main effect of time for all subjective appetite variables (all $P < 0.001$). With regard to the composite appetite score, the main effect of trial was not significant ($P > 0.05$). There was, however, a significant trial X time interaction effect ($P < 0.05$) in which, after adjustment for multiple comparisons, the PROCAL was lower than the CON at 45 min post-preload (**Figure 2A**).

Figure 1 Energy compensation during an ad libitum test meal 1 h after CONs, PROs, CALs, or PROCALs consumed by healthy adults. Values are individual differences (circles) and means (95% CIs) (horizontal lines). Labeled means without a common letter differ, $P < 0.05$; $n = 20$. CAL, high-calcium preload; CON, low-calcium and low-protein control preload; PRO, high-protein preload; PROCAL, high- protein and high-calcium preload.
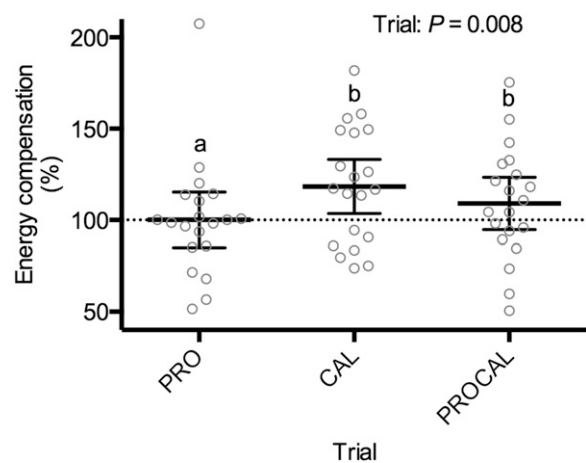
Figure 2 Composite appetite scores after CONs, PROs, CALs, or PROCALs consumed by healthy adults expressed over time (means ± SEMs) (A), as postprandial time-averaged (60 min) AUCs (means ± SEMs) (B), or as mean differences (95% CIs) (horizontal lines; circles are individual data) between the PRO, CAL, and PROCAL relative to the CON (C). Labeled means without a common letter differ, $P < 0.05$; $n = 20$. CAL, high-calcium preload; CON, low-calcium and low-protein control preload; PRO, high-protein preload; PROCAL, high-protein and high-calcium preload.
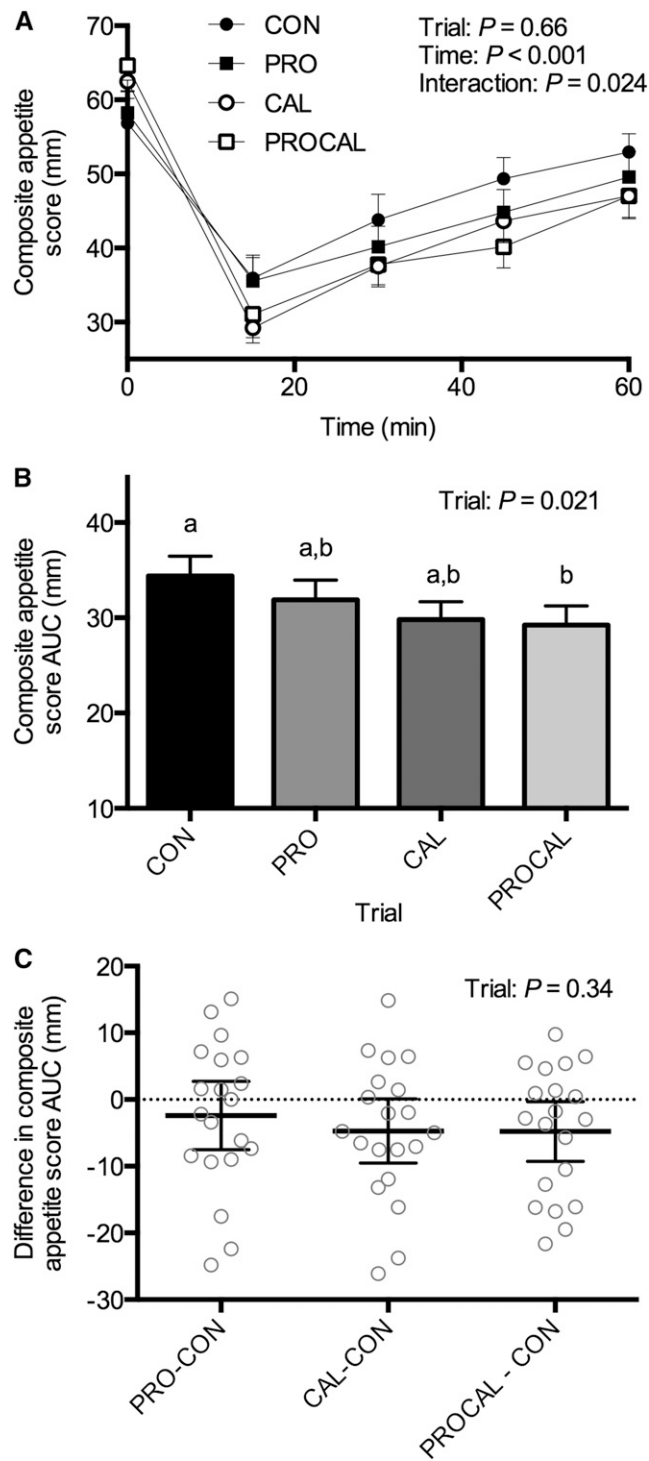
For all other appetite variables, there was no significant main effect of trial detected (all $P > 0.05$). Hunger, fullness, satisfaction, and prospective consumption all displayed significant interaction (trial 3 time) effects (all $P < 0.05$) (**Supplemental Figure 1**).

