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Metabolic impact of certain dietary proteins and/or amino acids - Glycaemic and hormonal responses to carbohydrate meals in healthy subject

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Metabolic impact of certain dietary proteins and/or amino acids

Glycaemic and hormonal responses to carbohydrate meals in healthy subjects

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Contents

Abstract

Re-occurring hyperglycaemic episodes promote subclinical low-grade inflammation and CVD in type 2 diabetes, emphasising the therapeutic role of tight blood glucose regulation. A tight blood glucose regulation is probably beneficial also in healthy subjects and mild elevations in postprandial glycaemia and triglycerides are associated with impaired flow-mediated dilation and increased markers of oxidative stress in young healthy subjects. Certain dietary proteins and amino acids (AA) have insulinogenic properties and might facilitate glycaemic regulation following a carbohydrate challenge. However, there is a lack of knowledge regarding the impact of different food proteins, and to what extent their effects can be influenced by supplementation with AA. Also, limited information exists with respect to influence of proteins/AA on metabolic response to carbohydrates in a composite meal.

The objective of the present thesis was to investigate the impact of whey and soy protein on postprandial blood glucose, plasma AA (p-AA) and hormonal responses when administered to healthy subjects in a glucose drink, as part of milk meals, or in combination with a composite carbohydrate meal. The effect of exchanging half of the protein for specific AA mixtures (5AA: isoleucine, leucine, lysine, threonine and valine) or (6AA: 5AA+arginine) was also examined. Additionally appetite rating in the postprandial phase was performed using VAS scales.

Whey protein (4.5-18g) reduced postprandial glycaemia, and increased insulinaemia and p-AA in a dose dependent way to a glucose challenge, and the p-5AA (iAUC 0-60 min) correlated to the insulin response (iPeak; $P < 0.009$). Lactose-equivalent amounts of bovine and human milk resulted in similar postprandial glycaemia and insulinaemia. A rapid response in GIP, GLP-1 and p-AA correlated to an early insulinogenic effect that was associated to reduction of glycaemia (iAUC 0-90 min; $P < 0.001$). Hydrolysed

and intact whey had similar effects on glycaemic responses when co-ingested with glucose, although hydrolysed way tended to be more insulinogenic, possible due to its higher early insulin and faster p-AA response compared with intact whey. Exchanging half of the intact or hydrolysed whey protein for 5AA magnified the insulinogenic effect and reduced postprandial glycaemia (iAUC 0-120min; $P < 0.05$).

Intake of whey or soy protein with or without addition of 5AA or 6AA, as a pre-meal protein drink (PMPD) prior a composite meal, considerably attenuated postprandial blood glucose incremental peak value (iPeak; $P < 0.05$). Also, all whey PMPDs with or without added AA reduced glycaemia (iAUC 0-120min; $P < 0.05$) and increased the Glycaemic Profile (GP; $P < 0.05$). Arginine had no additional effect on glycaemic responses when added to the 5AA mixture. Early GLP-1 and p-AA responses (iAUC 0-15 min) were associated with early insulin response (iAUC 0-15min). Early increment in insulin possibly explain the attenuation of over-all course of post-prandial glycaemia to the composite carbohydrate meal post the PMPDs. Interestingly, the lowering of glycaemic excursions was observed in the absence of elevated insulinaemic peak. Intake of a PMPD prior a composite meal had no effects on appetite rating (VAS) or plasma ghrelin.

Populärvetenskaplig sammanfattning

Förekomst av fetma och typ 2 diabetes ökar i Sverige, liksom globalt. Över 360 miljoner människor har diabetes och prognosen för år 2030 är drygt 550 miljoner (IDF 2011). Att arbeta med förebyggande åtgärder för att minska denna utveckling är därför nödvändigt. Något som har stor betydelse för hälsan är vilken typ av mat vi väljer att äta och dess näringsmässiga kvaliteter.

När vi äter går blodsockernivåerna upp. Återkommande höga blodsockersvar, som är vanliga vid typ 2 diabetes, ökar risken att drabbas av hjärtkärlsjukdom i framtiden. Därför är det extra betydelsefullt att kontrollera blodsockernivåerna hos typ 2 diabetiker. Även hos friska personer har låga blodsockernivåer i samband med måltider visat sig positiva, då risken att senare i livet drabbas av diabetes och hjärt-kärlsjukdom minskar.

Vissa komponenter i livsmedel har gynnsamma effekter som kan användas för att underlätta reglering av blodsockret. I den här avhandlingen har olika livsmedelsproteiner och aminosyrablandningar studerats. I måltidsstudier på friska försökspersoner visade det sig att om man drack vassleproteiner från mjölk tillsammans med en kolhydratrik måltid så minskade blodsockersvaret. Det visade sig också att tillsats av vissa enskilda aminosyror förstärkte denna effekt. Även sojaproteiner med tillsatta aminosyror hade en blodsockersänkande effekt. Effekten kunde förklaras av ett ökat insulinsvar. I blodet syntes också en ökning av vissa aminosyror och tarmhormoner. Att inta en liten dos av vassle/soja och aminosyror precis innan en måltid visade sig vara extra gynnsamt. Insulinet blev då effektivare och det behövdes mindre mängd insulin för att sänka blodsockret.

Resultaten från avhandlingen kan användas för utveckling av livsmedel och måltider med hälsofrämjande effekter i form av underlättad blodsockerreglering hos både friska och diabetiker. Behandling av typ 2 diabetes med vissa läkemedel kan ge oönskade biverkningar t.ex. plötsliga blodsockerfall. Användning av livsmedelsproteiner/aminosyror har troligtvis inte dessa nackdelar då den insulinstimulerande effekten bara uppkommer när blodsockret stiger efter en måltid.

List of papers

The thesis is based on the following papers:

Paper I — Effects of whey proteins on glycaemia and insulinaemia to an oral glucose load in healthy adults; a dose response study. **Gunnerud U**., Östman E., Björck I.
Submitted Manuscript 2013

Paper II — Glycaemic, insulinaemic and plasma amino acid responses to equi-carbohydrate milk meals; a pilot- study comparing bovine and human milk. **Gunnerud U.,** Holst J., Östman E., Björck I. *Nutrition Journal 2012. 11:83*

Paper III — Impact of hydrolysed versus intact whey proteins on post-meal glycaemic regulation. **Gunnerud U.,** Östman E., Björck I. *Manuscript 2013*

Paper IV — Effects of pre-meal drinks with protein and amino acids on glycemic and metabolic responses at a subsequent composite meal. **Gunnerud U.,** Heinzle C., Holst J., Östman E., Björck I. *PLoS One 2012, 7:e44731*

The author's contribution to the papers

Paper I

The author, U Gunnerud, was involved in the study design, coordinated the study, was responsible for the analysis of serum insulin and the plasma amino acids, evaluated the results and was responsible for writing the manuscript.

Paper II

The author, U Gunnerud, was involved in the sample collection, evaluated the results and was responsible for writing the manuscript

Paper III

The author, U Gunnerud, was responsible for the study design, developed the test products, coordinated the meal study, was responsible for analysis of serum insulin and plasma amino acids, evaluated the results, contributed significantly to interpretation of data and was responsible for writing the manuscript.

Paper IV

The author, U Gunnerud, was responsible for the study design, developed the test products, was responsible for the analysis of serum insulin and subjective satiety, evaluated the results, contributed significantly to interpretation of data, and was responsible for writing the manuscript.

Other publications related to the subjects of this thesis

The insulinogenic effect of whey protein is partially mediated by a direct effect of amino acids and GIP on beta-cells. Salehi, A., **Gunnerud U**., Muhammed S., Östman E., Holst J., Björck I., Rorsman P.

Nutrition and Metabolism 2012: 9:48.

Abbreviations

(i)AUC	(Incremental) area under the curve
AA	Amino acids
BCAA	Branched chained amino acids
EAA	Essential amino acids
GI	Glycaemic index
GP	Glycaemic profile
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide 1
II	Insulinaemic index
IGI	Insulinogenic index
IRS	Insulin resistance syndrome
iPeak	Incremental peak
p-AA	Plasma amino acids
p-glucose	Plasma glucose
PMPD	Pre-meal protein drink
S-PMPD	Soy pre-meal protein drink
T2D	Type 2 diabetes
VAS	Visual analogue scale
W-PMPD	Whey pre-meal protein drink
WWB	White wheat bread
5AA	isoleucine, leucine, lysine, threonine and valine
6AA	5AA + arginine

Background

Glycaemic regulation and insulin secretion

In the postprandial phase after a carbohydrate load, plasma glucose (p-glucose) levels increases within a few minutes, and the insulin secreting system is activated when blood glucose level reaches a concentration of ≈7mmol/l[1]. The main action of insulin is to facilitate glucose uptake into adipose and muscle cells where it is stored as either triglycerides or glycogen. Insulin also inhibits the endogenous hepatic glucose production.

Current knowledge suggests that in human pancreatic β-cells, glucose molecules enter the cells mainly via the glucose transporters GLUT1 and GLUT3[1, 2]. Glucose is then phosphorylated, and ATP generated via the TCA cycle [3]. ATP, when transferred out into the cytosol causes ATP-sensitive K^+-channels to close, initiating a depolarisation of the cell membranes and a concomitant opening of voltage sensitive Ca^{2+}-channels. The increased intracellular concentrations of Ca^{2+} finally triggers insulin exocytosis[3]. Once in the blood, insulin binds to the insulin receptors on the adipose and muscle cell membranes and induces a signal transduction cascade which mobilize the glucose transporter (GLUT4) to the membrane surface and enables transport of glucose into the cells[4].

It is well known that insulin is secreted in a biphasic manner[5], with a rapid first phase of a comparatively short duration (≈10 min), and with a peak within 5-7 minutes[6]. The second phase is more sustained and decreases slowly (2-3h) to baseline value[7, 8]. Dysfunction of the β-cell is present in type 2 diabetes (T2D), and especially defects in the first phase insulin secretion have been suggested to be an early sign of T2D pathogenesis[9-11].

Glucagon is another important hormone in the maintenance of glucose homeostasis. It is secreted from the α-cells of the Langerhans islets but the exact regulating mechanism is not yet fully elucidated. Glucagon release is stimulated by hypoglycaemia and suppressed by hyperglycaemia, but mechanisms related to β-cells and the central nervous system has also been implicated[12-14]. In T2D, hyperglucagonaemia is observed both in a fasting and postprandial phase, and it is involved in the pathogenesis of the disease[15, 16].

