Glycemia and insulinemia in healthy subjects after lactose-equivalent meals of milk and other food proteins: the role of plasma amino acids and incretins[1–3]

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ABSTRACT

Background: Milk products deviate from other carbohydrate-containing foods in that they produce high insulin responses, despite their low GI. The insulinotropic mechanism of milk has not been elucidated.

Objective: The objective was to evaluate the effect of common dietary sources of animal or vegetable proteins on concentrations of postprandial blood glucose, insulin, amino acids, and incretin hormones [glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1] in healthy subjects.

Design: Twelve healthy volunteers were served test meals consisting of reconstituted milk, cheese, whey, cod, and wheat gluten with equivalent amounts of lactose. An equicarbohydrate load of white-wheat bread was used as a reference meal.

Results: A correlation was found between postprandial insulin responses and early increments in plasma amino acids; the strongest correlations were seen for leucine, valine, lysine, and isoleucine. A correlation was also obtained between responses of insulin and GIP concentrations. Reconstituted milk powder and whey had substantially lower postprandial glucose areas under the curve (AUCs) than did the bread reference (−62% and −57%, respectively). Whey meal was accompanied by higher AUCs for insulin (90%) and GIP (54%).

Conclusions: It can be concluded that food proteins differ in their capacity to stimulate insulin release, possibly by differently affecting the early release of incretin hormones and insulinotropic amino acids. Milk proteins have insulinotropic properties; the whey fraction contains the predominating insulin secretagogue. *Am J Clin Nutr* 2004;80:1246–53.

KEY WORDS Glycemic index, insulin index, milk, whey, food proteins, insulinotropic amino acids, amino acids, incretin hormones

INTRODUCTION

It has been established that a diet characterized by carbohydrate-containing foods, which induce low glycemic responses after the consumption of a meal, has advantageous effects on the risk factors for type 2 diabetes and cardiovascular disease (1–4). In 1998, the FAO/WHO recommended that the bulk of carbohydrate-containing foods in the diet be those with a low glycemic index (GI), particularly for persons with glucose intolerance and diabetes (5). For many carbohydrate-rich foods there is a linear correlation between the GI and the insulinemic index (II) (6). Because hyperglycemia and hyperinsulinemia are both ramifications of insulin resistance, it could be argued that a low GI and a low II are both critical product characteristics for the observed metabolic benefits. As suggested by Augustin et al in 2002 (7), the link between a high-GI diet and diabetes may relate to elevated postprandial blood glucose peaks but also to an increased insulin demand. Insulin resistance and hyperinsulinemia are often observed concomitantly, and elevated insulin concentrations cause insulin resistance. Indeed, hyperinsulinemia—when induced experimentally over a 48–72-h period at normoglycemic conditions—may induce insulin resistance in healthy subjects (8).

Milk was recently shown to cause a discrepancy between GI and II in healthy subjects by producing considerably higher IIs than are expected from the low GI commonly reported for milk products (9, 10). Inconsistencies between glycemic and insulinemic responses to milk products have been reported previously in both type 2 diabetic patients (11) and healthy subjects (12).

Interestingly, there is epidemiologic evidence suggesting that overweight subjects with a high intake of milk and dairy products are at a lower risk of developing diseases related to the insulin resistance syndrome (13). However, the insulinotropic effect of milk has not been sufficiently acknowledged and the mechanism, as well as the potential health implications remain unclear.

The insulin response to milk products does not relate solely on the lactose component. Consequently, when testing pure lactose in healthy subjects, the II paralleled the GI, suggesting that some other noncarbohydrate component is responsible for the insulinotropic effect of milk, eg, the milk proteins. It is well known that different food proteins differ in their effect on glucose metabolism in humans (14–18), and several amino acids are potent in stimulating insulin secretion (19–23).

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[2] Supported by grants from The Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (project no. 50.0297-00), and Direktör Albert Påhlssons stiftelse för forskning och välgörenhet.

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Received February 10, 2004.

Accepted for publication May 4, 2004.

TABLE 1
Nutrient composition and serving size of the test meals and the white-wheat-bread (WWB) reference meal[1]

Meal	Amount of product	Added lactose[2]	Total carbohydrate	Total protein	Serving quantity of liquid
WWB	58.4	—	25.0	2.8	250[3]
GL	4.0	25.0	25.0	2.8	550[4]
GH	25.2	25.0	25.0	18.2	550[4]
Cod	74.7	25.0	25.0	18.2	250[5]
Milk	51.3	—	25.0	18.2	550[6]
Whey	28.0	19.4	25.0	18.2	550[6]
Cheese	52.6	25.0	25.0	18.2	250[5]

[1] GL, gluten low; GH, gluten high.
[2] Amount of lactose added to reach 25 g carbohydrate in the meals.
[3] Tap water was served in addition to the bread.
[4] Gluten and lactose were mixed in tap water.
[5] Lactose dissolved in tap water was served along with the protein source.
[6] Powder was dissolved in tap water.

Glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) are released in response to a meal and enhance insulin secretion (24), and protein has been shown to increase the GIP response in both type 2 diabetic patients and healthy subjects (25).

In the current study, common dietary sources of animal or vegetable proteins (whey, casein, dried skim milk, cod, and gluten) were evaluated concerning their influence on postprandial responses of glucose, insulin, amino acids, GIP, and GLP-1.

