

Dietary protein in the regulation of the somatotrophic axis

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**DIETARY PROTEIN in the REGULATION of the
SOMATOTROPIC AXIS**

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The studies presented in this thesis were performed at the Nutrition and Toxicology Research Institute Maastricht (NUTRIM), which participates in the graduate school VLAG (Food technology, Agrobiotechnology, Nutrition and Health Sciences), accredited by the Royal Netherlands Academy of Arts and Science.

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DIETARY PROTEIN in the REGULATION of the SOMATOTROPIC AXIS

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht
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Introduction

The somatotropic axis originates in the hypothalamus (brain region) and pituitary gland (beneath the hypothalamus). Through the central release of growth hormone (GH) and peripheral production of insulin-like growth factor 1 (IGF-1), the somatotropic axis is involved in linear growth and intermediary metabolism¹. Disorders as visceral obesity, sarcopenia and growth retardation in children are characterised by a low activity of the somatotropic axis²⁻⁵. Several amino acids, building blocks of protein, and protein itself are previously found to stimulate the activity of the somatotropic axis⁶⁻¹³. This stimulating potential is investigated in this thesis from a neuroendocrinological, physiological and pathophysiological perspective. This chapter gives an overview of the concepts underlying the studies, starting with the neuroendocrinology, i.e. the regulation of the somatotropic axis. It continues with the physiological effects of GH and IGF-1, including linear growth and intermediary metabolism, and the pathophysiology of the somatotropic axis, i.e. the consequence of a reduced somatotropic activity. Furthermore a section on dietary protein and its role in the regulation of the somatotropic axis is provided, finishing with an overview of the research questions investigated in this thesis.

REGULATION OF THE SOMATOTROPIC AXIS

The secretion of GH by the anterior pituitary is pulsatile and mediated by two neurohormones (GH releasing hormone (GHRH) and somatostatin), which are produced by the hypothalamus and continually influence the secretory rate of GH. Both hypothalamic peptides are secreted in independent waves and interact together to generate and control GH release. GHRH stimulates GH release, whereas somatostatin has an inhibitory effect¹⁴⁻¹⁷. Both GHRH and somatostatin are transported via a portal blood system from the hypothalamus to the anterior pituitary gland and bind to specific receptors in the pituitary to influence the production of GH by the somatotrophs (cells in the anterior pituitary gland that produce GH). The integrated effect of GHRH and somatostatin on the pituitary gland ultimately leads to GH secretion in a pulsatile pattern with about 10-20 secretory pulses per 24h¹⁸. Pulses of GH secretion occur at times of maximum GHRH and minimum somatostatin secretion^{19, 20}. Recently, ghrelin, a hormone from the stomach, is investigated suggesting a new mechanism for regulation of GH secretion via a specific receptor in the somatotrophs¹³.

Daily GH secretion rate varies from a maximum of nearly 2 milligram per day in late puberty to a minimum of 20 microgram per day in older and obese adults²¹. The differences in the amount of GH secretion during life are explained by changes in GH pulse amplitude, with unchanged GH pulse frequency. GH pulse amplitudes decline with age and GH concentrations in middle age are about 15% of pubertal

levels. Both men and women have an increased pulse frequency during the night time hours²¹. Fasting GH concentrations are higher in women than in men. Overall, women have more continuous GH secretion and GH pulses that are of more uniform size than men. Major GH secretory pulses, accounting for approximately 70% of daily GH secretion, occur in the deep sleep, i.e. slow wave sleep (SWS). The decline in SWS from early adulthood to midlife is paralleled by a major decline in GH secretion, suggesting that age-related alterations in the GH-IGF-1 axis may partially reflect decreased sleep architecture²¹.

Thus, the GH secretory pattern is characterized by diurnal pulses and therefore accurate quantification of GH secretion requires extra attention, to exclude missing pulses. Frequent sampling of every 20 min has been shown to be an appropriate manner to quantify the GH secretion²².

Stimulators and inhibitors of the somatotrophic axis

The balance of stimulating and inhibiting peptides determines GH release and is influenced by factors as sex, ageing, stress, sleep, body composition and food intake. These factors can be mediated on neural, metabolic and hormonal level. An overview of the somatotrophic regulation is shown in Figure 1.

Neural- Neural stimulators and inhibitors affecting GH release include neurotransmitters^{21,23}. Dopaminergic, alpha-adrenergic and serotonergic agents all stimulate GH release. For instance, levodopa (i.e. a dopaminergic agonist) and norepinephrine (alpha-adrenergic pathways) increases GH secretion and propranolol (i.e. beta-adrenergic agonist) inhibits GH secretion²³.

Metabolic- Changes in the levels of metabolic fuels play an important role in regulation of GH secretion. When glucose concentrations are decreased, i.e. hypoglycaemia, GH secretion is stimulated. Insulin-induced hypoglycaemia is used clinically as a test to provoke GH secretion²⁴. Acute ingestion of glucose results in an elevated plasma glucose concentration and inhibits GH secretion, although there is a rebound release 3-4 h later after glucose ingestion as a consequence of decreasing glucose concentrations^{25,26}.

With respect to the role of fat, there is clear evidence that free fatty acids (FFA) control the GH secretion. Pharmacological reduction of circulating FFA raises GH secretion²³ and FFA elevation induced by the combination of exogenous intralipid plus heparin reduces spontaneous GH secretion^{23,27} and abolishes GH responses to various stimuli^{23,28,29}. The inhibitory effect of FFA on GH secretion at the pituitary is rapid, within minutes and is dose and time dependent³⁰.

Furthermore, infusion or ingestion of certain amino acids (AA) increases GH secretion^{7,31}. The most well known GH promoting AA is arginine³¹. Intravenous administration of arginine is used clinically as a test to provoke GH secretion³²⁻³⁴. Also

other AA have been shown to promote GH release, in particular lysine, ornitine, glutamine, methionine, histidine, tyrosine, glycine and tryptophan, but not leucine and valine^{7, 9-11}. The influence of amino acids and protein on the somatotropic activity is extensively dealt within this thesis.

Hormonal- GH secretion is affected by numerous hormones. Estrogen enhances the GH secretory response to many stimuli, and the same holds for prolactin and gonadotropins (LH and FSH). Furthermore ghrelin stimulates the secretion of GH from the somatotrophs. Ghrelin is high just before a meal and is stimulated by ingestion of protein, but suppressed after a meal by ingestion of carbohydrates or fat^{35, 36}. GH release is suppressed by progesterone, glucocorticoid therapy, hypercortisolism, hyperthyroidism and hypothyroidism²³. Circulating leptin concentrations, which are positively correlated with abdominal and total body fat³⁷, are inversely related with serum GH concentrations^{38, 39}. Furthermore, GH itself exerts a negative feedback on its own secretion. Also elevated IGF-1 concentrations (as a result of elevated GH concentrations) inhibits the GH release by a negative feedback loop^{30, 40}. Both GH and IGF-1 stimulate somatostatin secretion and inhibit GHRH^{41, 42}.

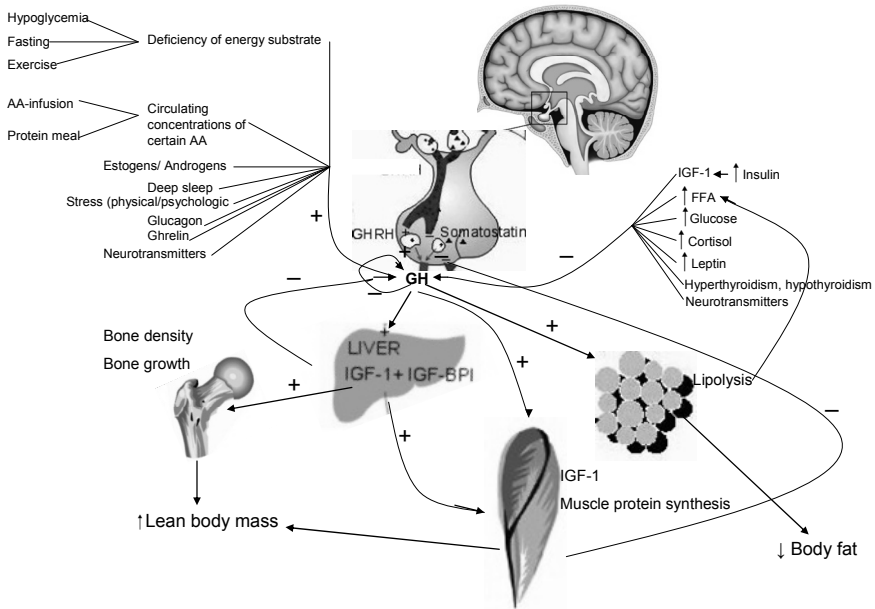


Figure 1: The regulation of the somatotropic axis.

Physiological effects of GH

The meaning 'growth hormone' is derived from its ability to produce an increase in linear growth. Furthermore, the hormone plays an important role in the regulation of metabolic processes. The physiological effects of GH are summarized in Figure 1.

Linear growth

Longitudinal growth occurs through a process of cell proliferation, the addition of new cells to the growth plate of the bone and hypertrophy, resulting in the expansion of the growth plate⁴³. Although the control of bone growth is not entirely understood, the somatotrophic axis plays a key role in these processes. IGF-1 receptors are found in proliferating bone condrocytes, and IGF-1 itself stimulates synthesis of collagen⁴⁴. Also from epidemiological studies it is known that the somatotrophic axis is associated with linear growth in infancy, childhood and puberty. Increased IGF-1 concentrations and subsequently an increased growth velocity determines the growth curves of each individual child⁴⁵⁻⁴⁹.

Metabolism

Besides the impact of the somatotrophic axis on linear growth, many metabolic effects that persist throughout life are affected by the hormones of the somatotrophic axis. More specific, GH has acute and chronic metabolic effects on lipid, glucose and protein metabolism. More prolonged GH exposure induces IGF-1 production⁵⁰, which is accompanied by an increase in lean body mass and a decrease in body fat, in particular the visceral component, in adults⁵¹. Metabolic effects of GH on fat, glucose and protein metabolism are described below.

Effects of GH on fat metabolism were already demonstrated in the 1930's when studies showed that the injection of rats with extracts from the anterior pituitary gland reduce body fat. Subsequent studies administrated GH subcutaneously and demonstrated that the decrease in body fat is caused by GH⁵²⁻⁵⁵. Injection of GH results in an increase in fat mobilization, oxidation, decrease in fat deposition and activation of hormone sensitive lipase, resulting in increased triglyceride hydrolysis to free fatty acids and glycerol (lipolysis)⁵⁶⁻⁵⁸, resulting in elevated blood FFA concentrations. The lipolytic action of GH is a direct effect and may underlie the ability of GH to decrease the excessive levels of adipose tissue, in particular in the visceral regions⁵⁹⁻⁶⁴. Furthermore, GH clearly shifts fuel utilization by increasing lipid oxidation and reducing carbohydrate and protein oxidation, probably by means of substrate competition⁶⁵.

In addition to the effects of GH on fat metabolism, several effects of GH on glucose and protein metabolism are reported. Acute effects of GH on glucose metabolism include an impairment in muscle glucose uptake, together with a decrease in glucose oxidation, an increase in glucose storage⁶⁵⁻⁶⁹ and a diminished gluconeo-

genesis⁵⁸. The increase in GH concentrations is suggested to play a role in mediating the switch from gluconeogenesis to lipolysis⁷⁰.

Somatotropic effects on protein metabolism include a stimulated protein anabolism in many tissues. GH, via IGF-1 increases protein synthesis by enhancing amino acid uptake and directly accelerating the transcription and translation of mRNA. As described above, GH tends to decrease protein catabolism by mobilizing fat as a preferred fuel source. This protein saving effect is an important mechanism by which GH promotes growth and development of lean body mass^{50, 70, 71, 72}. The anabolic effects of GH becomes more consistent with prolonged GH exposure, as also illustrated in acromegaly (a syndrome that results when the pituitary gland produces excess GH) resulting in an increased LBM^{51, 73}.

Overall, the metabolic effects of GH on the somatotrophic axis include an increase in the lipolysis, fat mobilisation, activation of hormone sensitive lipase, triglyceride hydrolysis and a decrease in fatty acid reesterification ultimately resulting in a reduction of fatmass and change of fat distribution from visceral towards peripheral fat. Furthermore, an increase in somatotrophic activity results in increased rates of whole body proteolysis, synthesis of protein, underlying an increase in lean body mass in the long term.

Pathophysiology of the somatotrophic axis

A diminished or absent secretion of GH from the anterior pituitary gland, also called GH deficiency (GHD), is characterised by a reduced linear growth in children, increased body fat, in particular in the visceral regions, and decreased lean body mass^{74, 75}. GHD occurs in congenital pituitary deficiencies, pituitary adenoma or other disorders that are characterised by chronic damage of the pituitary gland or hypothalamus.^{42, 76 77, 78}

Children with GHD usually present with short stature and low growth velocity for their age and pubertal stage⁷⁹. Furthermore their weight is normal for their height, however their absolute lean mass is reduced and their absolute fat mass is unchanged compared with controls, which implies an unfavourable body composition. The diagnosis GHD in childhood involves a multiple step diagnostic process, usually culminating in GH stimulation tests (GHST) to identify whether the pituitary gland is able to release a pulse of GH when provoked by various stimuli as arginine, clonidine, insulin, levodopa or GHRH^{80, 81}. In children, GHST are used to estimate the level of insufficient GH secretion in order to decide about starting GH therapy⁸². GH therapy consists of administration of recombinant human GH (rhGH) to restore IGF-1 concentrations^{83, 84}, resulting in an increase in linear growth in children who are too small for their age⁸⁵⁻⁸⁷, with an improvement in body composition development².

Besides in GHD, a diminished GH secretion can also occur in patients with a normal hypothalamus/pituitary gland, but with a chronic disease as the metabolic syndrome⁸⁸; this state is called hyposomatotropism. Similarities exist between patients with hyposomatotropism and GHD. For instance, adults with the metabolic syndrome and adults with GH deficiency are both characterised by relatively high visceral adipose tissue, insulin resistance, high serum triglyceride (TG), concentrations, low serum high-density lipoprotein (HDL) cholesterol concentrations and an increased prevalence of hypertension and increased mortality from cardiovascular diseases^{4, 88, 89}. With increased visceral fat mass, GH secretion is blunted with a decrease in the amount of GH secreted per burst but without any major impact on GH secretory burst frequency⁹⁰. Similar to GH concentrations, IGF-I concentrations are inversely related to the percentage of body fat⁹⁰, and in particular to the amount of visceral adipose tissue and not to the amount of subcutaneous fat mass⁹¹. Replacement therapy with rhGH has demonstrated favourable effects on most of the metabolic abnormalities of GHD in adults⁹². Similar to rhGH treatment in GHD, rhGH treatment in visceral obesity has shown to reverse the metabolic abnormalities, resulting in a decrease in visceral/abdominal fat mass, increase in lean mass, improvement of insulin sensitivity, but also improvement of the lipid profile and reduction of cardiovascular risk factors^{89, 93}.

DIETARY PROTEIN and SOMATOTROPIC AXIS

Dietary protein

Dietary protein has two general roles in nutrition, a specific role as source of amino acids (AA) and a non-specific role as energy source. Nine of the 20 nutritionally important AA are classified as essential AA, i.e. cannot be synthesised by the body and must be supplied with the diet and the remaining 11 AA are classified as non-essential, i.e. can be synthesised in the body from other AA. In humans, the essential AA are; histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. The non-essential amino acids are alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, proline, serine and tyrosine⁹⁴. Furthermore, conditionally essential AA are AA that need the donation of either a carbon or an accessory group from another amino acid in order to be synthesized. These AA include arginine, cysteine, glutamine, glycine, proline and tyrosine⁹⁵. AA that are essential for adults are also essential for children.

Dietary protein is found in almost all foods of animal and plant origin. Sources of dietary protein are thus all kinds of meat and fish, eggs, dairy products, cereals, vegetables and legumes, such as beans or peas. Dietary protein of different sources

are characterized by different AA compositions. Proteins are classified in relation to their nutritional quality, based upon the composition of AA and the amount of each amino acid. High-quality protein indicates an optimal amino acid composition of dietary protein, according to the needs of each amino acid. In general, animal sources of dietary protein, except gelatin, contain all essential amino acids and are therefore considered protein of high quality⁹⁶.

The protein requirement of an individual is defined by the WHO as the lowest levels of dietary protein intake that will balance the losses of nitrogen from the body in persons maintaining energy balance at modest levels of physical activity⁹⁷. The recommendation is 0.83 gram per kg body weight per day for adults and 1 gram per kg body weight for children between 6-10 y. Children from industrialised countries will typically have a protein intake that meets or exceeds their physiological requirement.

Dietary protein and somatotrophic axis

Amino acids are potent stimulators of GH secretion, either intravenously injected or orally ingested⁶⁻¹³. Arginine is the most well-described stimulant, although lysine, ornithine, glutamine, methionine, histidine, tyrosine, glycine and tryptophan are all considered effective GH releasers⁷. An oral mixture of arginine and lysine evokes a sevenfold increase of plasma GH concentrations compared with basal concentrations⁸, and a similar effect is observed after arginine-aspartate⁹⁸. The effect of AA on GH secretion appears to be exerted through suppression of hypothalamic SS release⁹⁹⁻¹⁰¹.

Since AA are the building blocks of protein, it is likely that also proteins are involved in the somatotrophic regulation, which is supported by epidemiologic studies in children and adults¹⁰²⁻¹⁰⁹. In intervention studies with extra protein consumption as intervention, IGF-1 concentrations were increased^{102, 103}. The same result is found in observational studies, in which the group with the highest tertile of milk intake showed higher IGF-1 concentrations than the group with the lowest tertile of milk intake^{104, 105}.

No short-term studies are performed investigating the somatotrophic regulation by protein. For some decades ago, some studies already investigated the effect of a meal on the somatotrophic axis. It has been reported that after a high protein meal, GH secretion is increased¹¹⁰ and after a high-carbohydrate meal, GH secretion is initially decreased, but increased in late postprandial phases¹¹¹. However, no change in GH concentrations were found after 1 week of a low carbohydrate, high fat and high protein diet¹¹². Another study found that high carbohydrate diets suppress GH secretion, whereas high fat diets and high protein diets of similar caloric value have no measurable effect²⁸. The reason for the inconsistency of the studies is unclear, it may be that the different techniques to assess GH secretion

result in inconsistency in the results. Furthermore it may be that differences in macronutrient composition are involved, as mentioned before, also the macronutrients glucose and fat are involved in the somatotrophic activity^{28, 110, 111, 113, 114}. No previous studies were performed investigating the specific effect of carbohydrate, fat or a combination of these, on the somatotrophic axis by protein.

There are indications that not only the amount of protein, but also the protein type is an important factor in the elevation of circulating IGF-1 concentrations, probably due to various AA compositions of different protein types. For instance, animal protein including milk, fish and poultry, but not red meat was associated with higher IGF-1 concentrations. Moreover ingestion of milk protein increased the IGF-1 concentrations, whereas the same amount of animal protein, ingested as meat, did not. In children IGF-1 concentrations were strongly associated with animal protein, but not with vegetable protein consumption^{106, 107, 109}. This, together with the observation that some AA are more potent GH stimulators than others, indicates that the somatotrophic activity of protein depends on the protein type. However, it is unknown which types of protein are more potent than others and if, ultimately, a possible variability in protein type differently influences linear growth and body composition. Hence this urges for systematic studies elucidating the capacity of various proteins, alone or as part of a meal to stimulate the somatotrophic axis.

Thus, to understand the role of dietary protein in the regulation of somatotrophic activity it is important to get insight in the somatotrophic activity of different protein types and of protein as part of a meal. Furthermore, it remains to be investigated whether the somatotrophic effects of protein can be used as tool in the diagnostic work-up of GHD or as nutritional therapeutic intervention to restore GH concentrations in hyposomatotropism.

OUTLINE OF THE THESIS

The studies described in this thesis investigate to what extent the dietary intake of protein affects the somatotrophic axis from a *neuroendocrinological, physiological and pathophysiological* perspective.

From literature it is known that AA are involved in the *neuroendocrine* regulation of the somatotrophic axis. Since AA are the building blocks of protein, it is likely that proteins, similar to AA, are involved in short term somatotrophic regulation. This hypothesis is tested in chapter 2, where we investigated the somatotrophic activity of protein, either in its complete form, hydrolysed form or as single AA. Since different proteins contain different AA compositions, we hypothesized that the various protein types differently affect the somatotrophic release (chapter 3).

A typical meal consumed by humans consists of a mixture of 3 macronutrients and each of the macronutrients may have an independent effect on the somatotropic release. Chapter 4 and 5 describe whether and how the somatotropic activity of protein is influenced by co-ingestion of carbohydrates and fat.

The *physiological* role of GH includes linear growth and body composition development. The hypothesis that protein and in particular the amino acids arginine and lysine stimulate the somatotropic axis, resulting in an increased linear growth (i.e. body height), increased lean body mass and decreased body fat is tested in epidemiological observational cohort studies (chapter 6 and 7).

A diminished or absent secretion of GH from the somatotrophs is a characteristic of the *pathophysiology* of the somatotropic axis resulting in a reduction in linear growth and an increase in visceral adipose tissue. Tests to evaluate the activity of the somatotropic axis in order to decide starting GH therapy are reviewed in chapter 8 and at the same time an alternative approach using dietary protein is suggested. In order to investigate the somatotropic activity of protein in lean subjects in comparison to that in a hyposomatotropic population, a study was executed to compare the GH promoting effect of dietary protein in lean subjects and visceral obese subjects, which is described in chapter 9.

Finally chapter 10 provides a general discussion that deals with the short term and long term effects of protein ingestion on the somatotropic axis and concomitantly on linear growth and body composition in lean and hyposomatotropic subjects.

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CHAPTER 1

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2

Effects of oral ingestion of amino acids and proteins on the somatotrophic axis

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ABSTRACT

Context: Growth hormone (GH) is an important regulator of growth and body composition. It has been shown that GH release can be promoted by intravenous as well as oral administration of various amino acids (AA), especially arginine and lysine (ARG+LYS), that are ample present in soy protein. The effects of dietary protein on GH secretion, however, are less well described.

Objective/design: In an experiment we compared the effects of oral ingestion of a mixture reflecting the amino acid composition of soy protein (AA), with oral ingestion of arginine + lysine, on GH-secretion in eight healthy women (BMI=19-25kg/m²; 18-24years). In a second experiment, we compared oral ingestion of hydrolysed soy protein and complete soy protein with the AA-mixture on GH secretion in eight healthy women (BMI=19-26 kg/m²; 19-36 years).

Both experiments were performed in a randomized single blind cross-over design. Serum GH, insulin glucose and plasma AA were determined every 20 minutes, during 3 h in the first experiment and during 5 h in the second experiment.

Results: Peak values of GH were higher after ingestion of the AA-mixture compared with ingestion of ARG+LYS ($p<0.05$). GH-responses, as determined by area under the curve (AUC), did not significantly differ after ingestion of the complete soy protein, hydrolysed soy protein or AA-mixture, but were all higher than after placebo ($p<0.05$).

Insulin-responses (AUC) were higher after ingestion of hydrolysed soy protein, complete soy protein and AA-mixture, compared with placebo ($p<0.05$). Glucose concentrations were unaffected.

Conclusion: Ingestion of soy protein, either hydrolysed or intact, as well as amino acids reflecting, soy protein stimulates GH release to a similar extent.

INTRODUCTION

Growth hormone (GH), a hormone originating from the anterior pituitary gland, is an important regulator of growth and body composition¹. GH exerts its growth promoting effect (typically of fat-free mass) through stimulation of the secretion of Insulin-Like Growth Factor I (IGF-I) from the target cells¹. Disorders as obesity, sarcopenia and growth retardation in children are characterized by low GH-concentrations¹⁻⁴.

It has been shown by many authors that GH secretion can be promoted by intravenous administration of various amino acids, including arginine, methionine, phenylalanine, lysine and histidine^{5,6}. Leucine and valine seem less potent, while isoleucine does not seem to affect plasma GH concentrations^{5,6}. In particular, the stimulatory effect of intravenous arginine on GH is clinically used as a method to assess the responsiveness of the GH secretory system, e.g., when GH deficiency is suspected⁷.

The stimulatory effect of amino acids on GH secretion is also present when amino acids are administered orally. Thus, ingestion of a dosage of 24 g of a mixture of essential amino acids increased plasma GH concentrations with 2,1 fold in comparison with basal values⁸. It is not clear whether all amino acids contribute equally to this phenomenon. Glutamine, arginine, glutamic acid and arginine plus lysine, have been shown to stimulate the GH response in several studies^{5,9}. After oral ingestion of glutamine or arginine, plasma GH-concentrations increased 2 - 4,5 fold higher in comparison with time controls^{9,10}, while a combination of arginine and lysine increased plasma GH-concentration 3-8 fold^{11,12}. In contrast, oral ingestion of aspartic acid or cysteine did not affect GH concentrations⁵.

Effects of amino acids on GH secretion, as mentioned above, may suggest a role of dietary protein in the regulation of plasma GH concentrations. However, only a limited number of studies focused on the effects of dietary proteins and diet composition on GH secretion. It has been shown that in children, dairy (but not meat) consumption is positively associated with growth and plasma concentrations of IGF-I¹³. Also, the GH responses to an arginine stimulus were lower during a high carbohydrate diet, than during a high fat or a high protein diet¹⁴.

Because the effects of dietary protein on GH secretion are less well documented, this study aims to investigate the effects of dietary soy protein ingestion, containing approximately equal amounts of arginine and lysine, on plasma GH concentrations. Amino acid mixtures are expected to be absorbed more rapidly than protein hydrolysates, which are expected to be absorbed more rapidly than intact proteins. This may result in different peak values of amino acids in the plasma, which may affect GH release.

METHODS & MATERIALS

Two series of experiments were performed to investigate the effects of dietary protein ingestion on plasma GH concentration. In the first experiment, the effects of ingestion of an amino acid mixture reflecting soy protein (AA) were compared with the effects of arginine and lysine (ARG+LYS). In the second series of experiments, the effects of oral ingestion of proteins (complete and hydrolysed) were compared with the effects of the AA-mixture on GH secretion.

Subjects

Subjects were recruited via advertisements at the university. In the first experiment as well as in the second experiment, eight healthy young females (experiment 1: age 21 ± 2.6 years, BMI 22.5 ± 2.5 kg/m², experiment 2: age 24 ± 5.5 years, BMI 22.7 ± 1.9 kg/m²) participated. Each subject participated once in this study. Subjects were in good health, non-smoker, using contraceptives, free of any other medication and spent no more than three hours a week on sport activities. Women came to the university in the same phase of their 'menstrual cycle'. The Medical Ethics Committee of Maastricht University approved the study protocol and all subjects gave their written informed consent before participating in the study.

Experimental design

A randomized cross-over study design was applied in both series of experiments. In the first experiment, subjects reported to the laboratory for consumption of 2 different testdrinks and in the second experiment for consumption of 4 different testdrinks, all testdrinks at separate test days. Each time, subjects arrived at the laboratory in a fasted state, in the morning. They were instructed to fast from 10 pm the night prior to the test day. A permanent cannula was inserted into a dorsal vein of the hand, which was placed in a thermoregulated (60°C) box for arterialized venous blood sampling¹⁵. Blood sampling began 60 min after placement of the cannula. Blood was sampled every 20 min for the next 3h in the first experiment and for the next 5h in the second experiment. During blood sampling, subjects remained awake and fasted and were allowed to drink water ad libitum. Immediately after obtaining the first blood sample, subjects received a testdrink. The testdrinks are described in Table 1. In the first experiment, subjects received the AA-testdrink and the ARG+LYS testdrink. In the second experiment, subjects received a drink containing a complete soy protein testdrink, a hydrolysed soy protein testdrink, an AA-testdrink (reflecting soy protein) and a placebo-testdrink. The AA in the AA-testdrinks were obtained from Bufa BV (Uitgeest, the Netherlands). The sugarfree syrup (Diaran, Cereal) added to the AA-drinks contained no proteins

and fat and a negligible amount of carbohydrates. The syrup was added to increase the taste of the testdrink. The protein-testdrinks were produced by NIZO (Netherlands Instituut Zuivel Onderzoek, Ede, the Netherlands).

Table 1: Composition of the testdrinks in experiment 1 and 2, based on a subject of 70 kg.

	Experiment 1		Experiment 2			
	AA	ARG+LYS	Hydrolysed soy protein	Complete soy protein	AA	Placebo
Volume (ml)	250	250	467	467	500	500
Cystein(g)	0.22	-	0.46	0.46	0.44	-
Methionine(g)	0.23	-	0.46	0.46	0.46	-
Aspartic acid(g)	2.06	-	4.00	4.28	4.12	-
Threonine(g)	0.67	-	1.30	1.40	1.36	-
Serine(g)	0.91	-	1.76	1.93	1.82	-
Glutamic acid(g)	3.38	-	6.34	7.04	6.76	-
Proline(g)	0.89	-	1.76	1.89	1.79	-
Glycine(g)	0.73	-	1.42	1.55	1.47	-
Alanine(g)	0.75	-	1.46	1.60	1.51	-
Valine(g)	0.88	-	1.72	1.84	1.76	-
Isoleucine(g)	0.92	-	1.68	1.80	1.84	-
Leucine(g)	1.49	-	2.81	3.02	2.98	-
Tyrosine(g)	0.71	-	1.26	1.39	1.42	-
Phenylalanine(g)	0.97	-	1.76	1.93	1.94	-
Histidine(g)	0.49	-	0.88	0.96	0.98	-
Lysine(g)	1.13	1.13	2.14	2.34	2.27	-
Arginine(g)	1.43	1.43	2.60	2.81	2.86	-
Tryptophan (g)	0.24	-	0.46	0.50	0.47	-
Sugar free syrup (ml)	20	20	-	-	40	40

Blood analysis

In both experiments, arterialized venous blood was collected in cloth tubes (Becton Dickinson Vacutainer system; Becton Dickinson, Franklin Lakes, NJ) and in the second experiment, arterialized venous blood was also divided into cloth tubes and into heparinized tubes (Becton Dickinson Vacutainer system; Becton Dickinson, Franklin Lakes, NJ). Blood in the clot tube (containing 'Silica Clot Activator') was allowed to clot for 30 minutes and was centrifuged at 3000 rpm, 4°C for 10 min to obtain serum. Serum in the first experiment was collected for determination of GH and in the second experiment for determination of GH, insulin, glucose and urea concentrations. Blood in heparinized tubes was kept on ice to minimize enzymatic reactions. Amino acid analyses were performed in plasma, which was obtained by centrifugation at 4°C for 10 min at 3000xg. Subsequently 250 µL plasma was deproteinized with 20 mg dry sulfosalicylic acid for analysis of plasma amino acids concentrations and enrichment. Each aliquot of serum and plasma was frozen immediately in liquid nitrogen and stored at -80°C, until analysis. All samples from one subjects were run in the same assay.

The hGH-concentrations were measured by using an ultrasensitive hGH chemiluminescence immunoassay (Beckman Coulter, Harbor Blvd. Fullerton, U.S.A.). The

intra- and inter-assay coefficients of variation were 1.4-2.1 and 6.8-8.6% respectively, at GH concentrations of 3.7-14.2 and 3.1-7.3 $\mu\text{g/l}$, respectively.

Insulin concentrations were measured by an electrochemiluminescence immunoassay (ELICA) (Roche Diagnostica, Hoffman-La Roche, Basel, Switzerland). Glucose concentrations were measured by using enzymatic assay (G6-PDH) (Roche Diagnostica, Hoffman-La Roche, Basel, Switzerland). Amino acid concentrations were measured by using a HPLC system (Pharmacia, Woerden, The Netherlands). Urea concentrations were measured spectrophotometrically on a COBAS Mira S (Roche Diagnostica, Hoffman-La Roche, Basel, Switzerland).

Statistics

All data are expressed as mean \pm SEM. Statistical significance was set at $p < 0.05$. Statview SE+Graphics (1988; Abacus Concepts, Berkely, CA, USA) was used for the analysis. In the first experiment, growth hormone responses were calculated as peak values. In the second experiment, growth hormone, insulin, glucose, urea and amino acid responses were calculated as area under the curve (AUC), as peak values and as time to peak values. Statistical analyses of the data were performed using repeated measures analysis of variance (ANOVA), correcting for multiple analyses. Post hoc, the Scheffe F-test was used to locate possible significant differences.

