Metabolic effects of amino acid mixtures and whey protein in healthy subjects: studies using glucose-equivalent drinks[1–3]

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ABSTRACT

Background: Milk protein, in particular the whey fraction, has been shown to display insulinotrophic properties in healthy persons and persons with type 2 diabetes. In parallel to the hyperinsulinemia, a pronounced postprandial rise of certain amino acids and of glucose-dependent insulinotrophic polypeptide (GIP) was observed in plasma.

Objective: The objective of the study was to determine to what extent the insulinotrophic properties of whey could be simulated by specific amino acid mixtures.

Design: Twelve healthy volunteers were served drinks consisting of pure glucose (reference drink) or glucose supplemented with free amino acids or whey proteins (test drinks).

Results: A test drink with the branched-chain amino acids isoleucine, leucine, and valine resulted in significantly higher insulin responses than did the glucose reference. A drink containing glucose and leucine, isoleucine, valine, lysine, and threonine mimicked the glycemic and insulinemic responses seen after whey ingestion. With consumption of this drink, the glucose area under the curve (AUC) was 44% smaller ($P < 0.05$) and the insulin AUC was 31% larger (NS) than with consumption of the reference drink. With consumption of the whey drink, the AUCs were 56% smaller (glucose; $P < 0.05$) and 60% larger (insulin; $P < 0.05$), respectively, than with the reference drink. The whey drink was accompanied by an 80% greater GIP response ($P < 0.05$), whereas the drinks containing free amino acids did not significantly affect GIP secretion.

Conclusion: A mixture of leucine, isoleucine, valine, lysine, and threonine resulted in glycemic and insulinemic responses closely mimicking those seen after whey ingestion in the absence of an additional effect of GIP and glucagon-like peptide 1. *Am J Clin Nutr* 2007;85:996–1004.

KEY WORDS Milk, whey, blood glucose, serum insulin, hyperinsulinemia, amino acids, incretin hormones

INTRODUCTION

Milk products are powerful acute stimulants of insulin secretion (1–3) and have the ability to enhance the insulin response also when supplied in a mixed meal (4, 5). The key mechanism probably is the action of the milk proteins, because neither lactose (1) nor milk fat (6) can account for the elevated insulinemia. In previous work, we found that whey proteins are particularly insulinotrophic, both compared with casein in cheese and with other proteins of animal or vegetable origin (7). The essential amino acids leucine, isoleucine, valine, lysine, and threonine were among the amino acids that showed pronounced postprandial increments in plasma after a whey drink and also the strongest correlation with insulin response. We thus concluded that these amino acids are particularly important in milk-induced hyperinsulinemia. In addition, the postprandial increment in glucose-dependent insulinotrophic polypeptide (GIP) was particularly pronounced after the whey drink. The experimental design, however, did not allow conclusive comparisons of the importance of the specific amino acids with the role of bioactive peptides containing these amino acids (7).

It is well known that various food proteins may stimulate insulin release (8–12). The rate at which the amino acids are released during digestion and absorbed into the circulation may differ significantly among different food proteins. Whey is considered a rapidly digested protein, which thus promotes higher concentrations of amino acids in postprandial plasma (13). Several amino acids may act as direct insulin secretagogues (14–20), and an increase in plasma amino acids appears to be associated with enhanced insulin response (21).

The aim of the current study was to determine the effect of specific amino acids on the postprandial insulin response in healthy subjects. The postprandial responses of blood glucose, serum insulin, GIP, and glucagon-like peptide 1 (GLP-1) were evaluated after the ingestion of drinks containing equicarbohydrate amounts of glucose without or with addition of free amino acids. Leucine, isoleucine, valine, lysine, and threonine were selected because of their predominant appearance in postprandial blood after whey ingestion, and they were included in amounts corresponding to their

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TABLE 1
Nutrient composition and serving size of the glucose reference drink and the test drinks with whey protein or amino acid mixtures[1]

Constituent nutrient	Reference drink	Test drinks			
		AA2	AA3	AA5	Whey
	g/serving	*g/serving*			
Glucose	25	25	25	25	25
Leucine	—	—	2.2	2.2	—
Isoleucine	—	—	1.1	1.1	—
Valine	—	—	1.1	1.1	—
Lysine	—	1.6	—	1.6	—
Threonine	—	1.4	—	1.4	—
Whey protein	—	—	—	—	18
ΣAmino acids		3.0	4.4	7.4	
Amount of liquid (mL)	250	250	250	250	250

[1] AA2, lysine and threonine; AA3, leucine, isoleucine, and valine; AA5, leucine, isoleucine, valine, lysine, and threonine.

TABLE 2
Contents of amino acids in the whey

Amino acid	Value
	mg/g whey
Aspartic acid	94.1
Threonine	61.1
Serine	38.8
Glutamic acid	141.4
Proline	46.7
Glycine	13.8
Alanine	42.1
Valine	59.3
Cysteine	22.8
Methionine	19.4
Isoleucine	57.3
Leucine	79.8
Tyrosine	20.8
Phenylalanine	21.3
Lysine	76.1
Histidine	18.7
Arginine	22.0

contents in whey. A glucose-equivalent whey protein drink was included as a reference.

SUBJECTS AND METHODS

Test drinks

Four test drinks and a reference drink were included in the study. Glucose in 250 mL water was used as the reference drink. The test drinks consisted of 1) lysine and threonine (AA2); 2) leucine, isoleucine, and valine (AA3); 3) leucine, isoleucine, valine, lysine, and threonine (AA5); and 4) whey protein (Semper AB, Stockholm, Sweden). All test drinks contained 25 g carbohydrates in the form of glucose. The whey contained <0.2% lactose according to the manufacturer. The whey drink was adjusted to contain 18 g protein, in accordance with an earlier study (7).