SUBJECTS AND METHODS

Test meals

All meals contributed 25 g carbohydrate and 18.2 g protein, except the gluten low (GL) and white-wheat-bread reference (WWB), which contained 25 g carbohydrate and 2.8 g protein (**Table 1**). The carbohydrate source was lactose in all test meals, whereas it was starch in the WWB meal. If the intrinsic lactose content was lower than 25 g per serving, lactose (Lactose 17296-500; Merck Eurolab, Stockholm) was added. Whey proteins and roller-dried skimmed milk were tested as a drink, whereas casein was administrated in the form of cheese. Gluten was tested in 2 different quantities, one corresponding to the protein content of the test meals based on animal-protein sources (gluten high, GH) and the other to meet the gluten content of the WWB reference (GL).

Roller-dried skim milk powder (SMP) was obtained from Arla Foods (Stockholm). According to the manufacturer, 96 g powder added to 937 g water equaled the composition of the original milk; 550 g of the reconstituted milk was served. Cheese (Västan 5% fat; Ostkompaniet, Stockholm) was sliced into cubes and served with 250 mL lactose solution. Spray-dried whey protein powder was obtained from Arla Foods. Before being served, 28 g powder was dissolved in 550 g tap water.

Gluten (Gluvital 21000; Cerestar, Mechelen, Belgium) at 2 different concentrations (GL and GH) was homogenized in water with a home hand mixer. Lactose was added to each gluten mixture. Frozen cod fillet was bought at the local market (Skärhamns torskfiléer, Skärhamns frys AB, Sweden). The fillet was cooked in a microwave oven before being served; 250 mL lactose solution was added to the cod meal. The WWB included in the reference meal was prepared in a home baking machine and standardized according to Liljeberg and Björck (26). Bread corresponding to 25 g available starch was served with 250 mL water.

Chemical analysis

The lactose content of milk and whey powder was determined by using β-galactosidase to hydrolyze lactose into glucose and galactose. Ten units β-galactosidase was added to milk, and whey samples corresponding to 50 mg lactose were dissolved in potassium phosphate buffer (pH 7.3) and incubated at 30 °C for 60 min. The liberated amount of glucose was then detected by using a glucose oxidase peroxidase reagent, dissolved in 0.5 mol trisphosphate buffer/L (pH 7.0; 5.6 g/100 mL), and analyzed spectrophotometrically at 450 nm. The protein content was analyzed by using the Kjeldahl procedure. The starch content of the WWB was determined according to the method of Holm et al (27).

A hydrolysis step was performed to analyze peptide-bound amino acids of the different food proteins. The proteins were dissolved in 6 mol HCl/L, containing 0.1% phenol, and kept at 110 °C for 20 h (28). Tryptophan, cysteine, and methionine were lost during acid hydrolysis; therefore, the contents of these amino acids were not measurable. In addition, glutamine and asparagine are converted to glutamic acid and aspartic acid, respectively, during the hydrolysis step.

The amino acids were analyzed with an amino acid analyzer (LC 5001; Biotronik, München, Germany) by using ion-exchange chromatography (29). The amino acids were separated by using standard lithium citrate buffers of pH 2.85, 2.89, 3.20, 4.02, and 3.49. The post column derivatization was performed with ninhydrin (30).

Subjects and study design

Twelve healthy nonsmoking volunteers (6 men and 6 women aged 20–28 y) with normal body mass indexes (21.9 ± 1.26 kg/m^2; $\bar{x}$ ± SD) and not receiving drug treatment participated in the study. All subjects had normal fasting blood glucose concentrations (4.1 ± 0.03 mmol/L; $\bar{x}$ ± SEM) and no history of lactose malabsorption. The meals were provided as breakfasts, on 7 different occasions, in random order with ≥1 wk between each.

In the evenings before each test, the subjects were instructed to eat a standardized meal consisting of white bread with water and

thereafter to refrain from ingesting anything but small amounts of water before the meal test the following morning.

When arriving at the laboratory, a peripheral catheter was inserted into an antecubital vein, and a fasting blood sample was drawn. At all time points, samples were taken in 2 tubes: one for serum and one for plasma (EDTA).

Immediately after withdrawal of the fasting sample, the test meal was served and a digital timer was started. The subjects were requested to complete the meal steadily over a 12-min period, and coffee or tea (150 mL) was served when the meal was finished. Each subject drank either coffee or tea throughout the study. All meals were well tolerated, and the subjects had no problems finishing eating within the 12-min period.

All test subjects gave their informed consent and were aware of the possibility of withdrawing from the study at any time they desired. The Ethics Committee of the Faculty of Medicine at Lund University approved the study.

Blood analysis

Venous blood samples were drawn at fasting and 7.5, 15, 30, 45, 60, 75, 90, 105, and 120 min after consumption of the meal began for the analysis of blood glucose and serum insulin. Samples were also taken at 0, 15, 30, 45, 60, 90, and 120 min for the measurement of free amino acids in plasma. Samples were also collected 0, 7.5, 15, 30, 45, and 60 min after the WWB, milk, cheese, whey, and cod meals for the measurement of plasma GIP and GLP-1.