A repeated measures ANOVA revealed a significant effect for the composite appetite AUC ($P < 0.05$) in which the PROCAL was lower than the CON (Figure 2B). The hunger AUC displayed a significant overall effect ($P < 0.05$), although after adjustment for multiple comparisons, there were no significant differences detected between specific trials (all $P > 0.05$). There was no overall effect for the satisfaction or prospective consumption AUC (both $P > 0.05$), although the main effect for fullness AUC approached significance ($P = 0.06$).

When expressed as the change in appetite sensations relative to control (mean difference $\pm$ 95% CI) (Figure 2C), the PRO did not suppress appetite sensations (23 mm; 95% CI: 28, 3 mm; $P > 0.05$), whereas the reduction in appetite with the CAL vs. the CON (25 mm; 95% CI: 29, 0 mm; $P = 0.06$) approached significance, and the PROCAL significantly reduced the composite appetite AUC relative to the CON (25 mm, 95% CI: 210, 21 mm; $P = 0.023$). However, no significant differences were observed between the PRO-CON vs. the CAL-CON vs. the PROCAL-CON (main effect: $P > 0.05$).

Plasma variables. Plasma insulin concentrations displayed a main effect of trial ($P < 0.01$) and a main effect of time ($P < 0.001$), with no significant interaction (trial X time) effect ($P > 0.05$) (Figure 3A). Plasma GIP_{1-42} concentrations also demonstrated a main effect of trial ($P < 0.01$) and a main effect of time ($P < 0.001$), with no significant interaction effect detected ($P > 0.05$) (Figure 3B). Likewise, plasma $GLP-1_{7-36}$ concentrations displayed main effects of trial ($P < 0.001$) and time ($P < 0.001$) with no significant interaction effect ($P > 0.05$) (Figure 3C).

A repeated measures ANOVA revealed a significant overall effect for the insulin, GIP_{1-42}, and $GLP-1_{7-36}$ AUC ($P < 0.01$, $P < 0.05$, and $P < 0.001$, respectively). After adjustment for multiple comparisons, the insulin AUC was higher with the PROCAL than with the CON (**Supplemental Figure 2A**). The GIP_{1-42} AUC was not significantly different between each trial (Supplemental Figure 2B), whereas the $GLP-1_{7-36}$ AUC was higher with the PRO and the PROCAL than with the CON (Supplemental Figure 2C).

There were no differences between the PRO, CAL, and PROCAL in the change in insulin AUC relative to the CON (**Figure 4A**); however, the PRO and PROCAL produced significantly more positive changes than did the CON, when compared with the CAL (Figure 4B, C).