The amino acids were provided by Bröste AB (Mölndal, Sweden). The branched-chain amino acids (BCAAs) were obtained as a mixture (BCAA211; Ajinomoto, Kawasaki, Japan) with the leucine-to-isoleucine-to-valine ratio being 2:1:1, whereas threonine (l-threonine; Ajinomoto) and lysine (l-lysine monohydrochloride; Ajinomoto) were provided as single amino acids. The compositions of the drinks are shown in **Table 1**.

The test drinks with added amino acids were based on the 5 essential amino acids, the appearance of which in postprandial blood had the highest correlation coefficients with the insulinogenic index in a previous study with whey (7). The total amount of these 5 amino acids in the test drink was thus similar to their content in the previously studied whey drink (7.37 g). However, not only the amount of amino acids ingested but also the postprandial amino acid profile may be important for the insulin response. Therefore, the ratios of the amino acids in the test drinks were, as far as possible, based on the incremental postprandial areas under the curve (AUC) in healthy subjects fed a whey drink (7). Because we used a commercially prepared mixture of the BCAA, the ratios between leucine, isoleucine, and valine were, however, fixed. The amino acid composition of the whey is shown in **Table 2**.

Subjects and study design

Twelve healthy nonsmoking volunteers (6 M, 6 F; aged 20–30 y) with body mass index (BMI; in kg/m^2) ranging from 19.5 to 25.7 ($\bar{x}$ ± SEM: 22.4 ± 0.6) and without drug treatment participated in the study. All subjects had normal mean fasting blood glucose concentrations (4.6 ± 0.04 mmol/L; **Table 3**).

The drinks were provided as breakfasts in random order on 5 different occasions with ≥1 wk between each test. In the evening before each test, the subjects were instructed to eat a standardized evening meal consisting of white wheat bread and water at ≈2100–2200 and thereafter not to ingest anything but water. When the subjects arrived at the laboratory the next morning, a

TABLE 3
Fasting values of blood glucose, serum insulin, and plasma glucose-dependent insulinotrophic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) in healthy subjects before ingestion of the reference glucose drink and whey and glucose drinks to which free amino acids were added[1]

Meal	Blood glucose	Serum insulin	Plasma GIP	Plasma GLP-1
	mmol/L	*nmol/L*	*pmol/L*	*pmol/L*
Reference	4.6 ± 0.1^a	0.046 ± 0.005^a	6.2 ± 1.0^a	8.8 ± 1.4^b
Whey	4.7 ± 0.1^a	0.041 ± 0.003^a	7.2 ± 1.6^a	11.1 ± 1.4^{a,b}
AA2	4.6 ± 0.1^a	0.048 ± 0.008^a	10.2 ± 2.4^a	12.8 ± 1.9^a
AA3	4.6 ± 0.1^a	0.047 ± 0.005^a	8.3 ± 1.8^a	9.8 ± 1.0^{a,b}
AA5	4.6 ± 0.1^a	0.047 ± 0.006^a	7.8 ± 1.8^a	9.7 ± 1.8^{a,b}

[1] All values are $\bar{x}$ ± SEM; $n = 12$. AA2, lysine and threonine; AA3, leucine, isoleucine, and valine; AA5, leucine, isoleucine, valine, lysine, and threonine. Values in the same column with different superscript letters are significantly different, $P < 0.05$ (ANOVA followed by Tukey's test).

TABLE 4
Glycemic and insulinemic responses [90-min area under the curve (AUC)] in healthy subjects after reference glucose drinks and glucose drinks to which whey or free amino acids were added[1]

Meal	Blood glucose AUC	Change	Serum insulin AUC	Change
	mmol · min/L	*%*	*nmol · min/L*	*%*
Reference	103.4 ± 21.0[a]		10.6 ± 1.3[a,b]	
Whey	45.8 ± 10.8[b]	−56	17.0 ± 2.0[c]	60
AA2	83.0 ± 14.1[a,b]	−20	9.4 ± 1.1[a]	−11
AA3	71.7 ± 14.1[a,b]	−31	14.8 ± 2.0[c]	40
AA5	58.1 ± 12.3[b]	−44	13.9 ± 1.6[b,c]	31

[1] All values are $\bar{x}$ ± SEM; $n = 12$. AA2, lysine and threonine; AA3, leucine, isoleucine, and valine; AA5, leucine, isoleucine, valine, lysine, and threonine. Values in the same column with different superscript letters are significantly different, $P < 0.05$ (ANOVA followed by Tukey's test).

peripheral catheter was inserted into an antecubital vein, and a fasting blood sample was drawn. Thereafter, the test drink was served, and the subjects were instructed to drink steadily over a 12-min period. After the drinks were finished, 150 mL tea or coffee was served. Each subject drank either tea or coffee throughout the study.

All test subjects gave written informed consent and were aware that they could withdraw from the study at any time they desired. The Ethics Committee of the Faculty of Medicine at Lund University approved the study.

Blood analysis

At all timepoints, blood was sampled into 3 tubes: one for serum, one for plasma (containing EDTA), and one for blood glucose analysis (using a tube containing sodium fluoride and potassium oxalate). For the analysis of blood glucose and serum insulin, venous blood samples were drawn at fasting and 15, 30, 45, 60, 75, 90, and 120 min after commencing the meal. Samples were also taken at 0, 15, 30, 45, 60, 90, and 120 min for the measurement of plasma GIP and GLP-1. Blood glucose concentrations were measured in whole blood by using a glucose oxidase peroxidase reagent. Serum was centrifuged for 15 min (2500 × *g* at 19 °C) and frozen at −20 °C until analyzed for insulin. The insulin measurement was performed on an integrated immunoassay analyzer (CODA Open Microplate System; Bio-Rad Laboratories, Hercules, CA) by using an enzyme-linked immunosorbent assay kit (Mercodia Insulin Elisa; Mercodia AB, Uppsala, Sweden).