Amino acid mediated insulin secretion

In addition to the glucose mediated insulin secretion referred to above, several amino acids (AA) may stimulate insulin secretion both *in vivo* [17-19] and *in vitro*[20], although the presence of glucose is required for the *in vivo* stimulation[21]. Practically all essential AA (EAA) appears to have insulin stimulating properties, although of different magnitudes[17]. AA can affect the pancreatic β-cell through different pathways; by stimulating TCA activity[21], causing depolarisation of the membrane directly[22] or through co-transportation with Na^+, leading to depolarization of the membrane[20].

The branched chained AA (BCAA); isoleucine, valine and leucine have been shown to be prominent insulin secretagouges[17], and in particular leucine seems to be especially potent[23]. Leucine and isoleucine appears to affect the β-cell in a similar way to glucose[24], and several studies indicate that leucine enters the mitochondria directly and then feeds the TCA-cycle, generating ATP, causing a membrane depolarization, and insulin exocytosis[21, 24]. Additionally, the BCAA also affect the mammalian target of rapamycin (mTOR) signalling pathway, thus inducing mRNA translation and promoting protein synthesis[25].

Venous infusion of arginine and phenylalanine stimulate insulin release[17]. In addition, arginine facilitates insulin release in the presence of glucose[22], and possibly also enhance leucine and glucose mediated insulin secretion[26].

Role of incretins

Several hormones, of which some are recognized as potent insulin secretagouges, are secreted in the gut in response to nutrients. Two of these are glucose-dependent insulinotrophic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) which are released into the circulation in response to carbohydrates, fats and proteins[27]. GIP is mainly secreted from the K-cells in the duodenum and jejunum[28] whereas GLP-1 is mainly secreted from L-cells in distal ileum and colon[29]. Even though the GLP-1 secreting cells are mainly located within the distal part of the intestine, it is suggested that secreting cells are located also in the proximal parts, since plasma levels of both incretins rises within minutes after food intake[30]. The insulin secretion is higher following orally ingested glucose compared to intravenously infuse. This insulin amplifications is caused by incretins, and the effect is known as "the incretin effect"[31]. Thus, an important property of GIP and GLP-1 is to stimulate insulin and regulate postprandial glycaemia. The incretins have been suggested to affect also other metabolic functions. GIP has been suggested to stimulate both lipogenesis as well as to be involved in bone formation[32]. GLP-1 decreases gastric motility and gastric emptying, also resulting in reduced postprandial glycaemia[33] and it improves insulin sensitivity[34]. Additionally GLP-1 has been shown to possibly affect satiety and weight regulation by suppressing appetite and food intake[32, 35, 36] and recent knowledge has implicated GLP-1 as both antidiabetic and antiobesogenic hormone[37].

Metabolic impact of dietary proteins and amino acids

Dietary protein

It has been shown that certain dietary proteins stimulate insulin secretion when co-ingested with carbohydrates both in healthy individuals[38, 39] and in T2D subjects[40, 41]. Depending on protein source the postprandial plasma AA (p-AA) pattern can differ considerably, even though the AA pattern of the food protein

is quite similar. Milk, cheese, whey and cod protein have similar AA profiles, with approximately the same concentration of BCAA, but still they appear at different rates in postprandial plasma (iAUC 0-45 min)[42].

Bovine milk contains approximately 34.5g/L protein[43], and consists of two main protein fractions; casein (≈80%) and whey protein (≈20%)[44]. In the late 90's, Boirie et al presented a classification of casein and whey in terms of slow and fast proteins, respectively[45]. The soluble whey protein was perceived as rapidly digested and absorbed, resulting in a quick p-AA response and therefore classified as a fast protein. In contrast to whey, casein precipitate in the stomach and thus delay the gastric emptying, digestion and absorption, resulting in a slower p-AA response. Therefore, casein is classified as a slow protein[45]. Whey is a high quality protein, rich in BCAA[46], and with a high leucine content[47]. It has been shown that whey proteins have insulinogenic effects and reduces postprandial glycaemia in both healthy and T2D subjects when co-ingested with lactose or included in composite meals[42, 48]. The underlying insulinogenic mechanism for this effect of whey protein was ascribed a rapid p-AA response of the BCAA, together with lysine and threonine.

Soy protein is characterised by high nutritional quality and a high content of EAA. It has been reported to have several potential beneficial health effects of relevance in relation to the metabolic syndrome. Soy protein has been suggested to reduce the risk of insulin resistance and obesity[49, 50], and to have positive effects on fatty acid metabolism and cholesterol homeostasis[51]. It has also been reported to have satiating properties[52]. Additionally, soy-based food produced low postprandial glycaemic responses in healthy subjects while maintaining comparative low insulin response compared with a glucose reference with no soy protein added[53]. However, it should be noted that the soy drinks in addition contained chocolate which may have contributed to the results, and cacao beans are rich sources of flavonoids[54], with potential effects on acute glycaemic and insulinaemic responses[55]. Hence, the acute glucoregulatory effects of soy protein in humans have not been completely investigated.

Several observational studies indicate that a high intake of dairy products may have a protective role against the metabolic syndrome, obesity and T2D[56-59]. Suggested protective mechanisms include a high content of calcium, magnesium and vitamin D[60-62], improved folate bioavailability[63] and/or presence of bioactive peptides[64]. Furthermore, it has also been proposed that particularly the whey proteins may affect appetite regulation and could thus have positive effects on weight regulation[65]. The satiating effect may be mediated through the release of insulin[66, 67], and/or p-AA or through an incretin effect mediated by these proteins[38, 68]. Milk has additionally been recognized as a low Glycaemic Index (GI) food[69]. The GI often correlates with the Insulinaemic Index (II), where a low GI corresponds with a low II and vice versa. Milk products however, typically displays low GI and a high II[69]. The insulinogenic properties of the milk have been ascribed the whey protein fraction[42], and possibly explain why the GI of lactose in milk is lower than that of pure lactose[69].

Dietary amino acids

Dietary AA administrated in combination with carbohydrates stimulates pancreatic insulin secretion[20]. Leucine has been put forward as a strong insulin sectretagouge[23]. In contrast, lysine and arginine has been shown to reduce postprandial glycaemia without significant effects on the insulin response, when ingested with a glucose load[70, 71]. It has been reported that intake of mixtures of AA are more efficient in stimulating insulin and reducing glycaemia than the single AA[17]. A previous study demonstrated that oral intake of a mixture of 5AA (BCAA, lysine and threonine) in combination with a glucose load was more efficient than combinations of 2-3 AA at the time, and mimicked the postprandial glycaemia and insulinaemia following co-ingestion of whey protein and glucose[72]. Of particular interest is that the ability of the β-cells to secrete insulin in response to protein/AA is maintained also in late stage of T2D; that is when the glucose mediated insulin stimulation is hampered[48].

The postprandial state

Re-occurring hyperglycaemic episodes, as present in T2D, promote subclinical low-grade inflammation[73], and hence increases the risk of cardiovascular disease (CVD)[74, 75]. CVD is the main cause of death in patients with T2D, and long term poorly regulated glycaemia is a known risk factor in the development of atherosclerosis and CVD in these patients[76]. Recent evidence has emphasised the importance of the postprandial phase and indicates that improved regulation of postprandial glucose levels might be more important than lowering HbA1c to decrease the risk of atherosclerosis[73, 77, 78]. On accordance, low GI diets improve risk markers of CVD in T2D subjects[79].

A tight blood glucose regulation appears to be advantageous also for healthy subjects. It was recently shown that even mild elevations in postprandial glycaemia and triglycerides are associated with impaired flow-mediated dilation and increased markers of oxidative stress in young healthy subjects[80]. Dietary measures to reduce glycaemic oscillations might thus have beneficial effects also within the normal range of blood glucose excursions. Accordingly, it has been postulated that pro-inflammatory transcription factors (nuclear factor κB) is less activated with low GI food compared to high GI foods in young healthy subjects[81]. Further evidence for health benefits of lowered glycaemic excursions stem from observations that a three month low GI diet ameliorate endothelial function compared to a high GI diet in obese non-diabetic adults[82]. Additionally there are also indications that a diet characterized by a low GI protects against the development of T2D[83, 84], and coronary heart disease[85, 86].

From above it can be suggested that tight regulation of postprandial blood glucose levels may prevent CVD and dysmetabolism secondary to T2D, and it might also contribute to the primary prevention of T2D in the general population. Few food proteins have been studied for this purpose, and little information is available concerning mechanisms. To gain further understanding of how dietary proteins impacts on glycaemic regulation, the influences of incretins and p-AA on the insulinogenic effects needs to be elucidated. Moreover, in light of the proposed classification of food proteins in

terms of slow and rapid, the impact of within meal timing of protein intake emerged as an interesting and new topic for research.

Objective

The general aim of the present work was to investigate the postprandial blood glucose regulatory properties of whey and soy protein with or without supplementation with specific amino acid mixtures. For this purpose four human meal studies have been performed in young healthy subjects with focus on postprandial blood glucose, hormones, and plasma amino acid responses.

More specific aims were to investigate.

- The dose-response relationship between whey protein intake and postprandial metabolic responses.
- Potential differences in the metabolic potencies of bovine and human milk; displaying differences in protein content.
- Potential differences in metabolic responses to whey protein differing in physical form (hydrolysed vs. intact).
- The effect of exchanging part of the whey or soy protein for a specific mixture of insulinogenic amino acids.
- If a pre-meal whey or soy protein drink positively affected glucose regulation to at subsequent composite meal.

Material and Methods

Test products

Proteins and amino acids

In papers I, III, IV a whey protein isolate (Lacprodan DI-9224, Arla Foods Ingredients amba, Viby J, Denmark) with approximately 90% protein and maximum 0.2% of lactose according to the manufacturer, was used. Whey protein concentrate (Lacprodan DI-8702, Arla Foods Ingredients amba, Viby J, Denmark) (85% protein and 0.2% lactose) was used in paper II. Additionally in paper II, casein concentrate and skimmed human milk (Arla Foods, Stockholm, Sweden, respectively), as well as bovine milk (1.5% w/v) obtained from the local market were used. An enzymatically hydrolysed whey protein (WE80BH, DMW International, Veghel, The Netherlands) with a lactose content of 4% and a protein content of 80% was used in paper III. In paper IV a soy protein isolate (Prisolate 601 EM, KG Food Partner AB, Karlshamn, Sweden) containing 90% protein was tested.