RESULTS

In the first series of experiments, GH-responses reached higher peak values after ingestion of the AA-mixture ($4.9 \mu\text{g/L} \pm 1.2$) than after ingestion of the arginine + lysine testdrink ($2.2 \mu\text{g/L} \pm 0.7$) ($p < 0.05$). In the second series of experiments, GH-responses, as determined by area under the curve (AUC) and peak values were higher after ingestion of hydrolysed soy protein, complete soy protein and the AA-mixture, compared with ingestion of the placebo testdrink ($p < 0.05$). No differences between GH-responses (AUC and peak values) were found after ingestion of hydrolysed soy protein, complete soy protein and the AA-mixture (Figure 1, Table 2).

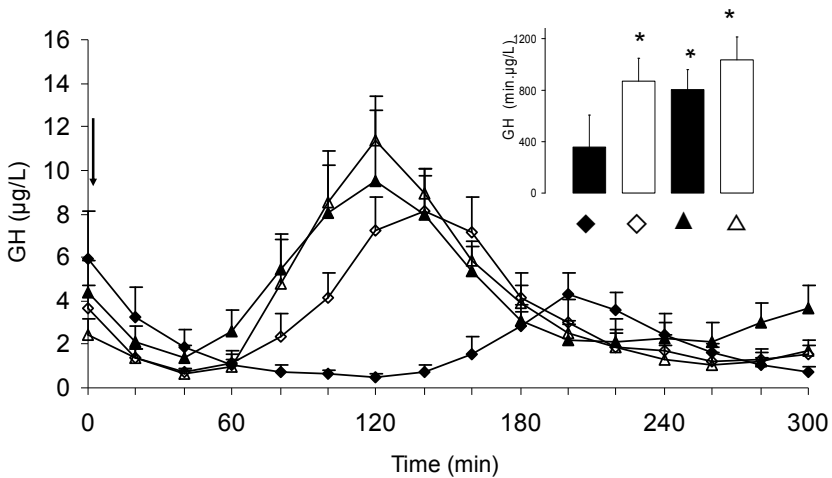


Figure 1: Growth hormone concentrations after ingestion of a placebo-testdrink (◆), AA-testdrink (◇), hydrolysed soy protein testdrink (▲), and complete soy protein-testdrink (Δ) (N=8). Data are presented as means (\pm SEM). \downarrow = ingestion of testdrink. * = $p < 0.05$ compared with placebo.

The insulin-concentrations, as determined by area under the curve (AUC) and peak values, were higher after ingestion of hydrolysed soy protein, complete soy protein and the AA-mixture, compared with ingestion of the placebo ($p < 0.05$). Ingestion of complete soy protein, hydrolysed soy protein or the AA-mixture did not affect insulin responses (AUC and peak values) (Table 2, Figure 2).

The glucose-response (AUC) did not differ between each of the test testdrinks (Table 2).

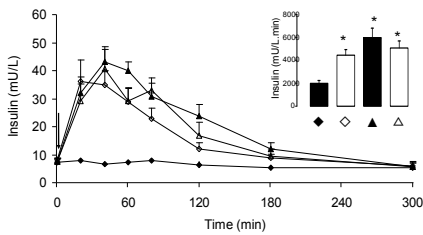


Figure 2: Insulin concentrations after ingestion of a placebo-testdrink (◆), AA-testdrink (◇), hydrolysed soy protein testdrink (▲) and complete soy protein-testdrink (Δ) (N=8). Data are presented as means (\pm SEM). \downarrow = ingestion of testdrink. * = $p < 0.05$ compared with placebo.

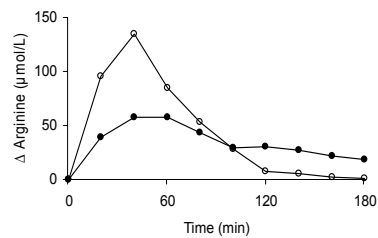


Figure 3: Arginine concentrations after ingestion of the Arg+Lys testdrink and AA testdrink (N=8). —● = Arg+Lys testdrink, —○ = AA testdrink

The arginine-concentration was higher after ingestion of the AA-mixture, compared with the arginine + lysine testdrink which was higher compared with the placebo ($p < 0.05$) (Figure 3). No differences were found in total amino acid peak-concentrations after ingestion of the protein and the AA-testdrink. Glutamic acid, aspartic acid and tyrosine, however, showed a higher peak after ingestion of the hydrolysed and complete soy protein testdrinks compared with the concentrations obtained after ingestion of the AA-testdrink ($p < 0.05$). Valine, phenylalanine and leucine peaks were lower after ingestion of the hydrolysed and complete soy protein testdrinks in comparison with the AA-testdrink ($p < 0.05$). In contrast with the total amino acid peak, total amino acids (AUC) in response to the different testdrinks was higher after ingestion of the hydrolysed and complete soy protein testdrink, compared with the AA-testdrink ($p < 0.05$). The difference in total amino acid concentrations (AUC) between the hydrolysed and complete soy protein testdrink in comparison with the AA-testdrink was also reflected in aspartic acid, glutamic acid, citruline, arginine, tyrosine, methionine, isoleucine and leucine responses ($p < 0.05$). Total amino acids (AUC) and each separate amino acid (except for taurine and ornithine) were higher after ingestion of the hydrolysed soy protein testdrink, complete soy protein testdrink and AA-testdrink in comparison with the placebo-testdrink ($p < 0.05$). Time to peak (TTP) was later after ingestion of hydrolysed and complete soy protein testdrink (85 min) than after ingestion of the AA-testdrink (45 min) ($p < 0.05$). This difference in TTP was also reflected by the TTP in serine, histidine, citruline, tyrosine, valine, methionine, isoleucine, phenylalanine, tryptophan and leucine ($p < 0.05$). Table 2 shows an overview of the differences in AA-concentration as measured by AUC, peak-values and time to peak, between the protein-testdrinks, AA-testdrink and placebo testdrink. Figure 4 shows each separate amino acid over time after ingestion of the testdrinks.

Urea-concentrations, as determined by area under the curve (AUC) did not differ between ingestion of hydrolysed soy protein, complete soy protein or the AA-mixture, but were higher than after ingestion of placebo ($p < 0.05$) (Table 2).

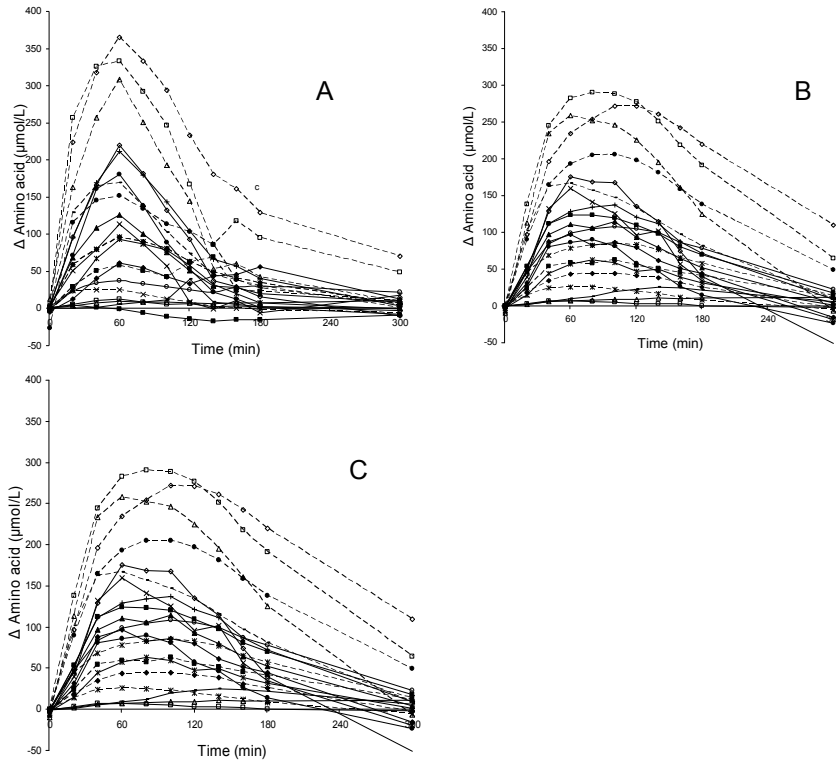


Figure 4: Amino acid concentrations after ingestion of the AA-testdrink (a), hydrolysed soy protein testdrink (b), and complete soy protein-testdrink (c) (N=8).

- ◆ = glutamine, —■ = aspartic acid, —▲ = serine, —× = glutamic acid,
- * = histidine, —● = glycine, —| = threonine, —▬ = citruline, —◇ = alanine,
- = taurine, —△ = aAB, —○ = tyrosine, —× = methionine, —* = phenylalanine,
- ◆ = tryptophan, ---■ = ornithine, ---▲ = arginine, ---● = isoleucine, ---◇ = valine,
- = leucine, ---△ = lysine

Table 2: Blood parameters after ingestion of a placebo-testdrink, AA-testdrink, hydrolysed soy protein testdrink and complete soy protein testdrink (n=8), expressed as AUC (a), peak-values (b) and time to peak, TTP (c).

a	AUC			
	Placebo	AA-mix	Hydrolysed	Complete
GH (min.µg/L)	357 ± 94	870 ± 176 ^a	804 ± 154 ^a	1035 ± 179 ^a
Insulin (min.mU/L)	1985 ± 268	4452 ± 649 ^a	5988 ± 822 ^a	5087 ± 605 ^a
Glucose (min.mmol/L)	874 ± 68	1044 ± 35	996 ± 39	998 ± 35
Urea (min.mmol/L)	62 ± 42	307 ± 60 ^a	426 ± 78 ^a	366 ± 53 ^a
Total AA (min.mol/L)	-23.0 ± 16.4	27.3 ± 4.7 ^a	43.0 ± 29.0 ^{a,b}	40.8 ± 22.9 ^{a,b}
Glutamine	-3.9 ± 1.6	11.4 ± 4.0 ^a	13.7 ± 1.5 ^a	12.4 ± 2.1 ^a
Aspartic acid	-1.3 ± 0.8	2.7 ± 0.4	22.4 ± 1.6 ^{a,b}	22.5 ± 1.0 ^{a,b}
Serine	-0.1 ± 0.9	13.7 ± 3.0 ^a	18.2 ± 2.5 ^a	19.3 ± 1.1 ^a
Glutamic acid	-2.8 ± 3.2	8.8 ± 3.5	18.3 ± 3.5 ^a	20.7 ± 3.2 ^a
Histidine	-0.2 ± 1.0	9.7 ± 2.8 ^a	9.8 ± 2.0 ^a	11.3 ± 1.3 ^a
Glycine	-0.2 ± 2.6	14.2 ± 2.5 ^a	9.5 ± 2.5 ^a	11.7 ± 1.7 ^a
Threonine	-2.9 ± 1.8	23.5 ± 2.5 ^a	23.2 ± 2.6 ^a	20.8 ± 2.3 ^a
Citruline	-0.4 ± 0.2	1.6 ± 0.5 ^a	4.3 ± 0.5 ^{a,b}	4.1 ± 0.4 ^{a,b}
ARG	0.6 ± 0.9	19.5 ± 2.5 ^a	27.9 ± 3.2 ^{a,b}	26.7 ± 3.4 ^a
Alanine	-16.6 ± 5.1	19.6 ± 3.4 ^a	20.0 ± 6.4 ^a	20.7 ± 3.4 ^a
Taurine	0.2 ± 0.2	1.3 ± 0.4 ^a	0.6 ± 0.2	1.2 ± 0.3
aAB	0.9 ± 0.2	2.5 ± 0.3 ^a	2.7 ± 0.4 ^a	2.7 ± 0.3 ^a
Tyrosine	-1.3 ± 0.8	8.1 ± 1.5 ^a	21.5 ± 1.5 ^{a,b}	18.6 ± 1.1 ^{a,b}
Valine	0.4 ± 0.9	55.4 ± 3.9 ^a	58.5 ± 2.4 ^a	48.2 ± 6.9 ^a
Methionine	-0.6 ± 0.1	1.8 ± 0.7 ^a	3.4 ± 0.4 ^{a,b}	3.1 ± 0.2 ^a
Isoleucine	1.6 ± 0.6	19.9 ± 7.6 ^a	40.6 ± 0.2 ^{a,b}	35.2 ± 1.9 ^{a,b}
Phenylalanine	0.0 ± 0.4	13.9 ± 3.0 ^a	16.5 ± 1.4 ^a	14.1 ± 1.0 ^a
Tryptophan	-1.3 ± 0.8	5.3 ± 1.6 ^a	7.6 ± 1.0 ^a	6.6 ± 0.6 ^a
Leucine	3.6 ± 1.1	46.1 ± 3.3 ^a	57.1 ± 3.1 ^{a,b}	49.7 ± 2.7 ^{a,b}
Ornithine	-6.5 ± 6.6	10.0 ± 1.9	28.0 ± 17.8	10.9 ± 0.9
LYS	-2.4 ± 3.9	33.2 ± 2.7 ^a	42.1 ± 4.1 ^a	42.4 ± 3.3 ^a

b	Peak			
	Placebo	AA-mix	Hydrolysed	Complete
GH (µg/L)	4 ± 3	12 ± 5 ^a	9 ± 3 ^a	13 ± 4 ^a
Insulin (mU/L)	.	40 ± 7	47 ± 7	44 ± 5
Glucose (mmol/L)
Urea (mmol/L)
Total AA (µmol/L)	.	4592 ± 229	4865 ± 200	4782 ± 221
Glutamine	.	171 ± 24	232 ± 19 ^b	230 ± 18 ^b
Aspartic acid	.	58 ± 4	182 ± 9 ^b	203 ± 11 ^b
Serine	.	247 ± 26	255 ± 113	251 ± 13
Glutamic acid	.	540 ± 17	597 ± 32	577 ± 27
Histidine	.	190 ± 21	161 ± 8	160 ± 9
Glycine	.	338 ± 28	292 ± 16	289 ± 16
Threonine	.	357 ± 21	294 ± 13	313 ± 22
Citruline	.	66 ± 22	49 ± 3	48 ± 3
ARG	.	234 ± 29	244 ± 35	237 ± 32
Alanine	.	455 ± 48	469 ± 23	471 ± 20
Taurine	.	51 ± 9	38 ± 2	44 ± 2
aAB	.	27 ± 2	31 ± 3	26 ± 3
Tyrosine	.	96 ± 12	160 ± 10 ^b	147 ± 6 ^b
Valine	.	563 ± 28	451 ± 17 ^b	418 ± 13 ^b
Methionine	.	50 ± 4	49 ± 4	46 ± 2
Isoleucine	.	277 ± 30	276 ± 14	258 ± 11
Phenylalanine	.	153 ± 12	139 ± 8	127 ± 5 ^b
Tryptophan	.	114 ± 26	98 ± 4	97 ± 4
Leucine	.	445 ± 23	407 ± 20	384 ± 16 ^b
Ornithine	.	138 ± 34	109 ± 8	97 ± 8
LYS	.	459 ± 35	437 ± 27	442 ± 40

c	TTP			
	Placebo	AA-mix	Hydrolysed	Complete
GH (min)	195 ± 8	120 ± 18 ^a	130 ± 9 ^a	113 ± 7 ^a
Insulin (min)	.	33 ± 7	40 ± 6	45 ± 7
Glucose (min)
Urea (min)
Total AA (min)	.	45 ± 4	88 ± 10 ^b	83 ± 8 ^b
Glutamine	.	75 ± 9	83 ± 11	70 ± 11
Aspartic acid	.	63 ± 37	75 ± 5	88 ± 11
Serine	.	45 ± 4	78 ± 6 ^b	78 ± 9 ^b
Glutamic acid	.	55 ± 7	70 ± 6	68 ± 6
Histidine	.	55 ± 5	95 ± 14 ^b	88 ± 7 ^b
Glycine	.	58 ± 5	75 ± 7	75 ± 7
Threonine	.	63 ± 9	198 ± 11	85 ± 9
Citruline	.	100 ± 7	135 ± 7 ^b	125 ± 7
ARG	.	55 ± 5	63 ± 9	78 ± 9
Alanine	.	65 ± 4	80 ± 7	70 ± 6
Taurine	.	65 ± 11	48 ± 6	60 b 7
aAB	.	83 ± 20	135 ± 13	110 ± 17
Tyrosine	.	53 ± 7	105 ± 8 ^b	95 ± 8 ^b
Valine	.	55 ± 10	115 ± 8 ^b	103 ± 6 ^b
Methionine	.	33 ± 6	78 ± 13 ^b	58 ± 8
Isoleucine	.	40 ± 4	98 ± 9 ^b	88 ± 8 ^b
Phenylalanine	.	40 ± 4	110 ± 7 ^b	90 ± 11 ^b
Tryptophan	.	48 ± 6	85 ± 10 ^b	88 ± 7 ^b
Leucine	.	43 ± 5	100 ± 8 ^b	93 ± 11 ^b
Ornithine	.	48 ± 4	80 ± 13	78 ± 12
LYS	.	53 ± 8	85 ± 13	83 ± 13

Data are presented as means (± SEM).

^ap<0.05 compared with the placebo-testdrink (repeated measures ANOVA).

^bp<0.05 compared with the AA-testdrink (repeated measures ANOVA).

DISCUSSION

Oral administration of soy protein appeared to stimulate GH secretion. This effect was preserved when soy protein was ingested as either hydrolysed soy protein or as free amino acids. These results suggested that the potency to stimulate GH secretion, was not influenced by the rate of amino acid absorption.

A 2.3 fold increase in GH concentrations was found after oral ingestion of a test-drink with approximately 1.2 gram arginine and 1.2 gram lysine in comparison with basal values. This finding confirms previous studies by Isidori and Suminski, which showed that oral ingestion of 1.2-1.5 gram arginine and 1.2-1.5 gram lysine increased GH release 3-8 fold in comparison with placebo. In addition, we found that the AA mixture, reflecting soy protein (17 gram AA), with equal amounts of arginine and lysine as in the ARG+LYS testdrink, increased GH concentrations 2.2 peak fold more than arginine and lysine alone. We concluded that in addition to arginine and lysine, other amino acids are involved in the observed stimulation of GH release, directly or indirectly via conversion to another AA. The somatotrophic effects of the amino acids reflecting soy protein composition, were unaffected whether these amino acids were orally administered as either intact or as partially hydrolysed soy protein.

To the best of our knowledge, this is the only human study performed analysing the effects of variable chain length of proteins on GH secretion. Only one other animal study, performed in rats in which casein is used as protein source in a meal or as free amino acids, also showed no different results in GH-concentrations after intake of a meal with dietary (casein) amino acids in free form or as complete casein proteins¹⁶.

The similar GH responses after ingestion of the AA-mixture and soy protein are associated with comparable peak values of amino acid concentrations (Figure 3). Absorption of the amino acids was slower after ingestion of the (intact or hydrolysed) proteins than after ingestion of the AA mixture, as indicated by the lower AUC and the lower time to peak of the plasma amino acid responses. This was however not associated with differences in GH responses, indicating that the absorption rate of the amino acids after oral ingestion was not of pivotal value for the GH responses. Hence, the underlying mechanism of the increased GH release after protein ingestion may be related to GH-secreting properties of insulin^{7, 17, 18}, since increased insulin responses to protein or amino acid ingestion may play a role in the observed GH responses^{19, 20}. This effect is independent of plasma glucose responses, which were unaffected by protein or amino acid ingestion.

We showed a clear effect of intact soy protein on GH secretion; the next step should be to assess the effects of soy protein as part of a meal. Previous studies on the effects of meals on plasma GH have produced controversial results. Some studies showed an increase of GH-concentrations after normal and high protein meals in humans^{6, 21-23}, while other studies showed decreased or unaffected GH-secretion after normal and high-protein meals^{14, 23-25}.

The clinical and physiological relevance of the results of this study remains to be investigated. These results show that in normal healthy women, GH-responses increase after oral ingestion of proteins. It is not known whether this effect also occurs in a GH-deficient population, such as in obese subjects, and if so, whether this has any physiological effects.

In conclusion, ingestion of soy protein, either hydrolysed or intact, as well as amino acids reflecting soy protein, stimulates GH release to a similar extent. Increased serum levels of insulin as well as peak amino acid concentration but not the absorption rate of AA, may be responsible for the somatotropic effects. Further investigations are necessary to elucidate the role of dietary protein in the regulation of plasma GH levels following meal ingestion.

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CHAPTER 2

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3

The effects of dietary protein on the somatotropic axis; a comparison of soy, gelatin, alpha- lactalbumin and milk

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ABSTRACT

Introduction: Growth hormone (GH) is an important regulator of growth and body composition. It has been shown that GH release can be promoted by administration of various amino acids, such as arginine and lysine, that are present in soy protein. We previously showed that oral ingestion of soy protein stimulates the GH release, it is not known however to which extent other proteins stimulate the GH secretion.

Methods: Ingestion of soy protein (soy), gelatin protein (gelatin), alpha-lactalbumin protein (α -lac) and milk protein (milk) were compared on their GH stimulating capacity. After oral ingestion of protein (0.6 gram protein per kg bodyweight), blood was sampled every 20 min for 5h to analyse GH-, amino acid-, insulin- and glucose-concentrations. The study was performed in eight healthy women (age: 19-26 years, body mass index (BMI): 19-26 kg/m²) in a randomized, single blind, placebo-controlled crossover design.

Results: GH responses were more increased after ingestion of gelatine (8.2 ± 1.1 μ g/L) compared with ingestion of soy, α -lactalbumin and milk (5.0 ± 0.8 μ g/L, 4.5 ± 0.6 μ g/L, 6.4 ± 1.0 μ g/L respectively) ($p < 0.05$). After ingestion of each protein, GH responses were higher compared with placebo ingestion ($p < 0.05$). Simultaneously ingestion of gelatin resulted in the highest serum-arginine concentrations (ARG) compared with after ingestion of the other proteins ($p < 0.05$). Insulin as well as glucose concentrations were not different after ingestion of the various proteins ($p < 0.05$).

Conclusion: The GH-promoting activity of protein depends on the protein source, in that, gelatin protein is the most potent GH stimulator. Arginine may be the responsible amino acid in the GH promoting effect of gelatin, although each protein may have its own specific AA-spectrum involved in the stimulation of the somatotropic axis

INTRODUCTION

Growth hormone (GH), a hormone originating from the anterior pituitary gland, is an important regulator of growth and body composition ¹. GH exerts its growth promoting effect through stimulation of the secretion of Insulin-Like Growth Factor I (IGF-I) from the target cells ¹. Low GH-concentrations may be involved in the pathogenesis of obesity, sarcopenia and growth retardation ¹⁻⁴.

It has been shown by many studies that GH secretion can be promoted by intravenous or oral administration of various amino acids (AAs), either alone or in combination ^{5, 6}. Especially ingestion of the combination of the AAs arginine (ARG) and lysine (LYS) in a ratio of 1:1 was found to be a potent GH secretor ⁷. Previously we found that ingestion of soy protein (SOY), containing relatively high amounts of ARG and LYS in approximately equal amounts, stimulates the GH secretion two fold more compared with a mixture of ARG and LYS ⁸. From this, it can be concluded that besides ARG and LYS, also other AA stimulate the somatotropic axis, as indeed was shown for the AAs methionine, phenylalanine, histidine and glutamic acid ⁶. Therefore, it is expected that also ingestion of other proteins than soy, with different ARG/LYS ratios and/or higher concentrations of certain AAs, influence the GH secretion.

The objective of the present study was to examine the GH response after ingestion of different proteins. SOY was hypothesized being a relatively strong GH stimulator among the proteins because of the relatively high and equal amounts of ARG and LYS. We compared this protein with gelatin (gelatin) and alpha lactalbumin (α -lac) because of their distinct ARG:LYS ratios. Thus, gelatin contains relatively high amounts of ARG, but relatively low amounts of LYS (ARG:LYS, 1:0.45), while α -lac contains relatively low amounts of ARG and high amounts of LYS (ARG:LYS, 1:5.8). In addition, we also tested milk protein (milk) as this protein is widely used in food products, and contains relatively high amounts of LYS, but lower amounts of ARG (ARG:LYS, 1:3.2).

METHODS

Subjects

Subjects were recruited via advertisements at the university. They underwent a screening procedure including medical history taking, measurement of bodyweight and height. Eight females were selected on the basis of being in good health, non-diabetic, non-smoker, non-vegetarian, using oral contraceptives, not using other medication, not spending more than three hours sport activities a week and at most moderate alcohol users (<10 alcoholic consumptions per week). Only females

were included, because in previous studies was found that females responded more consistently and with a greater increase in GH after ingestion of certain amino acids than men^{9,10}. Their mean age was 21 ± 1.5 years, and their body mass index (BMI) was 22 ± 1.8 kg/m². All tests were performed in the early follicular phase of each woman's menstrual cycle, to eliminate the confounding variable of changing serum estrogen concentrations. The Medical Ethics Committee of Maastricht University approved the study protocol and all subjects gave their written informed consent before participating in the study.

Experimental design

A randomized crossover study design was applied. Subjects reported to the laboratory for consumption of five different test drinks (soy, gelatin, α -lactalbumin, milk and placebo), at separate test days. Each time, subjects arrived in the morning at the laboratory in a fasted state. They were instructed to fast from 10 pm the night prior to the test day. A permanent canula was placed into a dorsal hand vein of the contralateral arm, and this hand was kept in a thermoregulated (60°C) box to sample arterialized venous blood¹¹. Blood sampling began 60 min after placement of the canula. Blood was sampled every 20 min for the next 5h. During blood sampling, subjects remained awake and were allowed to drink water ad libitum. Immediately after obtaining the first blood sample, subjects received a test drink. The test drink was based on 0.6 gram protein per kg bodyweight dissolved in 400 ml water with 20 ml sugarfree syrup (Diaran, Cereal). The placebo test drink contained 400 ml water with 20 ml sugarfree syrup. The syrup was added for the taste and contained no protein and fat and a negligible amount of carbohydrates. The AA-composition of the proteins is shown in Table 1.

Table 1: AA composition of the proteins tested. Amount in g per 100g of protein. α -lac = alpha lactalbumin

	Soy	Gelatin	α -lac	Milk
Arginine	6.8	8.4	1.8	3.7
Cysteine	1.1	0.0	4.9	0.7
Histidine	2.3	0.9	2.8	2.8
Isoleucine	4.4	1.5	5.8	5.6
Leucine	7.1	3.0	11.0	10.0
Lysine	5.4	3.9	10.5	8.5
Methionine	1.1	0.9	1.2	2.9
Phenylalanine	4.6	1.9	4.0	5.0
Threonine	3.3	1.9	4.9	4.7
Tryptophan	1.1	<0.1	3.8	1.5
Tyrosine	3.4	0.5	4.3	5.5
Valine	4.2	2.3	4.4	7.0

Blood analysis

Arterialized venous blood was collected in clot tubes, containing 'Silica Clot Activator' (Becton Dickinson Vacutainer system; Becton Dickinson, Franklin Lakes, NJ), and in heparinized tubes (Becton Dickinson Vacutainer system; Becton Dickinson, Franklin Lakes, NJ). Blood in the clot tubes was allowed to clot for 30 minutes and was centrifuged at 3000 rpm, 4°C for 10 min to obtain serum. Serum was collected for determination of GH and amino acid (AA) concentrations. Blood in heparinized tubes was kept on ice to minimize enzymatic reactions and centrifuged at 3000rpm at 4°C for 10 min to obtain plasma. Glucose and insulin analyses were performed in plasma. Each aliquot was frozen immediately in liquid nitrogen and stored at -80°C. All samples from the same subjects were run in the same assay.

The GH-concentrations were measured by using an ultrasensitive GH chemiluminescence immunoassay (Beckman Coulter, Harbor Blvd. Fullerton, U.S.A.). Insulin concentrations were measured by an electrochemiluminescence immunoassay (ELICA) (Roche Diagnostica, Hoffman-La Roche, Basel, Switzerland). Glucose concentrations were measured by using enzymatic assay (G6-PDH) (Roche Diagnostica, Hoffman-La Roche, Basel, Switzerland). The concentrations of the amino acids arginine, lysine, histidine, methionine, ornitine, phenylalanine and glutamic acid were measured using a HPLC system (Shimadzu, Duisburg, Germany). These AA were chosen to analyse, because these are supposed in previous studies to stimulate the GH response^{5-7, 12-14}.

Statistics

Data are presented as mean changes from baseline +/- standard error to the mean (SEM). Baseline ratings for GH, insulin, glucose and AAs were not statistically significantly different among treatments, therefore delta concentrations were used for statistical analysis. Growth hormone, insulin, glucose and AA responses were calculated as incremental area under the curve (IAUC), peak values and time to peak (TTP) values. Statistical analyses of the data were performed using repeated measures analysis of variance (ANOVA), correcting for multiple analyses. Post hoc comparisons were made with the Fisher PLSD-test. A p-value <0.05 was regarded as statistically significant. All statistical tests were performed by using Statview SE Graphics software (version 4.5; Abacus Concepts Inc, Berkeley, CA, USA).

RESULTS

GH responses as determined by incremental area under the curve (IAUC) and peak values were increased after gelatin ingestion when compared with ingestion of soy,

α -lactalbumin, milk and placebo ($p < 0.05$). After ingestion of soy, α -lactalbumin and milk, GH responses (IAUC and peak) were increased compared with placebo ($p < 0.05$). GH responses after ingestion of milk were delayed compared with GH concentrations after ingestion of the other proteins (Figure 1, Table 2).

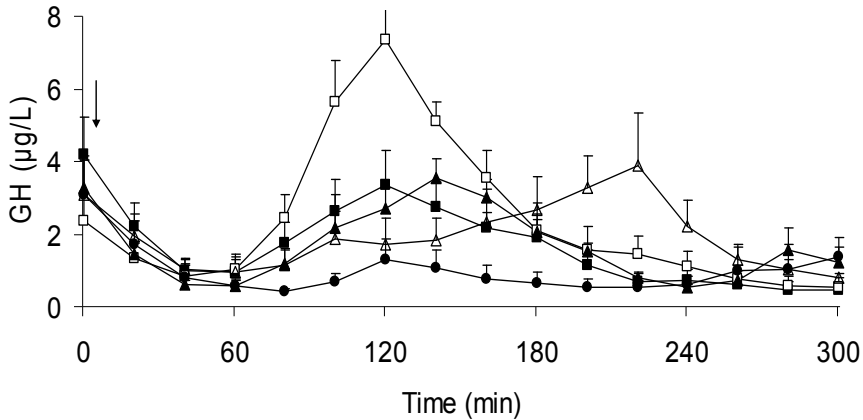


Figure 1: Growth hormone concentrations after ingestion of a testdrink containing soy protein (■), gelatin protein (□), alpha lactalbumin protein (▲), milk protein (Δ) and placebo (●) ($n=8$). Data are presented as mean (\pm SEM) in lines. \downarrow = ingestion of testdrink.

The insulin responses, determined as IAUC and peak values, were higher after ingestion of the proteins, compared with ingestion of the placebo drink ($p < 0.05$). There were no differences between the insulin concentrations after ingestion of the different proteins. There was no TTP difference between the insulin responses after ingestion of the different test drinks (Table 2).

The glucose concentrations, determined as IAUC and peak values were not differently affected by ingestion of the test drinks. There was no TTP difference between the glucose responses after ingestion of the different test drinks (Table 2).

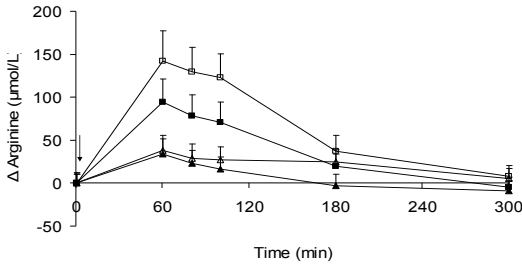


Figure 2: Changes in arginine concentrations after ingestion of a testdrink containing soy protein (■), gelatin protein (□), alpha lactalbumin protein (▲) and milk protein (Δ) (n=8). Data are presented as mean (± SEM) in lines. ↓= ingestion of testdrink.

AA responses to protein ingestion as determined by IAUC and peak values are shown in Table 2. Arginine (ARG) concentrations were significantly higher (IAUC and peak) after ingestion of gelatin, compared with the other proteins ($p < 0.05$). Furthermore, ARG concentrations were higher after ingestion of soy compared with α -lactalbumin and milk ($p < 0.05$) (Figure 2, Table 2). Ornithine (ORN) concentrations were significantly higher (AUC and peak) after ingestion of gelatin, compared with the other proteins ($p < 0.05$). Furthermore, ORN concentrations were high after ingestion of soy compared with α -lactalbumin and milk ($p < 0.05$). Histidine (HIS) concentrations, expressed as AUC and peak value were higher after ingestion of soy or α -lactalbumin compared with gelatin ($p < 0.05$). Lysine (LYS) concentrations were higher (AUC and peak value) after ingestion of α -lactalbumin compared with the other proteins ($p < 0.05$). Methionine (MET) concentrations (IAUC, peak value) were higher after ingestion of milk compared with the other proteins ($p < 0.05$). Peak values of phenylalanine (PHE) were higher after ingestion of soy or α -lactalbumin compared with gelatin or milk ($p < 0.05$). Peak values of glutamic acid (GLU) were higher after ingestion of gelatin compared with milk ($p < 0.05$) (Table 2).