The tubes with EDTA were allowed to rest for 30 min before undergoing centrifugation at 2500 × *g* and 19 °C for 15 min. Approximately 1 mL plasma was separated and stored in a frozen state at −20 °C before measurement of GLP-1 and GIP. Plasma (800 μL) was removed for the measurement of free amino acids.

GIP and GLP-1 concentrations in plasma were measured after extraction of plasma with 70% ethanol (by vol, final concentration). For the GIP radioimmunoassay (22), carboxyl terminal–directed antiserum R65 was used, which crossreacts fully with human GIP but not with the so-called GIP 8000, whose chemical nature and relation to GIP secretion are uncertain. Human GIP and ^{125}I human GIP (70 Bq/nmol) were used for standards and tracer. The plasma concentrations of GLP-1 were measured by using the method of Orskov et al (23) 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 (24). 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.

Free amino acids were purified by mixing 200 μL of 10% sulfosalicylic acid with 800 μL plasma to precipitate high-molecular-weight proteins according to the method of Pharmacia Biochrom Ltd (Cambridge, United Kingdom) The amino acid solutions were frozen at −20 °C before they were analyzed in an amino acid analyzer (Biochrom 30; Pharmacia Biochrom Ltd) by using ion-exchange chromatography. The amino acids were separated by using standard lithium citrate buffers of pH 2.80, 3.00, 3.15, 3.50, and 3.55. The postcolumn derivatization was performed with ninhydrin (25).

Calculations and statistical analysis

The mean (±SEM) incremental 0–90-min AUCs for glucose and insulin, 0–90-min AUCs for GIP and GLP-1, and 0–45-min AUCs for the different amino acids were calculated for each subject and meal by using GraphPad PRISM software (version 3.02; GraphPad Software Inc, San Diego). All AUCs below the baseline were excluded from the calculations.

Significant differences among the AUCs were assessed with a general linear model (ANOVA) followed by Tukey's multiple-comparison test using MINITAB statistical software (release 13.32; Minitab Inc, State College, PA). Values are presented as means ± SEM, and differences resulting in values of $P < 0.05$ were considered significant.

The differences between the products at different timepoints 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 was performed for each timepoint by using the MINITAB software.

RESULTS

Blood glucose and insulin responses

No significant differences were found in fasting blood glucose or serum insulin concentrations ($P < 0.05$; Table 3). The blood glucose responses after ingestion of the whey (−56%) and the

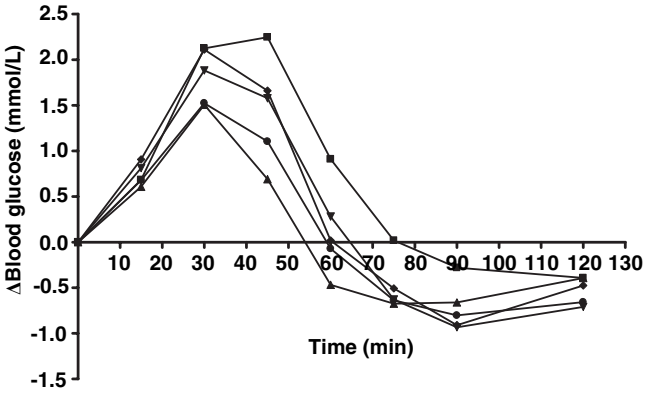

FIGURE 1. Mean (±SEM) incremental changes (Δ) in blood glucose in response to glucose (■); whey (▲); leucine, isoleucine, and valine (▼); lysine and threonine (♦); and leucine, isoleucine, valine, lysine, and threonine (●). $n = 12$ healthy subjects. A significant treatment effect ($P < 0.05$) but no treatment × time interaction ($P = 0.06$) was found.

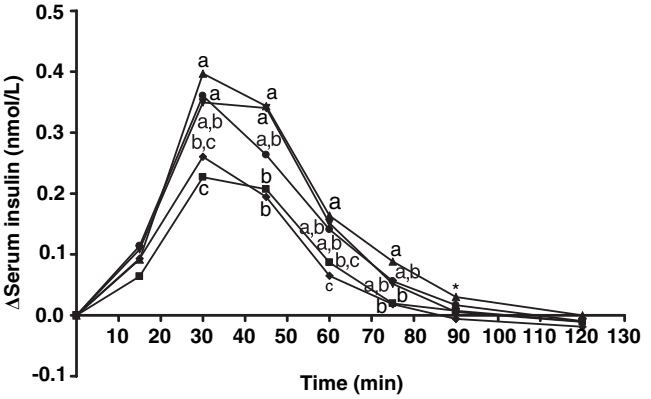

FIGURE 2. Mean (±SEM) incremental changes (Δ) in serum insulin in response to glucose (■); whey (▲); leucine, isoleucine, and valine (▼); lysine and threonine (♦); and leucine, isoleucine, valine, lysine, and threonine (●). $n = 12$ healthy subjects. A significant treatment effect ($P < 0.05$) and treatment × time interaction ($P < 0.05$) were found. Values with different letters are significantly different, $P < 0.05$ (Tukey's test). *At 90 min, whey induced a significantly higher insulin response than did the glucose reference or lysine and threonine.

AA5 (−44%) drinks were significantly lower than those after ingestion of the glucose reference drink ($P < 0.05$; **Table 4**). By contrast, although AA2 and AA3 tended to have lower glycemic excursions than did the glucose reference, no significant differences were seen (**Figure 1**). Furthermore, no significant differences were found between the 3 amino acid drinks (AA2, AA3, and AA5) with respect to postprandial blood glucose response.