In some of the test meals in papers III and IV, half of the whey doses were exchanged for AA mixtures. All AA were L-isomers and specified as food grad by the manufacturer (Ajinomoto, Kawasaki, Japan).

The amount of AAs (isoleucine, leucine, lysine, threonine and valine, 5AA) in paper III, were based on the ratio between the p-AA responses seen following intake of 18 of whey protein[42]. The composition of the AA mixture was as follows: isoleucine (620mg), leucine (1080mg), lysine (970mg), threonine (780 mg), valine (1050mg).

In paper IV, the AA mixture contained isoleucine (708.7mg), leucine (1282.7mg), lysine (900mg), threonine (900mg), valine (708.7mg) based on

previous findings[72]. In two of the test meals in paper IV, arginine (708.7mg) was added to the 5AA mixture and was set to match the AA with the lowest content. Arginine, isoleucine and threonine came from Ajinomoto, Kawasaki, Japan and leucine, lysine and valine were bought from Sigma-Aldrich AB, Stockholm, Sweden.

Glucose and lactose

Glucose (VWR International AB, Stockholm, Sweden) was added as a carbohydrate source in the whey drinks and was also used as reference drinks in papers I and III.

In paper II, lactose (no 17296-500, Merck Eurolab, Stockholm, Sweden) was added to all test drinks to balance the carbohydrate content of the drinks. All the test drinks contained the total amount of 25g lactose.

Bread products

In paper II a white wheat bread (WWB) was used as reference and it was baked in a bread baking machine as described earlier[87]. A commercial WWB (Dollar Storfranska, Lockarp, Malmö, Sweden) was used in the composite sandwich meal, and was also used as the reference meal in paper IV.

Water and coffee

The whey protein in paper I was dissolved in 250ml of water. In paper II 250ml of water was served to the WWB reference meal, whereas the amount of liquid in the test meals was in the range 379-510ml. In paper III, the whey and AAs were dissolved in 150ml of water and an additional 100ml of cold coffee (Gevalia brygg meallanrost, Kraft Foods Sverige AB, Upplands Väsby, Sweden) was added to the drinks prior to serving. In Paper IV, 100ml water was used to dissolve the pre-meal (PMPD) whey/soy protein and AA drinks and an additional 150ml of water was served to the composite meal.

Ham and butter

A standardized composite sandwich meal was served in paper IV and it contained butter (Bregott, Arla Foods, Stockholm, Sweden) and ham (Rökt Gästabudsskinka, Widerbergs kött AB, Lund, Sweden).

Meal studies

Test subjects

All subjects in the four papers were healthy non-smoking volunteers, aged 20-30 years old with body mass indices 22.8 ± 0.4 kg/m^2 (mean ± SEM) and not receiving and drug treatment. A total of 51 subjects participated in the four studies (24 men and 27 women) and they all had normal fasting p-glucose (p-glucose; 5.1 ± 0.0 mmol/L; mean ± SEM) and serum insulin (0.05 ± 0.0 nmol/L; mean ± SEM) levels and no history of lactose malabsorption. All subjects gave their informed written consent and were aware of the possibility to withdraw from the study at any time. The studies were approved by the regional ethical review board in Lund, Sweden.

Study design

Four randomized, single blind, within-subjects meal studies have been performed. All test meals were provided as breakfasts in random order, approximately one week between each test. The test subjects were instructed to eat a standardized meal in the evening (between 21.00 and 22.00) prior to each test day, consisting of WWB slices provided by us, and an optional drink. Thereafter they were instructed to avoid eating and drinking anything but small amounts of water (50ml) until the start of the test. Additionally they were asked to avoid alcohol, excessive physical activity and food rich in dietary fibers the day before each test. When the subjects arrived in the laboratory in the morning (at 07.45) a peripheral catheter (BD Venflon, Becton Dickinson, Helsingborg, Sweden) was inserted into an antecubital vein. After

drawing a fasting blood sample (t = 0 min) the randomised test meals were served. In papers I-III the subjects were instructed to finish their test meals within 12 min. In paper IV the test subjects were instructed to first a pre-meal protein drink (PMPD) as a bolus and then immediately start to eat the standardized composite sandwich meal. Altogether, the PMPD and the sandwich meal were to be consumed within 12 min.

Chemical analysis of the test products

Protein

The crude protein content in paper II was analysed in all the meals using the Kjeldahl procedure (Kjeltec Auto 1030 Analyser; Tecator, Höganäs, Sweden).

In papers I, III and IV the protein content was analysed using an elemental analyser (FlashEA 1112m thermo Fisher Scientific Inc., Waltham, MA, USA). Protein content was analysed on air dried and milled products.

Amino acids

The peptide-bound AA of the different food proteins were analysed using a hydrolysis step, performed to analyse peptide-bound AAs of the different food proteins. The proteins were dissolved in 6 mol HCL/L, containing 0.1 phenol, and kept at 110˚C for 20h[88]. Tryptophan, cysteine, and methionine were lost during acid hydrolysis; therefore, the contents of these AAs were not measurable. In addition, glutamine and aspargine are converted to glutamic acid and aspartic acid, respectively, during the acid hydrolysis step.

The AAs were analysed with an AA analyser (LC5001; Biotronik, München, Germany) using ion-exchange chromatography. The AAs were separated by using standard lithium citrate buffers of pH 2.85, 2.89, 3.20, 4.02 and 3.49. The post column derivatisation was performed with ninhydrin[89].

Starch and lactose

Available starch in the WWBs in papers II and IV were determined enzymatically according to the method of Holm et al[90]. Samples were incubated in a phosphate buffer with thermostable α-amylase (Termamyl, Novo Nordisk A/S, Denmark) in boiling water for 20 min, followed by a 30 min incubation with amylglucosidase (Roche Diagnostics GmbH, Germany) at 60˚C. Finally the liberated glucose was determined with a glucose oxidase peroxidase reagent (GLOX). The starch was calculated from the amount of glucose multiplied by 0.9.The lactose content of the test meals in paper II was analysed as galactose and glucose following enzymatic hydrolysis with β-galactosidase as described by Nilsson et al[42].

Physiological parameters and subjective rating of appetite

Blood samples were taken continuously for 2h (Papers I and II) or 3h (Papers III and IV). A schematic overview of the sampling series in the four studies is presented in **Table 1**. Capillary blood samples were used to determine blood glucose and venous blood was drawn for analysis of serum insulin and p-AA in all four studies. In some cases additional venous blood was taken for additional plasma incretins (Papers II and IV) and plasma ghrelin (Paper IV) analysis. All serum and plasma (EDTA) tubes were left on ice to rest for approximately 30 min before centrifugation (1800*g, 4˚C) and the samples were thereafter kept frozen at -20˚C until analysed.

Table 1. Sampling series of the blood collected in papers I-IV.

	Glucose and insulin			Amino acids	Incretins		Ghrelin
Paper	I	II	III, IV	I, II, III, IV	II	IV	IV
Time (min)							
0	X	X	X	X	X	X	X
7.5		X			X		
15	X	X	X	X	X	X	
30	X	X	X	X	X	X	X
45	X	X	X	X	X		
60	X	X	X	X	X	X	X
75		X			X		
90	X	X	X		X		X
105		X			X		
120	X	X	X		X	X	X
180			X			X	X

Plasma glucose

P-glucose was analysed immediately after sampling using HemoCue® B-glucose equipment (HemoCue AB, Ängelholm, Sweden).

Serum insulin

The analysis was done on an integrated immunoassay analyzer (CODA Open Microplate System; Bio-rad Laboratories, Hercules, CA, USA) using an enzyme immunoassay kit (Mercodia AB, Uppsala, Sweden).

Plasma amino acids

Free AA were purified by mixing 100µl of 10%sulfosalicylic acid with 400µl plasma to precipitate high-molecular weight proteins according to the method of Pharmacia Biochrome LTD (Cambridge, United Kingdom). The AAs solutions were filtered before analyzing with an AA analyser (Biochrome 30; pharmacia Ltd) by iso-exchange chromatography. The AAs were separated by using standard lithium citrate buffers of pH 2.80, 3.00, 3.15, 3.50 and 3.55. Postcolumn derivatisation was performed with ninhydrin[89].

Plasma GIP and GLP-1

The incretins were measured after extraction of plasma with 70% ethanol (by vol, final concentration). For GIP radioimmunoassay, a C-terminal direct antiserum R 65, that cross-reacts fully with human GIP but not with so called GIP 8000, whose chemical nature and relation to GIP secretion is uncertain, was used[91]. Human GIP and ^{125}I human GIP (70 MBq/nmol) were used for standards and tracers. The concentration of plasma GLP-1 was measured 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[92]. For both assays the sensitivity was < 1 pmol/L, intraassay CV < 6% at 20 pmol/L, and recovery of standard, added to plasma before extraction, ≈100% when corrected for losses inherent in the plasma extraction procedure.

Plasma ghrelin

Total ghrelin was analysed using a radioimmunoassay kit (Linco research Inc., St. Charles, MO, USA).

Subjective satiety

In paper IV the test subjects were asked to repeatedly rate their subjective feelings for hunger, satiety and desire to eat, using a 100 mm Visual Analogue Scale (VAS).

Calculations and statistical methods

The results are expressed as means ± SEM and values of ≤ 0.05 are considered statistically significant. The incremental areas under the curve (iAUC) were calculated for each subject and test meal, using the trapezoid rule (GraphPad PRISM, version 5.04; GraphPad Software Inc., San Diego). All areas below

the baseline were excluded from the calculations and each subject was her/his own reference.

The glycaemic index (GI) and insulinaemic index (II) were calculated from the 120 min postprandial iAUC for p-glucose and serum insulin, respectively, with WWB (Papers II and IV) or glucose (Papers I and III) as references[93]. Additionally, the incremental glucose and insulin peaks (iPeak) were calculated as maximum postprandial increase from baseline for each individual (Papers I, III, IV). In paper IV, the course of glycaemia was analysed by calculating the glycaemic profile (GP), defined as the duration (up to 180 min) of the glucose curve divided with the iPeak of glucose[94]. The insulinogenic index was used in paper IV and is defined as insulin iAUC 0-45 min divided with the glucose iAUC 0-45 min.