Table 2: Changes in GH, insulin, glucose and amino acids after ingestion of a test drink with soy protein, gelatin protein, alpha lactalbumin protein, milk protein and placebo (n=8), expressed as IAUC, peak-values and time to peak (TTP).

	IAUC				
	Placebo	Soy	Gelatin	Alpha lactalbumin	Milk
GH (min.µg/L)	184.0 ± 52.1	374.0 ± 49.6 ^a	641.4 ± 74.6 ^{a,b,c,d}	421.4 ± 44.2 ^a	477.2 ± 53.4 ^a
Insulin (min.mU/L)	-256.1 ± 166.0	2599.5 ± 254.8 ^a	1747.3 ± 459.1 ^a	2249.9 ± 344.1 ^a	1837.6 ± 423.1 ^a
Glucose (min.mmol/L)	8.9 ± 46.5	96.4 ± 51.6	32.5 ± 63.8	91.2 ± 40.2	22.8 ± 35.9
Arginine (min.mmol/L)		10.6 ± 1.7 ^a	18.7 ± 1.9 ^{b,c,d}	1.8 ± 1.0	6.2 ± 1.0 ^f
Lysine (min.mmol/L)		23.7 ± 3.5	17.0 ± 2.5	48.1 ± 3.8 ^{b,d,e}	25.2 ± 2.1
Histidine (min.mmol/L)		5.3 ± 1.5 ^e	1.0 ± 1.1	6.3 ± 1.5 ^e	4.8 ± 1.1
Methionine (min.mmol/L)		1.0 ± 0.3	1.0 ± 0.3	0.3 ± 0.3	4.8 ± 0.4 ^{b,c,e}
Ornithine (min.mmol/L)		7.9 ± 1.1 ^{a,d}	10.3 ± 1.3 ^{b,c,d}	-1.9 ± 0.8	3.5 ± 0.7 ^f
Phenylalanine (min.mmol/L)		7.6 ± 1.0 ^{c,d,e}	1.5 ± 0.6	5.0 ± 0.7 ^e	4.8 ± 0.3 ^f
Glutamic acid (min.mmol/L)		0.2 ± 2.3	-0.5 ± 1.2	-1.1 ± 1.4	-0.6 ± 1.5

	Peak				
	Placebo	Soy	Gelatin	Alpha lactalbumin	Milk
GH (µg/L)	2.2 ± 0.8	5.0 ± 0.8 ^a	8.2 ± 1.1 ^{a,b,c}	4.5 ± 0.6 ^a	6.4 ± 1.0 ^f
Insulin (mU/L)	8.5 ± 1.1	32.1 ± 7.7 ^a	32.3 ± 5.9 ^a	33.7 ± 4.2 ^a	35.3 ± 6.2 ^{a,b}
Glucose (mmol/L)	5.3 ± 0.1	5.5 ± 0.1	5.4 ± 0.1	5.3 ± 0.2	5.4 ± 0.1
Arginine (mmol/L)		204.5 ± 11.4 ^{a,d}	233.6 ± 20.9 ^{b,c,d}	129.2 ± 3.9	121.6 ± 11.0
Lysine (mmol/L)		354.4 ± 9.3	302.1 ± 20.6	546.0 ± 38.0 ^{b,d,e}	288.0 ± 16.56
Histidine (mmol/L)		138.4 ± 7.9 ^e	113.5 ± 5.0	142.8 ± 8.74 ^e	117.88 ± 5.8
Methionine (mmol/L)		39.3 ± 2.0 ^f	34.5 ± 1.7	33.8 ± 1.6	50.8 ± 3.5 ^{b,c,e}
Ornithine (mmol/L)		110.1 ± 7.0 ^{a,d}	146.5 ± 11.6 ^{b,c,d}	59.3 ± 3.4	72.8 ± 3.9 ^f
Phenylalanine (mmol/L)		125.1 ± 7.5 ^{a,e}	87.9 ± 3.9	117.1 ± 6.4 ^{d,e}	89.8 ± 2.8
Glutamic acid (mmol/L)		124.9 ± 7.9	132.3 ± 4.0 ^f	125.0 ± 8.8	104.2 ± 3.9

	TTP				
	Placebo	Soy	Gelatin	Alpha lactalbumin	Milk
GH (min)	180 ± 30	122 ± 12 ^{a,d}	122 ± 6 ^{a,d}	122 ± 10 ^{a,d}	190 ± 22
Insulin (min)	30 ± 18	30 ± 18	30 ± 18	26 ± 4	20 ± 0
Glucose (min)	32 ± 10	42 ± 18	36 ± 18	50 ± 18	32 ± 8
Arginine (min)		56 ± 18	58 ± 18	50 ± 18	60 ± 8
Lysine (min)		60 ± 7 ^f	52 ± 18	52 ± 18	42 ± 2
Histidine (min)		66 ± 18	48 ± 4	58 ± 18	58 ± 18
Methionine (min)		66 ± 18	56 ± 18	56 ± 18	56 ± 8
Ornithine (min)		62 ± 8	52 ± 18	46 ± 4	50 ± 8
Phenylalanine (min)		66 ± 18	56 ± 18	56 ± 18	56 ± 8
Glutamic acid (min)		62 ± 7 ^f	58 ± 5 ^f	58 ± 18	50 ± 8

Data are presented as mean (± SEM). ^aSignificantly higher than after placebo ingestion ^bSignificantly higher than after soy protein ingestion ^cSignificantly higher than after alpha lactalbumin protein ingestion ^dSignificantly higher than after milk protein ingestion ^eSignificantly higher than after gelatin protein ingestion

DISCUSSION

This study showed that oral intake of protein stimulates the GH response, moreover, the GH-promoting activity of protein depends on the protein-source, in that, gelatin protein (gelatin) ingestion stimulates the GH response to a higher extent than ingestion of the proteins; soy (soy), alpha lactalbumin (α-lactalbumin) and milk (milk).

Studies investigating the effect of protein on the somatotrophic axis mainly tested milk and found positive associations between milk intake and somatotrophic hor-

mones among adults and children¹⁵⁻²³. For instance, in Mongolian children, the GH response was increased after daily intake of 720 ml milk for 4 weeks, compared with baseline²³. Furthermore, twelve year old girls showed approximately 10% higher IGF-1 levels after consumption of 300 ml milk per day for 6 months, compared with their controls¹⁵. These findings are in accordance with our finding that milk intake increases the GH response when compared with placebo.

Furthermore a study by Ohsumi et al. investigated the influence of gelatin compared with casein protein on GH secretion. In contrast to our findings, they found reduced GH concentrations after ingestion of a lower quality of dietary protein (i.e. gelatine) compared with a higher quality dietary protein (i.e. casein) in aged rats²⁴. In their study, GH concentrations were measured once after an ingestion-period of 5h, which is different from our protocol where we measured GH concentrations every 20 min after ingestion of a constant amount of protein. The different protocols used may have influenced the results, since casein is known to be aggregated into a gel in the stomach which could delay the absorption rate of AA in the intestine and therefore delay the GH response after ingestion of casein protein, compared with gelatine protein^{25,26}.

The mechanism behind this source-dependent protein-induced GH secretion, with gelatin being the most potent stimulator of GH release, remains unclear. Gelatin is low in essential amino acids compared with the proteins soy, milk and α -lactalbumin, however it contains the highest amounts of arginine (ARG) among the tested proteins. ARG is widely known and used as a GH stimulating amino acid²⁷⁻²⁹ and therefore could clarify the GH promoting effect of gelatin, since ARG intake as well as serum concentrations of ARG were higher after intake of gelatin compared with the other proteins. Simultaneously with ARG concentrations, serum ornithine (ORN) concentrations were higher after ingestion of gelatin compared with the other proteins. ARG is converted into ORN in the urea cycle³⁰ and therefore it is not surprising that these amino acids are both present in a higher extent after ingestion of gelatin. Also ORN is known to stimulate the GH secretion⁶.

Furthermore, soy contains relatively high concentrations of ARG and both serum ARG and ORN concentrations were significantly increased compared with concentrations after intake of milk and α -lactalbumin. In our previous work we found that soy stimulates the GH secretion to a higher extent than a mixture of ARG and lysine (LYS), which indicates that also other AAs than ARG and LYS are involved in the GH secretion⁸. This result is confirmed by this study, showing that α -lactalbumin and milk also increase the GH response, but in IAUC and peak not different from soy. Alpha-lactalbumin contains higher amounts of LYS and intake of α -lactalbumin results in higher LYS concentrations compared with the other proteins. Milk contains higher amounts of methionine (MET) and the intake of milk results in higher MET concentrations compared with the other proteins. LYS as well as MET are known to stimulate the GH secretion⁶. This finding indicates that the presence of

specific GH stimulating AAs is more important than the ratio of ARG:LYS in a certain protein.

After ingestion of milk, the GH peak was delayed compared with GH peaks after ingestion of the other proteins. This could be explained by the effects of casein in the stomach. Casein is known to be aggregated into a gel in the stomach and therefore AA absorption could be delayed^{25, 26}. In our study we found that after ingestion of milk, AA-concentrations increase and stay at a certain level for a longer time, compared with the other protein, resulting in a AA-plateau which may explain the delayed GH peak after ingestion of milk (Figure 2). In spite of the delayed GH peak, GH IAUC and GH peak values were not different between soy, alpha-lactalbumin and milk.

Furthermore, in our results we did not find different glucose and insulin concentrations after ingestion of the various proteins, which indicates that glucose as well as insulin are not responsible for the differences in degree of GH stimulation after ingestion of the proteins.

The clinical and physiological relevance of the results of this study remains to be investigated. These results show that in lean healthy women, GH-responses increase after oral ingestion of proteins and in particular gelatin protein. It is not known whether this effect also occurs in a GH-deficient population, such as in obese subjects, and if so, whether this has any physiological effects.

Thus, GH concentrations are increased after ingestion of protein, however, the GH-promoting activity of protein depends on the protein-source, in that, gelatin protein is the most potent GH stimulator. Arginine may be the responsible amino acid in the GH promoting effect of gelatin, although each protein may have its own specific AA-spectrum involved in the stimulation of the somatotrophic axis.

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4

Somatotropic responses to soy protein alone and as part of a meal

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ABSTRACT

Context: Growth hormone (GH) is an important regulator of growth and body composition. We previously showed that GH release can be promoted by oral ingestion of soy protein, it is not known however whether these somatotropic effects of soy protein are also present when soy protein is ingested as part of a complete meal.

Objective/design: We compared effects of oral ingestion of soy protein alone with effects of a meal containing the same amount of soy protein on GH secretion in six healthy women (body mass index (BMI) = 19-26 kg/m²; 19-36 years), in a randomized crossover design. During the complete experiment serum GH, insulin and glucose were determined every 20 minutes.

Results: GH responses as determined by area under the curve (AUC) and peak values were lower after ingestion of the meal, in comparison with GH responses after the soy protein consumption alone ($p < 0.05$), and did not differ from the placebo condition. Glucose-responses and insulin-responses, both determined as area under the curve (AUC) and peak values, were higher after ingestion of the meal, compared with these after ingestion of the protein-drink or the placebo ($p < 0.05$).

Conclusions: The somatotropic effect of soy protein is reduced and delayed when soy protein is ingested as part of a complete meal. Dietary carbohydrates, by increasing serum levels of glucose and insulin concentration, as well as dietary fat, may have interfered with the somatotropic effects of soy protein.

INTRODUCTION

Growth hormone (GH), a hormone originating from the anterior pituitary gland, is an important regulator of growth and body composition ¹. GH exerts its growth promoting effect (typically of fat-free mass) through stimulation of the secretion of Insulin-Like Growth Factor I (IGF-I) from the target cells ¹.

It has been shown by many authors that GH secretion can be promoted by intravenous or oral administration of various amino acids ^{2,3}. The stimulatory effect of amino acids on GH secretion is also present when ingested as proteins. Our previous study showed that GH secretion did not differ after ingestion of an amino acid mixture from that of soy protein, with comparable amino acid content ⁴.

It is not known whether the somatotropic effect of soy protein is still present when it is ingested as part of a complete meal. No study compared the effect of protein alone with a meal on GH-secretion. Studies on the effects of meals in general on plasma GH showed controversial results. It has been reported that after a high protein meal, GH is increased ⁵ and after a high-carbohydrate meal, GH is first decreased, but increased in late postprandial phases ⁶. However no change in GH concentrations were found after 1 week of a low carbohydrate, high fat and high protein diet ⁷. Another study found that high carbohydrate diets suppress growth hormone secretion, whereas high-fat diets and high protein diets of similar caloric value have no measurable effect ⁸.

The reason for the inconsistency in above-mentioned studies is unclear. It may be that either the protein content of the meal was insufficient to increase GH or that other meal-components interfere with protein on GH concentrations. Therefore, this study aims to compare the effects of protein given alone or combined with carbohydrates and fat on GH secretion.

METHODS

Subjects

Subjects were recruited via advertisements at the university. Six healthy young females (age 24 ± 5.8 years, BMI 22 ± 2.5 kg/m²) participated. Each subject was in good health, non-smoker, using contraceptives, free of any other medication and spent no more than three hours a week on sport activities. The Medical Ethics Committee of Maastricht University approved the study protocol and all subjects gave their written informed consent before participating in the study.

Experimental design

A randomized crossover study design was applied. Subjects reported to the laboratory for consumption of 3 different test products (placebo, soy protein alone, or a meal containing soy protein, see Table 1), at separate test days. Each time, subjects arrived at the laboratory in a fasted state, in the morning. They were instructed to fast from 10 pm the night prior to the test day. A permanent cannula was inserted into a dorsal vein of the hand, for venous blood sampling⁹. Blood sampling began 60 min after placement of the cannula. Blood was sampled every 20 min for the next 5h. During blood sampling, subjects remained awake and fasted and were allowed to drink water ad libitum. Immediately after obtaining the first blood sample, subjects received a test product. The composition of the test products is described in Table 1.

Table 1: Composition of the tests conditions, based on a subject of 70 kg.

	Soy protein-drink	Meal	Placebo
Volume (g)	467	834	500
Soy protein (g)	42	42	-
Carbohydrates (g)	-	92.4	-
Fat (g)	-	14.92	-
Sugar free syrup (ml)	-	-	40
Viscosity	Liquid	Semi-solid	Liquid

Blood analysis

Arterialized venous blood was collected in cloth tubes (Becton Dickinson Vacutainer system; Becton Dickinson, Franklin Lakes, NJ) and was allowed to clot for 30 minutes and was centrifuged at 3000 rpm, 4°C for 10 min to obtain serum. Serum was collected for determination of GH, insulin and glucose concentrations. Each serum aliquot was frozen immediately in liquid nitrogen and stored at -80°C, until analysis. All samples from the same subjects were run in the same assay.

The GH-concentrations were measured by using an ultrasensitive GH chemiluminescence immunoassay (Beckman Coulter, Harbor Blvd. Fullerton, U.S.A.).

Insulin concentrations were measured by an electrochemiluminescence immunoassay (Roche Diagnostica, Hoffman-La Roche, Basel, Switzerland). Glucose concentrations were measured by using enzymatic assay (Roche Diagnostica, Hoffman-La Roche, Basel, Switzerland).

Statistics

All data are expressed as median \pm SEM. Statview SE+Graphics (1988;Abacus Concepts, Berkely, CA, USA) was used for the analysis. Growth hormone, insulin and glucose responses were calculated as area under the curve (AUC), peak values

and time to peak (TTP) values. Since the data was not normally distributed, Kruskal-Wallis tests (non-parametric), were performed. Mann-Whitney tests were performed to locate possible significant differences. Statistical significance was set at $p < 0.05$.

RESULTS

Growth hormone responses as determined by area under the curve (AUC) and peak values were increased after soy protein ingestion when compared with placebo ($p < 0.05$). GH responses were significantly higher at $t = 260$ and $t = 280$ after ingestion of the meal compared with placebo ($p < 0.05$). No differences in AUC and peak values were found in GH responses after ingestion of the meal compared with the placebo. GH responses (AUC and peak values) were lower after ingestion of the meal than after soy protein alone ($p < 0.05$). Time to peak concentration (TTP) was later after ingestion of the meal than after ingestion of the protein-drink and the placebo ($p < 0.05$) and TTP was later after the placebo than after the protein-drink ($p < 0.05$) (Figure 1, Table 2).

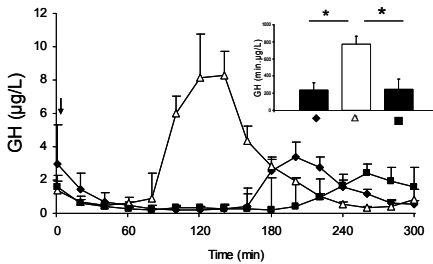


Figure 1: Growth hormone concentrations after ingestion of a placebo-testdrink (◆), soy protein drink (Δ), and meal (■) (n=6). Data are presented as medians (± SEM) in lines and bars(AUC). ↓ = ingestion of testdrink. * = $p < 0.05$

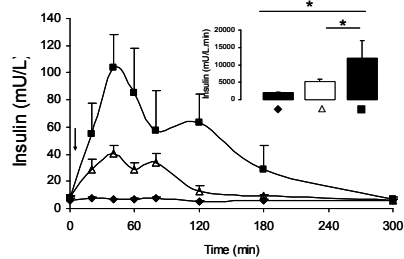


Figure 2: Insulin concentrations after ingestion of a placebo-testdrink (◆), soy protein drink (Δ), and meal (■) (n=6). Data are presented as medians (± SEM) in lines and bars(AUC). * = $p < 0.05$.

The glucose-concentrations and insulin-concentrations, both determined as AUC and peak values, were higher after ingestion of the meal, compared with ingestion of the protein-drink or the placebo ($p < 0.05$). Ingestion of the soy protein-drink did not affect glucose or insulin responses (AUC and peak values), compared with placebo. No difference between the conditions was found in TTP of glucose and insulin concentrations (Table 2).

Table 2: Blood parameters after ingestion of a placebo-testdrink, soy protein-drink and a meal (n=6), expressed as AUC, peak-values and time to peak, TTP.Data are presented as medians (\pm SEM).

	AUC		
	Placebo	Soy protein	Meal
GH ($\mu\text{g/L}\cdot\text{min}$)	235 \pm 87	772 \pm 96 ^a	245 \pm 122 ^b
Insulin ($\text{mU/L}\cdot\text{min}$)	1972 \pm 189	5185 \pm 753	11910 \pm 5042 ^{a,b}
Glucose ($\text{mmol/L}\cdot\text{min}$)	50 \pm 31	37 \pm 26	231 \pm 32 ^{a,b}
	Peak		
	Placebo	Soy protein	Meal
GH ($\mu\text{g/L}$)	4 \pm 2	10 \pm 5 ^a	5 \pm 4 ^b
Insulin (mU/L)	.	49 \pm 6	105 \pm 116 ^b
	TTP		
	Placebo	Soy protein	Meal
GH (min)	190 \pm 9	120 \pm 8 ^a	260 \pm 19 ^{a,b}
Insulin (min)	.	40 \pm 8	50 \pm 15
Glucose (min)	.	.	40 \pm 4

^ap<0.05 compared with the placebo-drink (Mann-Whitney).^bp<0.05 compared with the soy protein-drink (Mann-Whitney).

DISCUSSION

It was shown that the somatotrophic effect of soy protein, as had been shown before, was reduced and delayed when soy protein is ingested as part of a complete meal. This may indicate that besides soy protein, carbohydrates and/or fat play an important modulating role in the postprandial secretion of GH after ingestion of a high-protein meal. Thus, either carbohydrates or fat, or both carbohydrates and fat, are likely to have interfered with the GH-promoting effect of soy protein.

To the best of our knowledge, this is the only human study performed, assessing the effects of a soy protein alone and as part of a complete meal on GH secretion. Two previous studies compared the effects of intakes of glucose, protein and glucose plus protein on plasma GH concentrations. Pallotta and Kennedy showed a delayed increase of GH-concentrations (5h after ingestion) following glucose (100 gram) and starch (700 gram, equivalent to 100 gram glucose) after the blood sugar has fallen, and an acute increase of GH-concentrations (2h after ingestion) following the protein intake (60 gram). No effect was shown after ingestion of glucose plus protein¹⁰. Also Rabinowitz showed a delayed increase of GH after intake of glucose (100 gram) and an acute increase of GH after intake of beef (64 gram proteins, 3 grams fat), but a blunted response after a mixed-substrate meal (glucose plus beef)⁵. Based upon these studies, it is likely that carbohydrates may have contributed to the blunted GH- response of soy protein ingestion after intake of a mixed meal, presumably through elevation of plasma glucose concentrations. Indeed, hyperglycaemia is associated with a decrease in plasma GH¹¹, while hypo-

glycaemia is a potent stimulator of plasma GH¹². The results from these studies, the two previous and the present study, are similar and point into the direction of carbohydrates suppressing a protein stimulated GH response.

With respect to the role of fat intake as part of a meal, increasing plasma concentrations of free fatty acids (FFA), but not triglycerides, as a result of fat ingestion may also have been involved in the suppression of the GH-promoting effect of soy protein during meal ingestion, since FFAs have suggested to suppress GH-secretion^{13,14}.

Fineberg et al. showed that GH release by protein ingestion can be inhibited but not completely abolished by increases of postprandial FFA concentrations¹⁴. These studies show that also alterations in FFA concentrations modified GH secretion. Together, all previous mentioned studies show that both plasma glucose and plasma FFA concentrations influence GH secretion. Therefore, the question remains which of the macronutrients are most responsible for interference with the protein-induced GH secretion. More research, with studies that only add either carbohydrates or fat to the protein being assessed is necessary.

Some studies suggest an effect of gastric emptying on hormonal release^{15,16}. In this study, it is unlikely that gastric emptying has an influence on the reduced and delayed GH-secretion after ingestion of a meal, compared with the soy protein-drink, since we showed in a previous study where we compared ingestion of an amino acid mixture with complete protein on GH secretion, that the rate of the appearance of plasma amino acids (AA) is not related to GH responses⁴.

This study confirms previous findings that ingestion of dietary protein regulates GH secretion in the short-term. Moreover it also shows that the physiological role of food intake on the regulation of GH secretion involves complex interactions between the different macronutrients. Also in the long-term (after 7 days) low carbohydrate/ high protein diets increased IGF-1 concentrations and stimulated muscle protein synthesis and whole-body proteolysis⁷.

The clinical relevance of the results of this study however remains to be investigated. These results show that in normal healthy women, GH responses increase after oral ingestion of proteins, but not when proteins are combined with carbohydrates and fat. It is not known whether this effect also occurs in a GH-deficient population, such as in visceral obese subjects. In the visceral obese, GH replacement therapy reduced weight and in particular decreased fat mass and increased lean body mass^{17, 18}, more over it appeared that weight loss also restored GH concentrations to normal values¹⁹. It may be useful to explore the possibility of adding only one other macronutrient to soy protein in order to be able to design a diet that could stimulate GH secretion in such a population.

In conclusion, the short term somatotropic effect of soy protein is reduced and delayed if soy protein is ingested as part of a complete meal. Dietary carbohydrates, by increasing levels of glucose and insulin concentration, as well as free fatty acids may have interfered with the somatotropic effects of soy protein.

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Growth hormone responses to ingestion of soy protein with or without fat and/or carbohydrate in humans

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ABSTRACT

Background & Aim: Growth hormone (GH) is an important regulator of growth and body-composition. It has been shown that GH-release can be promoted by administration of various amino acids, such as arginine and lysine, that are present in soy protein. Soy protein ingestion increases GH-secretion more than ingestion of a combination of arginine and lysine. The GH promoting effect of soy protein however is not present when soy protein is ingested combined with carbohydrate and fat. The aim of this study is to investigate whether carbohydrate or fat eliminates the GH-promoting effect of soy protein.

Methods: We compared ingestion of soy protein plus carbohydrate (soy+carbohydrate) or soy protein plus fat (soy+fat) with ingestion of soy protein alone(soy), soy protein plus carbohydrate plus fat (soy+carbohydrate+fat) and a placebo. The study was performed in eight healthy women (age: 21 ± 1.5 years, body mass index (BMI): 22 ± 1.8 kg/m²) in a randomized, single blind, placebo-controlled crossover design. GH-, insulin-, glucose- and AA-concentrations were measured during 5h.

Results: Ingestion of soy+carbohydrate or soy+fat stimulated GH-responses to the same extent than soy (incremental area under the curve (IAUC); $374 \pm 50\mu\text{g/L}\cdot 5\text{h}$, $400 \pm 57\mu\text{g/L}\cdot 5\text{h}$, $403 \pm 73\mu\text{g/L}\cdot 5\text{h}$ respectively), but more than after ingestion of soy+carbohydrate+fat or placebo (IAUC; $226 \pm 43\mu\text{g/L}\cdot 5\text{h}$, $84 \pm 52\mu\text{g/L}\cdot 5\text{h}$ respectively) ($p < 0.05$). Insulin responses were higher after ingestion of soy+carbohydrate or soy+carbohydrate+fat, than after soy+fat or soy ($p < 0.05$). Compared with placebo, all conditions showed higher insulin concentrations ($p < 0.05$). Glucose responses were higher after ingestion of soy+carbohydrate than after the other conditions ($p < 0.05$). Peak plasma arginine-concentrations were higher after ingestion of soy compared with soy+carbohydrate+fat ($p < 0.05$).

Conclusion: The GH promoting activity of soy protein depends on the macronutrient composition, in that, fat and carbohydrate should not be present at the same time. Serum arginine concentrations may be involved in the somatotropic activity of protein. Glucose and insulin responses however are not involved.

INTRODUCTION

Growth hormone (GH), a hormone originating from the anterior pituitary gland, is an important regulator of growth and body composition and exerts its growth-promoting effect through stimulation of the secretion of Insulin-Like Growth Factor I (IGF-I)^{1,2}. Furthermore, GH has several metabolic effects, the most important one is an elevation of FFA by stimulation of the lipolysis^{3,4}, which could lead to the switching substrate metabolism from glucose and protein utilisation to lipid oxidation^{2,5}. With respect to glucose metabolism, increased GH concentrations decrease glucose oxidation and there is evidence that gluconeogenesis is stimulated by increased GH concentrations^{2,6}. The effects of GH on protein metabolism in basal state (i.e. after an overnight fast) are not unambiguous and perhaps of minor biological significance, however in stress states and pathological states, GH suggest anabolic actions, which may include increased protein synthesis and decreased breakdown at the whole body level^{2,7}.

The effects of GH secretion on fat, glucose and protein metabolism are recognized. On the other hand, there is also evidence that several macronutrients influence the GH secretion.

It has been shown by many authors that GH secretion can be promoted by intravenous or oral administration of various amino acids, either alone or combined^{8,9}. The stimulatory effect of amino acids on GH secretion is also present when the amino acids are ingested as a protein. Our previous study showed that GH secretion was similar after ingestion of an amino acid mixture or after ingestion of soy protein, with comparable amino acid content¹⁰. However, the GH promoting effect of soy protein was reduced and delayed when soy protein was ingested as part of a meal, containing protein, carbohydrate and fat¹¹. This may indicate that not only soy protein, but also carbohydrate and/or fat plays an important modulating role in the postprandial secretion of GH after ingestion of a high-protein meal.

Studies investigating the somatotropic effects of macronutrients have shown various results. Infusion or intake of glucose or carbohydrate resulted in reduced GH concentrations over a short-term period (2-3h), followed by increased GH concentrations after 3-5 h¹²⁻¹⁶. One study found that GH responses at 60 min and 120 min after the intake of glucose combined with protein were not different compared with ingestion of glucose alone¹⁷, while other studies found that GH responses were lower after the intake of carbohydrate combined with protein, than after intake of carbohydrate or protein alone^{15,16}. The rebound rise of GH after glucose administration is probably related to the amount of glucose given¹⁸. With respect to the role of lipids, lipid infusion suppresses GH secretion^{19,20}, even as lipid ingestion combined with heparin injection²¹, however no suppression of GH secretion is found when lipids, without heparin are ingested²¹. Also two other studies found that fat did not alter GH concentrations after an arginine stimulus²².

²³. Furthermore, controversial results of lipid administration on exercise induced GH release were found, while *Hunter et al* found no suppression of the exercise induced GH release after ingestion of fat, *Cappon et al* found a reduced exercise-induced GH release after ingestion of fat compared with placebo or carbohydrate intake before exercise ²⁴.

Taken together, previous studies showed an increase in GH secretion after ingestion of protein, however, this GH promoting effect of protein is eliminated when combined with carbohydrate and fat. Equivocal effects of carbohydrates and fat on GH secretion were found, therefore, the aim of this study is to investigate whether carbohydrate or fat or a combination is responsible for interference with the protein-induced GH secretion when protein is ingested combined with carbohydrates and fat. Ingestion of protein alone, combined with either carbohydrate or fat and protein combined with carbohydrate and fat are compared.

METHODS

Subjects

Subjects were recruited via advertisements at the university. They underwent a screening procedure including medical history taking, measurement of bodyweight and height. Eight females were selected on the basis of being in good health, non-smoker, non-vegetarian, using oral contraceptives, not using other medication, not spending more than three hours sport activities a week and at most moderate alcohol users (<10 alcoholic consumptions per week). Only females were included, because in previous studies was found that females responded more consistently and with a greater increase in GH after ingestion of certain amino acids than men ^{25, 26}. Their mean age was 21 ± 1.5 years, and their body mass index (BMI) was 22 ± 1.8 kg/m². All tests were performed in the early follicular phase of each woman's menstrual cycle, to eliminate the confounding variable of changing serum estrogen concentrations. The Medical Ethics Committee of Maastricht University approved the study protocol and all subjects gave their written informed consent before participating in the study.

Experimental design

A randomized crossover study design was applied. Subjects reported to the laboratory for consumption of five different test drinks, separated by at least one week. Each time, subjects arrived at the laboratory in a fasted state, at 8.30h. They were instructed to fast from 10 pm the night prior to the test day. A canula was placed into a dorsal hand vein of the contra lateral arm, and this hand was kept in a

thermoregulated (60°C) box to sample arterialized venous blood ²⁷. Blood sampling began 60 min after placement of the canula (t=0). Blood samples for determination of GH were obtained before and every 20 min for 5 hours after ingestion of the testdrinks, blood samples for determination of glucose and insulin were obtained before and 40,60,180 and 300 min after the intake of the testdrinks.

Blood samples for determination of amino acids were obtained before and 60, 80, 100, 180 and 300 min after intake of the testdrinks. During blood sampling, subjects remained awake and fasted and were allowed to drink water ad libitum. Immediately after obtaining the first blood sample (t=0), subjects received a test drink and had to be finished in 5 minutes. Subjects received a drink containing soy protein (Supro® 590, The Solae Company, St. Louis, MO, United States of America), soy protein plus carbohydrate (soy+carbohydrate), soy protein plus fat (soy+fat), soy protein plus carbohydrate plus fat (soy+carbohydrate+fat) and a placebo, which contained only water. Carbohydrate used in the testdrink was tapioca-starch (Farinex VA50T, AVEBE, Veendam, The Netherlands and Perfectamyl 3108 AVEBE, Veendam, The Netherlands), fat used was sunflower-oil (Reddy, NV Vandemoortele, Roosendaal, The Netherlands). The sugarfree syrup (Diaran, Cereal) added to improve the taste of the drinks, contained no protein and fat and a negligible amount of carbohydrate. Since the objective of this study was to investigate whether the carbohydrate or the fat part of a (high protein) diet is responsible for the elimination of the GH response to soy protein, the macronutrient amount of the test drinks was kept constant, resulting in a different energy content. The viscosity of the testdrinks was equal. The composition of the test drinks is described in Table 1.

Table 1: Composition of the tests conditions, based on a subject of 70 kg.

	Soy	Soy+CH	Soy+Fat	Soy+CH+Fat	Placebo
Volume (ml)	450	450	450	450	450
Soy protein (g)	42	42	42	42	-
Carbohydrate (CH) (g)	-	92	-	92	-
Fat (g)	-	-	15	15	-
Sugar free syrup (ml)	40	40	40	40	40

Blood analyses

Arterialized venous blood was collected in clot tubes, containing 'Silica Clot Activator' (Becton Dickinson Vacutainer system; Becton Dickinson, Franklin Lakes, NJ), and in heparinized tubes (Becton Dickinson Vacutainer system; Becton Dickinson, Franklin Lakes, NJ). Blood in the clot tubes was allowed to clot for 30 minutes and was centrifuged at 3000 rpm, 4°C for 10 min to obtain serum. Serum was collected

for determination of GH and amino acid (AA) concentrations. Blood in heparinized tubes was kept on ice to minimize enzymatic reactions. Glucose and insulin analyses were performed in plasma, which was obtained by centrifugation at 4°C for 10 min at 3000 rpm. Each aliquot was frozen immediately in liquid nitrogen and stored at -80°C, until analysis. All samples from the same subject were analysed in the same assay.