In an examination of the postprandial insulin responses, the whey and AA3 (containing leucine, isoleucine, and valine) drinks were found to induce significantly ($P < 0.05$) higher AUCs than did the glucose reference drink. Although the 31% difference in insulin response between AA5 (containing leucine, isoleucine, valine, threonine, and lysine) and white wheat bread was not significant, AA5 caused insulin responses similar to those seen after the whey drink. In contrast, the AA2 drink (containing lysine and threonine) induced a significantly ($P < 0.05$) lower insulin response than did the AA3 and AA5 drinks—one that was similar to that after the glucose reference drink.

Thirty minutes after the beginning of the meal, serum insulin responses after the whey, AA3, and AA5 drinks were significantly ($P < 0.05$) higher than those after the reference drink (**Figure 2**), and the whey and AA5 drinks also induced significantly ($P < 0.05$) higher serum insulin concentrations than did the AA2 drink. At 45 and 60 min after the beginning of the meal, the whey drink and AA3 caused significantly ($P < 0.05$) higher insulin responses than did AA2.

Postprandial glucose-dependent insulinotrophic polypeptide and glucagon-like peptide 1 responses

No significant differences were found in fasting plasma GIP concentrations between the drinks (Table 3). The 0–90-min AUC for plasma GIP concentrations was significantly higher after the whey drink ($P < 0.05$) than after the drinks containing free amino acids or the glucose reference drink (**Table 5**, **Figure 3**).

The fasting concentration of plasma GLP-1 was significantly higher before the AA2 drink than before the glucose reference drink ($P < 0.05$, Table 3). However, no significant differences were found in postprandial GLP-1 responses, expressed as 0–90-min AUCs, after ingestion of the different test drinks and the glucose reference drink. In addition, no significant treatment × time interaction ($P = 0.96$) was observed (**Figure 4**).

Postprandial plasma amino acids

Minor but significant differences were found in fasting values for some amino acids (**Table 6**). Seventeen amino acids were detectable in plasma after ingestion of each of the test drinks (**Table 7**). Cysteine was detected only after ingestion of the reference drink, and methionine was detected in the postprandial plasma only after ingestion of the AA2 drink. After ingestion of the other test drinks, the methionine and cysteine concentrations fell beneath the limit of detection.

After consumption of the glucose reference drink, increments in plasma amino acids were marginal, and no differences in response were found between the studied amino acids (Table 7). The whey drink resulted in significantly higher amino acid responses than did the glucose reference drink (Table 7; **Figure 5**). As expected, the AA2 drink elicited significantly higher concentrations of threonine and lysine than of isoleucine, leucine, and valine (**Figure 6**). After the AA3 drink containing the BCAAs, plasma concentrations of leucine, valine, and isoleucine increased significantly more than did those of other amino acids (**Figure 7**).

The AA5 drink, with leucine, isoleucine, valine, lysine, and threonine, induced higher postprandial responses of leucine and valine in the 30–120-min period than did the other amino acids ($P < 0.05$; **Figure 8**). In addition, the leucine concentration at 15 min was significantly higher than that of the other amino acids.

The postprandial serine response was more pronounced after the whey drink than after any of the other drinks. The tyrosine AUC was significantly higher after the whey drink than after the glucose reference or AA5 drink. A significant difference was also seen in histidine concentrations between the whey drink and the AA2 drink.

DISCUSSION

The current study concurs with the previous finding that whey-induced hyperinsulinemia is associated with a concomitant increase in postprandial amino acid (7). In that earlier report, we

TABLE 5
Plasma glucose-dependent insulinotrophic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) responses [90-min area under the curve (AUC)] in healthy subjects after reference glucose drinks and glucose drinks to which whey or free amino acids were added[1]

Meal	GIP AUC	Change	GLP-1 AUC	Change
	pmol · min/L	*%*	*pmol · min/L*	*%*
Reference	1733 ± 204[a]		472.3 ± 76.2[a]	
Whey	3121 ± 328[b]	80	578.1 ± 73.6[a]	22
AA2	1917 ± 282[a]	11	375.8 ± 75.9[a]	−20
AA3	2089 ± 268[a]	21	499.9 ± 88.2[a]	6
AA5	1779 ± 226[a]	3	498.6 ± 77.4[a]	6

[1] All values are $\bar{x}$ ± SEM; $n = 12$. AA2, lysine and threonine; AA3, leucine, isoleucine, and valine; AA5, leucine, isoleucine, valine, lysine, and threonine. Values in the same column with different superscript letters are significantly different, $P < 0.05$ (ANOVA followed by Tukey's test).

described significant correlations between several amino acids and the insulinogenic index. We concluded that, in particular, leucine, isoleucine, valine, lysine, and threonine are likely to act as insulin secretagogues after a whey drink. It is well known that amino acids stimulate insulin release in the pancreatic beta cell, alone or in combination (15–20). The BCAAs, in particular, have attracted interest (26), and leucine has been suggested as one of the most potent insulin secretagogues (27–30). The mechanism for amino acid–mediated insulin release is complex, and several metabolic pathways are activated in the β-cell, depending on the amino acid (29, 31).

As judged from the current study, increases in the concentrations of amino acids in plasma are the likely mediators of the stimulated insulin secretion after a protein drink. In accord with that probability, a protein hydrolysate was reported to result in a greater increment of plasma amino acids and, as a consequence, in a greater insulin response than was an intact protein (21).