Blood glucose was analyzed immediately in whole blood from EDTA-coated tubes with the use of a B-Glucose Analyzer (Hemocue AB, Ängelholm, Sweden). The tubes with EDTA were allowed to rest for 30 min before being centrifuged (2500 × *g*, 19 °C) for 6 min. About 1 mL plasma was separated and stored frozen at −20 °C before the measurement of GLP-1 and GIP; 800 μL plasma was separated for the measurement of free amino acids.

Free amino acids were purified by mixing 200 μL 10% sulfosalicylic acid with 800 μL plasma to precipitate high-molecular-weight proteins, according to Biotronik. The amino acid solutions were frozen at −20 °C before they were analyzed using an amino acid analyzer (Biotronik LC 5001) as described above. The plasma concentration of aspartic acid was close to the limit of detection and was therefore not evaluated.

Serum tubes were centrifuged for 15 min (2500 × *g*, 19 °C), and serum was frozen at −20 °C for the measurement of insulin. The serum insulin measurement was performed on an integrated immunoassay analyzer (CODA Open Microplate System; Bio-Rad Laboratories, Hercules, CA) by using an enzyme immunoassay kit (Mercodia Insulin Elisa; Mercodia AB, Uppsala, Sweden).

GIP and GLP-1

GIP and GLP-1 concentrations in plasma were measured after extraction of plasma with 70% ethanol (by vol, final concentration). For the GIP radioimmunoassay (31), we used the carboxyl-terminal directed antiserum R 65, which cross-reacts fully with human GIP but not with the so called GIP 8000, whose chemical nature and relation to GIP secretion is uncertain. Human GIP and ^{125}I human GIP (70 MBq/nmol) were used for standards and tracer. The plasma concentrations of GLP-1 were measured (32) against standards of synthetic GLP-1 7–36 amide by using antiserum code no. 89390, which is specific for the amidated carboxyl terminus of GLP-1 and, therefore, does not react with GLP-1–containing peptides from the pancreas. The results of the assay accurately reflect the rate of secretion of GLP-1 because the assay measures the sum of intact GLP-1 and the primary metabolite, GLP-1 9–36 amide, into which GLP-1 is rapidly converted (33). For both assays, sensitivity was <1 pmol/L, the intraassay CV was <6% at 20 pmol/L, and the recovery of standard (which was added to plasma before extraction) was ≈100% when corrected for losses inherent in the plasma extraction procedure.

Calculations and statistical methods

The incremental (0–90 min) areas under the curve (AUCs) for glucose and insulin were calculated for each subject and meal by using GraphPad PRISM (version 3.02; GraphPad Software Inc, San Diego). All AUCs below the baseline were excluded from the calculations. The AUCs were expressed as means ± SEMs.

Significant differences among the AUCs were assessed with a general linear model (ANOVA) followed by Tukey's multiple comparison test (MINITAB, release 13.32; Minitab Inc, State College, PA). Differences resulting in P values < 0.05 were considered significant.

The differences between the products at different time points were analyzed by using a mixed model (PROC MIXED in SAS release 8.01; SAS Institute Inc, Cary, NC) with repeated measures and an autoregressive covariance structure. When significant interactions between treatment and time were found, Tukey's multiple comparison test were performed for each time point (MINITAB, release 13.32; Minitab Inc).

The initial postprandial responses (0–30-min and 45-min AUCs) of GIP, GLP-1, and amino acids were calculated and correlations were made with insulin responses. These intervals were chosen because the insulin-stimulating mechanism occurs in the early postprandial phase.

To study whether the serum insulin concentrations correlated with the postprandial concentrations of any of the free amino acids, the 45-min AUC for insulin was calculated and divided by the 45-min AUC for blood glucose to obtain the insulinogenic index (34). Spearman's rank correlation was then used to study the relations between the insulinogenic index and each amino acid. A correlation for each subject was calculated and from these values the mean value of Spearman's correlation coefficient was obtained. To determine the P value, a permutation test was performed by using MATLAB with the null hypothesis that no correlation existed (the alternative hypothesis was that the data were correlated).

Plasma amino acid responses (45-min AUC) were correlated with the insulinogenic index rather than with insulin responses to distinguish between glucose-mediated insulin response and other possible insulin secretagogues present in milk.

It was difficult to get enough plasma from one of the test persons for the GIP and GLP-1 analysis; therefore, the results of the analysis of the incretin hormones are based on 11 subjects only. Differences in the responses of GIP and GLP-1 were tested on data based on the 45-min incremental AUC. To evaluate the relation between GIP, GLP-1, and insulin response, the increase in GIP and GLP-1 during the 0–30-min interval was correlated with the corresponding increase in insulin response by using Spearman's rank correlation as described above.

TABLE 2
Content of amino acids in the different meals[1]

	Meal						
Amino acid	WWB	GL	GH	Cod	Milk	Whey	Cheese
	mg/serving						
Asp	198	92	580	1831	1395	1848	1249
Thr	102	71	446	849	800	1268	665
Ser	198	142	895	877	1077	1005	1033
Glu	1546	658	4158	2493	3868	3024	3635
Pro	529	245	1550	644	1754	1148	1823
Gly	173	90	570	752	385	395	368
Ala	173	89	559	752	657	1053	368
Val	198	122	774	1097	1170	1725	1195
Ile	166	97	610	774	852	1016	798
Leu	318	191	1207	1364	1775	1764	1652
Tyr	148	95	600	627	816	549	963
Phe	215	148	937	709	867	652	882
Lys	138	48	305	1836	1395	1596	1414
His	106	66	418	397	487	381	522
Arg	176	100	630	1167	641	437	557

[1] WWB, white-wheat-bread reference meal; GL, gluten low; GH, gluten high.