The data in paper II was analysed using a mixed model of variance (ANOVA). In papers I, III and IV a mixed model analysis of covariance (ANCOVA) with subject set at as random variable and corresponding baseline (fasting values) as a covariate. Differences between groups were identified using Tukey's multiple comparisons tests (MINITAB, release 14, Minitab Inc., State College, PA, USA). In cases of unevenly distributed residuals (tested with Anderson-Darling test), Box-Cox transformations were performed in the data prior to the ANOVA and/or ANCOVA respectively.

In paper I, dose-response relations were testes using a mixed model ANCOVA where the whey dose and fasting values were set as covariates and subjects as random variable and the iAUC set as the response. To estimate the reduction in iAUC per gram increase of whey protein, linear regression was used[95].

Time × treatment interactions were analysed using a mixed model (PROC MIXED in SAS release 8.01, SAS institute Inc., Cary, NC, USA) with repeated measures and an autoregressive covariance structure. Subjects were modelled as a random variable (Papers I-IV) and corresponding baseline values (fasting values) were modelled as covariates (Papers I, III-IV). When significant interactions between treatment and time were found, Tukey's multiple comparisons test was performed for each time point by using the MINITAB software.

Correlations analysis were conducted to evaluate the relations among dependent measures with the use of Spearman's partial correlation coefficients controlling for subject (Papers I-IV) and corresponding baseline values (Papers I, III-IV) (two-tailed test; IBM SPSS Statistics software, version 20; SPSS Inc., Chicago, IL, USA).

Results and discussion

Glycaemic and hormonal responses in healthy humans to carbohydrate challenges with or without presence of protein/amino acids

The mean values for GI, glucose iPeak, II, insulin iPeak and insulin iAUC 0-15 min and 0-30 min for references and test products in papers I-IV are displayed below in **Table 2**.

Dose-response effects of whey protein

In the present thesis it was found that postprandial glucose, insulin and certain plasma amino acids (p-AA) responded in a dose dependent manner to whey protein when co-ingested with 25g glucose in a drink (Paper 1). The blood glucose response (iAUC 0-120 min) as well as the glucose iPeak were inversely related to whey intake ($r = -0.786$ $P < 0.001$; $r = 0.764$; $P < 0.001$, respectively). A regression analysis showed that for each gram of whey protein added, the postprandial glycaemia (iAUC) was reduced with -3.8±1.4 mmol·min/L ($P = 0.000$). Significant reductions in glycaemia were observed following the 9 and 18g doses of whey protein (**Table 2**). There was a non-significant trend so that the lowest whey dose (4.5g) tended to reduce the glycaemic response (iAUC 0-120 min) and iPeak with 25% and 17%, respectively, compared to the glucose reference drink. Postprandial insulin (iAUC 0-120 min) and iPeak responded in a positive dose dependent way to whey protein intake ($r = 0.836$; $P < 0.000$; $r = 0.875$; $P < 0.000$, respectively). As a general tendency all whey doses (4.5, 9 and 18g) resulted in increased postprandial insulin (iAUC 0-120 min), compared with the glucose reference

with no added whey, although only significant following the highest whey dose.

A previous study in healthy subjects has shown that p-glucose responses (iAUC 0-120 min) to a 25g carbohydrate load (WWB reference or lactose in the case of the test products) were significantly decreased in presence of 18g of whey protein[42]. In the present thesis (Paper I) also 9g whey reduced glucose responses (iAUC 0-120 min and iPeak), but without significant impact on postprandial insulin (iAUC 0-120 min; **Table 2**). In this thesis, we additionally show that also low doses of whey protein have insulinogenic properties since the 4.5g dose resulted in an increased insulin iPeak, compared to the glucose reference drink. Altogether it could be proposed that comparatively modest levels of whey protein supplementation can facilitate glycaemic regulation to a carbohydrate challenge through an insulinogenic effect in health subjects. Similar findings have also been reported for subjects with T2D where presence of 5g of whey derived peptides reduced postprandial glycaemia ($P < 0.001$) to a 50g glucose load[95]. The results in Paper I propose that co-ingestion of a similar amount of intact whey may affect glycaemic regulation also in healthy subjects.

In paper I it was further observed that all measured p-AA (iAUC 0-60 min), except glutamic acid, responded to whey in a dose dependent manner ($r = 0.633$-0.860; $P \leq 0.006$). Positive correlations between plasma responses of isoleucine, leucine, lysine, threonine and valine (5AA; iAUC 0-60 min), and insulin iPeak ($r > 0.494.$; $P < 0.009$) were found, in support of the involvement of these specific 5AA in postprandial insulin response following whey intake. In accordance, it has been reported that in comparison with other proteins, whey consumption promotes elevated levels of plasma AA (p-AA)[96, 97] and especially the 5AA in the acute phase[42]. These 5AA are potent insulin secretagouges, that possibly promotes insulin release directly by acting on the pancreatic β-cells, or indirectly by stimulating GIP[42, 72].

Table 2. GI, Glucose iPeak, II, Insulin iPeak and insulin iAUC 0-15 and 0-30 min for references and test products in papers I-IV.

Products	Glucose		Insulin			
	GI	iPeak	II	iPeak	iAUC 0-30min	iAUC 0-15min
	%	Δ mmol/L	%	Δ nmol/L	nmol/L	mmol/L
Glucose (ref)	100[a]	4.1±0.4[a]	100[a]	0.16±0.02[a]	2.2±0.4[a]	0,52±0,11[a]
18g whey	63±10[b]	2.8±0.2[b]	189±22[b]	0.28±0.02[b]	3.2±0.3[a]	0,70±0,10[a]
9g whey	72±10[b]	3.1±0.2[b]	139±19[a]	0.24±0.02[b]	3.3±0.4[a]	0,87±0,15[a]
4.5g whey	85±12[ab]	3.4±0.3[ab]	151±21[ab]	0.22±0.02[b]	3.0±0.6[a]	0,61±0,11[a]
WWB (ref)	100[a]	2.0±0.3[a]	100[a]	0.25±0.05[a]	2.1±0.4[a]	0,31±0,09[a]
Whey	61±15[b]	1.4±0.2[ab]	165±21[b]	0.41±0.08[b]	3.7±0.9[a]	0,29±0,10[a]
Casein	44±13[b]	1.0±0.2[b]	110±20[a]	0.30±0.05[ab]	2.8±0.6[a]	0,27±0,08[a]
Bovine	43±12[b]	1.3±0.2[ab]	88±10[a]	0.30±0.06[ab]	3.2±0.8[a]	0,44±0,13[a]
Human	57±11[b]	1.5±0.2[ab]	117±19[ab]	0.26±0.04[ab]	3.2±0.7[a]	0,45±0,11[a]
Glucose (ref)	100[a]	3.6±0.2[a]	100[a]	0.19±0.02[a]	3.1±0.7[a]	0,53±0,14[a]
HW	89±11[ab]	3.0±0.3[ab]	186±17[b]	0.35±0.03[b]	5.1±0.8[ab]	1,10±0,18[a]
HW+5AA	84±10[b]	3.0±0.2[b]	184±25[b]	0.39±0.04[b]	5.2±0.6[b]	0,86±0,15[a]
W	86±8[ab]	3.0±0.3[ab]	167±25[b]	0.32±0.03[b]	4.7±1.1[ab]	0,77±0,15[a]
W+5AA	79±11[b]	2.9±0.2[b]	178±23[b]	0.35±0.04[b]	4.8±0.5[ab]	0,99±0,16[a]
Ref (ham sandwich)	100[a]	2.9±0.2[a]	100[a]	0.35±0.04[a]	3.3±0.6[ac]	0,59±0,14[a]
W-PMPD	73±11[b]	1.7±0.2[b]	118±10[a]	0.38±0.04[a]	4.8±0.7[abc]	1,02±0,22[ab]
W-PMPD+5AA	53±8[b]	1.4±0.1[b]	123±14[a]	0.38±0.04[a]	5.2±0.6[b]	1,36±0,22[b]
W-PMPD+6AA	60±6[b]	1.4±0.1[b]	121±10[a]	0.34±0.04[a]	4.5±0.6[abc]	1,09±0,20[ab]
S-PMPD	67±9[ab]	1.9±0.1[b]	105±13[a]	0.31±0.03[a]	3.2±0.4[a]	0,60±0,12[a]
S-PMPD+5AA	74±9[ab]	1.8±0.2[b]	119±9[a]	0.38±0.05[a]	4.4±0.5[abc]	1,00±0,13[ab]
S-PMPD+6AA	91±18[ab]	1.9±0.2[b]	123±9[a]	0.39±0.03[a]	4.3±0.6[abc]	0,93±0,17[ab]

Values are means ± SEM, Products within each paper not sharing the same letters were signifcantly different , $P < 0.05$ (ANOVA followed by Tukey's test in paper II, ANCOVA followed by Tukey's test in papers I, III – IV). See abbrevation list for abbrevations .

Taken together, 9g of whey protein, and possibly even lower doses, may be efficient in reducing postprandial glycaemia following 25g glucose challenge in healthy subjects, and that the 5AA are important mediators of the insulinogenic effect. This prompted us to investigate the metabolic impact of these 5AA as a supplement to different protein drinks (see below paper III and IV).

Metabolic responses to human and bovine milk

In paper II, lactose equivalent amounts of human and bovine milk, as well as reconstituted whey- and casein drinks were examined with respect to glycaemia and insulinaemia in healthy subjects. White wheat bread (WWB) was included as a reference. No standardisation of protein content was performed, and the human milk thus contained less than 25% of the protein content of the other three test drinks (about 3.5g vs 16g of protein). All test drinks, including the human milk, reduced glycaemia (iAUC 0-120 min) to a similar extent compared to the reference meal (see **Table 2**). Since all test drinks contained lactose (GI 68)[69], and the reference was a starch based bread (GI 100)[69], the low glycaemic responses seen with all test drinks may be partly explained by their lactose content. Interestingly, the human milk displayed a 10% lower insulin response (iAUC 0-120 min) compared to the WWB reference, whereas the other test drinks (bovine milk, whey and casein drinks) instead tended to increase the insulin responses. However, the whey drink was the only test drink which resulted in a significant increase in insulin compared to the reference (iAUC 0-120 min; **Table 2**). When comparing the bovine and human milk, it appears as if the human milk was more efficient in regulating postprandial glycaemia to lactose per protein portion. The effect might possibly stem from the high proportion of whey in the protein of the human milk. The casein:whey ratio of human milk is reported to be 50:50-20:80, depending on the lactation period[98], compared with 80:20 for bovine milk[43]. Based on these figures the human milk portion contained 1.75-2.8g whey protein, whereas the bovine milk had about 3.2g whey protein per serving.