Among the amino acids included in the current study, the BCAAs seem to be major determinants of insulinemia and appeared to mimic the extent of hyperinsulinemia after a whey drink. Although AA3, containing only the BCAAs had a slightly greater insulin response than did AA5, the glycemic and insulinemic responses to AA5 more closely resembled the responses to the whey drink. AA5 induced not only a high insulin response but also a significantly lower glycemic response than did the glucose reference drink; this finding corresponds to the lowered glycemia seen after the whey drink. Moreover, AA5 well mimicked the plasma amino acid pattern seen after whey ingestion. No significant differences were seen in plasma amino acid responses (ie, AUCs) except leucine, tyrosine, and serine between the whey drink and the AA5 drink. The leucine response was significantly higher after the AA5 than after the whey drink.

No differences were found in GIP responses after the glucose reference drink or the drinks containing free amino acids, all of which had lower (33–44% AUC) increments than those seen after the whey drink. Only limited data regarding GIP responses after the ingestion of single amino acids are available in the literature. However, ingestion of lysine has been found to enhance GIP response, whereas no effect was seen with threonine in obese hyperglycemic (ob/ob) mice (32). An amino acid mixture has also been reported by Thomas et al (33) to stimulate GIP response and cause a subsequent insulin rise after interduodenal perfusion. The discrepancy between the results in the current study and those earlier findings may be due to the stimulating effects of the glucose present in our test drinks, which may have concealed the amino acid–induced GIP response. Thomas et al (33) also suggested that a threshold concentration may exist for amino acid–induced GIP release. However, it seems that GIP plays only a modest role in mediating insulin-releasing actions of amino acids (32).

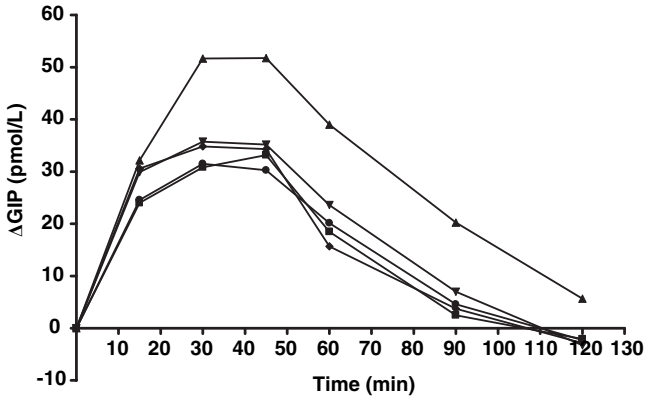

FIGURE 3. Mean (±SEM) incremental changes (Δ) in plasma glucose-dependent insulinotrophic polypeptide (GIP) in response to glucose (■); whey (▲); leucine, isoleucine, and valine (▼); lysine and threonine (♦); and leucine, isoleucine, valine, lysine, and threonine (●). $n = 12$ healthy subjects. A significant treatment effect ($P < 0.05$) but no treatment × time interaction ($P = 0.12$) was found.

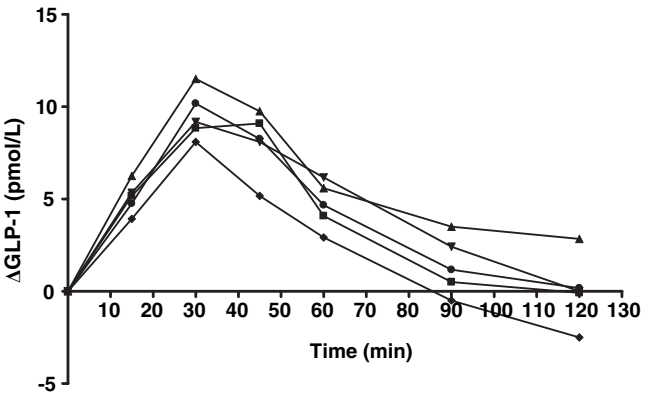

FIGURE 4. Mean (±SEM) incremental changes (Δ) in plasma glucagon-like peptide 1 (GLP-1) in response to glucose (■); whey (▲); leucine, isoleucine, and valine (▼); lysine and threonine (♦); and leucine, isoleucine, valine, lysine, and threonine (●). $n = 12$ healthy subjects. A significant treatment effect ($P < 0.05$) but no treatment × time interaction ($P = 0.96$) was found.

TABLE 6
Fasting values of the different amino acids in healthy subjects before ingestion of reference glucose drinks and glucose drinks to which whey or free amino acids were added[1]