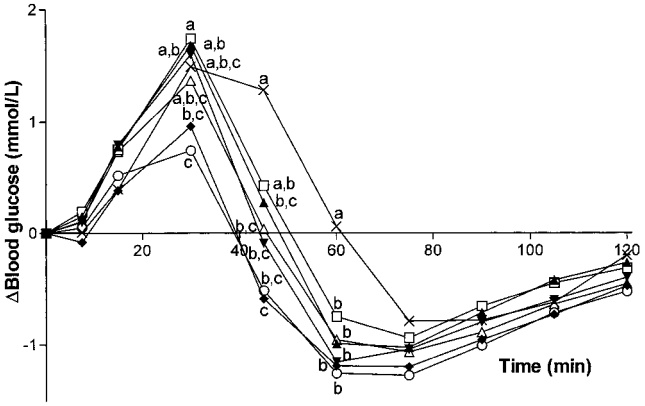

FIGURE 1. Mean (±SEM) incremental changes (Δ) in blood glucose in response to equal amounts of carbohydrate from a white-wheat-bread reference meal (×) and test meals of whey (○), milk (♦), cheese (△), cod (□), gluten-low (▲), and gluten-high (▼) meals. A significant treatment effect ($P < 0.0001$) and treatment × time interaction ($P < 0.0001$) were found at a given time. Values with different lowercase letters are significantly different, $P < 0.05$ (Tukey's test). $n = 12$ healthy subjects.

RESULTS

Amino acid content in the test meals

Concentrations of the amino acids in the test meals are presented in **Table 2**. The concentrations of branched-chain amino acids in the milk-based products and the cod meal were in the same range. However, the content of leucine was somewhat lower in cod than in milk, whey, and cheese. The cod meal contained almost the same amount of valine as milk and cheese, whereas the whey showed a considerably higher amount. Lysine was slightly more represented in cod than in the dairy products. GH contained somewhat lower amounts of lysine and the branched-chain amino acids compared with the other test meals.

Postprandial blood glucose and insulin responses

Milk powder and whey had lower postprandial glucose responses ($P < 0.05$), expressed as AUC (0-90min), than did the reference (**Table 3**). No significant differences in the AUC for blood glucose were found between the reference and the GL, GH, cod, and cheese meals.

Significant differences between treatments over the entire time course ($P < 0.0001$) and a significant treatment × time interaction ($P < 0.0001$) were found for blood glucose concentrations. A post hoc analysis showed that the blood glucose responses at 30 min were higher after milk and whey than after the cod meal (**Figure 1**). At the same time point, GH and GL had significantly higher glucose responses than did the whey meal ($P < 0.05$). Forty-five minutes after eating commenced, a higher blood glucose response was observed after the reference meal than after all test meals, except for cod. At 60 min, all test meals elicited lower glucose values than did the reference ($P < 0.05$).

Although the blood glucose responses after the whey meal were considerably lower than those after the reference meal (−57%), the serum insulin AUC (Table 3) was significantly higher (90%) ($P < 0.05$). The insulin response registered after whey deviated from all other test meals by being significantly higher. The milk and cheese meals showed significantly higher insulin AUCs than did the GL.

TABLE 3
Postprandial blood glucose and insulin areas under the curve (AUCs) and the insulinogenic index after the test meals and the white-wheat-bread (WWB) reference meal[1]

Meal	Glucose AUC (0–90 min)	Change[2]	Insulin AUC (0–90 min)	Change[2]	Insulinogenic index (0–45-min AUC)
	mmol · min/L	%	*mmol · min/L*	%	
WWB	50.2 ± 7.6[a,3]	—	8.0 ± 0.7[b,c]	—	0.16 ± 0.03[b]
GL	42.4 ± 6.8[a,b,c]	−16	6.2 ± 0.7[c]	−23	0.17 ± 0.03[b]
GH	35.4 ± 5.9[a,b,c]	−30	8.2 ± 0.9[b,c]	+3	0.25 ± 0.06[b]
Cod	43.9 ± 8.4[a,b]	−13	7.1 ± 1.0[b,c]	−11	0.14 ± 0.01[b]
Milk	19.3 ± 4.5[c]	−62	9.9 ± 1.2[b]	+24	0.55 ± 0.08[a]
Whey	21.8 ± 5.6[b,c]	−57	15.2 ± 1.6[a]	+90	0.72 ± 0.2[a]
Cheese	39.3 ± 10.1[a,b,c]	−22	10.0 ± 0.9[b]	+25	0.27 ± 0.05[b]

[1] $n = 12$. GL, gluten low; GH, gluten high. Values in the same column with different superscript letters are significantly different, $P < 0.05$ (ANOVA followed by Tukey's test).
[2] Change in postprandial response as a percentage of the WWB reference meal.
[3] $\bar{x}$ ± SEM (all such values).