One suggested mechanism for how certain dietary proteins causes insulinogenic effects is through incretin stimulation[32]. Previous studies has preferentially seen a GIP effect following whey intake[48], thus is it interestingly that the whey drink resulted in significantly higher GLP-1 and GIP responses compared to the reference and the other test drinks (iAUC 0-120 min; Paper II). Additionally, the human milk and the reconstituted whey protein elicited a higher early increase in plasma GLP-1 (iAUC 0-30 and 0-45 min) compared to the WWB reference. It was also noted that the human milk elicited a significant increase in plasma GIP already at 7.5 min. Both GIP and GLP-1 are

strong insulin secretagouges[32, 99], and stimulation of these incretins could thus add to the insulinogenic properties seen with whey protein. Previous data in both healthy and T2D subjects has mainly shown stimulation of GIP response after intake of whey protein (18g protein)[42, 48]. Limited information is available concerning the impact of whey intake on GLP-1. However, a markedly higher whey dose (48g) increased plasma GLP-1 levels when co-ingested with a considerable amount of butter (100g) and low amounts of carbohydrates[100], suggesting additive incretin stimulation from the fat content of the meal. According to Veldhorst et al, intake of 25E% whey protein resulted in significantly higher GLP-1 compared to soy and casein, when co-ingested with 55E% carbohydrates and 20E% fat[101]. In paper II, a significant increase in response of plasma GLP-1 and GIP (iAUC 0-120 min) were observed following 16g of whey protein. Furthermore, a strong positive correlation was found between early GLP-1 (iAUC 0-30 min) and early insulin responses, (iAUC 0-30 min; $r = 0.688$; $P < 0.001$), which further implicates the involvement of GLP-1 and also that an early incretin secretion may be important for the insulinogenic response. In support of such an opinion it was it recently seen in mice that an early GLP-1 responses might affect early plasma insulin concentrations, with reducing effects on postprandial glycaemia[102]. Compared with the whey drink, the human milk elicited more than 50% of the GLP-1 response (iAUC 0-30 min) despite having only 22% of the protein content. This suggests that human milk may be a strong GLP-1 stimulator, and despite its low protein content, human milk can be anticipated to contain almost the same concentration of whey as bovine milk. Interestingly, mixtures of whey protein, glucose and peanut oil reduced p-glucose response and increased peak insulin and GLP-1 levels compared with iso-caloric glucose ingestion in a mice experimental model[102]. It was concluded that the marked early insulin response to mixed meal ingestion, emanated from a synergistic, rather than an additive effect of the individual macronutrients in the mixed meal and that this was in part caused by increased levels of GLP-1. To my knowledge, this thesis is the first to report acute glycaemic, insulinaemic and incretin responses to human milk, and similarly to reconstituted whey, human milk appears to stimulate early GLP-1 as well as GIP response compared with a white wheat bread reference meal.

In paper II, p-AA (iAUC 0-60 min) tended to increase following all test drinks compared to the reference, although not significant in all cases. Plasma leucine, lysine and valine were significantly higher following the bovine milk, whey and the casein drinks, respectively. Plasma threonine was significantly increased following the whey and casein drinks, compared with the reference. However, there was only a tendency to increased p-AA following the human milk. When including all test products and the reference significant positive correlations were, however, found between postprandial p-5AA, and insulin and incretin levels (iAUC 0-60 min) respectively, as well as negative correlations between p-5AA and p-glucose responses (iAUC 0-60 min). This is in line with previous findings were a mix of BCAA, leucine and threonine, displayed strong insulin secreting properties in isolated Langerhans' islet from mice[103]. Although the human milk did not significantly increased overall p-AA responses compared to the reference, involvement of specific AA cannot be excluded. Additionally, the second most common AA in human milk is taurine, an organic acid containing an amino group (2-aminoethanesulfonic acid). Taurine has been reported with glycaemic regulating effects[104, 105], and could thus contribute to the metabolic response to human milk. The presently used experimental design (Paper II), does not allow conclusions regarding the potential role of taurine, since this acid was not analysed. Altogether data from papers I and II, suggest that p-AA are important factors for the insulinogenic properties of whey protein, and could possibly affect postprandial insulin levels through two different pathways; directly influencing the pancreatic β-cells, as well as indirectly through the incretin hormones GLP-1 and GIP.

Hydrolysed vs intact whey

In the present thesis, it was hypothesised that hydrolysed whey when co-ingested with glucose is digested and absorbed more rapidly than the intact form, resulting in an even more rapid p-AA and insulin response, with an even more efficient effect on acute glycaemia to a composite meal. A study was designed to investigate how 9g of hydrolysed and intact whey, respectively,

affected postprandial metabolic regulation following a 25g glucose load in healthy subjects (Paper III).

Both hydrolysed and intact whey tended to reduce postprandial glycaemia (iAUC 0-120 min) to a similar extent, ≈-23% (NS), compared to a glucose reference with no added protein. Both forms of whey also displayed similar reductions in glycaemic iPeak (3.0 Δmmol/L) compared to the glucose reference (3.6 Δmmol/L). Furthermore, both hydrolysed and intact whey significantly increased insulin responses (iAUC 0-120 min) compared to the reference, with the hydrolysed whey tending to be more insulinogenic (+53%) than the intact whey (+35%). Moreover, both whey forms resulted in significantly higher iPeaks for insulin (0.35 vs 0.32 Δmmol/L, respectively), compared with the reference (0.19 Δmmol/L; $P \leq 0.05$). The hydrolysed whey also resulted in a slightly higher early insulin response (+64%) than the intact whey (+52%) when compared to the reference, (iAUC 0-30 min, NS). In paper II, early insulin (iAUC 0-30 min) and GLP-1 (iAUC 0-30 min) correlated positively ($P \leq 0.05$), and the early insulin responses from the hydrolysed whey in paper III could thus be an indication of a more rapid incretin and p-AA response. Hydrolysed protein was found to be more rapidly digested resulting in a faster p-AA response[106] and has additionally been shown to significantly increase early insulin and GLP-1 (iAUC 0-30 min), compared to intact whey in T2D subjects[107]. It has been shown that a diminished secretion of GLP-1 could possibly lead to decreased insulin secretion[108] and that GLP-1 secretion is reduced in T2D subjects[109]. A stimulation of early GLP-1 from whey proteins could thus be beneficial for T2D. Interestingly has it also been proposed that BCAA, and especially leucine promotes GLP-1 secretion from the intestinal cells[110]. Contradicting to these results, data in the literature indicates differences in glycaemic regulating impact comparing hydrolysed and intact whey. Consequently, 10g of intact whey but not hydrolysed whey significantly reduced postprandial glycaemia (cumulative AUC 0-170min) to a preset pizza meal in healthy subjects[111]. However, the proteins were served 30 min before the pizza meal and without a carbohydrate source. Thus an early insulin response (iAUC 0-30 min), caused by a rapid p-AA response, when the proteins is co-ingested with carbohydrate might have been missed.

To mask the bitter taste of AA and small peptides in the hydrolysed whey, cold coffee (100ml) was added to the protein/glucose drinks. The glycaemic regulatory properties of coffee and caffeine are debated, and both positive[112, 113] well as negative effects[114] on glycaemic regulation have been reported. The fact that coffee was added to the protein drinks in paper III could possibly affect the results. However, coffee was added to all the test drinks and any possible interference was therefore compensated for.

Limited information is available concerning the potential impact of distributing the whey protein in intact vs hydrolysed form on parameters such as glycaemia, insulinaemia and p-AA, when co-ingested with a carbohydrate source. Whey protein has been referred to as a "rapid protein", based on its ability to cause a rapid increase in postprandial p-AA[45, 115]. It could be hypothesised that hydrolysed and intact protein fractions could differ in postprandial metabolic effects related to potential differences in rate of gastric emptying rate and/or release of its amino acids to the plasma. However, hydrolysed vs intact whey protein displayed similar gastric emptying rate in healthy subjects[116, 117]. However, in these studies the protein was served without[117], or with a very low amount of carbohydrates (< 5 g)[116]. In the absence of carbohydrates, ingestion of hydrolysed whey resulted in a more rapid total AA and BCAA release, and a higher insulin response (iAUC 0-120 min), compared to intact whey in healthy subjects[118]. On the contrary, a higher responses of plasma BCAA was reported following intake of intact whey intake compared with hydrolysed, but without similar effects on insulin[117].

Effects of combining dietary protein and amino acids

In vivo and in vitro studies have shown that AA stimulates insulin secretion[18, 19, 103, 119-121]. Evidence is further at hand suggesting that mixtures of AA are more efficient than single AA[17]. In Paper III, the effect of exchanging half of the protein in a whey/glucose drink for mixtures of specific AA was examined. The 5AA as mentioned above, were added to the intact or hydrolysed whey, respectively, providing in total 9g protein or protein/AA.

The two test drinks (hydrolysed and intact whey, respectively) without the added 5AA tended to reduce the glycaemia to a glucose load, but not significantly. Instead, both the 5AA-supplemented whey drinks resulted in a significant reduction in glucose responses (iAUC 0-120 min and iPeak) compared to the glucose reference (**Table 2**). Additionally, both the protein and protein/AA drinks significantly increased insulin responses (iAUC 0-120 min and iPeak), compared to the glucose reference. Consequently, exchanging half of the whey protein dose for the specific mixture of 5AA appeared to have a larger impact on the glycaemic regulation to a glucose load compared with hydrolysed or intact whey, only.