	Drink				
Amino acid	Glucose	Whey	AA2	AA3	AA5
			mmol/L		
Aspartic acid	0.046 ± 0.01[a]	0.065 ± 0.01[a]	0.034 ± 0.01[a]	0.035 ± 0.005[a]	0.032 ± 0.004[a]
Threonine	0.14 ± 0.01[a,b]	0.11 ± 0.01[b]	0.14 ± 0.02[a,b]	0.17 ± 0.02[a]	0.11 ± 0.01[b]
Serine	0.14 ± 0.02[a,b]	0.10 ± 0.01[a,b]	0.12 ± 0.01[a,b]	0.15 ± 0.01[a]	0.090 ± 0.01[b]
Asparagine	0.11 ± 0.02[a]	0.058 ± 0.01[b]	0.082 ± 0.01[a,b]	0.087 ± 0.003[a,b]	0.057 ± 0.01[b]
Glutamic acid	0.20 ± 0.02[a]	0.16 ± 0.03[a]	0.16 ± 0.01[a]	0.14 ± 0.02[a]	0.12 ± 0.02[a]
Glutamine	0.55 ± 0.04[a,b]	0.49 ± 0.06[b]	0.58 ± 0.07[a,b]	0.74 ± 0.05[a]	0.52 ± 0.07[a,b]
Proline	0.26 ± 0.05[a]	0.18 ± 0.02[a,b]	0.22 ± 0.03[a,b]	0.26 ± 0.02[a]	0.13 ± 0.02[b]
Glycine	0.21 ± 0.02[a,b]	0.26 ± 0.03[a]	0.19 ± 0.01[a,b]	0.23 ± 0.03[a,b]	0.16 ± 0.01[b]
Alanine	0.33 ± 0.02[a,b]	0.34 ± 0.04[a,b]	0.35 ± 0.03[a,b]	0.40 ± 0.03[a]	0.28 ± 0.02[b]
Valine	0.23 ± 0.02[a]	0.21 ± 0.02[a]	0.24 ± 0.02[a]	0.25 ± 0.02[a]	0.19 ± 0.02[a]
Cysteine	0.045 ± 0.007[a]	0.044 ± 0.01[a]	0.11 ± 0.03[a]	0.047 ± 0.01[a]	0.085 ± 0.02[a]
Methionine	< 0.01	< 0.01	0.045 ± 0.01	0.025 ± 0.01	< 0.01
Isolecine	0.065 ± 0.01[a]	0.062 ± 0.01[a]	0.064 ± 0.01[a]	0.065 ± 0.01[a]	0.055 ± 0.01[a]
Leucine	0.12 ± 0.01[a,b]	0.11 ± 0.01[b]	0.14 ± 0.01[a]	0.14 ± 0.01[a]	0.10 ± 0.01[b]
Tyrosine	0.089 ± 0.04[a]	0.044 ± 0.006[a]	0.067 ± 0.004[a]	0.079 ± 0.004[a]	0.039 ± 0.004[a]
Phenylalanine	0.053 ± 0.004[a,b]	0.048 ± 0.005[a,b]	0.050 ± 0.002[a,b]	0.055 ± 0.004[a]	0.038 ± 0.003[b]
Lysine	0.18 ± 0.02[a]	0.16 ± 0.01[a]	0.17 ± 0.01[a]	0.19 ± 0.01[a]	0.14 ± 0.01[a]
Histidine	0.10 ± 0.01[a]	0.11 ± 0.01[a]	0.13 ± 0.01[a]	0.12 ± 0.01[a]	0.10 ± 0.01[a]
Arginine	0.13 ± 0.02[a]	0.074 ± 0.01[a]	0.11 ± 0.05[a]	0.057 ± 0.01[a]	0.052 ± 0.004[a]

[1] All values are $\bar{x}$ ± SEM; $n = 12$. AA2, lysine and threonine; AA3, leucine, isoleucine, and valine; AA5, leucine, isoleucine, valine, lysine, and threonine. Values in the same row with different superscript letters are significantly different, $P < 0.05$ (ANOVA followed by Tukey's test).

In an earlier study, we (7) did not find any differences in GIP responses after the ingestion of milk and white wheat bread, despite the differences in insulinogenic properties between these food products. As judged from the absence of incretin stimulation after drinks with glucose and free amino acid mixtures in the current study, which contrasts with the presence of incretin stimulation seen with the pure glucose drink, our results indicate that GIP is not a major mediator of whey induced hyperinsulinemia. However, the whey drink caused a significantly enhanced GIP response, and it is possible that bioactive peptides present in

TABLE 7
Incremental postprandial areas under the curve (AUC) in plasma for the different amino acids from 0 to 45 min after the glucose reference or test drinks[1]

	Drink				
Amino acid AUC	Glucose	Whey	AA2	AA3	AA5
			mmol · min/L		
Aspartic acid	0.7 ± 0.5[a]	0.9 ± 0.4[a]	0.4 ± 0.2[a]	0.1 ± 0.1[a]	0.2 ± 0.1[a]
Threonine	0.5 ± 0.2[c]	2.6 ± 0.4[b]	4.1 ± 0.4[a]	0.7 ± 0.2[c]	2.1 ± 0.2[b]
Serine	0.4 ± 0.1[b]	1.5 ± 0.3[a]	0.4 ± 0.1[b]	0.6 ± 0.2[b]	0.5 ± 0.1[b]
Asparagine	1.1 ± 0.5[a]	2.0 ± 0.8[a]	1.3 ± 0.5[a]	1.5 ± 0.5[a]	0.4 ± 0.1[a]
Glutamic acid	0.4 ± 0.3[a]	1.0 ± 0.5[a]	0.3 ± 0.2[a]	0.3 ± 0.1[a]	0.2 ± 0.1[a]
Glutamine	2.3 ± 0.8[a]	7.3 ± 2.2[a]	4.3 ± 1.8[a]	4.1 ± 1.3[a]	2.7 ± 0.5[a]
Proline	1.4 ± 0.5[a]	3.1 ± 1.8[a]	0.7 ± 0.2[a]	0.5 ± 0.2[a]	1.2 ± 0.6[a]
Glycine	0.6 ± 0.4[a]	2.8 ± 1.7[a]	0.5 ± 0.2[a]	0.6 ± 0.3[a]	0.4 ± 0.2[a]
Alanine	2.0 ± 1.3[a]	2.9 ± 0.7[a]	1.5 ± 0.5[a]	1.1 ± 0.4[a]	1.3 ± 0.5[a]
Valine	0.4 ± 0.1[c]	5.3 ± 1.0[b]	0.4 ± 0.2[c]	7.9 ± 0.8[a]	5.2 ± 0.7[b]
Cysteine	1.5 ± 0.8	< 0.1	< 0.1	< 0.1	< 0.1
Methionine	< 0.1	< 0.1	0.3 ± 0.2	< 0.1	< 0.1
Isoleucine	0.4 ± 0.2[c]	2.9 ± 0.4[b]	0.4 ± 0.2[c]	5.0 ± 0.4[a]	3.6 ± 0.4[b]
Leucine	0.3 ± 0.1[d]	3.9 ± 0.5[c]	0.2 ± 0.1[d]	9.4 ± 0.8[a]	6.2 ± 0.6[b]
Tyrosine	0.2 ± 0.1[b]	0.7 ± 0.1[a]	0.3 ± 0.2[a,b]	0.4 ± 0.1[a,b]	0.1 ± 0.1[b]
Phenylalanine	0.4 ± 0.2[a]	0.7 ± 0.2[a]	0.4 ± 0.2[a]	0.7 ± 0.2[a]	0.3 ± 0.1[a]
Lysine	0.4 ± 0.2[b]	3.7 ± 0.6[a]	3.9 ± 0.3[a]	0.8 ± 0.3[b]	3.7 ± 0.5[a]
Histidine	0.4 ± 0.2[a,b]	1.2 ± 0.3[a]	0.2 ± 0.2[b]	0.6 ± 0.2[a,b]	0.5 ± 0.2[a,b]
Arginine	1.5 ± 0.5[a]	1.7 ± 0.7[a]	0.3 ± 0.1[a]	0.6 ± 0.3[a]	0.6 ± 0.2[a]