Associations between variables. The only correlations that were statistically significant were for the ΔCON composite appetite score AUC vs. ΔCON energy intake ($r = 0.37$, $P < 0.05$) (**Supplemental Figure 3A**), ΔCON plasma GIP_{1-42} AUC vs. ΔCON plasma $GLP-1_{7-36}$ AUC ($r = 0.46$, $P < 0.001$) (Supplemental Figure 3B), and ΔCON composite appetite score AUC and ΔCON plasma $GLP-1_{7-36}$ AUC ($r = -0.35$, $P < 0.05$) (Supplemental Figure 3C). Estimated habitual calcium intake (range: 253–2700 mg/d; median: 973 mg/d) did not correlate with either ΔCON plasma GIP_{1-42} AUC or ΔCON plasma $GLP-1_{7-36}$ AUC ($r = 20.04$, $P > 0.05$ and $r = 20.02$, $P > 0.05$, respectively).

Discussion
We demonstrated in this study that a high-protein preload produces almost perfect energy compensation, whereas a high-calcium preload (with and without protein) reduces appetite and results in overcompensation of subsequent energy intake (i.e., less energy intake relative to the energy in the preload). This coincided with an elevation in insulinemia, which could not be attributed clearly to the responses of the incretin hormones GIP_{1-42} and $GLP-1_{7-36}$.
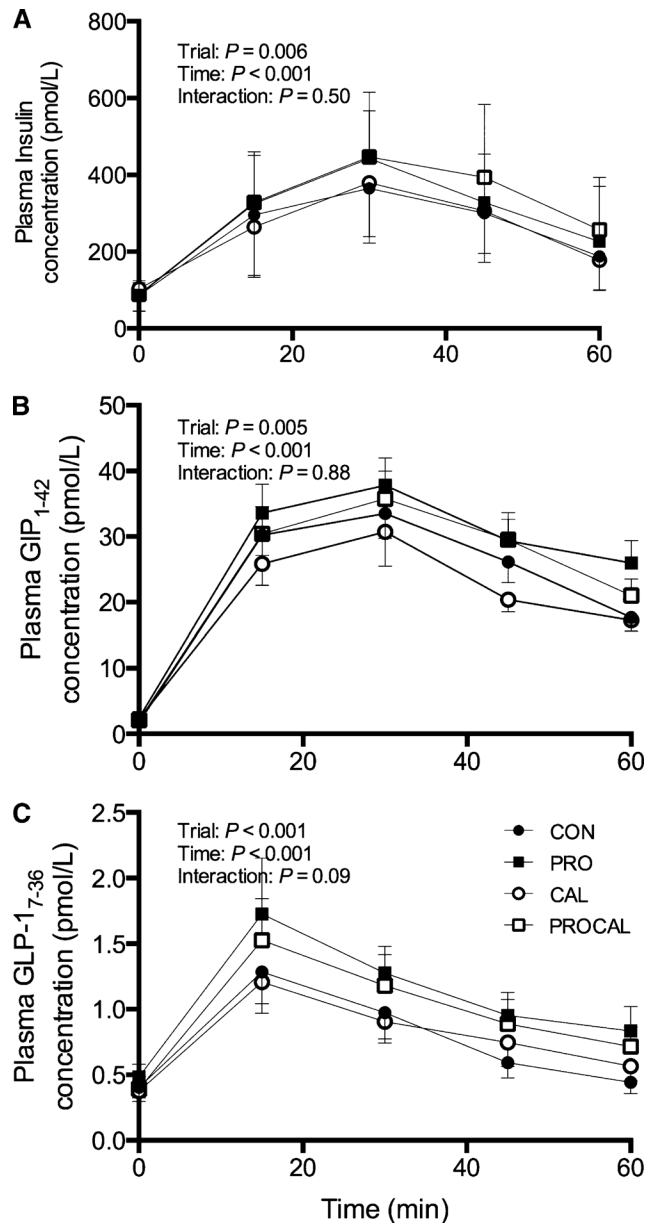
Figure 3 Plasma insulin (A), GIP$_{1-42}$ (B), and GLP-1$_{7-36}$ (C) concentrations after CONs, PROs, CALs, or PROCALs consumed by healthy adults. Values are means ± SEMs; n = 19. CAL, high-calcium preload; CON, low-calcium and low-protein control preload; GIP$_{1-42}$, glucose-dependent insulinotropic polypeptide$_{1-42}$; GLP-1$_{7-36}$, glucagon-like peptide-1$_{7-36}$; PRO, high-protein preload; PROCAL, high-protein and high-calcium preload.