The postprandial p-AA responses (iAUC 0-60 min) were significantly higher following all protein and protein/AA drinks, with a tendency to higher responses following the protein/AA, compared with the reference (Paper III). The plasma responses of the 5AA (iAUC 0-15 min) correlated positively to the early insulin responses (iAUC 0-15 min; $r = 0.419$; $P = 0.000$ and iAUC 0-30 min; $r = 0.385$; $P = 0.001$) and to the insulin iPeak ($r = 0.448$; $P = 0.000$). Additionally, the plasma responses of the 5AA (iAUC 0-15 min) correlated negatively to the glucose response (iAUC 0-120 min; $r = -0.342$; $P = 0.005$) and glucose iPeak ($r = -0.280$; $P = 0.023$). Similarly to the findings in papers I and II, the postprandial p-AA responses to the whey protein in paper III appeared to be related to the insulin (pos.) and glucose responses (neg.). It could be suggested that adding the 5AA-mixture to the whey protein further enhances the potency of the whey to regulate postprandial glycaemia, possibly by promoting increased levels in plasma of the 5AA, with enhanced insulin responses as a consequence. Furthermore, the results suggests that combining specific AA and whey protein appeared to more effective in reducing p-glucose than intact and hydrolysed whey alone.

Effects of a pre-meal protein/AA load

Most studies related to the impact of proteins and AA on acute insulin response and glycaemic regulation have been performed following administration of the protein moiety as part of either a carbohydrate containing

drink, or as a drink administered with a meal[39, 42, 48, 95, 122]. In several studies, as well as in the present thesis (Paper I-III) plasma insulin and p-AA starts to increase within the first 15 min in the postprandial phase[42, 48, 72, 123]. Based on this knowledge it was hypothesized that administration of protein with or without added AA prior to a meal could have advantageous effect on postprandial glucose regulation. A study was designed to evaluate the effects of pre-meal drinks containing protein, on glycaemic regulation and metabolic responses to a composite sandwich meal in healthy subjects (Paper IV). The protein source was either isolated whey or soy. Additionally the effect of exchanging half of the protein for two specific AA-mixtures was evaluated: the 5AA or the 5AA+arginine (6AA). Nine grams of soy (S) or whey (W) protein, with or without addition of 5AA or 6AA, was provided as pre-meal protein drinks (PMPDs) prior to a challenging composite ham sandwich meal contributing with 50g carbohydrates in the form of WWB. The PMPDs were taken as a bolus just before (< 1 min) the sandwich meal. The rational of including arginine were studies indicating arginine to possibly increase insulin sensitivity[124], and also when co-ingested with leucine, enhance the pancreatic insulin secretion[39].

All three W-PMPD resulted in significant reduction of glycaemia (iAUC 0-120 min) post the sandwich meal (**Table 2**). When including 5AA or 6AA-mixtures with the W-PMPD, there was a tendency to an additional reduction of glycaemic response (iAUC 0-120 min and iPeak), compared to the W-PMPD alone (NS). Consequently, the GI of the sandwich meal was non-significantly reduced from 73 with the W-PMPD to 53 (W-PMPD+5AA) and 60 (W-PMPD+6AA). Interestingly, the overall insulin responses and insulin iPeaks to all PMPD meals were not significantly higher than that of sandwiches served with only water. This absence of an elevated insulinaemic peak is of interest since insulin resistance might be triggered by hyperinsulinaemia[125]. However, the W-PMPD+5AA resulted in a higher early insulin response (iAUC 0-15 min and 0-30min) compared to the reference ($P < 0.005$). Interestingly, the early insulin response (iAUC 0-15 min) correlated negatively to glucose responses (GI, glucose and iPeak). In addition the early insulin (iAUC 0-15 min) also correlated to an increase in p-AA (of the 5AA and 6AA) and incretin

(GIP and GLP-1) responses (iAUC 0-15 min, respectively) and to the glycaemic profile (GP).

The three S-PMPDs were not as efficient as the W-PMPDs in reducing glycaemia. All S based PMPDs resulted in lower glucose iPeaks compared to the reference ($P < 0.005$), but no significance was observed for overall course of glycaemia (iAUC 0-120 min). Recent studies in T2D subjects suggest that soy protein co-ingested with 50g glucose, does not affect postprandial glycaemia [126], whilst other show a reducing effect[127]. Previous data in healthy subjects has shown that intake of > 40g soy protein in a composite meal containing 56g carbohydrates, reduced glycaemia (iAUC 0-120 min) compared to a cod protein containing meal[128]. The AA composition differs between the two proteins; whey protein being higher in BCAA, whereas soy protein is richer in arginine and phenylalanine. As previously mentioned, the BCAA in particular have been ascribed insulinogenic properties, and contribute to the insulin stimulating properties of whey protein[103]. However, at levels of inclusion, addition of the 5AA mixture to soy protein may not induce the specific p-AA responses seen in case of the whey+5AA. Benefits seen with large amounts of soy protein in the postprandial phase[128], may also partially be assigned to its residual content of potential bioactive components such genestein[129] and glyceollins[130].

Table 3. GP for references and test products in paper III and IV

	Products	GP min·mmol^{-1}·L^{-1}
Paper III	**Glucose (ref)**	28±3^{a}
	HW	29±3^{a}
	HW+5AA	29±3^{a}
	W	28±3^{a}
	W+5AA	29±3^{a}
Paper IV	**Ref (ham sandwich)**	55±5^{a}
	W-PMPD	89±7^{b}
	W-PMPD+5AA	127±22^{b}
	W-PMPD+6AA	125±16^{b}
	S-PMPD	78±7^{ab}
	S-PMPD+5AA	88±12^{b}
	S-PMPD+6AA	81±11^{ab}

Values are means ± SEM, Products within each paper not sharing the same letters were signifcantly different , $P < 0.05$ (ANCOVA followed by Tukey's test). See abbrevation list for abbrevations

A tentative measure of the course of postprandial glycaemia, the Glycaemic Profile (GP) has been introduced aiming at evaluating also the later postprandial period beyond the 120 min included in the GI[94]. The GP is defined as the duration for the incremental postprandial blood glucose response divided with the blood glucose incremental peak (min/mM), and a high GP thereby indicates a low glucose peak and a low prolonged late net increment in glycaemia. The concept of GP is based upon findings that a low but sustained postprandial glucose increment favours an improved glucose tolerance and lowered tri-glycerides levels are the time of the subsequent meal[131]. Also a course of glycaemia with high GP features appears to improve cognitive functioning in the late postprandial phase[132]. In **table 3**, GP values from paper III and IV are reported, and in **Figure 1 and 2** the postprandial blood glucose and serum insulin responses are displayed, respectively. Previous studies report a GP of 37 for WWB in healthy subjects[94]. In paper IV the reference WWB meal in combination with ham and butter resulted in a higher GP of 55 (**Table 3**). Additionally, when ingesting PMPDs prior to

starting the ham sandwich meal, the course of glycaemia was modulated towards higher GP's (**Figure 1 and Table 3**). All three W-PMPD, including the two with addition of 5AA or 6AA, and the S-PMPD+5AA resulted in higher GP ranging from 88-127, with the W-PMPD+5AA yielding the highest GP (127), which is higher than the range reported previously in studies examining courses of glycaemia to cereal products[94]. No significant differences in GP were observed between any of the test drinks and the glucose reference in paper III. Intake of a PMPD prior a composite meal (Paper IV) thus appears to affect the GP positively, compared to co-ingestion of protein and glucose (Paper III).

Worth mentioning is that different reference meals were used in paper III (25g glucose) and IV (50g WWB), although the amount of protein and AA were the same. Still the glucose peaks for both references reached the same magnitude (≈ Δ 3.0-3.5mmol). This is probably explained by the addition of ham and butter to the WWB (Paper IV), resulting in a slower gastric emptying and thus reducing the glycaemic response. In paper III, the reference and the test drinks resulted in a hypoglycaemic episode between 60-90 min (**Figure 1**); a physiological state that appears to be avoided if consuming the protein/AA prior a meal (as a PMPD), or in combination with a mixed meal.

In paper IV the insulinogenic index (IGI) was also calculated (**Table2**). IGI is a measure of the change in postprandial serum insulin divided by change in p-glucose, and is used in connection with an oral glucose tolerance test (OGTT) to measure β-cell function[133-135]. All PMPDs resulted in higher IGI estimated from the 45 min postprandial period, compared to the reference, with the W-PMPD+5AA showing the highest IGI (Paper IV). A high IGI could be interpreted as a high efficacy of insulin to lower postprandial glucose within the first 45 min.

Figure 1. **Postprandial plasma glucose responses following all products and references in paper III and IV**