The GH-concentrations were measured by using an ultrasensitive GH chemiluminescence immunoassay (Beckman Coulter, Harbor Blvd. Fullerton, U.S.A.). The intra- and inter-assay coefficients of variation were 1.4-2.1 and 6.8-8.6% respectively, at GH concentrations of 3.7-14.2 and 3.1-7.3 µg/l, respectively. Insulin concentrations were measured by an electrochemiluminescence immunoassay (ELICA) (Roche Diagnostica, Hoffman-La Roche, Basel, Switzerland). Glucose concentrations were measured by using enzymatic assay (G6-PDH) (Roche Diagnostica, Hoffman-La Roche, Basel, Switzerland). The concentrations of the amino acids; arginine, lysine, histidine, methionine, ornithine, phenylalanine and glutamic acid were measured using a HPLC system (Shimadzu, Duisburg, Germany).

Statistics

Data are presented as mean changes from baseline +/- standard error to the mean (SEM). Baseline ratings for GH, insulin, glucose and AAs were not statistically significantly different among treatments, therefore delta concentrations were used for statistical analysis. Growth hormone, insulin, glucose and AA responses were calculated as incremental area under the curve (IAUC), peak values and time to peak (TTP) values. IAUC is defined as the total AUC minus the baseline AUC. Peak values were defined as the highest mean value obtained within 300 min. Statistical analyses of the data were performed using repeated measures analysis of variance (ANOVA), correcting for multiple analyses. Post hoc comparisons were made with the Fisher PLSD-test. A p-value <0.05 was regarded as statistically significant. All statistical tests were performed by using Statview SE Graphics software (version 4.5; Abacus Concepts Inc, Berkeley, CA, USA).

RESULTS

GH-responses as determined by incremental area under the curve (IAUC) over 5h were higher after ingestion of soy alone, soy+carbohydrate or soy+fat, than after ingestion of soy+carbohydrate+fat or placebo ($p<0.05$) (Figure 1, Table 2). GH peak values after ingestion of soy alone, soy+carbohydrate or soy+fat were higher compared with ingestion of soy+carbohydrate+fat or placebo ($p<0.05$) (Figure 1, Table 2). GH peak values after ingestion of soy alone or soy+fat were higher com-

pared with soy+carbohydrate+fat. No difference in GH-response, AUC and peak-values, was found between ingestion of soy+carbohydrate+fat and placebo (Figure 1, Table 2). Time to peak values (TTP) were later after ingestion of soy+carbohydrate+fat or soy+fat than after ingestion of soy ($p<0.05$) and TTP was later after ingestion of the placebo than after ingestion of soy ($p<0.05$) (Figure 1, Table 2).

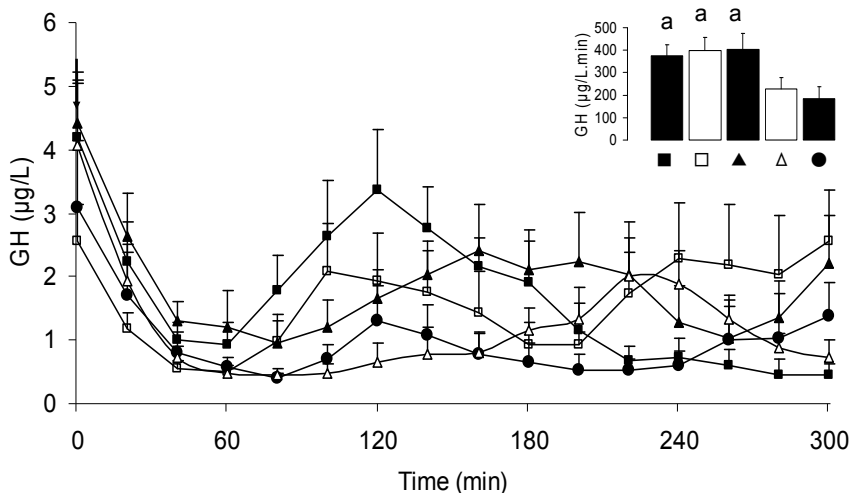


Figure 1: Growth hormone concentrations after ingestion of a placebo (●), soy protein (■), soy protein+carbohydrate (□), soy protein+fat (▲) and soy protein+carbohydrate+fat (△) ($n=8$). Data are presented as mean (\pm SEM) in lines and bars (IAUC). \downarrow = ingestion of testdrink. Peak values of GH were higher after ingestion of soy, soy+carbohydrate and soy+fat, compared with soy+carbohydrate+fat and placebo. $a = p<0.05$ compared with placebo and soy protein+carbohydrate+fat.

Insulin responses as determined by IAUC and peak values were higher after ingestion of soy or combined with either carbohydrate or fat, soy alone or soy+carbohydrate+fat than after ingestion of placebo ($p<0.05$). Ingestion of soy+carbohydrate and soy+carbohydrate+fat showed higher insulin responses (AUC and peak), compared with insulin responses after ingestion of soy+fat or soy alone. TTP of insulin was not significant different between the conditions (Figure 2, Table 2).

Glucose responses as determined by IAUC were higher after ingestion of soy + carbohydrate than after ingestion of soy + fat, soy + fat + carbohydrate, soy alone or placebo ($p<0.05$) (Figure 3, Table 2). Glucose peak concentration after ingestion of soy + carbohydrate was higher compared with soy + fat or placebo while glucose peak concentrations for soy + carbohydrate + fat were higher compared with ingestion of soy alone, soy + fat or placebo ($p<0.05$) (Table 2). TTP of glucose was not significant different between the conditions (Table 2).

With respect to serum AA-concentrations; AA-concentrations, determined as IAUC were not different between the testdrinks, soy alone, soy+carbohydrate, soy+fat and soy+carbohydrate+fat. Peak-concentrations of arginine, lysine, histidine, methionine, ornithine, and phenylalanine were higher after ingestion of soy compared with soy+carbohydrate+fat ($p<0.05$). Lysine, histidine, methionine, ornithine and phenylalanine concentrations were higher after ingestion of soy compared with soy+carbohydrate and soy+fat ($p<0.05$). Arginine concentrations were not different after intake of soy, soy+carbohydrate and soy+fat, but were higher compared with arginine concentrations after intake of soy+carbohydrate+fat ($p<0.001$). The time to peak of the AA-concentrations was not different after the ingestion of soy, soy+carbohydrate, soy+fat or soy+carbohydrate+fat. At 300 min after ingestion of the testdrinks, arginine, lysine and histidine concentrations were higher after ingestion of soy+carbohydrate than after ingestion of soy (Figure 4, Table 2).

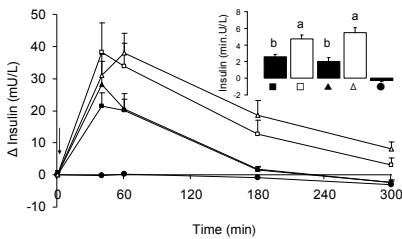


Figure 2: Delta insulin concentrations after ingestion of a placebo (●), soyprotein (■), soyprotein+carbohydrate (□), soyprotein+fat (▲) and soyprotein+carbohydrate+fat (Δ) (n=8). Data are presented as means (± SEM) in lines and bars (IAUC). ↓ = ingestion of testdrink. Peak concentrations of insulin are significant higher after ingestion of soy+carbohydrate and soy+carbohydrate+fat compared with soyprotein, soyprotein + fat and placebo. Insulin concentrations after ingestion of placebo are significant lower than after the other testdrinks. a = $p<0.05$ compared with soyprotein, soyprotein+fat and placebo. b= $p<0.05$ compared with placebo

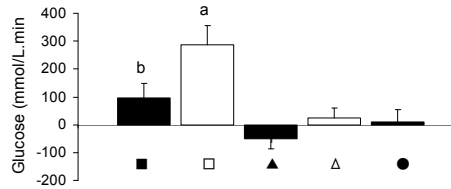


Figure 3: Glucose concentrations after ingestion of a placebo (●), soy protein (■), soy protein+carbohydrate (□), soy protein+fat (▲) and soy protein+carbohydrate+fat (Δ) (n=8). Data are presented as AUC over 5h. ↓ = ingestion of testdrink. a = $p<0.05$ compared with soy protein, soy protein+fat, soy protein+carbohydrate+fat and placebo, b = $p<0.05$ compared with soyprotein+fat.

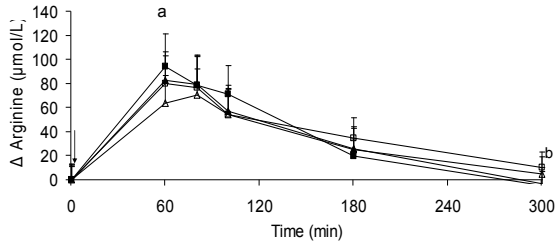


Figure 4: Delta arginine concentrations (ARG) after ingestion of soyprotein (■), soyprotein+carbohydrate (□), soyprotein+fat (▲) and soyprotein+carbohydrate+fat (Δ) (n=8). Data are presented as means (± SEM) in lines. ↓ = ingestion of testdrink. a = ARG concentrations at 60 min are higher after ingestion of soy, soy+carbohydrate and soy+fat compared with soy+carbohydrate+fat (p<0.001), b= Arginine-concentrations are higher at 300 min after ingestion of soy+carbohydrate than after ingestion of soy (p<0.05).

Table 2: Blood parameters after ingestion of a placebo, soy protein, soy protein+carbohydrate, soy protein+fat and soy protein+carbohydrate+fat (n=8), expressed as incremental area under the curve (IAUC), peak-values and time to peak (TTP). Data are presented as mean (± SEM).

	IAUC				
	Placebo	Soy	Soy+Carbohydrate	Soy+Fat	Soy+Carbohydrate+Fat
GH (min.µg/L)	184.04 ± 52.10	373.99 ± 49.56 ^{ab}	399.03 ± 57.11 ^{ab}	402.96 ± 72.62 ^{ab}	225.93 ± 43.00
Insulin (min.U/L)	-0.26 ± 0.17	2.60 ± 0.25 ^a	4.74 ± 0.44 ^{ab,cd}	2.03 ± 0.48 ^a	5.46 ± 0.63 ^{cd,de}
Glucose (min.mmol/L)	8.66 ± 46.51	96.36 ± 51.57 ^a	285.45 ± 69.51 ^{ab,cd,de}	-50.16 ± 37.77	74.55 ± 44.38
Arginine (min.mmol/l)		10.58 ± 1.72	12.73 ± 1.89	10.25 ± 1.18	10.64 ± 2.19
Lysine (min.mmol/l)		23.73 ± 3.54	21.82 ± 2.67	16.42 ± 1.44	17.56 ± 3.40
Histidine (min.mmol/l)		5.34 ± 1.52	4.80 ± 1.16	2.55 ± 0.78	3.31 ± 1.81
Methionine (min.mmol/l)		1.04 ± 0.31	0.59 ± 0.30	0.26 ± 0.22	0.30 ± 0.54
Ornithine (min.mmol/l)		7.85 ± 1.05	5.89 ± 0.93	15.24 ± 9.24	5.07 ± 1.28
Phenylalanine (min.mmol/l)		7.55 ± 1.04	6.61 ± 0.70	6.60 ± 0.46	6.37 ± 1.04
Glutamic acid (min.mmol/l)		0.16 ± 2.26	-2.52 ± 0.90	-2.19 ± 1.22	-1.89 ± 1.69

	Peak				
	Placebo	Soy	Soy+Carbohydrate	Soy+Fat	Soy+Carbohydrate+Fat
GH (µg/L)	2.19 ± 0.77	4.99 ± 0.81 ^{ab}	4.87 ± 0.88 ^{ab}	4.19 ± 0.68 ^{ab}	3.00 ± 0.56
Insulin (µU/L)	8.50 ± 1.07	32.10 ± 7.65 ^a	51.53 ± 9.14 ^{ab,cd}	33.84 ± 6.84 ^a	44.81 ± 6.51 ^{cd,de}
Glucose (µmol/L)	5.30 ± 0.14	5.50 ± 0.10	5.84 ± 0.13 ^{cd}	5.26 ± 0.22	5.95 ± 0.13 ^{cd,de}
Arginine (µmol/l)		204.50 ± 11.45 ^{ab}	204.50 ± 12.17 ^a	202.98 ± 8.73 ^a	141.88 ± 6.99
Lysine (µmol/l)		354.38 ± 9.32 ^{bc,cd}	313.00 ± 26.23	310.13 ± 18.62	299.25 ± 29.05
Histidine (µmol/l)		138.38 ± 7.92 ^{bc,cd}	130.50 ± 6.38	124.50 ± 14.98	123.13 ± 5.13
Methionine (µmol/l)		39.25 ± 1.99 ^{bc,cd}	33.63 ± 1.82	36.63 ± 1.96	34.88 ± 2.99
Ornithine (µmol/l)		110.15 ± 7.03 ^{bc,cd}	99.00 ± 5.47	104.75 ± 4.60	100.75 ± 9.41
Phenylalanine (µmol/l)		125.13 ± 7.47 ^{bc,cd}	115.00 ± 5.23	119.00 ± 2.81	108.88 ± 5.19
Glutamic acid (µmol/l)		124.88 ± 7.89	127.00 ± 9.25	136.88 ± 9.67	134.75 ± 14.61

	TTP				
	Placebo	Soy	Soy+Carbohydrate	Soy+Fat	Soy+Carbohydrate+Fat
GH (min)	180 ± 30 ^a	123 ± 11.60	168 ± 25	140 ± 14.20	193 ± 21 ^{ab}
Insulin (min)	37 ± 8	30 ± 2	29 ± 4	26 ± 3	34 ± 3
Glucose (min)	33 ± 9	43 ± 6	50 ± 8	43 ± 9	48 ± 9
Arginine (min)		55 ± 6	50 ± 5	48 ± 4	53 ± 4
Lysine (min)		60 ± 7	45 ± 3	50 ± 4	50 ± 4
Histidine (min)		65 ± 6	48 ± 4	58 ± 6	58 ± 6
Methionine (min)		55 ± 6	43 ± 3	45 ± 3	48 ± 4
Ornithine (min)		63 ± 7	50 ± 5	48 ± 4	55 ± 5
Phenylalanine (min)		65 ± 6	53 ± 5	53 ± 4	53 ± 4
Glutamic acid (min)		63 ± 7	50 ± 5	48 ± 4	58 ± 5

DISCUSSION

This study shows that the somatotrophic effect of soy protein was reduced by simultaneous co-ingestion of carbohydrate and fat, but not by co-ingestion of either carbohydrate or fat.

As mentioned in the introduction, carbohydrate as well as fat could interfere with protein's effect on GH secretion. Several studies, but not all¹⁷ found that carbohydrate ingestion alone increased the GH concentrations 3-5 h after ingestion¹²⁻¹⁶, and that fat ingestion did not change the GH response, however when heparin was injected combined with fat ingestion, GH concentrations were reduced, due to increased FFA concentrations^{21, 23}. Still only a few studies compared the GH responses after co-ingestion of carbohydrate or fat with protein^{15, 16} as we did in our study.

Two studies aimed to compare effects of protein or protein plus carbohydrate on GH response. They found a decreased GH response after ingestion of protein combined with carbohydrate compared with protein alone. The protein source used however contained also small amounts of fat^{15, 16}, and is therefore comparable with our test condition protein+fat. Hence, this confirms our results in which we found a reduced GH response when protein+carbohydrate+fat was ingested compared with protein+fat^{15, 16}.

To our knowledge, no further studies were performed comparing the ingestion of protein alone with protein plus carbohydrates. As we mentioned above, carbohydrate ingestion increases the GH response 3-5 h after ingestion¹²⁻¹⁶. In our results we found two GH peaks after ingestion of protein plus carbohydrates (Figure 1). The first peak may be a result of the protein induced GH secretion, as we also found after ingestion of protein alone. The second peak was not found after ingestion of protein alone, and must therefore be attributed to the simultaneous presence of carbohydrate. This could have resulted in changes in protein metabolism, as AA concentrations were higher at 300 minutes after ingestion of carbohydrate plus proteins, when compared with protein alone. These higher AA concentrations may, in turn, be involved in the increase of GH concentrations.

With respect to the role of fat combined with protein, we found that fat delays the GH response to protein, but the GH-AUC and GH-peak values after ingestion of protein plus fat were not different from that of protein alone, which is also observed in two studies, reporting that cornoil ingestion did not change GH concentrations. The delayed GH peak after ingestion of the test drinks containing fat compared with soy alone can not be clarified by insulin-, glucose- or AA-concentrations. Fat is known to delay gastric emptying, however this mechanism does not seem to be involved in the delayed GH response, since we did not find a delayed or lower insulin peak concentration, neither a delayed AA peak compared with ingestion of soy alone. A mechanism explaining the delayed GH peak after

ingestion of fat, may be a direct effect of fat on somatostatin which could delay the GH response after ingestion of the test conditions containing fat^{28, 29}.

The GH promoting effect of protein is still present when ingested combined with either carbohydrate or fat, but not when combined with carbohydrate and fat. The underlying mechanism of the eliminated GH promoting effect of soy after ingestion of soy combined with carbohydrate and fat remains unclear.

In line with our objective, the macronutrient amount of the testdrinks was kept constant, which resulted in different caloric contents of the testdrinks. We cannot rule out the possibility that the different energy content of the testdrinks affected the GH response, although a major role of energy content is not very likely, since no differences in AUC GH and PEAK GH responses were found between the ingestion of soy, soy+carbohydrate and soy+fat.

Insulin as well as glucose concentrations were higher after ingestion of soy combined with carbohydrate compared with soy alone, however GH concentrations (AUC) did not differ between the conditions, indicating that neither insulin nor glucose itself was instrumental in the decreased GH response after soy+carbohydrate+fat.

Amino acids that are known to stimulate the GH response are in particular arginine, but also lysine, histidine, ornithine, methionine, phenylalanine and glutamic acid^{8, 9, 30}. We found lower peak serum AA concentrations of arginine, lysine, histidine, ornithine, methionine and phenylalanine after ingestion of soy+carbohydrate+fat than after soy alone, which could explain the reduced somatotropic effect of soy protein after co-ingestion with carbohydrate and fat. Despite no differences in GH responses after ingestion of soy+carbohydrate, soy+fat and soy alone, serum concentrations of lysine, histidine, ornithine, methionine and phenylalanine were lower after ingestion of soy+carbohydrate or soy+fat compared with soy alone. This indicates that peak arginine, but not the other amino acids, may be involved in the reduced somatotropic effect of protein after co-ingestion with carbohydrate and fat. To provide more direct evidence that arginine is responsible for the reduced somatotropic effects, soy+carbohydrate+fat plus an arginine supplement could be tested.

Furthermore increased FFA concentrations are known to suppress the GH secretion. In this study we did not measure FFA concentrations, however we do not think that our results are explained by changing FFA concentrations, since increased insulin concentrations, known to suppress the lipolysis, were not different between soy+carbohydrate and soy+carbohydrate+fat. GH responses however were reduced after ingestion of soy+carbohydrate+fat, but not after soy+carbohydrate, compared with soy alone.

This study confirms previous findings that ingestion of dietary protein regulates GH secretion in the short-term. Moreover it also shows that the physiological role of food intake on the regulation of GH secretion involves complex interactions when

the different macronutrients are ingested together. Thus, the GH-promoting activity of soy protein depends on the presence of other macronutrients, in that fat and carbohydrate should not be present at the same time. Serum arginine concentrations may be involved in the somatotropic activity of protein, glucose and insulin responses however are not responsible for this effect.

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6

Association between intake of dietary protein and 3y-change in body growth among normal and overweight 6y old boys and girls (CoSCIS)

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ABSTRACT

Background: Growth-hormone (GH) affects linear growth and body composition, by increasing the secretion of insulin-growth-factor-1 (IGF-1), muscle protein synthesis and lipolysis. The intake of protein (PROT) as well as the specific amino-acids arginine (ARG) and lysine (LYS) stimulates GH/IGF-1 secretion.

Objective: To investigate associations between PROT intake as well as intake of the specific amino acids ARG and LYS, and subsequent 3y-change linear growth and body-composition among 6y-old children.

Methods: Data of 223 children were collected from Copenhagen (Denmark), during 2001-2 and again 3 y later. Boys and girls were separated into normal weight and overweight, based on body mass index (BMI) quintiles. Fat free mass index (FFMI) and fat mass index (FMI) were calculated. Associations between change (Δ) in height, FMI, FFMI respectively and habitual PROT-intake as well as ARG and LYS were analysed by multiple-linear-regressions, adjusted for baseline height, FMI or FFMI and energy intake, age, physical activity and socio economic status.

Results: High ARG-intake was associated with linear growth ($\beta=1.09\pm 0.54$ $p=0.05$) among girls. Furthermore in girls, Δ FMI was stronger inversely associated with high ARG-intake, if it was combined with high LYS-intake, instead of low LYS-intake ($p=0.03$). No associations were found in boys.

Conclusion: In prepubertal girls linear growth may be influenced by habitual arginine-intake and body fat gain may be relatively prevented over time by the intake of the amino acids arginine and lysine.

INTRODUCTION

Protein intake (PROT) is associated with greater velocity of linear growth in childhood¹ as well as with a more beneficial body composition²⁻⁴. Some studies suggest that the amount of protein intake, but also the protein source have a regulatory effect on body growth, for instance milk protein is stronger associated with growth in height if compared with meat or vegetable protein⁵. Growth hormone (GH) and Insulin-like growth factor 1 (IGF-1) play a central role in the regulation of growth in height and width^{5,6}. Besides the important role of GH/IGF-1 in regulating growth in height, it also regulates fat distribution and reduces fat mass⁷. Studies show an increased lipolysis and decreased fat mass after GH administration⁸⁻¹¹. Dietary protein may be involved in secretion of GH and IGF-1, because protein restriction results in low IGF-1 concentrations in healthy children, which returned to normal after refeeding with proteins¹². This finding is confirmed by a study showing that milk-protein, but not meat or vegetable protein, increases IGF-1 concentrations and growth in height⁵.

The effects of dietary protein on IGF-1 concentrations and growth^{1, 11, 13, 14} may be mediated by a direct GH-releasing effect of specific amino acids¹⁵⁻²¹, in particular arginine (ARG) and lysine (LYS)^{17, 19, 21}. Previous studies suggest that the amino acid composition is crucial in understanding the mechanism through which protein may influence GH and IGF-1 production in relation to growth and body composition, since specific proteins, as gelatin protein (which is high in ARG), stimulate the GH release more potently than other protein-sources²². However no previous studies have been performed investigating the relationship between the intake of specific amino acids and the development of fat mass and fat free mass in pre-pubertal children.

In this study we investigated associations between the intakes of protein and more specific the amino acids ARG and LYS in the habitual diet and subsequent changes in linear growth, body fat and fat free mass, among normal weight and overweight 6-year old boys and girls. High intakes of PROT, and more specifically a high intake of ARG, especially in combination with a high intake of LYS, was expected to increase linear growth and the fat free mass index and decrease fat mass index.

MATERIALS AND METHODS

Subjects

The data were derived from a four-year prospective intervention study of physical activity and health, the Copenhagen School Child Intervention Study (CoSCIS). Children from 46 preschool classes (6–7 years of age) in 18 schools in two suburban

communities in the Copenhagen area were invited to participate in the CoSCIS. In 2000 the community of Ballerup (10 schools, 27 classes) increased the number of physical exercise lessons from two to four a week for the first three years of school. The community of Taarnby (8 schools, 19 classes) was chosen as a control as it resembles Ballerup regarding the sociodemographic characteristics. The reported data in the present study are from the controls of this study (only community of Taarnby)^{23, 24}. A total of 706 children (69% of those eligible) volunteered in the study, and written informed consent was obtained from the parents/guardians. Of these 706 children, 415 from Ballerup and 291 from Taarnby participated at baseline, 614 children were followed-up at 3rd grade. The ethics committee of Copenhagen County approved the study. The measurements were performed from December 2001 until June 2002 preschool, at the 18 different schools and in the follow up from September 2004 until May 2005 at 3rd grade. All control children with complete information about food intake, physical activity, socio economic status, height, weight and skin fold-thickness at baseline as well as height, weight and skin fold-thickness at follow up were included. According to these inclusion criteria 94 boys and 109 girls respectively were included.

Measurements

Dietary intake

Parents recorded the dietary intake of their child for seven consecutive days in supplied booklets with pre-coded fixed answer possibilities supplemented with a possibility for open answers. Food portions sizes were estimated from household measures and a series of photographs. This booklet for dietary record has been developed and used in nationwide dietary surveys since 1995²⁵. The pre-printed diet records were delivered by the teachers in the schools in closed envelopes addressed to each child, who brought them home. The same logistic was used for return of the completed records.

Intakes of nutrients and foods were calculated using the General Intake Estimation System (Danish Institute for Food and Veterinary Research 2005) based on the Danish food database²⁶. The researchers computed the dietary information in a database, by which it was possible to calculate nutrient information (i.e. energy content, protein content, AA content) on the individual food items or whole meals and diets. Corrections for losses in cooking were made when calculating nutrient contents.

Reporting of dietary intake of energy was evaluated by comparing reported energy intake with estimations of energy expenditure (based on basal metabolic rate (Harris & Benedict) and physical activity level (PAL). The PAL in children varies between 1.3

and 1.9²⁷. Children who reported an energy intake below BMR*1.3 or above BMR*2.0 were excluded.

Anthropometry & other measurements

All measurements were carried out using standardised methods, which have been described in detail elsewhere^{23, 24}.

Height was measured by a Harpenden stadiometer to the nearest 1 mm. Body weight was measured to the nearest 0.1 kg using a SECA electronic scale. Both measurements were performed during school-time by experienced scientists. Bicipital, tricipital, subscapular, and suprailiac skinfolds were measured with a Harpenden skin fold calliper to the nearest 1 mm. The dominant side of the body was determined by asking the child to take a pen and write his/her name. The data shown in this study represent the mean of three measurements taken on the non-dominant side of the body. The sum of four skin folds (SFS) was used to calculate body fat percentage. Children were divided into quintiles according to their BMI, adjusted for sex and age²⁸. Analyses were first performed in the whole group of girls and boys and later the groups were divided in BMI quintile 1-4 and BMI quintile 5.

Habitual physical activity (PA) was measured by the MTI 7164 activity monitor (Manufactory Technology Inc, Fort Walton Beach, Florida, USA). This monitor has been validated in several studies and has shown both high mechanical reproducibility and good validity with respect to free living conditions in children²⁴. To allow familiarisation, the children had to wear the MTI monitor one day before recording. It was secured directly to the skin at the lower back using an elastic belt. The children were instructed to wear the monitor continuously except during water based activities or when sleeping. A mean count was calculated for each child²⁴.

The classification of socio-economic status (SES) was based on information regarding the education of the mother in the household at the first measurement point, since recent studies suggests that the risk factors considered in this study are stronger associated with the SES of the mother rather than that of the father²⁹⁻³¹. The education level of the mother was divided in 7 categories; (1) <7 yrs education, (2) 7-8 yrs education, (3) 9-10 yrs completed. elementary school, (4) 12 yrs. high school, (5) short non-university programmes under 3 y, (6) college 3-4y; (7) university degree over 4y.

Calculation of FFM and FM

The body fat percentage was estimated from the skin fold thickness using the equations from Weststrate³²;

- (i) girls aged 2–10 years: $\text{Fat\%} = ((562-1.1(\text{age}-2))/D) - (525-1.4(\text{age}-2))$ and
- (ii) girls aged 11-18 years: $\text{Fat\%} = ((533-7.3(\text{age}-10))/D) - (514-8.0(\text{age}-10))$ and
- (iii) boys aged 2–18 years: $\text{Fat\%} = ((562-4.2(\text{age}-2))/D) - (525-4.7(\text{age}-2))$

D = Total body density estimated from following equations: (i) girls aged 2–10 y: $D = (1.1315 + 0.0004 * (\text{age}-2)) - ((0.0719 - 0.0003 * (\text{age}-2)) * \log_4 \text{skin})$ and (ii) girls aged 11–18 y: $D = (1.1350 + 0.0031 * (\text{age}-10)) - ((0.0719 - 0.0003 * (\text{age}-2)) * \log_4 \text{skin})$ and (iii) boys aged 2–18 y: $D = (1.1315 + 0.0018 * (\text{age}-2)) - ((0.0719 - 0.0006 * (\text{age}-2)) * \log_4 \text{skin})$ where $\log_4 \text{skin} = \log(\Sigma \text{mean of the four skinfold thickness})$.

The fat mass (FM) and the fat free mass (FFM) were calculated from the body weight and the estimated body fat (%). FM and FFM were divided by squared height, to calculate fat-free mass index (FFMI) and fat mass index (FMI) respectively³³. These indices have been proposed as indicator of nutritional status, and because $\text{FMI} + \text{FFMI} = \text{BMI}$, their use allows to assess the contribution of fat mass and fat-free mass to BMI³³.

Table 1: Characteristics of boys and girls aged 6 years and 9 years according to BMI quintiles.

	Boys ¹ n=73		Boys ² n=21		Girls ¹ n=89		Girls ² n=20	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
6-year								
Weight (kg)	23.4	2.4	28.7	3.9	22.4	2.5	28.9	3.5
Height (cm)	122.8	5.3	125.4	5.9	121.1	4.3	123.7	5.0
Sum of Skinfold (mm)	30	6	46	14	35	8	59	15
BMI (kg/m ²)	15.4	0.7	18.2	1.1	15.2	0.7	18.9	0.9
FFMI (kg/m ²)	13.3	0.8	14.2	1.0	11.4	0.7	12.6	0.6
FMI (kg/m ²)	2.1	0.7	4.0	1.2	3.8	0.8	6.3	1.3
Physical activity score (counts)	126	40	131	40	116	26	117	26
SES 1/2/3/4/5/6/7 (%)	5/9/7/25/29/10/13		0/12/4/28/36/16/4		4/3/8/20/42/15/8		5/5/14/24/24/5/23	
Energy intake (MJ/d)	8.4	1.6	8.2	2.1	7.8	1.7	7.5	1.5
PROT intake (g/d)	69.5	17.8	71.3	15.7	63.4	15.2	59.7	13.7
ARG intake (g/d)	2.8	0.9	3.0	1.0	2.5	0.6	2.3	0.5
LYS intake (g/d)	4.5	1.3	4.6	1.1	4.1	1.1	3.8	1.1
9-year								
Weight (kg)	31.9	4.3	40.7	7.2	31.2	5.3	40.7	6.1
Height (cm)	138.9	6.1	141.7	6.7	137.8	4.3	141.5	5.7
Sum of Skinfold (mm)	26	9	46	18	32	10	62	22
BMI (kg/m ²)	16.5	0.9	20.3	1.4	16.3	1.1	19.1	1.5
FFMI (kg/m ²)	15.2	1.1	17.4	1.6	12.6	0.9	12.3	0.7
FMI (kg/m ²)	1.3	0.7	2.8	1.2	3.7	1.2	6.8	1.9

¹BMI-quintiles 1-4, ²BMI-quintile 5, PROT= Total protein, ARG = Arginine, LYS = Lysine, BMI = Body Mass Index, FFMI= Fat Free Mass Index, FMI= Fat Mass Index, SES = Socio Economic Status

Statistical analysis

Each dietary and anthropometric variable was treated as a continuous variable. The relationship between Δheight , ΔFMI , or ΔFFMI and intake of PROT as well as the intake of ARG or LYS at age 6y was assessed by a multiple regression model includ-

ing the covariates; baseline height, FMI or FFMI, age, energy intake (EI), PA and SES. The interaction of ARG with LYS was estimated in a model with the same covariates as the model described above. This model was converted to the final regression model, by dividing LYS into low and high LYS intake. Low LYS-intake was defined as LYS intake below median and high LYS-intake as LYS intakes above median. Since there was an interaction between the independent variables PROT, ARG, LYS and gender, data of boys and girls were analysed separately. After analysing data of boys and girls separately, they were divided in either the 1-4th quintile of BMI (leaner children) or in the 5th quintile of BMI (heavier children) to investigate if there is a difference in growth influenced by protein intake between the leaner children and the heavier children. To test the significance of the contribution of the variables, an F-test was used. The characteristics are expressed as mean \pm SD. All other values are expressed as mean \pm SE. A p-value <0.05 was considered as indicating statistical significance. All statistical analyses were performed using SPSS for WINDOWS, version 14.0; SPSS, Chicago, IL.