Previous evidence has suggested that dietary calcium may play a role in appetite control (6). However, the self-report nature of the measures used, combined with contradictory evidence (9, 21), make this somewhat equivocal. The data in the present study, acquired from a laboratory setting, suggest that calcium has the potential to acutely reduce postprandial appetite sensations and subsequent energy intake to a sufficient degree to offset any additional energy provided by the preload. Energy compensation was almost perfect (i.e., ~100%) in the PRO trial, whereas significant overcompensation occurred with the CAL and tended to occur with the PROCAL (Figure 1B). These data are consistent with the subjective appetite responses observed (Figure 2C), in which the PROCAL
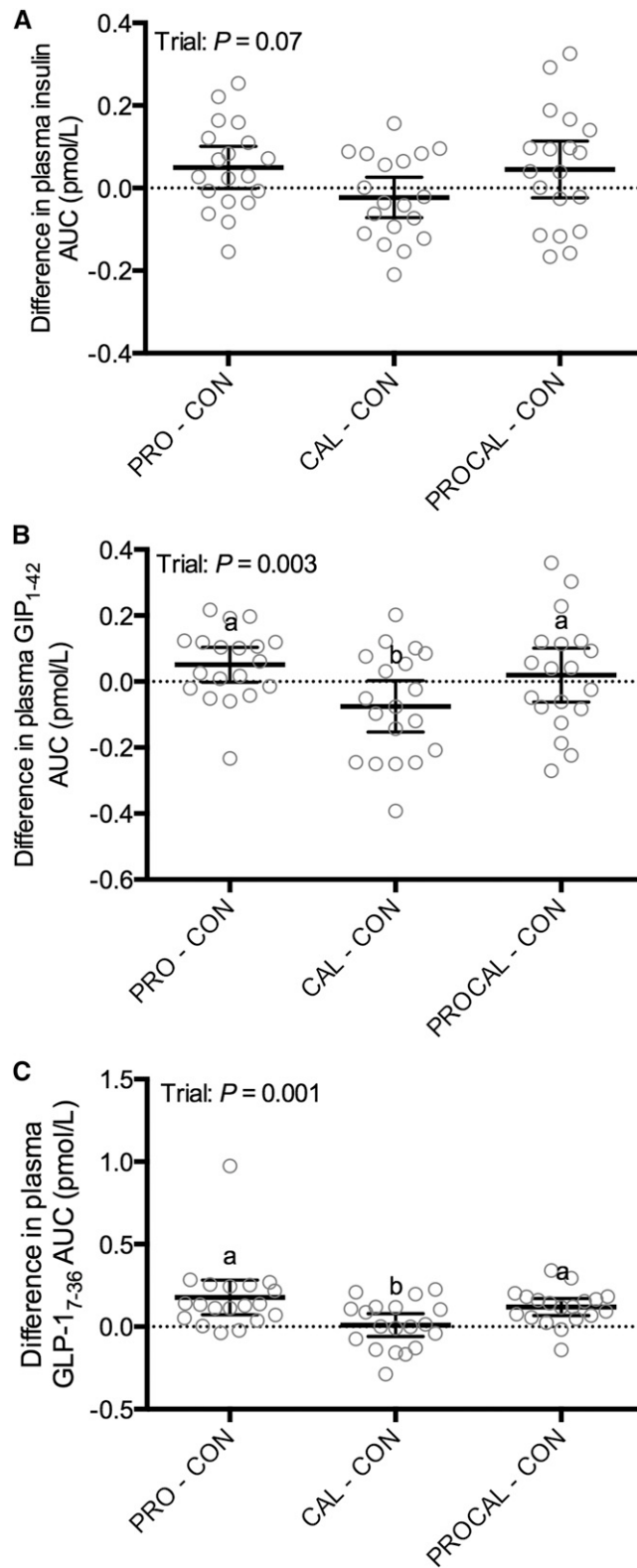
Figure 4 Plasma insulin (A), GIP$_{1-42}$ (B), and GLP-1$_{7-36}$ (C) postprandial time-averaged (60 min) AUCs after CONs, PROs, CALs, or PROCALs consumed by healthy adults. Values are individual differences (circles) and

mean differences (95% CIs) (horizontal lines) between the PRO, CAL, and PROCAL relative to the CON. Labeled means without a common letter differ, $P < 0.05$; $n = 19$. CAL, high- calcium preload; CON, low-calcium and low-protein control preload; GIP_{1-42}, glucose-dependent insulinotropic polypeptide$_{1-42}$; $GLP-1_{7-36}$, glucagon-like peptide-1$_{7-36}$; PRO, high-protein preload; PROCAL, high- protein and high-calcium preload.