[1] All values are $\bar{x}$ ± SEM; $n = 12$. AA2. lysine and threonine; AA3, leucine, isoleucine, and valine; AA5, leucine, isoleucine, valine, lysine, and threonine. Values in the same row with different superscript letters are significantly different, $P < 0.05$ (ANOVA followed by Tukey's test).

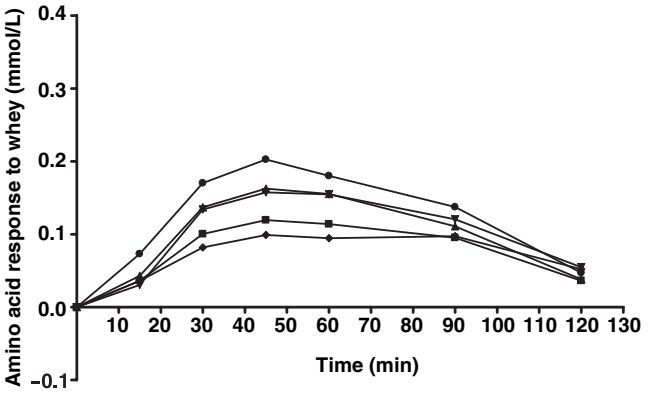

FIGURE 5. Mean (±SEM) incremental changes (Δ) in plasma concentrations of leucine (▲), isoleucine (■), valine (●), lysine (▼), and threonine (◆) in response to whey. $n = 12$ healthy subjects. A significant amino acid effect ($P < 0.05$) but no amino acid × time interaction ($P = 0.99$) was found.

whey or formed during digestion may specifically stimulate GIP secretion. The fact that the free amino acids did not stimulate incretin response may also explain why the insulin response tended to be slightly (but not significantly) lower after the AA5 drink than after the whey drink, because GIP and GLP-1 may enhance not only glucose-stimulated insulin secretion but also amino acid–induced insulin secretion (34).

Although the results of the current study allow us to propose that increases in the plasma concentrations of leucine, isoleucine, valine, lysine, and threonine have the greatest effect on whey-induced hyperinsulinemia, several other amino acids have also been described as efficient insulin secretagogues. Rocha et al (27) ranked the glucagon and insulin responses after the intravenous infusion of 20 amino acids in conscious dogs. Tryptophan, leucine, aspartate, isoleucine, and glutamate were found to be the most potent insulin secretagogues, whereas histidine, alanine, valine, aspargine, arginine, and serine were less efficient. Others have reported arginine as a potent stimulator of insulin release in healthy humans when administrated intravenously (16), but, when fed orally in realistic amounts, arginine seems to be a less potent insulin secretagogue (35).

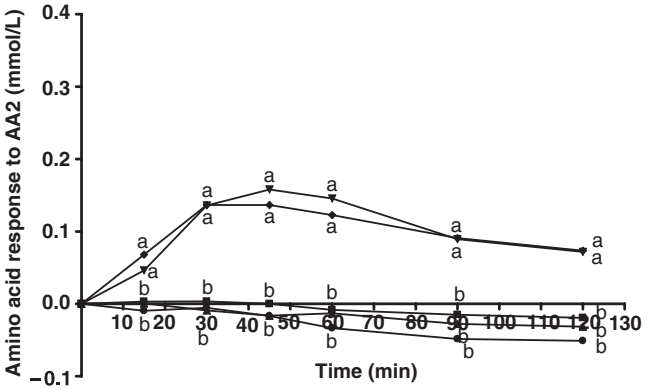

FIGURE 6. Mean (±SEM) incremental changes (Δ) in plasma concentrations of leucine (▲), isoleucine (■), valine (●), lysine (▼), and threonine (◆) in response to AA2 (lysine and threonine). $n = 12$ healthy subjects. A significant amino acid effect ($P < 0.05$) and amino acid × time interaction ($P < 0.05$) were found at a given time. Values with different letters are significantly different, $P < 0.05$ (Tukey's test).