RESULTS

The characteristics of the children, grouped by study year, gender and quintile of BMI are shown in Table 1.

The results are summarized in Table 2 and 3, with Δ height, Δ FMI and Δ FFMI as dependent variables and habitual intake of PROT as well as the specific amino acids ARG, LYS and the interaction between ARG and LYS as independent variables. Adjustments were made for the covariates; baseline height, FMI or FFMI, age, EI, PA, SES.

Association between change in height and habitual intake of PROT, and specifically ARG and LYS intake

Among girls, growth in height was associated with a high intake of ARG ($\beta=1.09\pm 0.54$ $p<0.05$), but intake of PROT or LYS was not associated with changes in linear growth (Table 2). Among boys this association did not reach significance.

Table 2: Adjusted models showing the associations between both ARG and LYS (g/day) intakes and changes in height (m) FMI and FFMI (kg/m²)

		Boys N=94			Boys ¹ N=73			Boys ² N=21		
		β	SE	p	β	SE	p	β	SE	p
Height (m)	PROT	0.04	0.02	0.11	0.04	0.03	0.11	-0.10	0.09	0.92
	ARG	0.61	0.36	0.10	0.60	0.46	0.19	0.84	0.88	0.36
	LYS	0.34	0.25	0.18	0.38	0.29	0.19	-0.09	0.82	0.92
FMI (kg/m ²)	PROT	0.00	0.00	0.79	0.00	0.00	0.51	-0.04	0.02	0.12
	ARG	0.02	0.05	0.72	-0.02	0.05	0.60	-0.02	0.21	0.92
	LYS	-0.01	0.04	0.78	-0.09	0.03	0.74	-0.57	0.13	0.01
FFMI (kg/m ²)	PROT	0.02	0.01	0.22	0.00	0.01	0.88	0.00	0.07	0.97
	ARG	0.40	0.23	0.09	0.00	0.21	1.00	0.60	0.64	0.39
	LYS	0.16	0.16	0.35	-0.02	0.14	0.90	-0.70	0.76	0.40

		Girls N=109			Girls ¹ N=89			Girls ² N=20		
		β	SE	p	β	SE	p	β	SE	p
Height (m)	PROT	0.02	0.03	0.59	0.02	0.03	0.63	0.12	0.12	0.33
	ARG	1.09	0.54	0.05	1.15	0.58	0.05	3.16	2.04	0.17
	LYS	0.21	0.32	0.52	0.19	0.35	0.59	1.69	1.20	0.20
FMI (kg/m ²)	PROT	0.00	0.01	0.79	0.00	0.01	0.88	-0.03	0.01	0.01
	ARG	-0.09	0.23	0.72	0.00	0.29	0.99	-0.51	0.11	0.01
	LYS	-0.06	0.13	0.66	-0.01	0.17	0.98	-0.30	0.06	0.01
FFMI (kg/m ²)	PROT	-0.01	0.01	0.26	0.00	0.01	0.80	-0.06	0.03	0.13
	ARG	-0.04	0.16	0.82	0.10	0.18	0.59	-0.63	0.51	0.28
	LYS	-0.09	0.09	0.31	-0.02	0.11	0.85	-0.42	0.29	0.22

¹BMI-quintiles 1-4, ²BMI-quintile 5, PROT= total protein, ARG = arginine, LYS = lysine, FFMI= Fat Free Mass Index (kg/m²), FMI= Fat Mass Index (kg/m²)

Table 3: Final models between the interaction of ARG and LYS (g/day) and height (m), FMI and FFMI (kg/m²)

		Boys N=94				Boys ¹ N=73				Boys ² N=21			
		β	SE	p	AxL ³	β	SE	p	AxL ³	β	SE	p	AxL ³
Height	Low LYS ⁴	1.03	0.94	0.28	0.53	1.01	1.06	0.35	0.42	1.41	3.70	0.71	0.85
	High LYS ⁵	0.44	0.51	0.39		0.17	0.73	0.82		0.64	1.13	0.59	
FMI	Low LYS ⁴	-0.01	0.13	0.94	0.65	-0.08	0.11	0.48	0.58	0.40	0.36	0.35	0.31
	High LYS ⁵	0.05	0.07	0.50		-0.01	0.07	0.85		-0.08	0.15	0.65	
FFMI	Low LYS ⁴	0.88	0.59	0.14	0.36	0.71	0.48	0.15	0.07	2.65	2.10	0.30	0.65
	High LYS ⁵	0.34	0.32	0.29		-0.17	0.32	0.59		1.48	0.98	0.23	

		Girls N=109				Girls ¹ N=89				Girls ² N=20			
		β	SE	p	AxL ³	β	SE	p	AxL ³	β	SE	p	AxL ³
Height	Low LYS ⁴	3.62	1.18	0.00	0.65	3.37	1.17	0.01	0.55	17.18	10.80	0.19	0.68
	High LYS ⁵	4.09	1.40	0.01		4.02	1.42	0.01		14.35	14.61	0.38	
FMI	Low LYS ⁴	0.22	0.50	0.66	0.03	0.21	0.56	0.71	0.03	-0.41	2.84	0.91	0.92
	High LYS ⁵	-0.74	0.60	0.22		-0.90	0.69	0.20		-0.57	3.75	0.91	
FFMI	Low LYS ⁴	0.50	0.36	0.17	0.93	0.50	0.37	0.18	0.78	8.24	5.50	0.38	0.51
	High LYS ⁵	0.47	0.43	0.28		0.41	0.46	0.37		10.17	6.78	0.37	

¹BMI-quintiles 1-4, ²BMI-quintile 5, ³Arginine X Lysine interactions, ⁴LYS < 4.25g/day, ⁵LYS > 4.25g/day, ARG = Arginine, LYS = Lysine, FFMI= Fat Free Mass Index (kg/m²), FMI= Fat Mass Index (kg/m²)

Association between change in FMI and habitual intake of PROT, and specifically ARG and LYS intake

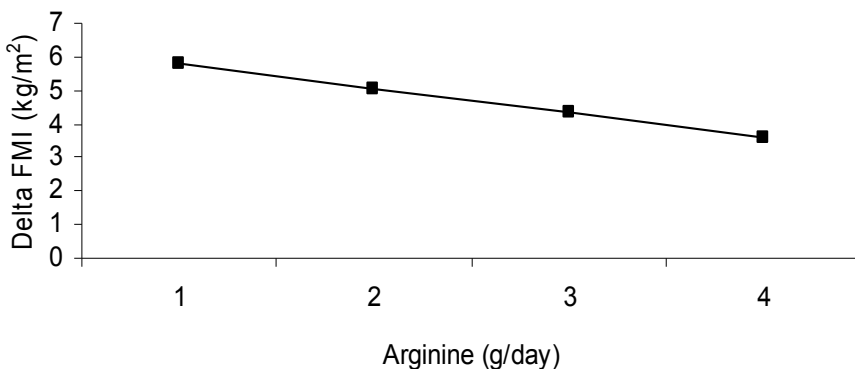
Significant associations were found between changes in FMI and habitual intakes of PROT as well as for the specific amino acids ARG or LYS separately or combined ($\beta = -0.03 \pm 0.01$ $p = 0.01$, $\beta = -0.51 \pm 0.11$ $p = 0.01$, $\beta = -0.30 \pm 0.06$ $p = 0.01$ respectively) among girls with a BMI in the 5th percentile, but not among the girls with a BMI in the 1-4th quintile (Table 2 and 3). Among boys, no associations were found between Δ FMI and the intake of PROT, significant associations however were found between Δ FMI and the intake of the specific amino acid LYS, among boys with a BMI in the 5th quintile ($\beta = -0.57 \pm 0.13$ $p = 0.01$) (Table 2).

Further analyses showed that ARG and LYS were not independently related to changes in Δ FMI ($p = 0.03$) (Table 3). The interaction analysis showed that among girls with a BMI in the 1-4th quintile, a stronger inverse correlation was found between Δ FMI and ARG intake if LYS intake was high, than if LYS intake was low ($p = 0.03$) (Fig 1).

Association between change in FFMI and habitual intake of PROT, and specifically ARG and LYS intake

No associations were found between change in FFMI and habitual intake of PROT, ARG or LYS (Table 2).

Figure 1: Interaction of ARG and LYS (g/day) and FMI (kg/m^2) among girls. -■-. high LYS ($> 4.25\text{g}/\text{day}$)
ARG = Arginine, LYS = Lysine, FMI = Fat Mass Index



DISCUSSION

The present study shows that linear growth as well as the development of body composition may be influenced by habitual dietary intake of the amino acids ARG and LYS, among pre-pubertal girls.

The association between linear growth and the intake of ARG, may be explained by the stimulation of the somatotrophic axis by ARG, since ARG is a potent stimulator of GH secretion¹⁶ and GH plays an important role in linear growth^{5, 34}. This association only reached significance in girls ($p < 0.05$), but also in boys the association had the same direction and magnitude ($p = 0.10$). For the intake of PROT in general or LYS the association with linear growth was weaker, indicating that in particular ARG is an important dietary determinant of linear growth. We could, however, not confirm the involvement of the somatotrophic axis within this study as plasma IGF-1 concentrations were not available in this cohort. Previous studies, investigating the effects of dietary protein source on growth in height showed increased IGF-1 concentrations as well as growth in height after intake of milk protein, but not after intake of animal or vegetable protein, which suggests an influence of specific amino acid concentrations or combinations influencing IGF-1 concentrations and growth in height^{5, 13}.

Besides the association between ARG and linear growth, high intakes of ARG, combined with high intakes of LYS, is associated with a relative decrease in FMI. This may also be explained by stimulating effects of these amino acids on GH secretion^{16, 17}, since GH is known to decrease body fat mass index³⁵. The sub-group analyses were based on small numbers which may have prevented the finding of significant associations between protein intake and FMI for the boys. All associations however were in the same direction for boys and girls, which suggest that protein was of importance for growth.

The interactions between the amino acids suggest that the effect of proteins is depending on the available amount and combination of specific amino acids, such as ARG and LYS as demonstrated in the present study. The somatotrophic regulation of growth and body composition could thus be modulated by privileged amino acids and proteins¹. In a recent study, we found remarkable differences in the potency of various dietary proteins to stimulate the somatotrophic activity, with gelatin (high in ARG) showing the largest increase on GH secretion among other proteins²².

In the present study, the mean intake per day of PROT was 66 ± 16 gram/day (2.6 gram/kg/day), which is considerably above the requirement of 0.95 g/kg/day [NHANES III]. However, the same protein intake was also seen in other studies among Danish children^{1, 21}. The mean intake per day of ARG was 2.6 ± 0.8 gram/day and for LYS 4.3 ± 1.2 gram/day, which is in accordance with the requirements for adults [NHANES III]. For children no accepted requirement values are published

[WHO report 2007]. ARG is present in large amounts in products like nuts, seeds, fish and meat, and particularly high concentrations are present in walnuts, sesame-seeds and shell-fish. LYS is present in meat and milk-products and is found in particularly high amounts in beef and cheese.

It should be noted that information on dietary intakes was obtained using a 7-days food record, and that measurement error may have occurred, as also indicated by the lower total energy intake among the children in the 5th BMI quintile compared with the lean children. However, all dietary instruments used to assess habitual diet intake have random as well as specific errors³⁶. On the other hand studies have shown that although energy intake is frequently underreported, protein reporting is more accurate³⁷. Therefore, we believe that the present data, especially with respect to protein intake, indeed largely reflect true intake levels. Moreover, in order to be able to take energy intake into account as covariate, we excluded clear under reporters and over reporters. Misreporters consisted of a few under reporters, they were clearly inaccurate reporters. Over reporting occurred more often, since parents/ caretakers report on children^{38, 39}. The ability of the parents to quantify portion sizes causes errors in quantification, for instance, parents report what they believe their child should eat, rather than what they actually do eat³⁸.

The results of the present study suggest that a physiological role of habitual PROT intake may depend on the amount and the combination of specific amino acids that may add to our understanding of how to perform future dietary intervention among pre-pubertal children, on growth in height and the regulation of body composition in later life. This has to be further investigated by experimental designs.

In conclusion, in prepubertal girls linear growth may be influenced by habitual ARG intake and body fat gain may be relatively prevented over time by a high intake of the combination of the amino acids ARG and LYS.

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Association between dietary protein and change in body composition among children (EYHS)

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ABSTRACT

Background & Aim: Growth hormone affects body composition by a relatively reduced fat-mass and increased fat-free-mass. The intake of protein as well as the specific amino-acids arginine and lysine potently stimulate GH secretion. This study investigated associations between intakes of protein, arginine, lysine and subsequent 6-year change in body-composition among 8-10-year old children.

Methods: Data of 364 children were collected from Odense, Denmark, during 1997-1998 and 6-year later as part of the European Youth Heart Study. Body Mass Index among children was subdivided by fat free mass index (FFMI) and fat mass index (FMI), based on skinfold-measurements. Dietary intake was estimated via 24h-recall. Associations between intakes of protein as well as arginine, lysine and change in FFMI and FMI were analysed by multiple linear regressions, adjusted for social economic status, puberty stage and physical-activity-level.

Results: Among lean girls inverse associations were found between protein as well as arginine and lysine intake and change in FMI ($\beta=-1.12\pm 0.56, p=0.03$, $\beta=-1.10\pm 0.53, p=0.04$, $\beta=-1.13\pm 0.51, p=0.03$ respectively). Furthermore among girls with a body mass index in the 5th quintile, protein intake was associated with Δ FFMI ($p=0.04$), and more specific when LYS-intake was high, ARG-intake was associated with Δ FFMI ($p=0.04$).

Conclusion: Among girls high protein intakes may decrease body fat gain and increase fat free mass gain, depending on the available amounts and combinations of arginine and lysine.

INTRODUCTION

The prevalence of obesity is rapidly increasing worldwide among adults, adolescents and children. Characteristics of overweight and obesity include higher percentages of body fat and lower concentrations of growth hormone (GH) and Insulin like Growth Factor-1 (IGF-1) ¹. GH plays an important role in regulating fat distribution and reducing fat mass¹ and studies show an increased lipolysis and decreased fat mass after GH administration ^{1, 2}. Dietary proteins ^{3, 4}, in particular gelatin [submitted data of our previous work], as well as specific amino acids (AAs) ^{5, 6}, particularly arginine (ARG) alone or in combination with lysine (LYS) ^{4, 5}, have been shown to stimulate the somatotrophic axis. This protein induced somatotrophic activity may influence the development of body composition and could be underlying in the finding that PE% (protein intake as percentage from the energy intake) is negatively associated with ponderal index ⁷.

This study aimed to investigate the relationship between the intake of proteins during the prepubertal phase on the development of fat-mass (FM) and fat-free-mass (FFM) towards puberty. Since studies suggest that amino acid composition is crucial in understanding the mechanism through which protein may influence GH and IGF-1 production in relation to growth and body composition, we also investigated the role of specific amino acids, i.e., ARG and LYS, on the development of FM and FFM.

For this purpose, we studied the associations between the intakes of protein and more specifically the amino acids ARG and LYS in the diet and subsequent changes in FM and FFM, among 9 years old children.

MATERIALS AND METHODS

Subjects

The data were derived from the Danish part of the European Youth Heart Study (EYHS). The subjects all lived and attended school in the community of Odense in 1997-2003, and participated in both EYHS-I, as 8–10 year old third grade children, and in the follow-up study, EYHS-II, in 2003 as 14–16 year old ninth grade adolescents.

Altogether 711 8- 10 year old children, who attended schools in the county of Odense, Denmark, were invited to participate in the study, along with their parents. Sampling went as follows; Schools were stratified according to location (urban, suburban, rural) and socioeconomic profile of uptake area (high, middle, low). From each stratum, a proportional, two-stage cluster sample of children was selected. The primary units were the schools. The sampling frame for schools was a

complete list of public schools in Odense, from which schools were sampled using probability proportional to school size. The secondary units were the children within the schools, and equal numbers of children were sampled from each school. The children were allocated code numbers and randomly selected using random number tables.

Twenty-eight out of 35 schools were asked to participate and 25 agreed. The only two urban low-income area schools in Odense were sampled, 20 out of 24 schools from middle-income areas, two out of four schools in high-income areas, and four out of five rural area schools were sampled. Of the three nonparticipating schools, one was rural, one was urban from middleclass area, and one was urban from a low-income area. All these three schools gave interference with the educational process as reason for not participating.

In total 771 third grade children participated in EYHS-I and 384 of these children (214 girls and 170 boys) were re-examined in EYHS-II as ninth grade students, corresponding to 49.8 % of the original sample. One of the reasons for non-participation in the follow-up study was reluctance to travel to the city of Odense for the examinations by several of the participants in EYHS-I, who no longer lived in the community of Odense at the time EYHS-II was initiated. If the coefficient of participation is calculated only taking into account subjects who still attended school in the municipality of Odense in 2003, the coefficient rises from 49.8 % to 57.0 %. In total 180 girls and 148 boys with complete information on all variables were included in the present study.

Measurements

Dietary intake

Information on habitual dietary intake was obtained by a 24h recall, supported by a qualitative food record at the first measurement point. The children completed a qualitative food record at home, followed the next day by a face to face interactive interview carried out by the same interviewer for all children. Interviews were carried out on school days, so dietary intake was not obtained for Fridays and Saturdays. The quantity of foods eaten and meals was estimated using common household measures and food pictures^{8,9}. Afterwards, the interviewer computed the dietary information (i.e. energy content, protein content, AA content) in a database, by which it was possible to calculate nutrient information on the individual food items or whole meals and diets via the Danish national food composition tables¹⁰.

Calculation of FFM and FM

Skinfold thickness (SFT) was measured at the first and second measurement point (age 8-10 year and 14-16 year respectively) with a Harpenden calliper (John Bull; British Indicators Ltd, Birmingham, UK). SFT was obtained for each child twice on the left-hand side at four locations, the triceps brachii and biceps brachii muscles, subscapularly, and superior to the anterior superior iliac spine^{11, 12}, and were finally expressed as the sum of the four skinfold thicknesses in mm (Σ SF). The same male investigator made all the skinfold measurements on the boys, whereas two female investigators made all the measurements on the girls. The inter- and intra-test Pearson correlation coefficients were 0.988 and 0.998 between skinfold measurements.

The body fat percentage (fat%) was estimated from the skinfold measurements using the equations given by Weststrate et al (in 1989).;

- (i) girls aged 2–10 years: $\text{Fat}\% = ((562 - 1.1(\text{age} - 2))/D) - (525 - 1.4(\text{age} - 2))$ and
- (ii) girls aged 11-18 years: $\text{Fat}\% = ((533 - 7.3(\text{age} - 10))/D) - (514 - 8.0(\text{age} - 10))$ and
- (iii) boys aged 2–18 years: $\text{Fat}\% = ((562 - 4.2(\text{age} - 2))/D) - (525 - 4.7(\text{age} - 2))$

D=Total body density estimated from following equations: (i) girls aged 2–10y: $D = (1.1315 + 0.0004 * (\text{age} - 2)) - ((0.0719 - 0.0003 * (\text{age} - 2)) * \log 4\text{skin})$ and (ii) girls aged 11–18y: $D = (1.1350 + 0.0031 * (\text{age} - 10)) - ((0.0719 - 0.0003 * (\text{age} - 2)) * \log 4\text{skin})$ and (iii) boys aged 2–18y: $D = (1.1315 + 0.0018 * (\text{age} - 2)) - ((0.0719 - 0.0006 * (\text{age} - 2)) * \log 4\text{skin})$ where $\log 4\text{skin} = \log(\Sigma \text{mean of the four skinfold thickness})$ and age in years.

FM and FFM were estimated from the body weight and the estimated fat%. Both FM and FFM were divided by height square (HT)², to calculate FFM index (FFMI) and FM index (FMI).

Other measurements

Physical activity was estimated with a questionnaire at the first measurement point. In this study, the question 'Exercise, which is most like you?' with possible answers; 'I don't exercise', 'I exercise sometimes but not regularly' and 'I exercise regularly' was used since this question was the most appropriate about the free-living physical activity. Results after using CSA-accelerometer as covariate showed the same results as using the questionnaire as covariate for physical activity. However only in 60% of the total sample we had measurements of PA by CSA accelerometer, therefore we decided to use the questionnaire as covariate for physical activity in the analysis.

The classification of socio-economic status (SES) was based on information regarding the adult female in the household at the first measurement point, since recent evidence suggests that the risk factors considered in this study are stronger associated with the SES of the mother rather than that of the father.

Assessment of puberty status was based on Tanner's pubic hair stages for boys and Tanner's breast development for girls.

Table 1: Characteristics of boys and girls aged 8-10 years and 14-16 years according to BMI quintiles

	Boys ¹ n=148			Boys ² n=118			Boys ³ n=30			Girls ¹ n=186			Girls ² n=148			Girls ³ n=38		
	Mean	±	SD	Mean	±	SD	Mean	±	SD	Mean	±	SD	Mean	±	SD	Mean	±	SD
8-10-year-old																		
Weight (kg)	33.6	±	5.8	31.6	±	5.9	41.4	±	5.3	33.1	±	4.7	31.0	±	4.4	41.1	±	5.8
Height (cm)	139.7	±	6.0	138.8	±	6.1	143.4	±	5.8	138.2	±	6.4	137.8	±	6.6	139.6	±	5.7
BMI (kg/m ²)	17.0	±	0.8	16.3	±	0.8	20.0	±	1.0	18.2	±	0.9	17.4	±	0.8	21.1	±	1.2
BMI z-score (min-max)	-2.54	-	1.78	-1.63	-	3.54	-0.97	-	2.74	-1.89	-	4.12	-2.16	-	1.97	-1.15	-	3.12
Sum of Skinfold (mm)	33	±	10	28	±	8	54	±	19	39	±	12	33	±	10	64	±	20
FFMI (kg/m ²)	13.9	±	0.8	13.7	±	0.8	14.9	±	0.8	12.8	±	0.8	12.5	±	0.8	13.9	±	0.8
FMI (kg/m ²)	3.1	±	0.8	2.6	±	0.7	5.1	±	1.4	5.4	±	1.1	4.9	±	1.0	7.2	±	1.7
SES (blue/white) %	45/55			44/66			47/53			40/60			38/62			47/53		
PA (1/2/3) %	7/31/62			7/30/63			9/38/53			9/44/47			8/42/50			10/51/39		
Tanner (1/2/3/4/5) %	100/0/0/0/0			100/0/0/0/0			100/0/0/0/0			71/28/1/0/0			80/20/0/0/0			38/57/5/0/0		
Energy intake (MJ/d)	9.5	±	2.6	9.5	±	2.5	9.4	±	3.0	8.8	±	2.1	9.0	±	2.2	8.1	±	1.8
Carb intake (g/d)	301.7	±	87.1	302.7	±	83.8	298.1	±	99.9	277.5	±	75.7	283.8	±	77.8	252.1	±	61.2
Fat intake (g/d)	82.9	±	30.1	83.4	±	28.4	80.8	±	36.5	77.8	±	26.0	79.3	±	26.5	71.9	±	22.9
PROT intake (g/d)	71.7	±	21.4	72.0	±	21.3	70.6	±	21.8	67.8	±	19.0	69.2	±	18.8	62.3	±	20.0
ARG intake (g/d)	3.0	±	1.2	3.0	±	1.2	2.9	±	1.2	2.8	±	1.0	2.9	±	1.0	2.6	±	1.0
LYS intake (g/d)	4.4	±	1.8	4.4	±	1.8	4.2	±	1.6	4.1	±	1.5	4.2	±	1.5	3.8	±	1.6
14-16-year-old																		
Weight (kg)	65.6	±	9.4	62.7	±	9.1	77.2	±	10.4	58.5	±	8.5	56.2	±	7.7	67.4	±	11.5
Height (cm)	176.3	±	7.2	175.5	±	7.4	179.5	±	6.5	165.2	±	6.7	165.2	±	6.7	165.1	±	6.8
BMI (kg/m ²)	21.1	±	1.2	20.3	±	1.2	24.1	±	1.2	18.0	±	1.5	16.3	±	1.3	24.7	±	2.2
BMI z-score min-max	-1.16	-	3.15	-1.21	-	2.89	-0.95	-	2.54	-1.56	-	2.30	-2.33	-	1.34	-1.21	-	3.98
Sum of Skinfold (mm)	35	±	18	30	±	14	55	±	32	52	±	15	48	±	13	70	±	23
FFMI (kg/m ²)	17.8	±	1.2	17.5	±	1.2	19.0	±	1.2	13.5	±	1.4	12.5	±	1.3	17.5	±	1.9
FMI (kg/m ²)	3.3	±	1.2	2.8	±	1.2	5.1	±	1.2	4.5	±	1.6	3.8	±	1.3	7.2	±	2.6

BMI= body mass index, ¹BMI-quintiles 1-4, ²BMI-quintile 5, ARG = arginine, LYS = lysine, FFMI= Fat Free Mass Index, FMI= Fat Mass Index, Carb= carbohydrate

Power calculation

A power calculation is verified, based on a number of 334 subjects, a two-sided significance level of 0.05 and a population correlation (ρ) between protein intake and body fat % of 0.14 for 8-10 y old children^{13, 14}. Using the equation for a regression analysis design, a power of 78% was calculated.

Statistical analysis

Each dietary and anthropometric variable was treated as a continuous variable. To improve the normality of their distribution, ARG, LYS and ZSF were logarithmically transformed. The relationship between intakes of PROT and more specific ARG or LYS at age 9 year and Δ FMI or Δ FFMI was assessed by a multiple regression model including baseline FFMI or FMI, age, energy intake, physical activity, SES, and puberty-stage as covariates. The interaction of ARG with LYS was estimated in a model with the same covariates as the model described before. This model was converted to the final regression model, by division of LYS into low and high LYS intake. Low LYS-intake was defined as LYS intakes below the median, and high LYS-intake as LYS intakes above the median. After analysing data of boys and girls, we divided boys and girls on BMI in either the 1-4th quintile or the 5th quintile. To test

the significance of the contribution of the variables, an F-test was used. All values are expressed as mean \pm SEM.

A p-value <0.05 was considered as indicating statistical significance. All statistical analyses were performed using SPSS for WINDOWS, version 14.0; SPSS, Chicago, IL.

RESULTS

The characteristics of the children, grouped by study year, gender and quintile of BMI are shown in Table 1.

The results are summarized in Table 2, with Δ FMI and Δ FFMI as dependent variables and habitual intakes of protein as well as the specific amino acids ARG, LYS as independent variables. Adjustments were made for baseline FMI or FFMI, age, energy intake, physical activity, SES and puberty-stage.

Association between intakes of protein, and specifically ARG and LYS intakes and changes in FMI and FFMI

Boys

Overall no significant associations were found for protein or for ARG or LYS intake and Δ FMI or Δ FFMI (Table 2).

Girls

The crude as well as the adjusted models showed associations between intakes of LYS and Δ FMI ($\beta = -1.16 \pm 0.56$ $p = 0.04$) (Table 2).

When dividing the girls according to BMI-quintiles, associations between PROT intake and Δ FMI ($\beta = -1.22 \pm 0.56$ $p = 0.03$), as well as ARG or LYS intake and Δ FMI ($\beta = -1.10 \pm 0.53$ $p = 0.04$, $\beta = -1.13 \pm 0.51$ $p = 0.03$ respectively) were found among those with a BMI in the 1-4th quintile (lean) (Table 2).

High PROT intake was positively associated with Δ FFMI in girls with a BMI in the 5th quintile ($p = 0.04$) (Table 2). In this group of girls, a stronger positive association was found between ARG intake and Δ FFMI when LYS intake was high (LYS intake above median), than when LYS intake was low (LYS intake below median) ($\beta = 8.87 \pm 0.52$, $\beta = -3.53 \pm 4.65$ respectively) ($p = 0.04$).

Table 2: Adjusted models showing the associations between both ARG and LYS(g/day) intakes and changes in FMI and FFMI(kg/m²)

		Boys			Boys ¹			Boys ²		
		β	SE	p	β	SE	p	β	SE	p
FMI (kg/m ²)	PROT	0.11	0.55	0.84	0.17	0.47	0.72	-0.4	2.76	0.89
	ARG	-0.32	0.49	0.56	-0.49	0.47	0.30	-0.90	2.01	0.66
	LYS	0.20	0.51	0.62	-0.45	0.42	0.29	-0.99	1.81	0.59
FFMI (kg/m ²)	PROT	0.27	0.62	0.67	0.16	0.63	0.81	0.00	2.66	1.00
	ARG	0.21	0.63	0.74	0.01	0.48	0.99	2.34	1.80	0.20
	LYS	0.12	0.57	0.84	0.15	0.47	0.75	2.01	1.54	0.20
		Girls			Girls ¹			Girls ²		
		β	SE	p	β	SE	p	β	SE	p
FMI (kg/m ²)	PROT	-0.92	0.64	0.15	-1.22	0.56	0.03	-3.21	2.72	0.25
	ARG	-1.12	0.60	0.06	-1.10	0.53	0.04	-0.22	2.57	0.93
	LYS	-1.16	0.56	0.04	-1.13	0.51	0.03	-0.29	2.27	0.90
FFMI (kg/m ²)	PROT	0.06	0.53	0.90	0.24	0.50	0.64	3.99	1.87	0.04
	ARG	0.20	0.51	0.70	0.13	0.68	0.85	0.27	1.84	0.89
	LYS	0.06	0.48	0.90	0.07	0.61	0.91	0.54	1.64	0.75

¹BMI-quintiles 1-4th, ²BMI-quintile 5th, ARG = arginine, LYS = lysine, FFMI= Fat Free Mass Index, FMI= Fat Mass Index

DISCUSSION

The present study shows that in girls dietary protein and the specific amino acids ARG and LYS may have influenced the development of body composition during puberty development.

Higher intakes of protein and specifically the amino acids ARG or LYS, were associated with less gain in FM over 6 years among 9 year old lean girls. This observations are in accordance with somatotrophic effects of protein and the specific amino acids ARG and LYS⁴⁻⁶, which may result in decreased serum levels of triglycerides and relative amount of FM^{7,15}.

A high protein intake was associated with the biggest increase in FFMI in girls with a BMI in the 5th quintile. This may be explained by the stimulating effects of proteins and AA on muscle protein synthesis¹⁶, which, in turn, may partially be mediated by the GH-stimulating properties of proteins and AA combined, but not when the AA were consumed separately⁵.

The association between Δ FMI and the intake of ARG or LYS, as well as interactions between ARG and LYS on Δ FFMI suggest that the effects of proteins may depend on the available amount and combination of specific AA, such as ARG and LYS. The somatotrophic regulation of growth could thus be modulated by privileged AA.

The described associations were found to be significant among girls only, although the associations in boys were in general in the same direction. The stronger associations could be explained by gender-differences in body composition development among pubertal children. Girls' FFM continue to increase until the age of 15y,

FM until 17 year. In boys FFM increases until 19 year and FM decreases after 13 year. Since the children in our study were measured at age 8-10 year and 14-16 year, it is plausible that gender-differences in the development of body composition influenced outcomes.

In this study, the mean intake per day of protein was 70 ± 21 gram/day (2.1 gram/kg/day), which is considerably above the requirement of 0.95 g/kg/day [NHANES III]. ARG and LYS intake per day were in accordance with requirements for adults [NHANES III]. ARG and LYS concentrations are both high in products like milk, meat and fish. Nuts and seeds consist of large amounts of ARG. Beef and cheese contains high amounts of LYS. The present study found that a high intake of ARG combined with high intakes of LYS was associated with the maximum increase in FFMI during growth. Such a diet may be composed by a high consumption of the above mentioned products.

The dietary assessment method has some incorporated problems and it should be noted that measurement error may have occurred by using a 24 h recall method, as also indicated by the relatively low total energy intake and fat intake among the children within the highest BMI quintile, compared with the leaner girls. However, other studies have shown that although energy intake may be underreported in obese subjects, protein reporting seems relatively more accurate¹⁷⁻¹⁹.

The advantage of 24 h recall methods above 3-day food records, is the person contact that contributes to the reliability of the collected data. There is no literacy requirement and the respondent burden is relatively small. The administration time is short and the procedure does not alter food intake patterns. The disadvantage of recall methods is the under and over estimation of portion size²⁰. For the quantification of portion sizes a picture book, including country-specific dishes, with additional household measures and other relevant measurements is recommended. In our study, portion size is estimated using common household measures and food pictures, as is described in the method section, in order to reduce the under and over estimation. The 24h dietary recall is validated in several studies, with doubly labelled water, and have been shown to be representative of energy expenditure in children^{18, 21}. Furthermore in third grade children, the 24h recall method was validated with observational data collected at the same moment as the 24h recall²² and with a 3 days dietary record²³. The 24h recall provide a relatively good characterization of the population's average nutrient intake²⁴, as is also found in our sample. In the present study, the means as well as tenth and ninetieth percentile intakes of energy and macronutrients were of the same magnitude as those reported by children (7–10 years) and adolescents (15–18 years) in the Danish nationwide survey (1995) in spite of the fact that the nationwide survey used a 7 days estimated diet record²⁵. The estimation of the energy intake with the equation of Harris and Benedict, multiplied by a physical activity level of 1.7 results in an energy intake of the same magnitude as the reported energy intake in the present study.