lowered appetite relative to the CON and the CAL tended to lower appetite relative to the CON.

The lack of any detectable increase in incretin hormone concentrations with protein-calcium coingestion could be due to either the habitual calcium intake of the participants or the blood-sampling site. A double-blind, placebo-controlled study demonstrated that 3 wk of calcium supplementation (1000 mg/d) results in a potentiation in postprandial plasma $GLP-1_{7-36}$ concentrations in response to a high-calcium meal, relative to a low-calcium control meal (22). This effect was not seen after 3 wk of placebo supplementation. Therefore, a high habitual calcium intake may be required to observe an acute effect from calcium intake on plasma incretin hormones. We attempted to explore this in the present study by examining the association between self-reported habitual calcium intake and the change in plasma incretin concentrations with the PROCAL vs. the CON. No significant correlation was observed between either GIP_{1-42} or $GLP-1_{7-36}$, and habitual calcium intake. The limitations associated with FFQs make it difficult to draw firm conclusions from these observations.

With regard to the sampling site, veins in the antecubital fossa may not provide a representation of the major site of action. As previously mentioned, GIP_{1-42} and $GLP-1_{7-36}$ are secreted by enteroendocrine cells in the gastrointestinal tract. DPP-IV in the endothelium acts immediately, reducing the quantity of $GLP-1_{7-36}$ entering hepatic circulation by ~75% from that which is originally secreted (10). Upon passing through the liver, degradation leaves 10–15% to enter the systemic circulation (10), where further degradation by DPP-IV in plasma and secreted by adipose tissue can take place (23). It is postulated that $GLP-1_{7-36}$ may be able to activate neurons in the intestine and liver (10), which permits central effects (on appetite and insulin secretion) independent of the systemic circulating concentration. Thus, to what degree the concentration measured in an antecubital vein reflects that in the enterocyte and hepatoportal region, which may be the sites of most interest, is unclear.

In addition, it should be acknowledged that numerous other putative mechanisms may also contribute to the appetite effects of protein and calcium intake, including delayed gastric emptying (24), plasma amino acid concentrations (25), and the concentrations of other gastrointestinal hormones such as cholecystokinin (26), peptide YY (12), and gastrin (27). Notwithstanding this, we chose to concentrate on the incretin hormones, given the insulin responses previously observed in humans (7, 8, 18) and in vitro/ex vivo (11, 12).

The design and timing of the preload before energy intake assessment (1 h), was chosen based on previous observations that calcium intake displays a time-dependent suppressant effect on appetite sensations in this period (7, 8), and also because this time period typically produces close to 100% compensation with preload designs (28) and is validated somewhat by the almost 100% compensation seen in the PRO trial. This does, however, constrain the applicability of the findings to this time period, and extrapolation to longer time periods are not recommended without further research. In addition, the quantity of calcium provided in preloads is equivalent to ~800 ml milk. Therefore, the practical application of these findings currently lies in fortification, rather than in normal milk composition. Nonetheless, this does provide a proof of principle and may be used to augment the satiety effects of premeal high-protein snacks (29, 30), and a dose-response study would be a logical progression. The primary outcome was determined as energy intake at the test meal; however, the PRO and PROCAL preloads also contained additional energy (Table 2), which means that any subsequent reduction in energy intake should be interpreted as appropriate energy compensation rather than as a reduction per se.

In conclusion, the consumption of a preload containing additional protein results in almost perfect energy compensation, whereas the addition of calcium, with or without protein, suppresses appetite and energy intake such that overcompensation ensues with no apparent protein-calcium synergy. It remains unclear whether these responses are attributable to changes in plasma insulin, GIP_{1-42}, or $GLP-1_{7-36}$ concentrations.

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