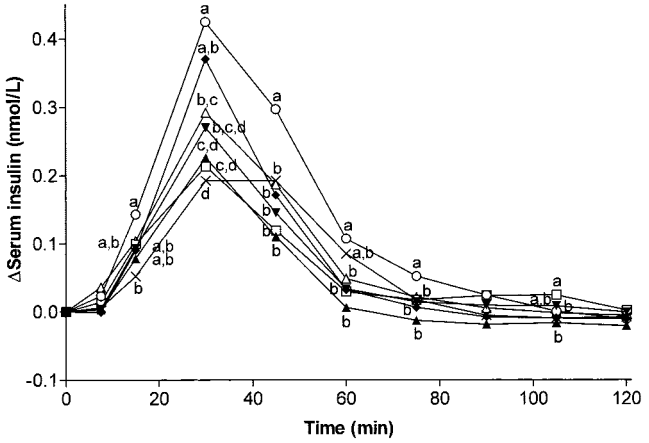

FIGURE 2. Mean (±SEM) incremental changes (Δ) in serum insulin in response to equal amounts of carbohydrate from a white-wheat-bread reference meal (×) and test meals of whey (○), milk (◆), cheese (△), cod (□), gluten-low (▲), and gluten-high (▼) meals. A significant treatment effect ($P < 0.0001$) and treatment × time interaction ($P < 0.0001$) were found at a given time. Values with different lowercase letters are significantly different, $P < 0.05$ (Tukey's test). $n = 12$ healthy subjects.

Significant differences between treatments over the entire time course ($P < 0.0001$) and a significant treatment × time interaction were found for insulin concentrations ($P < 0.0001$). Compared with the reference, whey resulted in increased insulin concentrations at 15, 30, 45, and 75 min (**Figure 2**). Also at 30 min, the insulin responses after the milk and the cheese meals were significantly higher than after the reference ($P < 0.05$). Serum insulin concentrations increased 15, 45, 60, and 75 min after whey ingestion compared with all other test meals. At 30 min, insulin concentrations were higher after the whey meal than after the other test meals, excluding milk.

Postprandial plasma amino acids

After the reference meal, only proline reached 0.02 mmol/L in postprandial blood. After the other meal with a low protein concentration (GL), glutamine and alanine were the only amino acids that had a plasma concentration >0.02 mmol/L. The other gluten meal (GH) elicited higher (>0.04 mmol/L) plasma amino acids concentrations of proline, glutamine, and alanine. Proline and glutamine reached peak values at 120 min, whereas alanine reached the highest concentration between 75 and 120 min.

After the cheese meal, proline and alanine had the highest plasma concentrations, with peaks at ≈0.12 mmol/L and 0.10 mmol/L, respectively. The milk meal resulted in the highest responses for leucine, proline, glutamine, valine, lysine, isoleucine, and alanine, with peak concentrations from 0.07 to 0.14 mmol/L.

Of all the test meals, the whey meal resulted in the most pronounced amino acid responses in postprandial blood; leucine, alanine, lysine, valine, threonine, isoleucine, and proline yielding the highest peaks, which ranged from 0.13 to 0.17 mmol/L.

After the cod meal, plasma concentrations of all amino acids were <0.06 mmol/L, except for lysine and alanine, which reached their highest values after 120 min: 0.12 and 0.10 mmol/L, respectively.

The 45-min AUC for each amino acid after the different test meals and WWB reference meal are shown in **Table 4**. A positive correlation was seen between all amino acids and the insulinogenic index (**Table 5**). The postprandial amino acid responses in plasma after the WWB and GL meals were almost negligible. The highest correlation coefficients were found for leucine, valine, lysine, and isoleucine.

GLP-1 and GIP

The postprandial AUCs for GLP-1 were not significantly different ($P < 0.05$) between the test meals (**Table 6**). No significant treatment effect ($P = 0.92$) or treatment × time interaction ($P = 0.67$) was seen after GLP-1 over the entire time period (**Figure 3**). However, the AUCs for plasma GIP concentrations were significantly higher after the whey meal than after the other test meals and the reference meal (Table 6).

An examination of GIP over the entire time period showed that both the treatment effect and the treatment × time interaction were significant ($P < 0.0001$ and $P = 0.0068$, respectively). A

TABLE 4
Incremental postprandial areas under the curve (AUCs) for the different amino acids from 0 to 45 min after the meals[1]