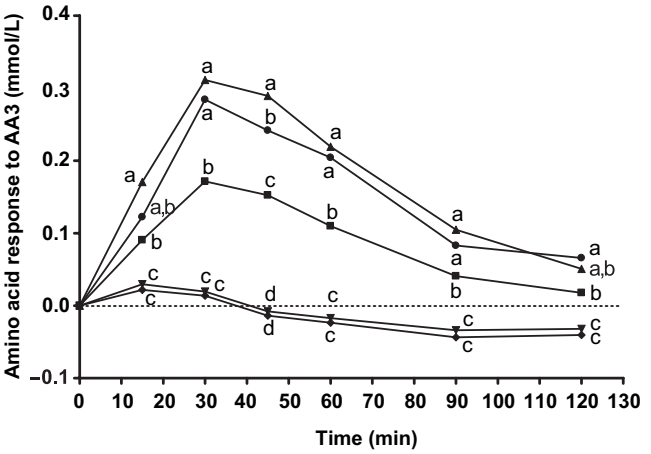

FIGURE 7. Mean (±SEM) incremental changes (Δ) in plasma concentrations of leucine (▲), isoleucine (■), valine (●), lysine (▼), and threonine (◆) in response to AA3 (leucine, isoleucine, and valine). $n = 12$ healthy subjects. A significant amino acid effect and amino acid × time interaction ($P < 0.05$ for both) were found at a given time. Values with different letters are significantly different, $P < 0.05$ (Tukey's test).

The higher glutamine concentrations seen after all drinks are probably mediated through endogenous output. The BCAAs may serve as precursors for glutamine synthesis (36), and leucine administration is known to stimulate the release of glutamine and alanine from the muscles (37). It has also been reported that the degradation of leucine to glutamine is enhanced in an experimentally induced insulin-resistant state (38). In addition, lysine may serve as a precursor for the de novo synthesis of glutamine in skeletal muscles when present in the physiologic range of 0.1 to 0.5 mmol/L, whereas a high concentration of threonine (10 mmol/L) was found to decrease glutamine formation (39). Moreover, it has been suggested that the conversion of glucose to glutamine increases during hyperinsulinemic, euglycemic conditions and enhances the glutamine output from skeletal muscles into plasma (40). It cannot be ruled out that glutamine is involved in whey-induced hyperinsulinemia, because glutamine enhances the glucose-stimulated insulin release through several mechanisms (41). One mechanism may be the key stimulus-secretion coupling factors generated during glutamine metabolism within the beta cell,

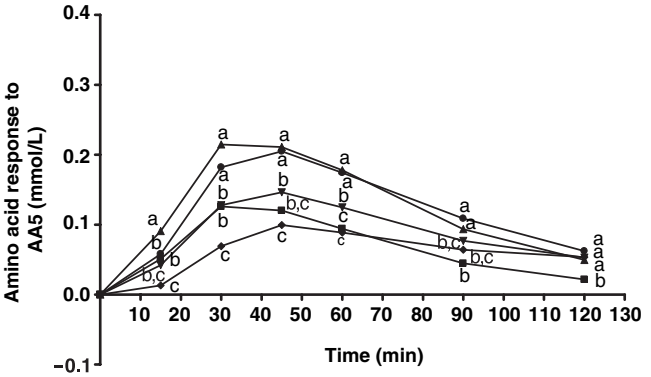

FIGURE 8. Mean (±SEM) incremental changes (Δ) in plasma concentrations of leucine (▲), isoleucine (■), valine (●), lysine (▼), and threonine (◆) in response to AA5 (leucine, isoleucine, valine, lysine, and threonine). $n = 12$ healthy subjects. A significant amino acid effect and amino acid × time interaction ($P < 0.05$ for both) were found at a given time. Values with different letters are significantly different, $P < 0.05$ (Tukey's test).

which interact with several of the metabolic pathways that control insulin release (42).

As judged from previous studies in our laboratory (7), the mechanism for whey-induced hyperinsulinemia seems to be ≥2 separate pathways, one connected to the significant increment in certain amino acids and the other connected through the incretins, where GIP, in particular, is stimulated. The current study strengthens earlier findings that postprandial increments of leucine, isoleucine, valine, threonine, and lysine in the blood are important to the insulinotrophic effect of whey. The modulation of the glycemic and the insulinemic responses after a glucose drink containing these 5 amino acids and after a glucose-equivalent drink containing whey proteins did not differ significantly. In fact, the mixture of glucose and the 3 amino acids isoleucine, leucine, and valine (AA3) also increased insulin, whereas the glucose drink with lysine and threonine had no effect. However, it cannot be excluded that not only specific amino acids but also the total plasma amino acid response is important for the insulin response. Hence, the drinks that caused the highest total plasma amino acid increments in the current study also caused the highest insulin responses. In comparisons of different amino acids, leucine has been found to be particularly insulinotrophic (27, 28, 43). Although the leucine content in postprandial plasma was higher after AA3 and AA5 drinks than after the whey drink, the whey drink induced a higher insulin response than the AA3 and AA5 drinks. Furthermore, despite a 30% difference ($P < 0.05$) in plasma leucine AUC between AA3 and AA5, no significant differences were noted in insulinemia. This lack of difference could indicate that the insulinotrophic effect of whey is better mimicked by mixtures of amino acids than by leucine alone.

Neither of the drinks containing free amino acids and glucose stimulated the GIP response, whereas the pure glucose drink did do so. The involvement of GIP or a bioactive peptide (or both) in the insulinotrophic effect of whey cannot be completely ruled out, and the whey drink tended to produce somewhat higher insulinemia than did the amino acid mixtures. However, the overall results of the current study indicate that the preferential increase in specific amino acids in postprandial plasma is the key mechanism for the protein-induced hyperinsulinemia seen after a whey drink.

MN and IMEB designed the study; IMEB secured the funding for the study; MN coordinated the study and was responsible collection and analysis of the data and for the amino acid analysis; JJH was responsible for the incretin analysis; MN was responsible for the statistical analysis; all authors contributed to the writing of the manuscript. None of the authors had a personal or financial conflict of interest.

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