However, there is day-to day variation in our dietary pattern, which can influence the results of the 24h recall. We agree that this is a limitation of the 24h recall, however we were particularly interested in the protein intake, which is known to have a lower variation between days ²⁶. Especially fibre intake and cholesterol intake showed a day to day variation ²⁴. Hence, the 24 h recall method used in the present study seems adequate in relation to the estimation of dietary protein intake.

The results of the present study may suggest that a physiological role of protein intake may depend on the amount and the combination of specific amino acids. This may add to our understanding of how to perform future dietary intervention on the regulation of body composition, even if this has to be further investigated by experimental designs.

In conclusion, among girls high intakes of protein may decrease body fat gain and increase fat free mass gain. The influence of a high protein intake seems furthermore dependent on the available amounts and combinations of the specific amino acids ARG and LYS.

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CHAPTER 7

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Pharmacological and physiological growth hormone stimulation tests to predict successful GH therapy in children

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ABSTRACT

Although the current use of growth hormone (GH) stimulation tests (GHST) is still subject to debate, the tests are widely used to diagnose GH deficiency. This literature review evaluates primarily the sensitivity, specificity and reliability of GHST and secondarily their convenience. The single pharmacological tests typically address only a single pathway in the complex physiological regulation of GH secretion and are therefore characterized by lower sensitivity, specificity and reliability than the combined pharmacological tests or physiological tests. In spite of the high levels of sensitivity, specificity and reliability, physiological tests require considerably more effort to perform, from the physician as well as from the child. Therefore, a need for an alternative, convenient, physiological GHST still remains. Oral ingestion of dietary protein is practically convenient and may induce more physiological stimulation of GH secretion, hence may be a promising valuable addition to the existing GHSTs in GH deficiency.

INTRODUCTION

Growth hormone (GH) stimulation tests are widely used to diagnose GH deficiency (GHD). The aetiology of GHD in children varies from the complete absence of growth hormone, leading to severe growth retardation, to a partial deficiency, leading to short stature^{1, 2}. The cause of GHD may be congenital or acquired. Congenital abnormalities range from neuro-endocrine dysfunction of GH secretion to isolated growth failure, with unknown aetiology. Acquired causes are a range of hypothalamic-pituitary axis insults, including tumours³. The exact definition of GHD remains controversial. In short and poorly growing children, GH secretion varies between a negligible GH secretion to a high GH secretion. An arbitrary, non scientific border was set between GHD and non-GHD as is described below.

Children with GHD usually present with short stature and low growth velocity for their age and pubertal stage⁴. It can be difficult to diagnose GHD, because of its variety in grades of severity, its broad space of physiological level and the fact that GH release is influenced by many factors such as age, pubertal stage, body fat mass, caloric intake and emotional stage^{5, 6}.

In October 1999 in Eilat, Israel, the Growth Hormone Research Society (GHRS) formulated Consensus guidelines used for diagnosing and treatment of children with GHD. In these guidelines, the diagnosis of GHD in childhood is defined as a multifaceted process requiring comprehensive auxological and clinical assessment, combined with radiological evaluation and biochemical tests of the GH- Insulin-like growth factor-1 (IGF-1) axis (also called GH stimulation tests (GHSTs)), as described below⁷.

Auxological assessment is based on parental height and growth velocity. Clinical assessment could involve the use of Magnetic Resonance Imaging (MRI) to identify abnormalities in the hypothalamic-pituitary region. Genetic disorders also have to be taken into account⁷. Radiological (x-ray) evaluation of the bone age should be used as part of the routine evaluation of children with growth failure over 1 yr of age.

Biochemical tests (i.e. single pharmacological GHSTs) are tests to evaluate the GH secretion capacity. These tests should be performed after an overnight fast using a standardized protocol. A canula is inserted in a vein and after 30 min the stimulating agent is administered orally or intravenously, dependent on the agent. Blood is sampled every 15 or 30 min for 3 hours. Stimulating agents include arginine, clonidine, glucagon, insulin and levo-dopamine. According to the Consensus, the diagnosis of GHD requires the traditionally peak GH concentration below 10 µg/L by radioimmunoassay (RIA), however the GHRS also mention the critics on this cut-point as the overlap between GHD and non-GHD in GH responses after GHSTs. In suspected GHD, without clear patho-physiological cause, two GH stimulation tests are required, whereas one suffices in children with a central nervous system pa-

thology, history of irradiation, multiple pituitary hypothalamic diseases or genetic defect. The use of 22-kDA hGH assay with monoclonal antibodies is recommended, to determine serum GH concentrations⁷.

In the absence of a gold standard, it is important for the clinician to integrate all available data before diagnosing, in order to prevent misdiagnosis and unnecessary, expensive therapy⁸. Although the use of GHSTs as biochemical tests to diagnose GHD has been a subject of debate for a long time, nevertheless, GHSTs are still essential instruments in diagnosing GHD, because there are no better alternatives available for evaluation of GH secretion^{7,9,10}.

This review describes literature reports of pharmacological and physiological GHSTs used in practical settings, and assesses them in terms of sensitivity, specificity, reliability and convenient for use in practice. First the requirements for GHSTs are described, followed by an overview of the current available tests, assessed in terms of the requirements. The outcomes are discussed, followed by a proposal for a new physiological GHST.

REQUIREMENT OF GHSTs

Requirements for GHSTs include primarily a high validity (sensitivity and specificity), reliability and secondarily convenience to use in practice. Each requirement is defined in this section and in the next section the requirements are used to assess GHSTs.

A valid test is defined as a test which measures what it is supposed to measure. A test must have an agreement with the diagnosis, as regulated by other measures or standards^{11,12}. In this review, validity is assessed by the specificity and sensitivity of a test. The sensitivity of the test is calculated as the number of children diagnosed with GHD who have a positive test result (the cut-off level is not exceeded in a child diagnosed with GHD) divided by the total number of children diagnosed with GHD. Specificity is the number of non-GHD children who have a negative test (the cut-off level is exceeded in a child defined as non-GHD) divided by the total number of children diagnosed as non-GHD. Sensitivity and specificity are expressed as percentages.

Reliability is defined as the consistency of a set of measurements or a measuring instrument. A reliable test will show the same result when the same test is repeated in the same subject on multiple occasions¹¹. Reliability is expressed as percentages of identical diagnose, as well as correlation coefficients (CC) and coefficients of variation (CV). The CV and CC seem to be more suitable for the evaluation of reliability than the percentage of identical diagnose because they are independent of the arbitrary cut-off levels.

In addition, convenience to use in practice is a general term, which includes the costs of the test, the duration and time spent by the doctor to perform the GHSTs as well as the impact on the child and the time it takes for the child to undergo the GHSTs in the hospital, preparations at home, invasivity and side-effects.

DESCRIPTION AND ASSESSMENT OF THE TESTS

Many different GHSTs have been used in children. Sizonenko et al.³ described at least 34 GHSTs that have been proposed and executed in 189 different combination protocols. The most common GHSTs and their characteristics are listed in table 1.

Many studies have been performed to assess the validity (sensitivity and specificity) and reliability of pharmacological and physiological GHSTs. Their results are summarized in Table 2. We assessed seven single stimulation tests (arginine (ARG), insulin-induced hypoglycaemia (ITT), clonidine, Levo-Dopamine, Growth Hormone Releasing Hormone (GHRH), propranolol, glucagon) as well as 2 combined stimulation tests (GHRH + propranolol, ARG + GHRH) and 2 physiological tests (sleep, exercise). The table shows details of each study, as used cut-off levels, assay used and handling of pubertal status, as well as the definition of GHD used in each study (reference value). In the assessment of validity, the definition of GHD used in the study is important, since the definition is the reference value for calculating sensitivity and specificity. Studies have defined GHD differently, varying from GH concentrations below 7 µg/L after ITT to a multiple diagnosis including clinical and biochemical measurements. Because of the inconsistency in the definition of GHD, as well as the inconsistency in cut-off levels used, assays used, and population (different ages, puberty stages and way to handle with priming), it is difficult to compare studies statistically in terms of sensitivity and specificity.

Although comparisons between studies are difficult, the ITT is widely described as the most sensitive and specific single stimulating test¹³. When comparing two tests with identical cut-off levels and definitions used to diagnose GHD, the ARG-test is more sensitive and specific than the clonidine test^{14, 15}. A very high validity of the clonidine test was found by Silva et al. and Zadik et al.^{16, 17}. This high validity in the study by Silva et al. is caused by the fact that these authors testing study-populations consisting of children with congenital GHD who showed very low GH concentrations¹⁶. The sensitivity of glucagon and Levo-Dopamine tests has not been tested as frequently. The specificity of the glucagon test is comparable with that of the ARG test and the specificity of the Levo-Dopamine test is comparable with that of the ITT, as was found by Ghigo et al.¹⁸.

Tests combining 2 stimulators, such as GHRH + ARG or pyridostigmine + GHRH are characterized by a high sensitivity and specificity^{13, 18} compared with the single stimulation tests. The physiological sleep test shows a higher validity than the ARG

test and is comparable in validity with the ITT^{19, 20}. The physiological exercise test shows a high sensitivity, but a low specificity²¹. Various studies have shown that increasing the cut-off level decreases the specificity of single stimulation tests and increases the sensitivity of the tests. However, the specificity of combined stimulating tests changes less by varying the cut-off levels. The most appropriate cut-off level of a test could be that which induces the highest sum of sensitivity and specificity, however this cut-off level seems to be different for each individual GHST^{12, 18, 21, 22}. Overall, the sensitivity and specificity of the GHSTs seem to be better in the combined pharmacological stimulation tests and sleep tests, compared with the single pharmacological stimulation tests.

Reliability of the GHSTs is shown in Table 3. We assessed six single stimulation tests (ARG, ITT, clonidine, Levo-Dopamine, ornithine, glucagon), 2 combined stimulation tests (ARG + ITT, glucagon-propranolol) and 2 physiological tests (sleep, continuous blood-sampling). The table shows details of each study, such as the cut-off level used, type of assay used, pubertal status at the first test and the time between the first test and the second test (retest). Overall the single GHSTs show a wide range of reliability outcomes; the coefficient of variation is high, while the percentage of identical diagnose and the regression coefficient between the two repeated tests are low, regardless of the time between test and retest^{9, 23-31}. The reliability of the GHSTs seems to be better in physiological tests than in single pharmacological tests^{17, 27, 28, 31-34}.

With respect to convenient use of the tests in practice, the effort from the physician and the effort from the patient, including side effects for the patient were evaluated. The characteristics of the tests are summarized in table 1. The single stimulation tests are characterized by a short duration, from 90 to 120 minutes (table 1). The exercise test also takes no more than 120 minutes^{18, 21}. The sleep test and the 12 or 24 h physiological GH-secretion test required an overnight admission³². Moreover, multiple sampling is inconvenient and time consuming^{20, 35}. Although no side-effects of these physiological tests are mentioned^{20, 21}, the single pharmacological GHSTs as well as the combined GHSTs however are well-known to induce side effects, such as hypoglycaemia, hypokalaemia, hypertension, nausea and vomiting^{14, 20, 32, 33, 36}, although not in all studies these side effects are reported. Several countries do not perform the ITT because of its dangerous adverse effects; severe hypoglycaemia and hypokalemia which may induce cardiac arrhythmia³⁷. Furthermore, single and combined stimulation tests require preparations for the patient at home, for instance no food intake is allowed during the night and in the morning before the test. Such preparations are not needed to perform the physiological tests.

For the physician, the physiological tests require more effort, as the patients has to stay in hospital overnight. In addition the physiological sleep test and 12-24 h continuous blood sampling test are quite expensive, because of the hospitalisation and the assays required for multiple samples^{32, 33, 38}. The exercise test is difficult to perform, because the level of exhaustion is arbitrary²¹.

Table 1: Description of the different growth hormone stimulation tests (GHST)

GHSTs (reference) Arginine (ARG) ^{15, 20, 21}	Brief description Infusion of ARG (0.5g/kg) in 30 min	Blood sampling -30, 0, 15, 30, 45, 60, 90, 120	Side-effects Vomiting	Disadvantage of the test Fasting after 22.00 h the night before the test	Possible mechanism ARG reduces SS and possibly stimulates α -adrenergic receptors with GHRH-release Clonidine is an α -adrenergic agonist and acts probably through stimulation of GHRH	Duration of the test (min) 120
Clonidine ^{14, 16, 18, 22, 24}	100 $\mu\text{g}/\text{m}^2$ -150 $\mu\text{g}/\text{m}^2$ orally	-30, 0, 30, 60, 90, 120, (150)/ every 15 min	Hypertensive effects	Fasting after 22.00 h the night before the test	Glucagon stimulates GH by the secondary hypoglycaemia following the hyperglycaemia induced by glucagon. Levo-dopa increases the GH secretion through dopaminergic and α -adrenergic pathways. ITT is based on pituitary responsiveness to hypoglycaemia. ITT suppresses SS and stimulates the α -adrenergic receptors	120/180
Glucagon ^{18, 21, 24}	Infusion of glucagon intramuscular (30-50 $\mu\text{g}/\text{kg}$)	(-30), 0, 30, 60, 90, 120, (150), 180/ every 15 min	Nausea, abdominal pain often	Fasting after 22.00 h the night before the test		180
Levo-dopamine ¹⁸	1.25, 250 or 500 mg orally	Every 15 min	Nausea, vomiting, vertigo	Fasting after 22.00 h the night before the test		180
Insulin tolerance test (ITT) ^{14, 15, 18, 21, 24, 36}	Infusion of insulin (0.05-0.1U/kg) Blood glucose is monitored throughout the test	-30, 0, (15), 30, (45), 60, 90, 120	Hypoglycaemia Hypokalaemia Arrhythmia	Fasting after 22.00 h the night before the test The test was considered valid if the plasma glucose conc. fell to a nadir which was less than half that of the fasting level, or was lower than 2.2 mmol/L)		120
Pyridostigmine (PD) ¹⁸ GHRH ¹⁸ GHRH- Pyridostigmine(PD) ¹⁸ GHRH-ARG ^{18, 18, 54}	60 mg orally 1 $\mu\text{g}/\text{kg}$ iv PD orally 60 min before GHRH Infusion of GHRH at start (time 0) and ARG over time from time 0 over 30 min. Propranolol was given (orally) 2 hr before the glucagon injection	Every 15 min -30, 0, 15, 30, 45, 60, 75, 90 -30, 0, 15, 30, 45, 60, 75, 90 -15, 0, 15, 30, 45, 60, 75, 90, -30, 0, 30, 60, 120, 150, 180	Cramps Facial flushing Cramps, flushing See ARG, facial flushing Nausea, vomiting, abdominal pain	Fasting after 22.00 h the night before the test Fasting after 22.00 h the night before the test Fasting after 22.00 h the night before the test Fasting after 22.00 h the night before the test		180 90 90 90 300
Sleep ^{20, 21, 42}	Before sleep, a canula was inserted. The time of the onset of sleep was observed	Insertion of the canula and at 15 min intervals, between 30 en 120 min after sleep onset or when a EEG is used, in 10 min intervals when stage IV is reached	-	One night in the hospital		App.12 h
Physical exercise ^{18, 21}	30 min rest between inserting the canula and start of exercise. Workload was increased step by step by increasing velocity and ascended angle until individual exhausting was achieved (Heart rates>180/5 mins>160/Respirator Quotient<1) Every 20-30 minutes for 12 or 24 h.	0, (10), 20, 30	-	The degree of physical fitness affects a GH release to exercise. For practical reasons it is difficult to formally exercise small children and the latency between the initiation of the exercise and the GH peak observed varies with the level of fitness of the patient. One night in the hospital		120 12/24h

GHRH = Growth Hormone Releasing Hormone, SS = Somatostatin

GHSTS UNDER DEBATE

The previous section reported higher validity and reliability of combined stimulation tests and physiological tests (sleep-test, 12-24h continuous blood sampling tests), compared with the single pharmacological stimulation tests, although the comparison between the tests are problematic due to the use of different protocols and target-groups. This section discusses the main criticisms on current used single pharmacological GHSTs (arginine, clonidine, glucagon, insulin and Levo-dopamine), a combined pharmacological test (GHRH+ARG) and physiological GHST (sleep test, exercise test, 12-24h sampling). We first discuss general criticisms, followed by specific criticisms on the single pharmacological test, combined pharmacological tests and physiological tests.

General criticisms

Using GHSTs in general raises problems. Firstly, the cut-off level of GH-concentration to distinguish between non-GHD and GHD is arbitrary, since there is no clear definition about the normal increase of GH concentration after GHST^{32, 39, 40}. For instance, some children grow perfectly well with a maximum peak of 7 µg/L^{31, 41} after a single pharmacological stimulation test, while poor or delayed growing children could have a maximum peak of 12 µg/L with the same test. From table 2 it becomes clear how cut-off levels determine the sensitivity and specificity of the test¹¹. Each test, however, has its own mechanism to stimulate GH secretion and therefore should need a matched cut-off level to distinguish between GHD and non-GHD^{9, 10, 36}. Conversion factors to transfer from one test to the other test may be necessary to make test-outcomes comparable¹⁰. The current arbitrary cut-off level used by the GHRS of 10 µg/L seems to be insufficient to distinguish non-GHD from GHD children with the currently used pharmacological GHSTs.

Secondly, there are variations in the measurement of GH concentrations in serum, resulting in a different diagnosis from the same sample due to the use of different assays, analyses in different laboratories and variety in unit conversion factor (mU/L to mcg/L)⁴²⁻⁴⁵. One of the causes of these variations is the use of various protocols of immunoassays regarding the interacting assay component³³. A mixture of antibodies with different affinity and specificity for the GH molecule is used in a polyclonal assay while a monoclonal assay uses either one or two monoclonal antibodies. As a result, total concentration of GH measured by a polyclonal assay and a monoclonal assay may not be equal⁴⁶. Moreover, half of GH in the circulation is bound to binding proteins which can interfere with the result and give either falsely high or falsely low GH readings, depending on the type of assay^{6, 46}. An example is given by a study from Levin et al., in which six of the samples showing normal results (>10 µg/L) measured with the polyclonal radioimmunoassay, were

classified as GHD ($<10 \mu\text{g/L}$) based on the results from a Hybritech radioimmunoassay⁴⁶. The newer assays, for instance immunofunctional assay (IFA), can give GH concentrations 2-3 times lower than the older immunoradiometric assay (IRMA), including magnetic immunoassay (MIA) and radio immunoassay (RIA)^{16, 44}. For the newer assays, the right border should be found⁴⁷. Although the GH Research Society recommends an assay which measures 22-kDa GH and uses monoclonal antibodies^{7, 45, 48}, there are still various GH assays used for the analysis of GH concentrations.

Thirdly, several factors influencing GH secretion may affect the results of the GHSTs, for example age, puberty, weight, stress, but also metabolic status and extent of spontaneous GH secretion before testing^{18, 39, 43}. Several studies reported that children who failed to increase their GH-concentrations above cut-off values with GH stimulation tests, showed normal responses after administration of sex steroids or after spontaneous puberty^{32, 33, 49}. Priming with sex steroids could reduce the amount of misdiagnosed patients with more than 50%⁵⁰, however, if GH secretion becomes normal after priming, it would be unclear whether to treat the patient or not, since the cause of deficiency cannot be clearly identified²³. The current Consensus of the GHRS does not give clarity about priming in pre-puberty⁷. Besides stage of puberty, also age is a factor influencing GH secretion⁵¹. Furthermore the amount and distribution of fat-mass and fat-free-mass influence GH secretion^{9, 10}. Stress, as emotional deprivation, is another important factor decreasing GH concentrations in children. Furthermore, the time interval since the last spontaneous gGH peak is influencing the response to GHST, for instance if there has been a GH peak shortly before the test, the response on the test is blunted¹⁰. Finally the metabolic status of the child is important when interpreting the GH concentration after a GHSTs. In single and combined stimulation tests, a child has to be fasted before and during the test.

Single pharmacological tests

Besides the general difficulties in distinguishing non-GHD from GHD, pharmacological tests have some specific issues. Single pharmacological stimulation tests do not present normal secretion dynamics and are non-physiological. Each stimulator has its own mechanism of influencing the GH secretion^{3, 52} which results in different ways of artificial and non-physiologic stimulation⁴³ and in discordant results when different tests are used in the same patients^{3, 11}. For instance, when a patient shows a low GH-response to a clonidine-test, where the mechanism is probably via GHRH, it can not be presumed that the GH response to an arginine-test (where the mechanism is probably via suppression of somatostatin) is also diminished.

Table 2: Sensitivity and specificity of Growth Hormone Stimulation Tests (GHSTs)

Author	Y	Test	Age	N	Information about pubertal status	Peak GH-response (µg/ml)	Assay	Cut-off level	Sensitivity %	Specificity %	Diagnoses GHD is defined as/ is based on
Youton ¹⁵	1	ARG	15 m - 17 Y	Group 1: 19 GH >7 mug/ml Group 2: 18 GH <3mug/ml Group 3: 23 >3x7mug/ml	3 patients; minimal evidence of puberty	ARG peak G1: 13.8 G2: 1.7 G3: 9.6	Doubly antibody method	7 (µg/L)	75	85	ITT-results below 7 µg/L
Health services human growth hormone committee ¹⁴	1	CLO	4.7 - 16.7 Y	64 short stature	No information	ITT positive: 28 mU/L ITT negative: 5 mU/L	IRP immunoassa	20 (µg/L)	65	69	ITT-results below 20 mU/L
Datani ¹⁶	1	ITT	3.9 - 11.9 Y	Group 1: 85 GHD Group 2: 63 non-GHD	All prepubertal	CLO positive: 32 mU/L CLO negative: 6 mU/L	Hybiteth Tandem-R IRMA	10 (µg/L) 13.5 (µg/L) 15 (µg/L) 20 (µg/L)	51 64 69 82	79 70 63 49	Growth velocity SDS < 0.8.
Tillmann ¹²	1	ARG (n=90) GLU (n=65) CLO (n=38)	10.3 - 4.8	Group 1: 60 GHD Group 2: 110 non-GHD	78 pre-pubertal/ early puberty, 24 primed 54 not	Group 1: 7.2 (0.5-42.2) Group 2: 18.5 (0.9-53.6)	Two-side IRMA	All tests together 5 (µg/L) 7.5 (µg/L) 10 (µg/L)	All tests together 53 73 85	All tests together 92 85 76	2x GHST < 10 ng/mL
Bright ⁶⁰	1	Various types of tests, no distinguish	8.5 ± 3.2	Group 1: 172 GHD Group 2: 64 non-GHD	All prepubertal	No information	No information	10 (µg/L)	82	25	Delta height SDS > 0.5 after 1 year of therapy
Silva ¹⁶	2	CLO	Group 1: 1.8 - 22.5 Group 2: 2.5 - 10.8	Group 1: 26 GHD Group 2: 30 non-GHD	All prepubertal	Group 1: 0.56-0.58 Group 2: 12.87-5.8	Monoclonal immuno-fluorometric (IFMA)	3.3 (µg/L)	100	93	Congenital GHD, 12 had isolated GHD and 14 had multiple hypothalamic-pituitary hormone deficiencies
Donaubauer ²¹	3	Group 1: ARG Group 2: ITT Group 3: EX Group 4: ARG	Group 1: 10.0 (5.2-16.1) Group 2: 10.7 (4.5-14.9)	Group 1: 30 GHD Group 2: 47 non-GHD	68 in Tanner 1 9 in Tanner 2-4	Group 1: ARG/ITT: 5.5 ± 0.5 EX: 4.1 ± 0.7 Group 2: ARG: 16.1 ± 1.3 EX: 5.0 ± 0.6	Synrelga	10 (µg/L)	EX: 90	EX: 11	2x GHST < 10 µg/L
Ciamparini ²²	2	CLO ARG GHRH+ARG (only in 13 ISS)	Group 1: 6.8 ± 4.0 Group 2: 9.2 ± 3.1	Group 1: 33 GHI Group 2: 56 ISS	Group 1: Except 1, all prepubertal Group 2: 15 pubertal T2-3, others	No information	Immuno-radiometric assay (IRMA)	ARG and CLO 7 (µg/L) 10 (µg/L)	ARG and CLO 100 66	ARG and CLO 57 78	clinical and biochemical ground

GROWTH HORMONE STIMULATION TESTS IN CHILDREN

Study	Test	Age	Subjects	Prepubertal	Information	RIA	GHRH+ARG (µg/L)	ITT	GHRH+ARG	Notes
Hindmarsh ¹⁹	1 ITT	9 m - 16y	Group 1: 31 GHD Group 2: 36 non-GHD	All prepubertal	No information	RIA	20 (µg/L) 15 mU/L	ITT 94 Sleep 97	GHRH+ARG 46	A low growth velocity (height velocity SDS for chronological age < 1SD)
	9 Sleep-test (with EEG)									
	8 Sleep-test (with EEG)									
King ²⁰	1 Sleep-test ARG	2 - 16y	28	Children over 12 y and prepubertal; primed with androgens or estrogens	GHD group: ARG; 4.7 Sleep: 3.4 Non-GHD group: ARG; 24.8	RIA-IRP	15 mU/L	ARG 100 Sleep 100	ARG 72 Sleep 95	Clinical and biochemical ground
	9 Sleep-test ARG									
	8 Sleep-test ARG									
Ghigo ¹⁸	1 -PE	11.2 ± 0.1	472 children	269 prepubertal 203 pubertal Stage 2-4	Sleep: 24.8 PE: 12.7 ± 1.1 IH: 13.2 ± 1.2 Arg: 16.7 ± 1.2 L-d: 13.0 ± 1.1 Glu: 16.9 ± 1.9 PD: 13.5 ± 1.0 GHRH: 28.8 ± 2.0 PD+GHRH: 47.8 ± 1.9	IRMA (HGH-CPK)	7/10	-	PE: 85-64 IH: 76-51 Arg: 87-67 Clo: 90-78 L-d: 76-64 Glu: 90-65 PD: 85-64 GHRH: 91-85 PD+GHRH: 100 ARG+GHRH: 100	Growth velocity < -0.8SDS, lower bone age and lower IGF-1 levels.
	9 -IIT									
	9 -ARG									
	6 -CLO									
	-L-D									
	-GLU									
	-PD									
	-GHRH									
	-PD+GHRH									
	-ARG+GHRH									
Maghnie ¹⁵	2 GHRH+ARG	9.6 ± 3.1	GHD: 36; male, 24 female	No information	ITT: 2.1 ARG: 1.5 GHRH+ARG: 8.3	IFMA	GHRH+ARG 20 (µg/L) 24.2 (µg/L)	GHRH+ARG 75 86.1	GHRH+ARG 96.4 95.2	2x GHST < 10 ng/mL Insulin and Arginine
	0									
	2									

ARG=arginine, CLO=clonidine, ITT=insulin tolerance test, GLU=glutamine, EX=exercise, GHRH=growth hormone releasing hormone, m=months, y=years, GHD=growth hormone deficiency, µg/L=microgram/litre, mU/L=microunits/liter, PD= Pyridostigmine

Table 3: Reliability of Growth Hormone Stimulation Tests (GHSTs)

Author	Year	First Test	Age	N	Pubertal status	Assay	Cut-off level	Reliability	Time between test-retest
Tassoni ²⁸	1991	ARG L-D	11.2 ± 2.4	49	short stature No information	RIA	Arginine / L-dopa: 8 µg/L mean Sleep concentration 3.3 µg/L	CV %: ARG 86.5 % L-D 77.6 % Sleep 31.3 %	1-3 week
Nicholson ²⁶	1991	ITT ARG	11.1	32	Idiopathic GHD; Radiation induced GHD;	Two-side IRMA	9 mU/L 20mU/L	Percentage of same diagnose; Idiopathic GHD 9 mU/L,69% 20 mU/L 77% Radiation induced GHD 9 mU/L,100%	After completing GH therapy, mean age: 17.5
Wacharasindhu ³⁰	1991	ITT	8.5 -15.6	8	idiopathic GHD	IRMA	15 mU/L	Percentage of same diagnose; 22%	Retest 3 months after completion of linear growth and stop GH-treatment, age: 15.1-19.6
Loche ²⁵	1991	CLO ARG ITT	5.2 - 10	33	All prepubertal	IFMA	10 µg//L	Percentage of same diagnose; 15 %	1-6 months
Hilzner ²⁴	1992	CLO GLU	10.1 ± 3.1	84	No info	Two chemoluminescence enzyme immunometric assay	10 µg/L	Correlation: CLO: r = 0.22. Within subject variability of GH peak was 43.3%. GLU: r = 0.08. Within subject variability of GH peak was 50.3%	1.8 ± 1.4y
Tauber ²⁹	1992	ARG/L-D/ CLO-betaxolol	16.7 ± 1.7	131	A small number of patients were primed with sex steroids before therapy	RIA / IRMA	10 µg/L	Percentage of same diagnose; Partial GHD 29 % Complete GHD 64 %	Retest in the first year following the therapy and at least 15 days after the last GH injection
Carel ⁹	1993	-ITT -AI -GP	10.5 ± 4.9	3233	Prepubertal 2585 Pubertal 648	No information	Not important for CC (R)	Correlation: ITT/ITT: r = 0.72 AI/AI: r = 0.60 GP/GP: r = 0.37	Both tests performed before treatment with GH
Zadik ³¹	1993	24h ICGH CLO ITT ARG	9.1 ± 1.8	40	No information	Combined antibody assay	-	Correlation: 24hr r = 0.859 CLO r = 0.487 ITT r = 0.453 Arg r = 0.326	4 weeks

Cacciari ²³	1	ARG/L-D	9.6 ± 2.8	184	140 prepubertal 40 puberty (annerstage ≥ 2)	Solid-phase sandwich IFMA	ARG/L-dopa: 8 µg/L Sleep 3.3 µg/L	Percentage of same diagnose 1 st retest ARG/L-D: 79 % Sleep: 75 % 2 nd retest compared with first test ARG/L-D: 71 % Sleep: 75 %	Retest 2.8 ± 1.14 weeks after start therapy
	9	Sleep							
	9								
	4								
Ropelato ²⁷	1	Spontaneous	10.5 ± 2.0	15	All prepubertal	Polyclonal combined antibody RIA	Arginine/Clonidine: 10 µg/L Spontaneous GH secretion; maximum spontaneous GH/peak/12h: 10 µg/L	Spontaneous GH secretion; 36 % CLO: 71 % ARG: 65 %	1 week
	9	GH secretion							
	9	CLO							
Donaldson ³⁴	6	ARG	5-16	55	All prepubertal	Monoclonal hybritech IRMA	4 µg/L (max peak) 0.7 µg/L (pooled GH)	Percentage of same diagnose; Max peak: 96% Pooled GH: 99%	One day
	1	Sleep							
	9								

ARG=arginine, CLO=clonidine, ITT=insulin tolerance test, GLU=glutamine, EX=exerdis, GHRH=growth hormone releasing hormone, L-D=Levo-Dopa, AI=ITT+ARG, GP=GLU+propranolol, 24h IC-GH=24h continue growth hormone sampling, CC%=correlation coefficient, r=regression coefficient, CV%= coefficient of variation, p<0.05=significantly, GHD=growth hormone deficiency, µg/L=microgram/litre, mU/L=microunits/liter

Furthermore all pharmacological agents stimulating GH secretion have side-effects, including nausea, somnolence and hypotension, hypoglycemia, hypokalaemia, cardiac arrhythmia^{33 36}.

Combined pharmacological tests

In practice, stimulation tests combining GH stimulation drugs, i.e. GHRH+ARG, GHRH+pyridostigmine, ITT+ARG or somatostatin+GHRH seem to be more appropriate, compared with the single GH stimulation tests, because of a reduced variability of the GH response⁵³. The reduced variability in GH response is caused by a more physiological way of GH secretion (due to two stimulation agents) and less influence of age and puberty status¹⁸. Some authors suggest to consider these tests as most reliable tests to assess GH secretory status in children, because these tests are able to assess the maximum secretory capacity of somatotrophic cells^{3, 18, 54}. These more physiological tests are however less tested in children, and appropriate cut-off limits need to be applied¹³. Although side-effects of the combined pharmacological tests are not well known, it could be that the same side-effects as the single pharmacological tests will occur.