	Meal						
Amino acid AUC	WWB	GL	GH	Cod	Milk	Whey	Cheese
				mmol · min/L			
Thr	0.2 ± 0.04^{b}	0.3 ± 0.1^{b,c}	0.5 ± 0.2^{b,c}	0.3 ± 0.1^{b}	1.4 ± 0.2^{b,c,d}	2.9 ± 0.3^{b,c,d}	1.0 ± 0.2^{b,c,d,e}
Ser	0.2 ± 0.1^{b}	0.1 ± 0.1^{b,c}	0.6 ± 0.2^{b,c}	0.3 ± 0.1^{b}	0.9 ± 0.9^{c,d}	1.7 ± 0.2^{e,f}	0.8 ± 0.2^{c,d,e}
Gln	0.5 ± 0.1^{a}	1.0 ± 0.5^{a}	1.6 ± 0.4^{a}	0.9 ± 0.2^{a}	2.0 ± 1.4^{a,b}	2.2 ± 0.4^{d,e}	1.6 ± 0.3^{a,b,c}
Pro	1.3 ± 0.5^{a}	0.6 ± 0.2^{a,b}	1.3 ± 0.3^{a}	0.5 ± 0.2^{b}	2.6 ± 0.5^{a}	2.8 ± 0.4^{c,d,e}	2.4 ± 0.4^{a}
Gly	0.2 ± 0.05^{b}	0.2 ± 0.1^{b}	0.4 ± 0.1^{c}	0.4 ± 0.1^{b}	0.7 ± 0.2^{d}	1.2 ± 0.6^{g}	0.3 ± 0.1^{c}
Ala	0.3 ± 0.1^{a,b}	0.8 ± 0.2^{a}	1.1 ± 0.3^{a,b}	1.1 ± 0.2^{a}	1.9 ± 0.3^{a,b}	3.2 ± 0.5^{a,b,c}	1.9 ± 0.3^{a,b}
Val	0.3 ± 0.1^{a,b}	0.2 ± 0.1^{b,c}	0.5 ± 0.1^{b,c}	0.3 ± 0.1^{b}	2.3 ± 0.3^{a,b}	3.1 ± 0.3^{a,b,c,d}	1.8 ± 0.4^{a,b,c}
Ile	0.4 ± 0.1^{a,b}	0.1 ± 0.02^{b,c}	0.4 ± 0.1^{c}	0.2 ± 0.1^{b}	2.1 ± 0.4^{a,b,c}	3.2 ± 0.3^{a,b,c}	1.8 ± 0.6^{a,b,c,d}
Leu	0.3 ± 0.1^{a,b}	0.2 ± 0.1^{b,c}	0.5 ± 0.1^{c}	0.2 ± 0.1^{b}	2.8 ± 0.3^{a}	3.9 ± 0.3^{a}	1.8 ± 0.4^{a,b,c}
Tyr	0.2 ± 0.1^{b}	0.3 ± 0.1^{b,c}	0.3 ± 0.1^{c}	0.2 ± 0.1^{b}	0.9 ± 0.2^{c,d}	0.7 ± 0.1^{g}	0.5 ± 0.2^{d,e}
Phe	0.1 ± 0.04^{b}	0.1 ± 0.03^{c}	0.3 ± 0.1^{c}	0.1 ± 0.02^{b}	0.5 ± 0.1^{d}	0.4 ± 0.07^{g}	0.6 ± 0.2^{d,e}
Lys	0.1 ± 0.05^{b}	0.2 ± 0.1^{b,c}	0.3 ± 0.1^{c}	0.5 ± 0.1^{b}	2.1 ± 0.4^{a,b}	3.7 ± 0.3^{a,b}	1.9 ± 0.4^{a,b}
His	0.1 ± 0.02^{b}	0.1 ± 0.04^{b,c}	0.3 ± 0.05^{c}	0.2 ± 0.04^{b}	0.5 ± 0.1^{d}	0.4 ± 0.1^{g}	0.4 ± 0.1^{d,e}
Arg	0.5 ± 0.2^{a,b}	0.2 ± 0.05^{b,c}	0.6 ± 0.2^{b,c}	0.4 ± 0.1^{b}	0.8 ± 0.2^{c,d}	1.1 ± 0.2^{f,g}	0.7 ± 0.3^{d,e}

[1] All values are $\bar{x}$ ± SEM; $n = 12$; WWB, white-wheat-bread reference meal; GL, gluten low; GH, gluten high. Values in the same column with different superscript letters are significantly different, $P < 0.05$ (ANOVA followed by Tukey's test).

TABLE 5
Spearman's correlation coefficients and *P* values for the relations between plasma amino acids [45-min area under the curve (AUC)] and the insulinogenic index (45-min AUC)

Amino acid	*r*	*P*
Thr	0.53	0.022
Ser	0.49	0.009
Gln	0.37	0.013
Pro	0.55	0.005
Gly	0.35	0.030
Ala	0.47	0.013
Val	0.63	0.005
Ile	0.58	<0.001
Leu	0.67	0.003
Tyr	0.48	0.003
Phe	0.47	0.007
Lys	0.62	0.005
His	0.32	0.054
Arg	0.38	0.020

post hoc analysis showed that the GIP concentration after whey was significantly higher than that after milk and the reference meal 15 min after ingestion ($P < 0.05$; **Figure 4**). At 30 min, a higher GIP concentration was found after whey than after cod. Between 45 and 60 min, whey induced a greater GIP response than did both cod and milk. At 60 min, the milk and cod meals resulted in lower GIP concentrations than did the reference bread ($P < 0.05$). An evaluation of the data for all meals, including the reference meal, showed a positive correlation between the GIP and insulin responses between 0 and 30 min (**Table 7**).