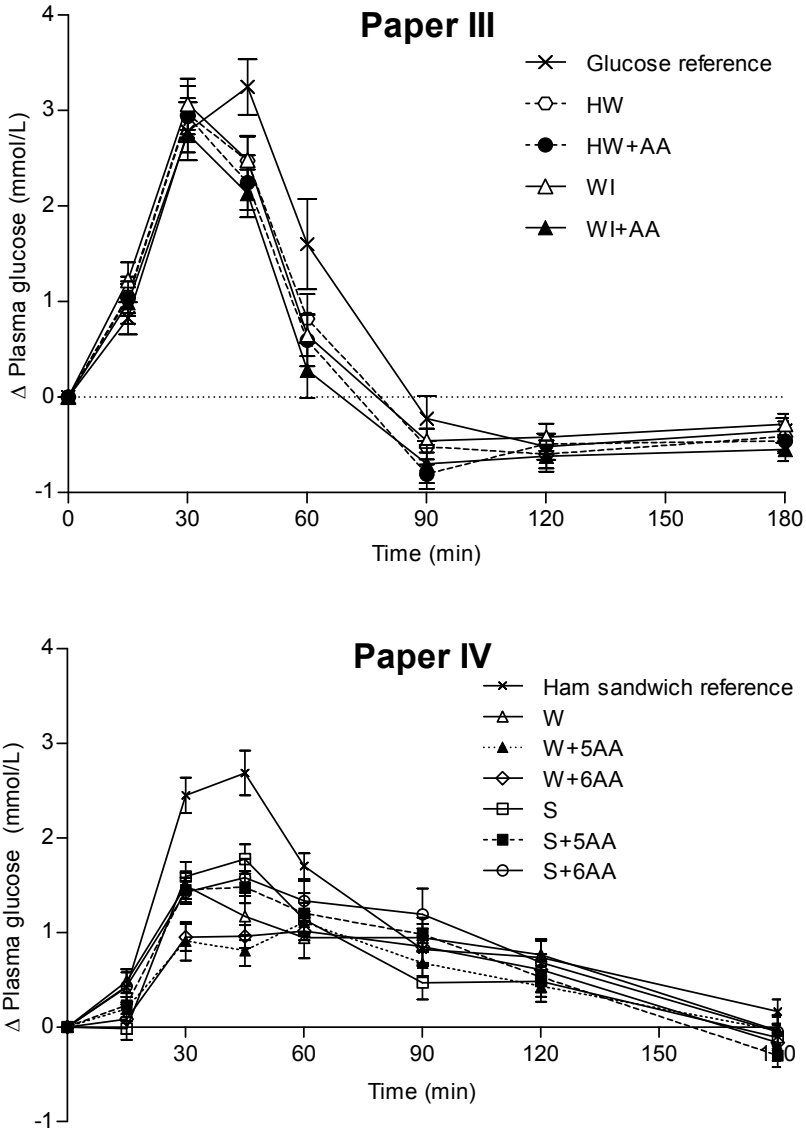
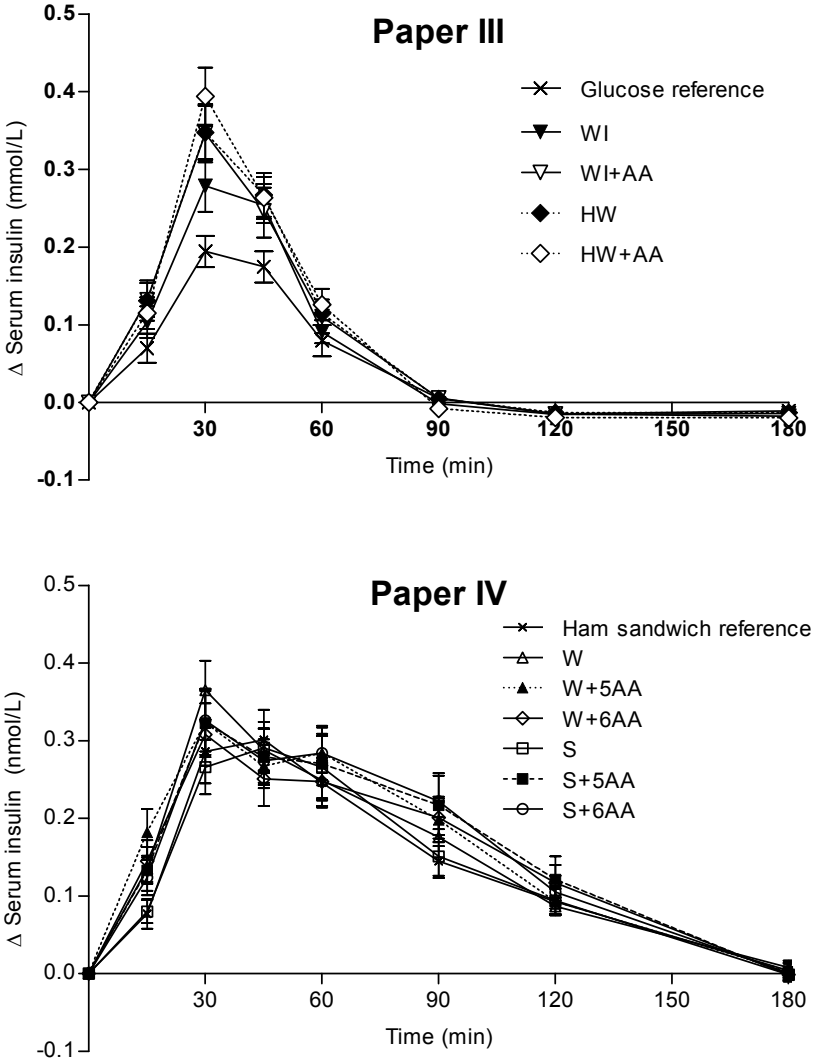


Figure 2. **Postprandial serum insulin responses following all products and references in paper III-IV**



Effects of soy- and whey protein and/or amino acids on subjective rating of hunger/satiety

Whey protein and certain AA[65, 101], as well as soy protein[51] have been associated with satiating properties. In paper III, subjective satiety to 9g of hydrolysed vs intact whey was studied in an experimental setting using equi-carbohydrate amounts of glucose, and a glucose reference with no protein. Subjective rating using VAS was performed in the postprandial period up to 180 min in young healthy subjects. Additionally, subjective rating of satiety to one and the same composite sandwich meal preceded by different PMPDs was estimated, also in healthy subjects up to 180 min following the composite meal. In paper IV, plasma ghrelin was also measured. No significant effects were observed in either paper III or IV within the time frame studied in any of the VAS parameters measured, nor in plasma ghrelin. A possible reason for the absence of effects on satiety/hunger measures, could be that both studies were powered to detect differences in postprandial plasma glycaemic and insulinaemic responses as primary outcome variables, and the number of test subject (n = 14-16) was set to match this design[93]. A higher participant number is possibly required to reach statistical power in the case of subjective ranking of satiety/hunger using VAS-scales. Additionally, the tested protein doses (total 9g protein) were quite low, and in paper IV a possible satiating effect of the protein/AA may have been concealed by the subsequent ham sandwich meal containing another 13g protein.

It has been observed that whey protein (50g) results in higher perceived feelings of satiety compared to tuna, egg or turkey, and the suggested mechanism is the rapid and high insulin response to the whey[136]. Even though insulin is a satiating hormone, blood glucose levels have been found to be a better predictor of satiety following whey intake[111]. Furthermore, it has been observed that a pre load of low whey doses (5-20g protein) in liquid form induced higher satiety but did not affect the energy intake (EI) after 2 h, compared to a water control[137]. Several factors have been suggested to affect satiating properties of whey protein, with insulin[66, 67] and incretin stimulating

properties[36], and also a more potent release of postprandial AA as proposed mediators of satiety and possible facilitated weight regulation[138, 139].

General discussion

A tight regulation of blood glucose after a meal reduces the risk of future complications in T2D subjects[78, 140, 141]. Postprandial glycaemic excursions may also be of relevance in healthy subjects, and epidemiological studies have linked low GI diets to reduced risks of CVD[79] and T2D[142].

In this thesis, significant differences were observed in glycaemia to a carbohydrate challenge in the presence of certain food proteins in young healthy subjects. Consequently, whey protein, both with and without added 5AA or 6AA, and soy protein with added 5AA reduced postprandial blood glucose excursion. A blunting effect on glycaemia following supplementation of glucose or lactose with whey protein has been observed previously[42, 95]. However, the present study underpin that exchanging part of the whey protein for the specific 5AA (isoleucine, leucine, lysine, threonine and valine) magnified the insulinogenic effect with a subsequent additional reduction of postprandial glucose response, compared to whey protein alone. A novel finding in the present thesis was the considerable attenuation of glycaemic response (-47% to -27%), and improvement in glycaemic profile (GP; +162% to - +143%) to a composite regular meal, when ingesting whey, or whey+5AA as a pre-meal load. Interestingly, these modulatory effects on postprandial glycaemia were obtained in the absence of elevated over all course of insulinaemia, and mainly the initial insulin response (iAUC 0-30 min) was increased (+58%) compared to reference meal.

The results from the present thesis support previous findings that the insulinogenic property of whey protein is partly mediated by a rapid plasma response of the 5AA. Oral intake of a mix of the specific 5AA can be anticipated to further enhance the rate by which the 5AA appears in plasma. Protein sources with similar AA patterns may still show different p-AA responses. Cod and whey protein have approximately the same amount of

BCAA, yet they result in significantly different p-AA responses, with the whey protein resulting in significantly higher p-AA and insulin responses[42]. Thus, the availability of the 5AA in the ingested protein might be of importance for the rapid p-AA response. Comparatively rapid p-AA responses are additionally observed following orally ingested AA such as leucine[143] and lysine[70], although not always accompanied with significant effects on insulin responses. Also intake of a mixture of AA results in rapid p-AA response[72]. From this thesis, it can be concluded that combining mixtures of specific AA with comparatively small doses of whey protein reduced postprandial glycaemia, both when ingested together with a carbohydrate load (Paper III), and prior to a composite meal (Paper IV).

It was shown that early insulin concentrations (iAUC 0-15 min) correlated positively to the corresponding GLP-1 and GIP responses (iAUC 0-15 min) (Paper IV). Also in paper II, early insulin (iAUC 0-30 min) correlated to GLP-1 (iAUC 0-30 min). These observations are in line with recent findings in mice, were early insulin and GLP-1 were enhanced following a mixed meal including whey protein, compared to a glucose load alone[102]. Also, the GLP-1 responses (Papers II and IV) were inversely related to the glycaemic response, suggesting the involvement of incretins in the glycaemic modulatory potential of whey protein and 5AA. It has been suggested that first phase insulin is blunted in T2D subjects[144], and that such a reduction is an early sign of impaired insulin secretion[145, 146]. Using dietary protein and AA to stimulate early insulin response could be an effective approach in reducing post meal glycaemic excursions. The results of using protein/AA resembles that of pharmaceuticals (e.g. Repaglinide and Glipizid) aiming to stimulate first phase insulin secretion in order to lower postprandial glucose levels in T2D treatment[147, 148].

The early GLP-1 secretion observed in the present thesis could result from stimuli of the L-cells in the more proximal parts of the gut[29]. The GLP-1 secreting effect is impaired in T2D subjects[109, 149]. Interestingly, GLP-1 is suggested to preserve pancreatic β-cells[150], and is also important for maintaining optimal β-cell function and insulin secretion. Such properties have led to an interest in GLP-1 analogues that are now often used in treatment of

T2D[151]. GLP-1 is rapidly inactivated by the enzyme dipeptidyl dipeptidase IV (DPP-4)[28]. Of particular interest in this context are recent data suggesting that whey protein may act as a DPP-4 inhibitor[152]. Whey protein could thereby promote an incretin effect and also prolong GLP-1 activity.