Physiological tests

Physiological tests such as the sleep test, exercise test and 24h continuously blood sampling are not frequently used, because of practical problems as described in the previous chapter. Although, the sleep test shows in common a higher sensitivity, specificity and reliability, compared with the single pharmacological tests and some authors have suggested physiological assessment of GH secretion may be a more relevant method of identifying children who might benefit from treatment with GH than the single pharmacological tests^{8, 19, 28, 34, 36}. The 24h continuously blood sampling test showed a high reliability^{27, 34, 49}, although authors may experience the test to distinguish normal from partial GHD difficult, because of the small differences in the 24h GH secretion, especially in puberty^{5, 10, 38, 43, 52, 53}. Furthermore inconvenience to use in practice as described in the previous section is a critic on the 24h continuous blood sampling^{20, 32, 33, 38}.

This review shows many problems in the tests used to diagnose GHD, which is most problematic in the diagnosis of partial GHD. Still single pharmacological stimuli of GH secretion with arginine, insulin, glucagon, GHRH, clonidine and L-dopamine are frequently used and considered in the Consensus of the GHRS to predict successful GH therapy.

NEW APPROACH FOR GHSTS

Besides the two established physiological GH stimulators sleep and exercise, nutrition also influences the GH secretion. Previous studies have shown that oral ingestion of amino acids (AAs), in particular oral ingestion of arginine (ARG) combined with equal amounts of lysine LYS (ARG:LYS in a ratio of 1:1), stimulates GH secretion potently⁵⁵. We found that ingestion of soy protein (containing relatively high amounts of arginine and lysine in a ratio of ARG:LYS 1:1) increases the GH secretion even to a higher extent than similar amounts of ARG combined with LYS supplemented as single amino acids⁵⁶. The observation that protein, ingested in a physiological dose of 0.6 gram protein per kg bodyweight, simulates the GH secretion to the same physiological level as either exercise or sleep^{21, 23}, suggests valuable application in the use of protein as stimulation agent in GHSTS.

We did not experience adverse effects during previous studies with such dosages of protein in young women. Ingestion of proteins is cheap, compared with the pharmacological stimuli and it is as easy to ingest as a drink yogurt.

It may be hypothesized that ingestion of dietary protein affects GH secretion through multiple mechanisms, including gastrointestinal signals such as ghrelin⁵⁷, other endocrine signals such as insulin⁵⁶, as well as changes in plasma amino acid concentrations⁵⁶. This makes dietary protein ingestion a more multifactorial and physiological stimulation of GH secretion than the unifactorial pharmacological GHSTS. Given the higher specificity, sensitivity and reliability of physiological tests in general, or compared with the single pharmacological GHSTS, the above described test may be a promising valuable addition to the existing GHSTS in diagnosing GHD.

However, the described test has not yet been applied in children and has to be further validated with respect to validity and reproducibility. This includes amongst other studies on the predicting value of protein-induced elevation of GH levels for the efficiency of GH treatment in children suspected of GHD.

SUMMARY/ CONCLUSION

Since the original observation of low or undetectable serum GH levels in fasting normal children and the extensive overlap in auxological parameters between GHD- and normal- children, the use of physiological or pharmacological stimuli in a provocation test has been the cornerstone for the diagnosis of GHD^{32, 53}. The current Consensus of the GH Research Society advises among other parameters, to choose 2 pharmacological stimulation tests to predict successful GH therapy⁷. Many factors, however, influence the outcomes of the current used single pharma-

colological stimulation tests, resulting in more confusing than clarifying the issue^{11, 19, 58}. Therefore, improving the quality of GH stimulation tests is important. The quality of the currently used tests can be improved by reducing the tests to maximally 2 or 3 standard tests, depleting a cut-off level valid for each separate test, using the same international protocol and assay for all tests in all laboratories⁴⁵. Moreover, factors such as age, puberty stage, stress, metabolic status must be considered, probably by using matched cut-off levels for different groups. Despite these quality-improvement suggestions, the single stimulation tests remain non-physiological, which results in different ways of stimulation and the induction of a high inter-individual variability between tests. An alternative, more physiological approach to diagnose GHD in children should be applied or developed to improve measurement- quality in predicting successful therapy. Tests combining two stimulation agents stimulate GH-secretion more physiological than tests using one agent. A sleep test does stimulate GH secretion in a physiological way, but has some practical difficulties. Therefore, a need for an alternative, convenient, physiological GHST still remains. Oral ingestion of dietary protein is practically convenient and may induce a more physiological stimulation of GH secretion. Hence it may provide a valuable addition to the existing GHSTs in GHD.

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Acute ingestion of gelatin protein increases blunted GH concentrations in visceral obesity

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ABSTRACT

Growth hormone (GH), a hormone originating from the anterior pituitary gland, is an important regulator of metabolism and body composition. Low GH secretion is associated with features of the metabolic syndrome, in particular increased visceral body fat and decreased lean body mass. It has been shown that GH release can be promoted by ingestion of protein, in particular gelatin protein. The question remains; is the GH-promoting effect of gelatin protein is also present in a population with blunted GH response, such as in visceral obesity.

Eight lean women (age: 23 ± 3 y, BMI: 21.6 ± 2.0 kg/m²) and eight visceral obese women (age: 28 ± 7 y, BMI: 33.8 ± 5.5 kg/m²) were compared with regard to their GH response after oral ingestion of gelatin protein (gelatin) (0.6 gram protein per kg bodyweight), placebo (water), or injection of growth hormone releasing hormone (GHRH) (1μ /kg body weight), in a randomized crossover design.

GH response after placebo, gelatin and GHRH were higher ($p < 0.05$) in lean subjects compared to that in visceral obese subjects ($p < 0.05$). Ingestion of gelatine ($p < 0.05$) increased GH response in both visceral obese (182.1 ± 81.6 μ g/L.5h vs. 28.4 ± 29.8 μ g/L.5h) and lean (631.7 ± 144.2 μ g/L.5h vs. 241.0 ± 196.8 μ g/L.5h) subjects. GH concentration in visceral obese subjects after ingestion of gelatin did not differ from those in lean, placebo-treated subjects ($p = 0.45$).

Thus, the dietary protein gelatin stimulates GH secretion in visceral obese subjects, to the levels found in placebo-treated lean subjects. Further research is necessary to investigate the relevance of protein as diagnostic tool in hyposomatotropism or in long term as improvement of metabolic abnormalities in hyposomatotropism.

INTRODUCTION

Growth hormone (GH), a hormone originating from the anterior pituitary gland, is an important regulator of metabolism and body composition^{1,2}. Low GH concentrations are associated with increased body fat, in particular in the visceral regions, and decreased lean body mass^{3,4}, known as independent risk factors for cardiovascular disease and type 2 diabetes mellitus, often clustered with insulin resistance, hypertension and dyslipidemia, and defined as the metabolic syndrome⁵. Diurnal GH concentrations are reduced by 75% in obese men compared to age-matched normal weight subjects⁶. The proportion of visceral fat of total body fat has been found to be a stronger predictor of diurnal GH release than the proportion total body fat of body mass⁷.

In addition to low 24h GH concentrations, decreased responsiveness to GH stimulation tests (GHST) has been demonstrated in subjects with obesity and in particular in those with abdominal adiposity⁸⁻¹⁰. GH response to the GHRH-arginine GHST is negatively associated with indices of central abdominal obesity, including waist circumference, trunk fat, and visceral adipose tissue¹¹. The state of visceral obesity, with reduced basal and 24h GH release as well as low responsiveness to GH stimulation tests is defined as hyposomatotropism^{12,13} and has several similarities with adult GH deficiency, including increased abdominal fat depots, insulin resistance, high serum levels of triglycerides (TG) and low serum levels of high-density lipoprotein (HDL)-cholesterol^{7,14,15}. GH administration has shown to reverse the metabolic abnormalities in adult hyposomatotropism and thereby decrease visceral fat mass, increase muscle mass and improve insulin sensitivity^{16,17}.

It has been shown that GH secretion can be promoted by intravenous or oral administration of various amino acids (AAs), either alone or in combination^{18,19}. Especially ingestion of the AA arginine (ARG) was found to be a strong stimulator of GH secretion^{20,21}. Recently, we reported that ingestion of various dietary proteins, including soy, milk, alpha lactalbumin and gelatin, stimulates the GH secretion in lean subjects, with the ARG-rich gelatin being the most potent stimulator (a 4 fold increase compared with baseline)²². It remains, however, to be established if this GH-promoting effect of gelatin protein is also present in a population with blunted GH secretion, such as in visceral obesity. This would offer a non-pharmacologic option to counteract the negative consequences of hyposomatotropism. To examine this question, lean and obese subjects were compared with regard to their somatotropic response to a growth hormone stimulation test (i.e. growth hormone releasing hormone), oral ingestion of gelatin protein and a placebo drink.

METHODS

Subjects

Subjects were recruited via local advertisements. Sixteen females participated. Eight had a BMI below 23 kg/m^2 and a waist circumference below 80 cm and eight a BMI above 30 kg/m^2 and a waist circumference above 90 cm. Each subject was in good health, non smoking, and used oral contraceptives. They were free of any other medication and spent less than three hours a week on sport activities. All tests were performed in the early follicular phase of the menstrual cycle, in order to eliminate confounding by changing serum estrogens concentrations. The Medical Ethics Committee of Maastricht University approved the study protocol and all subjects gave their written informed consent before participating in the study.

Experimental design

A randomized crossover study design was applied. Subjects reported to the laboratory at three different days in the morning. A standard GHRH stimulation test was performed once and subjects received a test drink (gelatin or placebo) twice. They were instructed to fast from 10 pm the night prior to the test day. A permanent canula was placed into a dorsal arm vein of the contralateral arm.

During the standard GHRH stimulation test, 1 microgram GHRH (FERRING B.V., Hoofddorp, The Netherlands) per kg bodyweight was administered, 40 min after placement of the canula. Just before and at 15, 30, 45, 60, 75 and 90 min after the injection of GHRH, blood was sampled to analyse GH, insulin and glucose concentrations

During the other 2 test days, blood sampling started 40 min after placement of the canula. Immediately after obtaining the first blood sample, subjects received a test drink, containing placebo or gelatin. The gelatin test drink contained 0.6 gram gelatin protein (Solugel LMC/3, 100PB Gelatins GmbH, Nienburg/Weser, Germany) per kg bodyweight dissolved in 400 ml water with 20 ml sugarfree syrup (Diaran, Cereal). The placebo test drink contained 400 ml water with 20 ml sugarfree syrup. The syrup was added for the taste and contained no protein and fat and a negligible amount of carbohydrates. Blood was sampled every 20 min for the next 5h to determine GH concentrations. Insulin and glucose concentrations were determined just before ingestion of the test drink and 40, 60, 180 and 300 min, after ingestion of the test drink. During blood sampling, subjects remained awake and were allowed to drink water ad libitum.

Anthropometric assessment

Measurement of waist circumference was performed in triplicate at the iliac crest in a standardized fashion with the subject in an upright position. Measurement of hip circumference was performed at the maximum circumference of the buttocks with the subject in an upright position. Body fat and fat free mass were determined by DXA (Discovery A, Hologic (Bedford, MA, USA)) testing. Measurements of regional adiposity using DEXA were standardized. The technique has a precision error of less than 1% for fat mass and lean body mass. BMI was calculated by total body weight/height², fat mass index (FMI) was calculated by absolute fat mass/ height².

Blood analysis

Venous blood was collected in clot tubes, containing 'Silica Clot Activator' (Becton Dickinson Vacutainer system; Becton Dickinson, Franklin Lakes, NJ, USA), and in heparinised tubes Becton Dickinson Vacutainer system; Becton Dickinson, Franklin Lakes, NJ, USA). Blood in these tubes was allowed to clot for 30 minutes and was centrifuged at 3000 rpm, 4°C for 10 min to obtain serum. Serum was collected for determination of GH concentrations. Blood in heparinised tubes was kept on ice to minimize enzymatic reactions and centrifuged at 3000 rpm at 4°C for 10 min to obtain plasma. Glucose and insulin analyses were performed in plasma. Each aliquot was frozen immediately in liquid nitrogen and stored at -80°C. All samples from the same subjects were run in the same assay.

The GH-concentrations were measured using an ultrasensitive GH chemiluminescence immunoassay (Beckman Coulter, Harbor Blvd. Fullerton, U.S.A.). The intra- and inter-assay coefficients of variation were 1.4-2.1 and 6.8-8.6% respectively, at GH concentrations of 3.7-14.2 and 3.1-7.3 µg/l, respectively. Insulin concentrations were measured by an electrochemiluminescence immunoassay (ELICA) (Roche Diagnostica, Hoffman-La Roche, Basel, Switzerland). Glucose concentrations were measured using enzymatic assay (G6-PDH) (Roche Diagnostica, Hoffman-La Roche, Basel, Switzerland).

Table 1: Characteristics of study subjects

	Lean women	Visceral obese women	p
n	8	8	
Age (yr)	23 ± 3	28 ± 7	0.12
Anthropometrics			
Height (cm)	169.8 ± 7.8	166.3 ± 3.3	0.26
Body weight (kg)	62.3 ± 7.7	93.3 ± 15.7	<0.001
BMI (kg/m ²)	21.6 ± 2.0	33.8 ± 5.5	<0.001
Waist circumference (cm)	71.7 ± 3.8	100.0 ± 10.3	<0.001
Hip circumference (cm)	93.6 ± 4.8	119.5 ± 17.2	<0.001
Waist:Hip (WHR)	0.7 ± 0.0	0.8 ± 0.0	<0.001
DEXA			
Total fat (kg)	15.5 ± 4.9	35.6 ± 9.5	<0.001
Trunk fat (kg)	6.1 ± 2.6	17.9 ± 5.8	<0.001
% Fat mass	25.4 ± 6.2	37.6 ± 6.6	<0.001
% Trunk fat from total fat mass	38.5 ± 6.2	49.7 ± 5.1	<0.001
FMI	5.3 ± 1.6	12.9 ± 3.3	<0.001
Insulin (basal) (mU/L)	8.1 ± 1.9	18.2 ± 3.0	<0.05
Glucose (basal) (mmol/L)	4.9 ± 0.2	5.8 ± 0.3	<0.05

FMI= fat mass index; (absolute fat mass/ height³)

Statistics

All data are expressed as mean +/- standard deviation (SD). Growth hormone, insulin and glucose responses were calculated as incremental area under the curve (i.e. area under the curve – baseline (Δ IAUC)) and peak values (i.e. peak – baseline (Δ peak)). The nonparametric Mann-Whitney U test was used to test for differences between lean subjects with visceral obese subjects. Repeated measures analysis of variance (ANOVA) was performed to compare between test conditions within one group. Associations between GH concentrations after injection of GHRH and ingestion of gelatin were calculated using the Peasons correlation. Coefficient of variation (CV%) in GH response is the ratio of the SD to the mean. A p-value <0.05 was regarded as statistically significant. All statistical analyses were performed using SPSS for WINDOWS, version 15.0; SPSS, Chicago, IL.

RESULTS

As shown in Table 1, visceral obese and lean subjects differed with respect to body weight, BMI, waist circumference, hip circumference, waist to hip ratio (WHR), total fat mass, trunk fat mass, % fat mass, % trunk fat mass from total fat mass and fat mass index (FMI) (p<0.001).

GH responses expressed as incremental area under the curve (IAUC) as well as Δ peak-values after i.v. injection of GHRH, were significantly higher in lean subjects compared to those with visceral obesity (p<0.05) confirming a blunted GH response to GHRH stimulation in our subjects with visceral obesity (Figure 1).

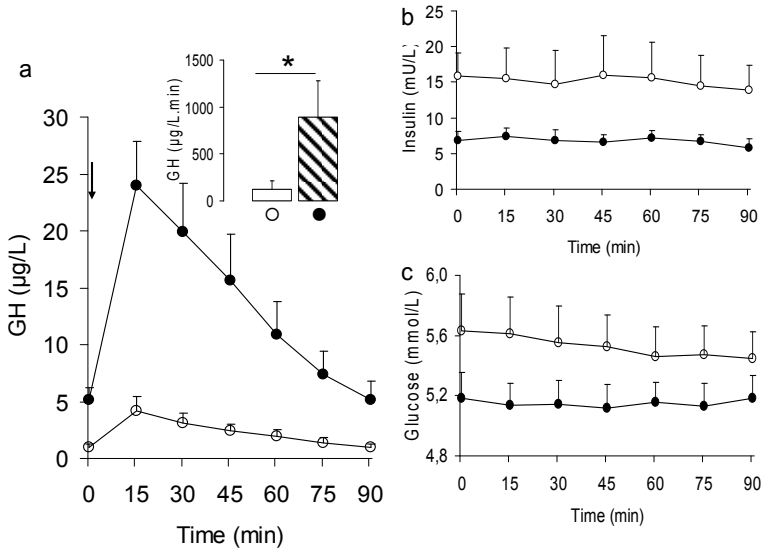


Figure 1: Growth hormone concentrations (a), insulin concentrations (b) and glucose concentrations (c) after i.v. injection of GHRH in lean subjects (●) (n=8) and visceral obese subjects (○) (n=8). Data are presented as mean (\pm SD) in lines. * = $p < 0.05$

In both visceral obese and well as in lean subjects, growth hormone responses (IAUC and Δ peak) were significantly higher after ingestion of gelatin compared to placebo ($p < 0.05$) (Figure 2). Basal GH-concentrations were significantly lower in visceral obese subjects, compared with lean subjects ($p < 0.05$). GH-responses (IAUC and Δ peak) after ingestion of gelatin protein or placebo, were significantly higher in lean subjects compared with visceral obese subjects ($p < 0.05$). GH responses as determined by IAUC as well as Δ peak-values after the placebo ingestion in lean subjects did not differ significantly from those after gelatin ingestion in visceral obese subjects ($p = 0.45$) (Figure 2). In the whole group, individual AUC and peak values of GH concentrations after GHRH injection correlated significantly with AUC and peak values of GH concentrations after gelatin ingestion (AUC; $r = 0.71$, $p < 0.01$, Peak; $r = 0.68$, $p < 0.01$). The coefficient of variation (CV%) in GH response in the lean subjects after GHRH was 39% and after gelatin protein 22%. In the obese subjects, the CV% in GH response after GHRH was 67% and after gelatin protein 43%. Insulin responses expressed as IAUC and Δ peak values were higher after ingestion of gelatin protein compared with placebo ($p < 0.05$). Basal insulin-concentrations (obese; 18.2 ± 3.0 mU/L vs. lean; 8.1 ± 1.9 mU/L) and basal glucose concentrations (obese; 5.8 ± 0.3 mmol/L vs. lean; 4.9 ± 0.2 mmol/L) were significantly higher in visceral obese than in lean subjects ($p < 0.05$) suggesting decreased insulin sensitivity in the visceral obese subjects.

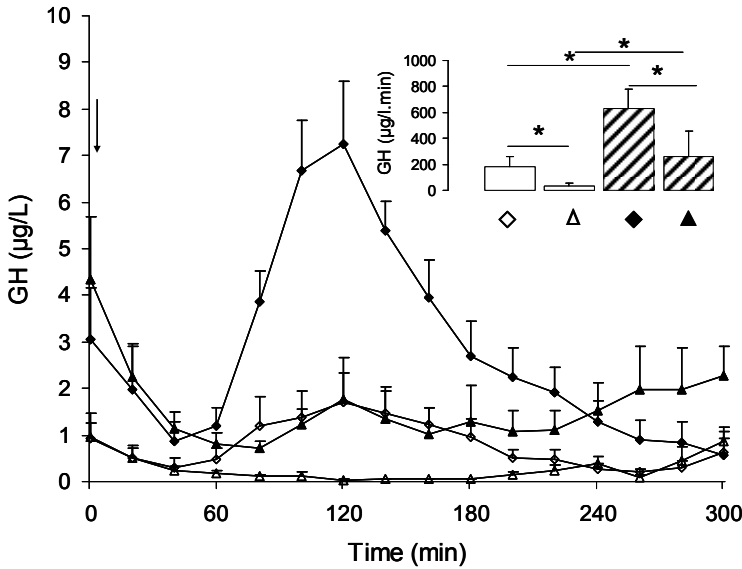


Figure 2: GH concentrations after ingestion of a test drink containing gelatin protein (◆=lean, ◇=visceral obese) or placebo (▲=lean, Δ=visceral obese). Data are presented as mean (± SD) in lines and bars (AUC). ↓= ingestion of test drink. * = p<0.05

DISCUSSION

This study shows that oral intake of a dietary protein (gelatin) stimulates the GH secretion in a visceral obese population, albeit to a lesser extent than in lean subjects. This resembles the blunted GH responses to GH stimulation tests (GHST) in visceral obese subjects as shown by others^{10,11}, and confirmed in this study. The GH concentrations after ingestion of gelatin protein in visceral obese subjects stimulates the GH release to the same levels found in placebo treated lean subjects.

We previously showed that protein ingestion stimulates GH secretion in healthy lean women, with gelatin protein as the strongest GH stimulator among different types of protein²². The present study shows that this GH promoting activity of dietary protein prevailed in the visceral obese subjects. The reduction in GH response to ingestion of gelatin in visceral obese subjects compared with the lean subjects is associated with reduced GH responses to GHRH stimulation. These data may imply an overall hypothalamic and pituitary mediated decreased GH secretory response and capacity in visceral obese subjects. Since GHRH is widely used as stimulating agent for a GH stimulation test (GHST) in the diagnosis of GHD, one may speculate that oral ingestion of a dietary protein such as gelatin is a potential alternative physiologic GH stimulator that can be used to diagnose GHD. The CV%

in GH response after GHRH is higher than after gelatin protein. Less variation may indicate that the test outcome distinguishes better between groups (hyposomatotropic and non-hyposomatotropic). In view of the complications and limitations of the current GHST's ²³, further studies on the validity and reliability of dietary protein ingestion in the diagnosis of GHD (i.e. in children suspected of GHD) seem appropriate.

Gelatin ingestion in visceral obese subjects increased circulating GH concentrations to the level of placebo-treated lean subjects. As shown in several studies, GH therapy, i.e., repeated i.v. injection of GH, can reverse the metabolic abnormalities shown in adults with hyposomatotropism ^{3, 13, 16, 17, 24-27}. A recent meta-analysis of *Mekala et al. 2009*, showed beneficial effects of GH therapy on body composition, including a decrease in body fat, increase in lean body mass, improvement in body fat distribution, as well as a decrease in WHR and visceral adiposity. GH therapy may also favourably affect lipid profile, leading to a decrease in total and LDL-cholesterol ²⁷. It seems reasonable to suggest that stimulation of endogenous GH secretion by other GH secretagogues may restore the reduced somatotrophic activity in visceral obese subjects, leading to improved values of parameters of cardiometabolic risk. A study in HIV patients with reduced GH concentrations investigated long term effect of GHRH administration ^{27, 28} and found normalised IGF-1 concentrations after administration of GHRH for 26 weeks, simultaneously reduced visceral adiposity and improvements in dyslipidemia were found ²⁸. Although the current study shows that ingestion of dietary protein acutely restores decreased plasma GH concentrations in visceral obese subjects, more long-term studies are required to establish whether dietary protein ingestion can also be used to chronically improve somatotrophic activity in visceral obesity and hyposomatotropism in general, and whether this strategy is effective in (partially) reversing the associated metabolic abnormalities.

Glucose concentrations, especially at baseline, were higher in visceral obese subjects compared with lean subjects (5.8 ± 0.3 mmol/L vs. 4.9 ± 0.2 mmol/L respectively), however it is unlikely that this affects the results, since none of the subjects were hyperglycaemic and hyperglycaemia is known to reduce GH secretion. The tendency to higher delta insulin peak values in visceral obese subjects compared with lean subjects may partly explain the reduced GH concentrations, since insulin is known to stimulate IGF-1, which is known to inhibit GH secretion via a negative feedback loop ²⁹.

In conclusion, ingestion of dietary protein (gelatin) stimulates GH secretion in visceral obesity, to the levels found in placebo-treated lean subjects. Further research is necessary to investigate whether dietary protein can be used as an appropriate and physiological stimulator, in the diagnostic work-up of hyposomatotropism and whether prolonged use of dietary proteins with GH secretory capacity is able to improve metabolic abnormalities in visceral obesity in the long term.

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10

General discussion

The somatotrophic axis plays a central role in the regulation of linear growth and body composition. A dysfunction of the somatotrophic axis results in a reduced linear growth in children, a reduced muscle and bone mass and increased fat mass in children and adults. The effects of dietary protein on the regulation of the somatotrophic axis in short term, long term and in pathophysiological conditions are discussed in this chapter.

SHORT TERM NEUROENDOCRINE REGULATION OF THE SOMATOTROPIC AXIS BY PROTEIN

Amino acids

The release of the somatotrophic axis is stimulated by i.v. injection or oral ingestion of several amino acids (AAs). Arginine in combination with lysine, have been shown to stimulate the GH release by 8 fold^{1, 2}. In our studies, we found that ingestion of a mixture of 20 AAs reflecting a dietary protein (i.e. soy protein) is even more potent in stimulating GH release than ingestion of a mixture of arginine and lysine³. In both conditions the same amounts of arginine and lysine were present, however serum arginine concentrations were higher after ingestion of the complex AA mixture compared with the mixture of arginine and lysine. This indicates that in addition to arginine and lysine, other AAs are indirectly involved in the observed stimulation of GH release, possibly via conversion into arginine. Several non-essential AAs are known to convert into arginine, for instance histidine and proline can convert from glutamine/glutamate to ornithine, citruline and finally into arginine⁴. Furthermore, other AAs than arginine and lysine are also known to stimulate the GH release directly⁵.

Although the AA composition of the test drinks influences the somatotrophic response, the chain length of protein was not of pivotal value for the GH response, since GH responses were similar after ingestion of a mixture of single AAs, a hydrolysed protein and a complete protein. Despite no differences between GH secretion over time after ingestion of an AA mixture, hydrolyzed protein and complete protein, total AA absorption was faster when single AAs were ingested³. However, the time to the peak serum concentration of arginine and lysine was not different between the conditions, suggesting that serum concentrations of certain AAs as arginine and lysine are more important than the total serum AA concentrations.

Protein sources

The above mentioned studies suggest that the GH secretory response to dietary protein is dependent on ingestion of the amount and type of AAs. Soy was hypothesized being a relatively strong GH stimulator among the proteins because of its relatively high content of arginine and lysine. However, this hypothesis was not confirmed by a study in which we compared ingestion of 4 different protein types and studied the effect of these different AA compositions on the somatotrophic release. Gelatin protein, which contains the highest amounts of arginine, is a stronger GH stimulator than soy protein, alpha lactalbumin protein and milk protein⁶. We found that serum arginine concentrations were higher after gelatin protein ingestion than after ingestion of the other protein sources. We also found that serum arginine concentrations were higher after soy protein ingestion than after alpha lactalbumin protein or milk protein ingestion, although ingestion of soy protein, alpha lactalbumin protein or milk protein evoked similar GH concentrations. This indicates that a high concentration of arginine stimulates the release of GH, although in addition to arginine other AAs are involved in GH release such as for instance methionine (high in milk protein) and lysine (high in alpha lactalbumin protein)⁶.

Presence of macronutrients

Serum concentrations of specific AAs influence the GH release, furthermore the presence of free fatty acids (FFA) and glucose in blood affect the somatotrophic release. Elevated plasma FFA concentrations⁷, as well as elevated plasma glucose concentrations are known to suppress the release of GH⁸. Since a typical meal consumed by humans consists of a mixture of 3 macronutrients and not of protein alone, the interaction of ingested carbohydrates and fat with the GH promoting effect of protein is studied. We found that the somatotrophic effect of protein was eliminated when soy protein was ingested combined with carbohydrate and fat, however remained unchanged over 5 hours when protein was combined with either carbohydrate or fat^{9,10}. This indicates that in addition to protein, the combination of carbohydrate and fat plays a modulating role in the postprandial secretion of GH after ingestion of a high protein meal.

Ingestion of carbohydrates alone does not affect the 5 hour GH response after protein ingestion, however it changes the secretory pattern of protein induced GH release; a second peak occurs when protein is combined with carbohydrates, approximately 4 hours after ingestion. The addition of carbohydrates to protein resulted in changes in protein metabolism, since the AA concentrations stay higher for a longer time when protein was co-ingested with carbohydrates than after

protein ingestion alone, which may explain the second GH peak. A possible explanation is that gluconeogenesis is higher when only protein is ingested compared with ingestion of protein plus carbohydrates, resulting in a faster decrease of serum AA concentrations after the combined ingestion of protein alone than after ingestion of protein plus carbohydrate ¹¹.

We reported that fat delays the protein induced GH peak, however it does not affect the GH response. The delayed GH peak is neither explained by the serum AA concentrations, nor by the peak insulin concentrations. Possibly a direct effect of fat intake on somatostatin secretion could cause delay in the GH response ^{12, 13}.

It remains unclear why the quantity of GH release after protein ingestion is not affected by co-ingestion of either carbohydrate or fat, whereas it is eliminated when protein is co-ingested with carbohydrate and fat. Again serum arginine concentrations are likely to be involved, since serum arginine concentrations are reduced after co-ingestion of carbohydrate and fat compared with ingestion of protein alone or co-ingestion with carbohydrate or fat, which indicates an interaction of carbohydrate, fat and protein with serum arginine concentrations. Another explanation for the reduced GH secretion after protein co-ingested with carbohydrate and fat compared with protein alone or combined with either carbohydrate or fat, may be the influence of the hormone ghrelin, known as GH secretagogue ¹⁴. Ghrelin concentrations are decreased postprandially, dependent on the energy content of the meal. Protein combined with carbohydrate and fat will result in lower ghrelin concentrations postprandially compared with protein or protein plus carbohydrate or fat, due to the higher energy content ¹⁵ and consequently will result in lower GH concentrations. With respect to insulin concentrations, we could not confirm their involvement in the eliminated GH response after soy plus carbohydrate plus fat, since insulin concentrations are equally increased after ingestion of the conditions protein plus carbohydrate and protein plus carbohydrate plus fat, although GH concentrations are only increased after ingestion of protein plus carbohydrates, but not after protein plus carbohydrate plus fat.

Thus the short term regulation of the somatotrophic axis by protein is dependent on the AA composition of protein and the presence of other macronutrients, in which serum arginine concentrations seem the strongest and most clear GH stimulator. However, also other AAs are also involved. This conclusion is based on young women using oral contraceptives. Future research should focus on the underlying mechanisms to understand the release of GH after different protein types and in the interaction with intake of other macronutrients. Postprandial protein metabolism influenced by ingestion of different protein types as well as protein in the presence of carbohydrates and/or fat has to be further investigated. It remains to be elucidated, for instance, why AA concentrations are higher 300 min after inges-

tion of protein plus carbohydrates than after protein alone and why the AA concentrations are decreased after co-ingestion of protein, carbohydrate and fat, compared with ingestion of protein alone. Furthermore the involvement of gender, estrogens, GHRH, somatostatin, FFA and ghrelin in the GH secretory response to ingestion of dietary protein intake (whether or not combined with other macronutrients) has to be investigated, in order to clarify the short term neuroendocrine regulation of the somatotropic axis by dietary protein.

PHYSIOLOGY OF THE SOMATOTROPIC AXIS INFLUENCED BY PROTEIN

Linear growth

We reported that ingestion of dietary protein stimulates the GH secretion acutely, dependent on its AA composition^{3, 6, 9, 10}. Furthermore, epidemiological studies have shown positive relationships between certain protein sources, linear growth and plasma levels of IGF-1 in children¹⁶⁻¹⁹. Our hypotheses that the best described GH promoting AA combination, i.e. arginine and lysine, would be associated with linear growth, is partly confirmed by our epidemiological study indicating a positive relationship between arginine intake, -but not protein or lysine intake- and linear growth in prepubertal girls²⁰. This association between the intake of arginine and linear growth may be explained by the stimulation of the somatotropic axis by arginine, since arginine is a potent stimulator of GH secretion²¹ and GH plays an important role in the stimulation of linear growth^{18, 22}. The finding that only arginine intake was associated with linear growth, indicates that the ingestion of those protein sources, high in arginine, are the most potent GH stimulators, affecting linear growth in the long term. Relatively high amounts of arginine are available in products like nuts, seeds, milk, meat and fish. This finding may be used to propose a diet containing higher amounts of arginine, to stimulate linear growth in children who are too small for their age and have relatively low levels of GH, but who are not classified as GH deficient. These children are not receiving GH therapy, but may still benefit from restoration of the low GH levels. Whether increasing arginine intake may be a suitable intervention in this group remains to be investigated.