DISCUSSION

Although the postprandial blood glucose response after the test meal with reconstituted skim milk powder was low, the insulin response after milk was not significantly distinguishable from that after the WWB reference. Thus, the present results confirm those from a previous study in which the ingestion of pasteurized milk resulted in a discrepancy between blood glucose (GI = 30) and the insulin response (II = 90), which was not present after a carbohydrate equivalent load of pure lactose (GI = 68; II = 50) (9). In that study, it was hypothesized that

TABLE 6
Postprandial glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) areas under the curve (AUCs) after the test meals and the white-wheat-bread (WWB) reference meal[1]

Meal	GLP-1 AUC (0–45 min)	Change[2]	GIP AUC (0–45 min)	Change[2]
		%		%
WWB	253 ± 61[a,3]	—	656 ± 97[b]	—
Cod	281 ± 72[a]	11	601 ± 120[b]	−8
Milk	285 ± 48[a]	13	605 ± 125[b]	−8
Whey	289 ± 77[a]	14	1097 ± 151[a]	67
Cheese	251 ± 84[a]	−1	790 ± 178[b]	21

[1] Values in the same column with different superscript letters are significantly different, $P < 0.05$ (ANOVA followed by Tukey's test).
[2] Change in postprandial response as a percentage of the WWB reference meal.
[3] $\bar{x}$ ± SEM (all such values).

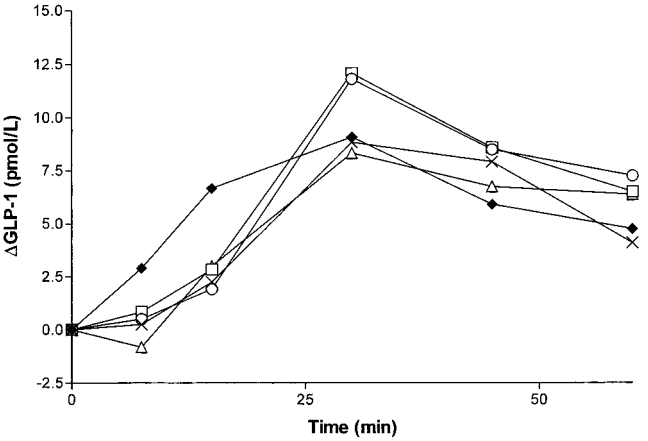

FIGURE 3. Mean (±SEM) incremental changes (Δ) in glucagon-like peptide 1 (GLP-1) in response to equal amounts of carbohydrate from a white-wheat-bread reference meal (×) and test meals of whey (○), milk (◆), cheese (△), and cod (□). No significant treatment effect ($P = 0.92$) or treatment × time interaction ($P = 0.67$) was found. $n = 11$ healthy subjects.

some milk component in addition to lactose appears to stimulate insulin secretion. As judged from similar and high IIs for reconstituted skim milk (<0.1% fat, present study) and pasteurized 3%-fat milk (9, 10), neither the fat content per se nor the drying process appears to be involved in the insulinotropic mechanism. Instead, we supposed an involvement of milk proteins. About 80% of milk proteins are casein and 20% are whey. When rennet (used in cheese making) is added to milk, casein proteins aggregate and form a gel but whey proteins remain soluble. Also, when the pH is decreased, casein proteins clot; hence, the acidity in the stomach makes casein, but not whey, to aggregate into a gel.

It was previously observed that the ingestion of milk and other food proteins may stimulate insulin secretion (11, 12, 35). In the current study, the insulin response to the whey meal was even more pronounced than that to milk, which indicated that the insulinotropic component may be connected to the soluble milk proteins. Assuming that the protein fraction of milk contains an

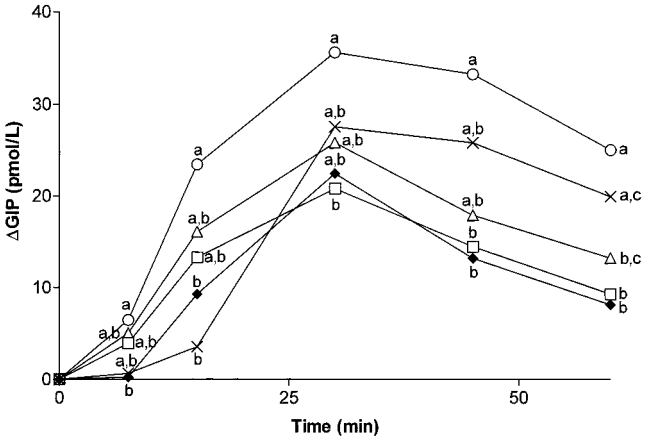

FIGURE 4. Mean (±SEM) incremental changes (Δ) in glucose-dependent insulinotropic polypeptide (GIP) in response to equal amounts of carbohydrate from a white-wheat-bread reference meal (×) and test meals of whey (○), milk (◆), cheese (△), and cod (□). A significant treatment effect ($P < 0.0001$) and treatment × time interaction ($P = 0.0068$) were found at a given time. Values with different lowercase letters are significantly different, $P < 0.05$ (Tukey's test). $n = 11$ healthy subjects.

TABLE 7
Spearman's correlation coefficients and *P* values for the relations between increments in glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) concentrations and the corresponding insulin increment (0–30 min)

	r	*P*
GIP	0.41	0.008
GLP-1	0.16	0.083

insulin secretagogue, the stimulating effect might be mediated through bioactive peptides or by specific amino acids released during digestion. Several amino acids are potent stimulators of insulin release, either when taken as a protein orally or when infused intravenously (21), and certain amino acids (eg, the branched-chain amino acids) are more insulinogenic than are others. van Loon et al (36) showed that the insulin response in healthy subjects was positively correlated with plasma leucine, phenylalanine, and tyrosine when ingested orally in the form of drinks in combination with glucose. Furthermore, it was concluded that protein hydrolysates stimulate insulin secretion to a higher extent than do intact protein because of a more rapid increase in postprandial plasma amino acid concentrations. In addition, Calbet and MacLean (37) described a close relation between the insulin response and the increase in plasma amino acid response, especially for leucine, isoleucine, valine, phenylalanine, and arginine. These findings indicate that the postprandial pattern of plasma amino acids may be an important entity for the insulinogenic properties of food proteins.