In the present work (Paper IV), both early GIP and GLP-1 (iAUC 0-15 min) correlated strongly to the p-5AA responses. Data in the literature have put forward that AA might have stimulatory effects on GIP secretion[153, 154]. Additionally, recent data from isolated pancreatic mouse islets incubated with human serum following whey protein intake showed an involvement of GIP, GLP-1 and p-AA on insulin secretion[103]. In the present work, GIP was not significantly affected (Paper IV), but stimulation of GIP from whey protein intake, co-ingested with 25g glucose, have been observed in previous studies[42]. A possible GIP effect from the whey/AA could possibly be concealed by a GIP effect from the fat and carbohydrates in the ham sandwich meal, where both macronutrients are known GIP stimulators[32]. A very early GIP response (7.5 min) was observed following human milk intake (Paper II). However, it should be mentioned that, GIP has been reported as intrinsic to human milk[155], and the very early GIP response might emanate from oral intake of the incretin. Very little is known regarding the activity of orally ingested incretins, and whether it can withstand the acidic milieu in the stomach remains to be elucidated. Possibly the importance of intrinsic hormones is less relevant in relation to bovine milk, since it is subject to pasteurisation or UHT processing.

Both hyperglycaemia and hyper-insulinaemia are parts of the pathogenesis in T2D[156], and it might be considered unwise to further enhance/stimulate the insulin secretion in these patients, in that it may lead to pancreatic exhaustion. However, long-term intensive treatment with sulfonylurea has been shown to reduce late complications in T2D subjects[157]. Interestingly, the whey protein and AA used in this thesis appears to have similar insulin stimulating effects on the pancreas as sulfonylurea. Furthermore, epidemiological studies indicate that high consumption of food rich in whey such as milk and dairy products, protect against development of T2D[158], the metabolic syndrome[159], and overweight and obesity[65, 160]. Those results suggest that dairy foods do not promote metabolic dysregulation. Additionally, when fed to mice over a

period of 8 weeks, the 5AA studied in the present thesis resulted in improved insulin sensitivity[161]. Another recent mouse trial showed that supplementation of dietary BCAA for 3 months increased longevity[162], which is also in favour of a protective role.

Mixtures of EAA have been shown to reduce fasting glycaemia and insulinaemia and improve metabolic control in poorly regulated elderly T2D subjects[163, 164]. There are also indications in the literature that dietary supplementation only with leucine may improve metabolic control in T2D[165]. Of interest in this context is that the capacity to secrete insulin to certain AA is maintained in the more advanced stages of the diabetic disease where the capacity to respond to glucose is severely decreased[163]. In addition to facilitating postprandial glycaemic regulation, supplementation with EAA might also increase muscle mass in elderly diabetic subjects. This in turn, may improve overall insulin sensitivity[166]. Supplementation of dextrose with EAA has been reported to improve glucose tolerance after an OGTT also in healthy overweight subjects[167]. Taken together, the observation that whey and AA may promote increased GLP-1 levels[110, 123], combined with reports of the antidiabetic and antiobesity potential of this incretin[37, 168], indicate benefits from utilising certain proteins and AA as meal or pre-meal supplement for facilitated glycaemic regulation. Thus it can be suggested that the use of protein and/or specific essential AA as insulin secretagouge to facilitate glycaemic regulation is safe, and should be evaluated both as a therapeutic tool, and with respect to prevention.

As a novel approach, the present thesis set out to investigate the potential effect of supplying a PMPD (pre-meal protein drink) prior to intake to a composite meal (Paper IV). The PMPD supplemented meals reduced postprandial glucose in the absence of a hyperinsulinaemic peak. Such an effect was not observed when the protein was served in combination with a carbohydrate source (Papers I-III). Interestingly it has been shown in mice that a bolus supplementation of leucine for seven days resulted in reduced fasting glycaemia, compared to continuous infusion via gavage[169]. Although difficult to translate such an effect to the meal situation in humans, these animal data suggest that a bolus load of AA may affect metabolism different from

continuous intake. Consequently, constant elevation of p-AA, as seen in T2D may not be advantageous[170-172], but further studies are needed to fully understand the mechanisms. The very few studies available addressing the influence of timing of whey protein ingestion has focused on appetite regulation and used larger protein doses[101, 173] or studied impacts of whey in the absence of a carbohydrate source[137]. However, recent data showed that milk consumption, both 30 and 120 min prior a meal reduced pre- and post-meal glucose levels, compared to other protein rich pre-meal drinks, in healthy subjects[174]. The effect was suggested to originate from the protein content and composition. It has also been observed that 10-40g of whey reduced postprandial glucose when served 30 min prior to a preset meal[111]. Insulin significantly increased up to 30 min prior to the meal intake compared to the control, and the pre-meal protein could be expected to exert an even better effect if ingested closer than 30 min to the subsequent meal.

Serving the protein/AA as a PMPD (Paper IV) was an attempt to exploit the potential of pre-meal loads of protein to reduce the glycaemic peak and increase the GP. The correlation between high early insulin (iAUC 0-15 and 0-30 min) and early incretin response (iAUC 0-15 min) and the fact that the peak for insulin occurs at 30 min, could be interpreted as if the optimal time of ingestion lies within this time frame. In the present thesis all studies were performed in healthy subjects. The optimal timing of ingestion might be different for different target groups, such as T2D and IRS subjects, where insulin sensitivity[156, 175], and blunted first phase insulin secretion[133] exist. These metabolic perturbations are factors that could possibly influence the insulinogenic effects from protein/AA.

Potential effects of increased protein intake

In the present thesis 4.5-18g of protein has been given to healthy subjects and it was found that 9g of protein was sufficient to achieve reducing effects on post meal glycaemic responses. Possibly also lower whey doses have positive effects on postprandial glycaemia. It could be debated whether a regular addition of 9g protein to the main meals could cause any side effects.

According to the National Food Agency in Sweden a diet for healthy, normal weight, active persons should provide approximately 65-120g of protein (10-20E% of protein) daily. An addition of 4.5-9g of protein/AA 2-3 times a day, as suggested by the results from the present thesis, induces a moderate increase of about 15-20% of total protein intake/day. As an example, 250ml of milk (2.5% fat) contains 8.75g protein and a 9g whey/AA drink thereby matches a glass of milk to each meal.

Data in the literature speaks in favor of an increased protein/AA intake. A five week intervention with an increase from 15E% to 30E% of daily protein intake, was reported to improve glycaemic control (24h integrated p-glucose area), in T2D subjects[176]. Another 3 weeks intervention with a high protein diet (2.0g/kg bw/day), significantly improved glycaemic control in T2D subjects compared to a low protein intake (0.8g/kg bw/day)[177]. Additionally did the DIOGENES study show that a modest increase of protein and decrease in GI over 26 week period could facilitate weight control in healthy subjects[178].

Recent metabolomics data in adult obese subjects have implicated that fasting plasma levels of AA as well as of acylcarnitine (C3 and C5 especially) are elevated, and also strongly correlated to decreased insulin sensitivity which is a key feature of T2D pathogenesis[179]. Acylcarnitines are metabolites in the BCAA catabolism, and it could thus be argued that high intakes of BCAA promote the development of T2D. On the contrary, data in young obese subjects instead indicates, that increased p-AA were positively correlated to β-cell function and insulin sensitivity[180, 181]. During a catabolic state, p-AAs are increased as a secondary effect[182]. Furthermore, a catabolic state causes insulin resistance[183], and it is thus difficult to predict if high p-AA levels induces insulin resistance or if it is the other way around. Interestingly, several positive effects are observed from increased intake of the BCAA. Sarcopenia is a problem related to aging[184], and the decline in skeletal muscle mass is linked to the development of T2D[185]. Leucine activate mTOR signalling and thus also protein synthesis, via both insulin-dependent and independent pathways[186]. Evidence speaks in favour of leucine supplementation as a tool for muscle preservation in elderly[187]. It was recently concluded that intake of > 3g of leucine to a meal, increased muscle synthesis in elderly[165]. Furthermore, intake

of 2-5g of leucine diminished postprandial glycaemia in T2D. Taken together, including the effects of whey and AA intake on GLP-1 and p-AA and the positive effects on postprandial glucose, meal supplementation with additional protein and 5AA intake is probably safe.

Conclusions

From the present studies performed in healthy subjects it can be concluded that;

- Whey protein (4.5-18g) co-ingested with glucose (25g) affected postprandial blood glucose, serum insulin and plasma-AA in a dose dependent manner, with decreasing glycaemia (iAUC 0-120 min) with increasing whey dose. P-5AA iAUC 0-60 min correlated to insulin iPeak.
- Equi-carbohydrate amounts of human- and bovine milk produced similar postprandial glycaemia and insulinaemia.
- Hydrolysed and intact whey had similar effects on glycaemic responses to a glucose challenge, although hydrolysed whey tended to be more insulinogenic.
- Exchanging half of the whey protein for a mixture of 5AA in a glucose challenge magnified the insulinogenic effect and lowered postprandial glycaemia iAUC 0-120 min.
- Whey protein with or without addition of 5AA or 6AA in a pre-meal load, considerably attenuated the postprandial glycaemia (iAUC 0-120 min and glucose iPeak), and increased GP, to a composite meal. Addition of arginine to the AA mix did not further influence glycaemia.
- The lowering of glycaemia following a pre-load intake of whey and soy protein with added 5AA and 6AA, were observed in the absence of a hyperinsulineamic peak.

- Soy protein with addition of 5AA ingested as a pre-meal load, reduced postprandial glucose iPeak and increased GP to a composite meal.
- Early GLP-1 and p-AA responses were associated with early insulin response after whey/AA intake, and possibly explain the attenuation of over-all course of post-prandial glycaemia to a carbohydrate challenge.

Future perspective

In this thesis the main focus has been on postprandial glycaemic responses in healthy subjects in an acute perspective. Of interest in the future will be to evaluate longer-term effects on metabolic risk markers in at risk subjects e.g. elderly or over-weight subjects following supplementation of meals with mixtures of specific proteins and AA. Also of interest is to evaluate the therapeutic potential in subjects with overt T2D. To further understand the mechanisms behind the postprandial glycaemic regulatory properties of the protein/AA several, complementary biochemical analysis need; C-peptide, glucagon and as well as analysis of insulin secretion, and insulin sensitivity. The specific impact of timing of the protein/AA meal supplement warrants further studies, and it is foreseen that meal supplements or pre-meal foods may be developed for this purpose.

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