Body composition

Since GH influences several metabolic functions such as promoting lipolysis and protein synthesis, we hypothesized that the best described GH promoting AA combination, i.e. arginine and lysine, would be associated with a relatively lower fat mass index (FMI) and higher fat free mass index (FFMI). The results of our epidemiological studies point into the direction that the intake of arginine and lysine, indeed is negatively associated with FMI and positively with FFMI. At the same time, also associations were found between total protein intake and FMI or FFMI, which indicates that there is an effect of arginine and lysine, but also an effect of protein intake in general.

These observations are in accordance with the somatotrophic effects of dietary protein in general^{3, 6, 9, 10} and the specific AAs ARG and LYS^{1, 3, 5} as well as with the concomitant effects of GH on the intermediary metabolism such as on glucose and fat oxidation and the indirect effect on muscle protein synthesis²³. GH suppresses glucose oxidation and concomitantly stimulates lipolysis, resulting in a decreased fat mass index. Furthermore GH stimulates the production of IGF-1, which stimulates the signalling of the PI-3 kinase/ mTOR pathway, resulting in an increase in muscle protein synthesis rates^{24, 25} and in an increase in FFMI in the long term. Other mechanisms through which protein stimulates muscle protein synthesis can be attributed to the potential of AAs to stimulate insulin secretion²⁶. Insulin is known to stimulate muscle protein synthesis^{25, 27}. Furthermore, AAs may directly stimulate muscle anabolism via an activation of the mTOR pathway, resulting in enhanced protein synthesis^{24, 28-30}.

Hence, dietary protein intake, as well as intake of the specific AAs arginine and lysine is negatively associated with fat mass index, indicating a role for dietary protein in GH secretion and concomitantly in the stimulation of lipolysis, resulting in a decrease in fat mass index in the long term. Furthermore, the positive association between protein intake as well as that of the specific AAs arginine and lysine, and FFMI may be explained by its effect on GH and IGF-1 secretion, but also by the insulin tropic action of protein and the direct effect of serum AAs on the activation of the mTOR pathway. Future research should focus on intervention studies to clarify whether total protein ingestion or specific AAs as arginine and lysine are determining the above mentioned long term effects on body composition.

PATHOPHYSIOLOGY OF THE SOMATOTROPIC AXIS INFLUENCED BY DIETARY PROTEIN

Diagnosis of GH deficiency

GH stimulation tests (GHSTs) are widely used to diagnose GH deficiency (GHD). Recombinant human GH (rhGH) is prescribed as therapy in children with GHD to stimulate their linear growth and improve their body composition. However, it is still unclear how to identify those patients who will benefit from this therapy³¹. Current GHSTs are pharmacological, stimulating GH release directly via only one single pathway in the complex physiological regulation of GH secretion, for instance via suppressing somatostatin release, stimulating GHRH release or via a decline in glucose concentrations (Figure 1). Stimulation of GH release via single pathways results in the induction of a high inter individual variation in test outcomes. The use of dietary protein as a stimulator of GH secretion in a GHST induces a more physiological way of stimulation, because oral ingestion of dietary protein affects GH secretion indirectly through multiple mechanisms, including gastrointestinal signals such as ghrelin³², endocrine signals such as insulin³, as well as changes in serum AA concentrations³, resulting in both a suppression of somatostatin, stimulation of GHRH and decline in glucose concentrations (Figure 1).

In adult hyposomatotropism we found less variation (CV%) in GH response after protein ingestion used as a GHST compared with the traditional use of GHRH in a pharmacological GHST³³. Less variation in the test outcomes may indicate that the test outcome distinguishes better between groups, resulting in a higher sensitivity, specificity and reliability of the test.

The use of dietary protein as GH stimulation test may be a valuable addition to the existing GHSTs in the diagnostic work-up of GHD. Further research in children is necessary to evaluate the use of dietary protein as stimulus in a GHST with respect to sensitivity, specificity and reproducibility, which can be calculated one year after start of rhGH therapy, based on the increase in linear growth. Furthermore, the boundary between subjects who will and who will not benefit from rhGH therapy has to be examined. The cut-off level for GH concentrations after dietary protein ingestion as stimulus in a GHST should be lower than the cut-off level after pharmacological stimulators, i.e. approximately 4 µg/L in stead of 10 µg/L respectively. The administered dosage of protein is physiological, i.e. orally ingested in amounts which could be present in our habitual diet, whereas the administered dosage of the pharmacological stimulators is injected intravenously in non-physiological dosages (i.e. dosages which are not naturally present in the body in these amounts), which results in different magnitudes of GH stimulation.

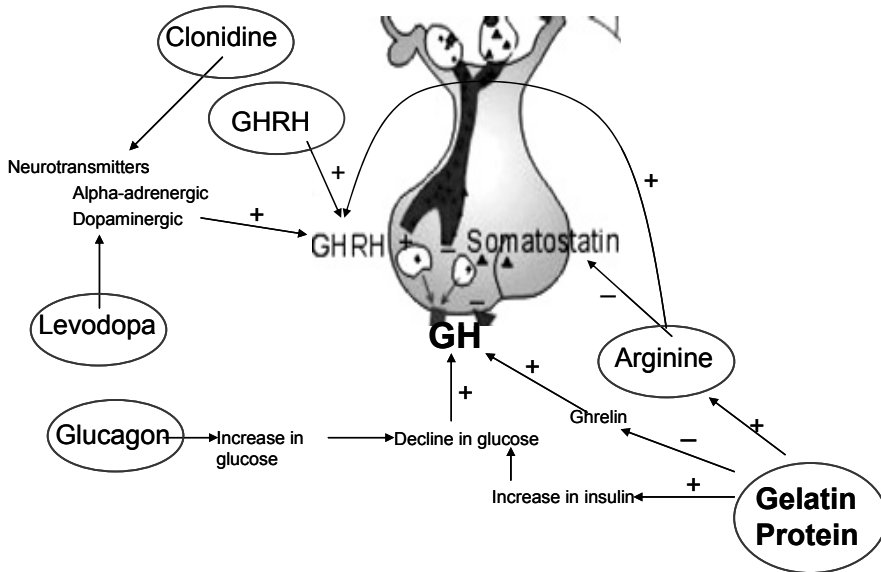


Figure 1: GH stimulation tests (GHSTs) are diagnostic tools to diagnose GHD in order to prescribe GH therapy in children. Pharmacological GHSTs advised by the consensus of the GH Research Society (1999) are: arginine, GHRH, clonidine, levo-dopa and glucagon. Arginine is suggested to stimulate the GH release via a suppression of somatostatin and a stimulation of GHRH; GHRH stimulates GH release via an increase in GHRH; clonidine and levo-dopa stimulate GH secretion via neurotransmitters involving GHRH secretion; glucagon stimulates GH secretion via an increase of endogenous glucose production, resulting in a decline in glucose, which directly results in a stimulated GH secretion.

Gelatin protein as alternative physiological GHST influences GH secretion indirectly via: 1. an increase in arginine concentrations, resulting in a suppression of somatostatin and a stimulation of GHRH; 2. an increase in insulin secretion, resulting in a decline of glucose concentrations and subsequently in a stimulated GH secretion and; 3. a decrease in ghrelin, resulting in a decline of GH secretion.

Visceral obesity

Visceral fat mass is negatively related to the secretion of GH; this condition of low GH secretion is denoted hyposomatotropism. Concomitantly with increased visceral fat mass, many other metabolic dysfunctions appear as reduced insulin sensitivity and unfavourable lipid profiles, resembling the metabolic syndrome³⁴. Prolonged administration of rhGH results in reduced visceral adipose tissue, an improvement of insulin sensitivity and an improvement of lipid profiles. Since we found that dietary protein ingestion stimulated the somatotrophic axis^{3, 6, 10}, we hypothesized that dietary protein also could stimulate release of somatotrophic axis in hyposomatotropism. We indeed confirmed this in one of the studies in this thesis³³. Reduced basal GH concentrations are a typical characteristic in hyposomatotropism, however protein ingestion stimulates the GH secretion to concentra-

tions comparable with GH concentrations in placebo-treated lean subjects with a normal functioning somatotrophic axis.

The explanation for the reduced activity of the somatotrophic axis in visceral obesity is not clear. On the one hand it may be that the somatotrophic release is diminished for instance due to activation of the hypothalamic pituitary adrenal (HPA) axis as a result of increased cortisol concentrations in visceral obesity, resulting in increased concentrations of corticotrophin releasing hormone (CRH), decreased production of GH and consequently a decreased lipid oxidation and an excessive gain of fat tissue³⁵. On the other hand it may be that an increase in fat mass indirectly reduces the secretion of GH, for instance due to increased FFA concentrations, which is a characteristic of visceral obesity³⁶ or due to increased insulin concentrations and peripheral insulin resistance, which also is a characteristic of visceral obesity, resulting in increased IGF-1 concentrations and therefore via a negative feedback to the suppression of GH release³⁷⁻³⁹. Furthermore, it could be that increased concentrations of leptin as a result of gain in fat mass^{40, 41}, reduce the GH secretion, as also increased cortisol concentrations as a result of gain in visceral fat mass³⁵ may result in a reduced GH secretion.

The GH promoting activity of dietary protein in hyposomatotropism probably acts by the same mechanisms as in lean subjects. However, the above-mentioned factors such as higher FFA concentrations, higher insulin concentrations, and higher leptin and cortisol concentrations in visceral obese hyposomatotropic subjects probably prevent a peak concentration comparable to that in lean subjects. Despite the magnitude of stimulation of the somatotrophic axis in hyposomatotropism is lower than in lean subjects, GH concentrations are increased, and reach levels that are found in placebo-treated, non-obese subjects. In the long term, this restoration of circulating GH concentrations probably will result in a reduction of fat mass (especially visceral fat mass), and a change of fat distribution from visceral to peripheral fat via an increase in the lipolysis, fat mobilisation, activation of hormone sensitive lipase, triglyceride hydrolysis and a decrease in fatty acid reesterification⁴²⁻⁴⁴. Furthermore this restoration of GH concentrations by dietary protein ingestion may result in an increase in fat free mass, via increased rates of protein synthesis^{23, 45}.

The GH promoting effect of dietary protein in hyposomatotropism indicates a role of protein as nutritional intervention to improve body composition. Nowadays, GH therapy or oral ingestion of GHRH is used to improve body composition in hyposomatotropism, as visceral obese patients, sarcopenic patients or patients with human immunodeficiency virus (HIV)⁴⁵⁻⁴⁷. At this moment, only pharmacological interventions are available. We are the first in investigating a nutritional intervention to stimulate GH release. However this requires attention to the dietary pat-

tern, since we know from our previous studies that the protein induced GH response is eliminated when protein is ingested combined with carbohydrate and fat and that the protein induced GH response occurs approximately 120 min after ingestion of protein. This results in a dietary pattern in which every 4 hours food is ingested as a two macronutrient meal high in protein. In the 1980s, Michael Montignac developed a diet where menus are designed to ingest limited combinations of macronutrients, which means that a meal should only consist of either a combination of protein with carbohydrates or protein with fat. Protein was allowed to be served at any time and indiscriminately with fat or carbohydrates. However the combination of carbohydrates and fat with protein should be avoided. Furthermore Montignac's method describes that meals should be ingested with at least 3-4 hours in between⁴⁸. Such a diet is an example for a diet aiming to stimulate the GH release. However, in order to develop a diet stimulating GH release, the protein source is paramount, as different protein sources are known to differentially stimulate the GH secretion. Soy, alpha lactalbumin and milk protein stimulate the GH secretion to the same extent, gelatin protein is a stronger GH stimulator⁶. Furthermore, epidemiological studies reported that milk protein was associated with increased IGF-1 concentrations, as vegetable proteins and meat protein were not^{17, 18}. Moreover the specific quantity of fat or carbohydrate combined with protein intake may affect the GH promoting effect of protein. In our study the protein plus carbohydrate meal consisted of 69 En% carbohydrate and 31 En% protein. The protein plus fat meal consisted of 44 En% fat and 56 En% protein¹⁰. In summary the GH promoting activity of dietary protein is also present in hyposomatotropism, resulting in a restoration of GH concentrations to a level comparable with placebo-treated non-hyposomatotropic subjects. A nutritional challenge to restore GH concentrations in hyposomatotropism should consist of four meals containing two macronutrients with at least 4 h in between.

GENERAL CONCLUSIONS

Effects of protein ingestion on enhancing the somatotrophic activity were studied from a neuroendocrinological, physiological and pathophysiological perspective.

Short term neuroendocrine activation of the somatotrophic axis by dietary protein intake is dependent on the AA composition of protein and the simultaneous intake of fat and carbohydrate, but independent on the chain length of protein or the intake of either carbohydrate or fat.

Habitual dietary protein intake is associated with growth in height and body composition. Arginine intake in particular is positively associated with an increase in

linear growth. Protein intake in general, as well as intakes of arginine and lysine are associated with a decrease in fat mass index and an increase in fat free mass index in children.

At the level of the pathophysiology of the somatotrophic axis, dietary protein ingestion as stimulus in a GH stimulation test (GHST) stimulates GH secretion in a physiological and multifactorial way, resulting in a lower inter individual variability of test outcomes compared with the unifactorial pharmacological stimuli traditionally used. Dietary protein ingestion as a diagnostic tool obviously discriminates between adult hyposomatotropism and non-hyposomatotropism with a low inter individual variability, and therefore seems to be an appropriate GHST in order to decide starting GH therapy in children with GHD.

Based upon our findings that dietary protein ingestion restores the low GH concentrations in hyposomatotropism, regular ingestion of milk or soy protein combined with either carbohydrates or fat with at least 4 hours in between the meals can be a valuable strategy to restore the somatotrophic activity and concomitantly improve the body composition.

FUTURE RESEARCH

More specific short term as well as long term intervention studies on the GH promoting action of dietary protein need to be performed in order to develop recommendations to stimulate the somatotrophic activity in hyposomatotropism. In our studies we found an involvement of serum AA concentrations in the short term somatotrophic regulation by dietary protein, however some results remain unclear, for instance the reason why AA concentrations are higher 300 min after simultaneous ingestion of protein and carbohydrates than after protein alone and why the AA concentrations are decreased after simultaneous intake of protein, carbohydrate and fat, compared with ingestion of protein alone. Studies to investigate the AA metabolism with stable isotope techniques after ingestion of dietary protein, combined with other macronutrients will contribute to the understanding of the short term regulation of the somatotrophic axis by dietary protein. Furthermore, serum concentrations of somatostatin, leptin, ghrelin, GHRH, cortisol, estrogens and FFA may play a role in the short term somatotrophic activation by dietary protein and measuring these concentrations is of pivotal value in future short term experiments in order to clarify the neuroendocrinological short term regulation of the somatotrophic axis by dietary protein.

From our studies it is known that dietary protein ingestion in the short term stimulates the somatotrophic activity in a non-hyposomatotropic as well as in a hyposo-

matotropic population. This effect however is dependent on the protein source and the presence of carbohydrate and fat. We also found in children that in the long term habitual arginine intake is positively associated with linear growth, and that dietary protein intake as well as the intake of the specific AAs arginine and lysine are associated with a decrease in fat mass index and an increase in fat free mass index. Future research should focus on the long term effects of a diet restoring circulating GH concentrations and concomitantly stimulating linear growth in hyposomatotropic children and improve body composition in hyposomatotropic (such as visceral obese) children and adults. Such a diet should consist of 4 meals, each with 4 h in between. Meals should consist of not more than two macronutrients, i.e. protein with either carbohydrate or fat. The protein source should be preferably milk, gelatin or soy. Linear growth in hyposomatotropic children, body composition, fat distribution, insulin sensitivity and lipid profiles in hyposomatotropic children and adults have to be measured before, 6 weeks, 3 months and 6 months after starting the intervention.

In our review we gave an overview of the sensitivity, specificity and reliability of the currently used unifactorial, pharmacological GHSTs and we suggested an alternative multifactorial physiological GHST, with dietary protein as stimulus. Concomitantly, we found that dietary protein as stimulus in a GHST obviously discriminates between adult hyposomatotropism and non-hyposomatotropism. A next step is to investigate whether dietary protein as a GH stimulus in a GHST is appropriate to predict the effect of rhGH on linear growth in children who are too small for their age. After one year of rhGH therapy, the predictive value of dietary protein (including sensitivity and specificity) as stimulus in a GHST with respect to the effect of rhGH on linear growth should be evaluated to examine the boundary between subjects who will benefit and will not benefit from GH therapy.

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Summary

Samenvatting

Summary

The somatotrophic axis, also known as the growth hormone (GH) - Insulin-like growth factor-1 (IGF-1) axis, originates in the hypothalamus and pituitary gland. Through the central release of GH and peripheral production of IGF-1, the somatotrophic axis is involved in linear growth and intermediary metabolism. Disorders as visceral obesity and growth retardation in children are characterised by a low activity of the somatotrophic axis. Repeated injection of GH, also named GH therapy, decreases visceral adipose tissue, increases muscle mass and bone density and furthermore stimulates linear growth in children. In addition to GH therapy, sleep, physical activity and nutrition influences the endogenous GH secretion. The latter is investigated in this thesis from a neuroendocrinologic, physiologic and pathophysiologic perspective.

From literature it is known that oral or intravenous administration of AA is involved in the *neuroendocrine* regulation of the somatotrophic axis. Arginine in combination with lysine, has been shown to stimulate the GH release potently. Since AA are the building blocks of protein, it is likely that proteins, similar to AA, are involved in short term somatotrophic regulation. In our studies we investigated the GH promoting effects of a mixture of 20 AAs, reflecting a dietary protein compared with a mixture of arginine and lysine. In both conditions the same amounts of arginine and lysine were present. We found that ingestion of a mixture of AA is more potent in stimulating GH release than ingestion of a mixture of arginine and lysine, which indicates that in addition to arginine and lysine, other AAs are directly or indirectly involved in the observed stimulation of GH release. Subsequently, we investigated the influence of the AA mixture, partially hydrolysed and complete protein on their GH secretory capacity and concluded that the chain length of protein is not of pivotal value for the GH response, since GH responses were similar after ingestion of a mixture of single AAs, a hydrolysed protein and a complete protein (chapter 2). The above-mentioned studies suggest that the GH secretory response to dietary protein is dependent on ingestion of the amount and type of AAs. This indication resulted in a study investigating the GH secretory capacity of different protein sources. The GH secretion after ingestion of soy, gelatin, algalactalbumin and milk protein was investigated. Soy protein was hypothesized being the strongest GH stimulator among the proteins because of its relatively high content of arginine and lysine. However, this hypothesis was not confirmed by our study, in which we found that gelatin protein is a stronger GH stimulator than soy protein, alpha lactalbumin protein and milk protein. Alpha lactalbumin, milk and soy protein stimulate the GH response to a similar extent (chapter 3).

Since a typical meal consumed by humans consists of a mixture of 3 macronutrients and not of protein alone, the interaction of ingested carbohydrates and/ or fat with the GH promoting effect of protein is studied. We found that the somatotropic effect of protein was eliminated when soy protein was ingested combined with carbohydrate and fat, however remained unchanged over 5 hours when protein was combined with either carbohydrate or fat. This indicates that in addition to protein, the combination of carbohydrate and fat plays a modulating role in the postprandial secretion of GH after ingestion of a meal (chapter 4 + 5).

In addition to the short term neuroendocrine effects of protein ingestion on GH secretion, we studied the long term effects of protein ingestion on the somatotropic axis from a *physiological* perspective. Epidemiological observational cohort studies, each consisting of approximately 250 children, were used to investigate the associations between protein intake and in particular the amino acids arginine and lysine and linear growth, lean body mass and body fat mass in prepubertal and pubertal children over 3-6 years. The results of these studies indicate a positive relationship between arginine intake and linear growth and point into the direction that the intake of arginine and lysine, indeed is negatively associated with fat mass index (FMI) and positively with fat free mass index (FFMI). At the same time, also associations were found between total protein intake and FMI or FFMI, which indicates that there is an effect of arginine and lysine, but also an effect of protein intake in general (chapter 6 + 7).

At the level of the *pathophysiology* of the somatotropic axis, we reviewed current used pharmacological tests to evaluate the activity of the somatotropic axis in order to diagnose GH deficiency in children who are too small for their age (chapter 8). Children with GH deficiency receive GH therapy. Current tests are pharmacological, stimulating GH release directly via only one single pathway in the complex physiological regulation of GH secretion. Stimulation of GH release via one single pathway results in the induction of a high inter individual variation in test outcomes. In this thesis, a new test, the dietary protein test, is proposed. The use of dietary protein as a stimulator of GH secretion induces a more physiological way of stimulation, because oral ingestion of dietary protein affects GH secretion indirectly through multiple mechanisms, resulting in a lower inter individual variability of test outcomes compared with the unifactorial pharmacological stimuli traditionally used.

In order to investigate whether the somatotropic effect of dietary protein is also present in (adult) hyposomatotropism, and whether ingestion of dietary protein can be used as a diagnostic tool to discriminate between adult hyposomatotropism and non-hyposomatotropism, we executed a study to compare the GH promoting effect of dietary protein in lean subjects (non-hyposomatotropic) and visceral obese subjects (hyposomatotropic). The hyposomatotropic state was confirmed by measuring the GH response to GHRH. This study shows that oral intake of a dietary

protein stimulates the GH secretion in lean as well as in visceral obese subjects. Ingestion of dietary protein showed to be an adequate method to discriminate between adult hyposomatotropism and non-hyposomatotropism, with a low inter individual variability compared to GHRH administration, and therefore may be an appropriate test in order to diagnose GHD (chapter 9).

Reduced basal GH concentrations are a typical characteristic in hyposomatotropism, however, protein ingestion stimulates the GH secretion in visceral obese subjects to concentrations comparable with GH concentrations in placebo-treated lean subjects with a normal functioning somatotrophic axis (chapter 9).

Based on the results reported in this thesis, it is concluded that the short term neuroendocrine activation of the somatotrophic axis by dietary protein intake is dependent on the AA composition of protein and the simultaneous intake of fat and carbohydrate, but independent of the chain length of protein or the intake of either carbohydrate or fat. Habitual dietary protein intake is associated with growth in height and body composition. Arginine intake in particular is positively associated with an increase in linear growth. Protein intake in general, as well as intakes of arginine and lysine are associated with a decrease in fat mass index and an increase in fat free mass index in children. Furthermore, dietary protein ingestion stimulates GH secretion in a physiological way, resulting in a lower inter individual variability of test outcomes compared with the unifactorial pharmacological stimuli traditionally used and therefore seems to be an appropriate test in order to decide starting GH therapy in children with GHD. Finally, based upon our findings that dietary protein ingestion restores the low GH concentrations in hyposomatotropism of visceral obesity, regular ingestion of milk or soy protein combined with either carbohydrates or fat can be a valuable strategy to restore the somatotrophic activity and concomitantly improve the body composition.

Samenvatting

De somatotrofe as, ook wel de groeihormoon (GH) / insulin-like growth factor-1 (IGF-1) as genoemd, heeft zijn oorsprong in de hypothalamus en hypofyse. GH wordt afgegeven door de hypofyse en stimuleert de afgifte van IGF-1 in de lever en spieren, wat invloed heeft op de lengtegroei en het intermediaire metabolisme. Lage GH afgifte komt onder andere voor bij individuen met viscerale obesitas en bij kinderen die te klein zijn voor hun leeftijd. Het herhaaldelijk injecteren van GH, ook wel GH therapie genoemd, leidt tot een daling van de viscerale vetmassa, verhoging van de spiermassa en botdichtheid, en tevens tot een toename van de lengtegroei bij kinderen. Naast het injecteren van GH kan de GH afgifte endogeen gestimuleerd worden door slaap, fysieke activiteit en door voeding. Dit laatste aspect is in dit proefschrift onderzocht vanuit een *neuro-endocrinologische, fysiologische* en *pathofysiologische* invalshoek.

Uit de literatuur is bekend dat orale of intraveneuze toediening van aminozuren (AA) de *neuro-endocriene* regulatie van de somatotrofe as beïnvloedt. Voornamelijk de combinatie van de AA arginine en lysine is bekend als een sterke stimulator van de GH afgifte. Eiwitten bestaan uit AA en daarom is het aannemelijk dat eiwitten, net als AA, betrokken zijn bij de korte termijn regulatie van de somatotrofe as. Allereerst hebben we onderzocht of een AA oplossing, waarin alle 20 AA van een eiwit aanwezig zijn, de GH afgifte sterker stimuleert dan een oplossing van arginine en lysine. In beide oplossingen waren gelijke hoeveelheden arginine en lysine aanwezig. We namen waar dat de afgifte van GH sterker was na inname van de AA oplossing dan na de arginine en lysine oplossing, wat duidt op een direct of indirect effect van ook andere AA dan arginine en lysine op de GH afgifte. Vervolgens onderzochten we het effect van ketenlengte van een eiwit op de GH afgifte. Hiertoe werd de GH afgifte na inname van de AA oplossing, gedeeltelijk gehydrolyseerd eiwit en compleet eiwit bepaald. Er was geen verschil in GH afgifte na de AA oplossing, het gehydrolyseerde eiwit en het complete eiwit, wat betekent dat de ketenlengte van een eiwit niet van invloed is op de GH afgifte (hoofdstuk 2).

Bovenstaande experimenten suggereren dat de aanwezigheid van bepaalde AA bepalend is voor de mate van GH afgifte. Deze aanwijzing resulteerde in de studie naar de invloed van verschillende eiwitten (en dus verschillende AA samenstellingen) op de afgifte van GH. De GH afgifte na inname van soja, gelatine, albumine en melk eiwit werd vergeleken. De hypothese stelde dat soja eiwit de GH afgifte het sterkst stimuleert in vergelijking met andere eiwitten, omdat soja eiwit relatief rijk is aan arginine en lysine. Deze hypothese werd niet bevestigd door onze studie, waar we vonden dat na inname van gelatine eiwit de GH afgifte het sterkst

steeg in vergelijking met de andere eiwitten, en dat alpha lactalbumine, melk en soja eiwit in gelijke mate in staat zijn de postprandiale GH respons te verhogen (hoofdstuk 3).

Omdat eiwitten in het dagelijks leven worden ingenomen in combinatie met koolhydraten en vetten, is onderzocht of het effect van eiwitten op de stimulatie van GH afgifte ook aanwezig is wanneer eiwitten worden ingenomen in combinatie met koolhydraten en/ of vetten. In deze studies vonden we dat de respons van eiwit inname op de GH afgifte was verdwenen wanneer inname van eiwit werd gecombineerd met inname van koolhydraten en vetten. Dit respons werd echter niet beïnvloed wanneer inname van eiwitten werd gecombineerd met inname van koolhydraten of vetten. Deze bevindingen zijn een aanwijzing dat naast eiwitten, de aanwezigheid van koolhydraten en vetten bepalend is in de afgifte van GH na een maaltijd (hoofdstuk 4+5).

Naast de neuro-endocriene effecten van eiwit inname op de acute afgifte van GH hebben we de lange termijn effecten van eiwit inname en de inname van de AA arginine en lysine op de somatotrofe as bekeken vanuit een *fysiologisch* perspectief. In deze studies hebben we met behulp van twee databases, elk bestaande uit ongeveer 250 kinderen, mogelijke associaties bestudeerd tussen de inname van eiwitten en de specifieke AA arginine en lysine en de 3-6 jaars ontwikkeling van lichaamssamenstelling en lengtegroei bij prepuberale en puberale kinderen. In deze studies is een positieve associatie gevonden tussen de inname van arginine en de lengtegroei. Verder is gevonden dat de totale eiwit inname, net als de inname van de AA arginine en lysine, geassocieerd is met een afname van de vetmassa en een toename van de vet vrije massa (gecorrigeerd voor de lichaamslengte) wat duidt op een effect van arginine en lysine, maar ook op een effect van de totale eiwit inname op de lichaamssamenstelling (hoofdstuk 6+7).

Met betrekking tot de *pathofysiologie* van de somatotrofe as, hebben we in een overzichtartikel verschillende testen geëvalueerd, die de activiteit van de somatotrofe as weergeven (hoofdstuk 8). Deze testen worden gebruikt in het diagnostisch proces om GH deficiëntie vast te stellen bij kinderen die te klein zijn voor hun leeftijd. Kinderen met GH deficiëntie komen in aanmerking voor GH therapie. De afgifte van GH is complex, wat betekent dat deze op vele manieren wordt beïnvloed. De huidig gebruikte instrumenten zijn op farmacologische basis en stimuleren de afgifte van GH op slechts één manier, wat leidt tot een hoge inter-individuele variatie van de testuitslag. In dit proefschrift wordt een nieuwe test voorgesteld om GH deficiëntie vast te stellen. In deze test worden eiwitten oraal toegediend waarna de postprandiale GH respons bepaald wordt. Eiwitten stimuleren de GH afgifte op verschillende manieren, wat leidt tot een meer fysiologische wijze van GH stimulatie en daardoor tot een kleinere inter-individuele variatie van de testuitslag in vergelijking met de farmacologische testen.

Vervolgens hebben we onderzocht of de bovengenoemde effecten van eiwitten op de stimulatie van GH afgifte ook in populaties met een verminderde werking van de somatotrofe as (hyposomatotroof) te vinden zijn en of orale inname van eiwit gebruikt kan worden als diagnostisch instrument om een onderscheid te maken tussen personen met en zonder hyposomatotropisme (hoofdstuk 9). Hiertoe werden slanke volwassen personen (zonder hyposomatotropisme) en visceraal obese personen (hyposomatotroof) met elkaar vergeleken op de GH afgifte na inname van eiwitten. De mate waarin personen in staat zijn om GH af te geven werd gemeten door de GH afgifte na intraveneuze toediening van GH releasing hormone (GHRH test) te bepalen. Gebleken is dat de GH afgifte na eiwit inname, net als in slanke personen, is gestimuleerd in visceraal obese personen en dat deze stimulatie de verlaagde GH concentraties in visceraal obese personen verhoogt tot concentraties vergelijkbaar met de niet-gestimuleerde GH concentraties in slanke personen. Tevens hebben we ontdekt dat orale inname van eiwit een duidelijk onderscheidt maakt tussen mensen met hyposomatotropisme en mensen zonder hyposomatotropisme met een lagere inter individuele variatie van de testuitslag in vergelijking met de GHRH test. Hieruit concluderen we dat de eiwit test een geschikt instrument lijkt om GH deficiëntie te diagnosticeren (hoofdstuk 9).

Uit de resultaten beschreven in dit proefschrift kan worden geconcludeerd dat het korte termijn effect van eiwitten in de voeding op de afgifte van GH af hangt van de eiwit bron en de aanwezigheid van koolhydraten en vetten in de voeding, maar niet af hangt van de eiwit ketenlengte of de aanwezigheid van koolhydraten of vetten. Dagelijkse eiwit inname is geassocieerd met groei in de lengte en breedte. Arginine inname is geassocieerd met groei in de lengte. Inname van eiwitten en inname van arginine en lysine zijn geassocieerd met een afname van de vet massa index en toename van de vetvrije massa index. Verder werd gevonden dat voedingseiwit de GH afgifte fysiologisch stimuleert, wat resulteert in een lage inter individuele variabiliteit van de testuitslag, vergeleken met de huidige farmacologische testen. Eiwit inname lijkt dus een geschikt diagnostisch instrument om GH deficiëntie vast te stellen. Tenslotte, gebaseerd op de vinding dat voedingseiwitten de lage GH concentraties herstellen in hyposomatotropisme bij visceraal obsitas, kan gesteld worden dat regelmatige inname van melk of soja eiwit, gecombineerd met koolhydraten of vetten een adequate strategie is om de verminderde werking van de somatotrofe as in visceraal obesitas te herstellen met als doel de lichaamssamenstelling te verbeteren.

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Op naar de toekomst!

Curriculum Vitae

Anneke van Vught was born on May 24th 1982 in Boxmeer. In 1999, she completed secondary school at the 'Elzendaal College' in Boxmeer. In the same year she started her bachelor education of nursing at the HAN University of Applied Sciences, where she graduated in July 2003. In August 2003 she started her master education; Health Sciences with specialization Movement Sciences at Maastricht University, where she graduated in 2005.

In January 2006, Anneke started working at the department of Human Biology, Maastricht University on a project of Top Institute Food & Nutrition. She studied the effects of dietary protein in the regulation of the somatotrophic axis, under supervision of Prof. M.S. Westerterp-Plantenga, Prof. R-J.M. Brummer and Dr. A.G. Nieuwenhuizen. The research performed in this period is described in this thesis. In 2008, she was awarded a 2nd place 'Foppe ten Hoor' young investigator award by the Netherlands Organisation of Scientific Research (NWO) for her work on the somatotrophic effects of soy protein in the presence of carbohydrate and/or fat.

Currently Anneke is working on the master program of Physician Assistant at the HAN University of Applied Sciences.

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