Of the 7 amino acids that reached the highest increments after the whey meal in the current study, the branched-chain amino acids (leucine, valine, and isoleucine), lysine, and threonine are all known to stimulate insulin secretion (20, 36, 38). Alanine might also have insulinotropic effects under select experimental conditions (39).

Whereas whey, milk, and to some extent cheese ingestion resulted in obvious amino acid responses, the remaining meals (GH, GL, cod, and WWB) resulted in only small increases in plasma amino acids. Generally, the amino acid responses to the cod meal occurred 60 min after ingestion. In contrast, peak amino acid responses to milk, whey, and cheese occurred more rapidly—within 30–45 min after ingestion—which indicated that milk proteins are highly digestible and result in a rapid release of amino acids into the circulation.

Instead of being related to amino acids per se, the insulinotropic effect of milk proteins might be related to bioactive peptides either present in the milk or formed during digestion in the small intestine. A possible pathway in the case of peptides may include the activation of the incretin system (24). Previous studies showed a protein-stimulated insulin response in type 2 diabetic patients (40) and healthy subjects (41) that did not parallel the rise in amino acids in the circulation, which suggests the involvement of the incretin hormones in protein-stimulated insulin release.

Conversely, Schmid et al (22) concluded that gut factors are only of minor importance and that amino acids are the major insulin secretagogue in the absence of carbohydrates. Whereas the GLP-1 responses to all of the test meals were similar in the current study, whey induced a particularly elevated GIP response. Thus, the higher GIP response after whey may have been one contributing factor to the observed elevated postprandial insulin response. The degree to which the GIP response explains the insulinotropic effect of whey proteins can, however, not be elucidated from the present data. Surprisingly, the GIP response to the milk meal was not elevated compared with the response to the reference meal. Similarly to whey, milk also showed an insulinogenic effect, although it was of a lower magnitude. This finding indicates that the stimulation of the incretin system may not solely explain the insulinotropic effects of whey.

In contrast with milk and whey, the postprandial blood glucose response after the meal consisting of cheese and lactose was not significantly different from that obtained after the WWB meal. However, serum insulin concentrations after the cheese meal were not significantly different from those after milk, although they were lower than those after whey. It is likely that cheese contains not only casein but also the remnants of whey proteins, and either this small amount of whey in the cheese curd is capable of enhancing insulin concentrations or the casein fraction itself may contain an insulin secretagogue. However, it is known that casein is more slowly digested than is whey (42, 43), and the different digestion rates of the proteins may effect the insulin response.

Wheat gluten in high and low amounts (the GH and GL meals, respectively) and cod affected glycemia and insulin response similarly to the reference meal, which suggests that both wheat gluten and cod have a poor capacity to stimulate insulin secretion. The lack of effect of wheat protein on the insulin response agrees with the consistency reported in GIs and IIs for a range of wheat products (6).

A synergistic effect of carbohydrates and proteins in stimulating insulin has been reported in diabetic subjects (44), whereas this effect was not seen in healthy persons (41). Although an additive effect of protein and carbohydrates (45) after the cod meal would be possible, the rise in plasma amino acids after the cod meal was modest compared with that after the milk and whey meals and presumably was too low to evoke an amino acid–induced insulin response.

Although whey and cod proteins are similar with respect to the content and distribution of amino acids, the postprandial plasma pattern of amino acids differed substantially after the test meals containing these proteins, most probably because of the different digestion and absorption rates of these proteins. It is especially interesting that several of the known insulinotropic amino acids (leucine, valine, isoleucine, lysine, and threonine) were among those amino acids that were observed to increase after the whey meal.

It can be concluded that food proteins differ in their capacity to stimulate insulin release, possibly by affecting the early postprandial concentrations of insulinotropic amino acids and incretin hormones differently. It cannot be excluded that an elevated plasma amino acid response is merely an indicator of the rapid digestion and absorption of whey proteins.

The results of the current study show that milk proteins have insulinotropic properties, with the whey fraction being a more efficient insulin secretagogue than casein. It remains to be shown whether the insulinotropic effect of whey and milk depends on an optimal and rapid postprandial release of certain amino acids to the blood, the release of a bioactive peptide, or an activation of the incretin system, particularly by enhancing GIP secretion. Also, the potential long-term effects of a noncarbohydrate–mediated insulin stimulus on metabolic variables should be evaluated in healthy persons and in persons with a diminished capacity for insulin secretion.

MN coordinated the study and was involved in the study design, the collection and analysis of the data, the statistical analysis, and the evaluation and the writing of the paper. MS was responsible for the amino acid analysis. AHF was involved in the design of the study, the acquisition of blood samples, and the evaluation of the data. JJH was responsible for the incretin analysis and was involved in the evaluation and the writing of the paper. IMEB secured the funding for the study and was involved in the design, evaluation, and writing of the paper. None of the authors had a conflict of interest.

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