Endocrine responses to the oral ingestion of a physiological dose of essential amino acids in humans

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Abstract

The response of insulin, human growth hormone (hGH), cortisol, leptin and ghrelin, in addition to various metabolic parameters, was measured at 10 minute intervals following the oral ingestion of a standardised physiological dose of essential amino acids (AA). Twenty-eight healthy male, fasted volunteers (aged 18–40 yrs, BMI 18.0–24.5 kg/m²) took part in the study; 13 volunteers in the AA group, nine subjects in an iso-caloric control group, and a further six subjects served as fasting controls.

Twenty minutes after ingestion, insulin reached peak concentrations that were up to 500% higher than basal values ($P < 0.0001$). The AA group and iso-caloric control group showed a similar insulin response.

AA ingestion led to an increase in hGH secretion with maximum concentrations being $2100 \pm 1013\%$ higher than the basal values ($P < 0.0001$). In contrast, no changes in hGH concentrations were observed in the iso-caloric controls; in the fasting controls only a slight

increase in hGH was found towards the end of the fasting period.

While cortisol decreased significantly ($P < 0.01$) during the study in the AA group, neither control group showed a significant change in this parameter.

Changes in leptin levels remained insignificant in all three groups, whereas ghrelin showed a different profile in each of the three groups, i.e. a continuous rise towards the end of the study period ($P < 0.001$) in the AA group, a less significant effect for the fasting group, and no effect at all in the iso-caloric control group.

There was no significant correlation between the concentrations or the area under curve of the hormones measured in any of the groups.

The endocrine data provided in this study indicate that a single bolus of essential AA in fasted individuals is associated with both anabolic and catabolic hormonal responses.

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Introduction

Metabolism is the continuous balance of anabolic and catabolic processes enabling the organism to adapt to physiological needs. Whereas carbohydrates and fatty acids are used preferentially as metabolic fuels, the uptake of amino acids (AA) is a prerequisite for anabolism, as in protein synthesis and growth.

In metabolic disorders such as phenylketonuria or maple syrup disease, patients lack particular enzymes to further metabolise one or more essential AA. These patients are given special diets, restricting the intake of these AA, and are supplemented with all the other AA (Dashman & Sansaricq 1993, Levy 1989). Another, non-clinical, application of AA supplementation was developed for an increasing number of sporting requirements, mainly in order to gain muscle growth (Rubinstein & Federman 2000, Chromiak & Antonio 2002).

The aim of our study was to investigate the influence of a standardised AA ingestion on the endocrine parameters involved in metabolic processes. Some hormones, such as

insulin (Santora *et al.* 1979, Fukagawa *et al.* 1986, Liao & Lane 1995) and growth hormone (hGH) (Copeland & Nair 1994), are known to mediate AA uptake in peripheral tissues, whereas catabolic hormones such as the glucocorticoids act by stimulating the enzymes of gluconeogenesis (Tayek & Katz 1997, Khani & Tayek 2001). The uptake of AA leads to a further synthesis of androgens (Kingston *et al.* 1986, Nagata *et al.* 1999) which additionally support protein synthesis and muscle growth.

We were therefore interested in the extent to which certain hormones, i.e. insulin, cortisol, hGH, leptin and ghrelin, are influenced by the uptake of a standardised physiological AA dosage in a homogenous group of healthy volunteers.

The hypothesis was that AA cause a shift towards anabolic processes which is initiated by increased insulin (Fukagawa *et al.* 1986, Tessari 1994) and hGH, and accompanied by a decrease in glucocorticoids (Louard *et al.* 1994). The effect of a single AA bolus on leptin and ghrelin was of particular interest, as kinetic data on the effects of special diets on these two peptide hormones are

either controversial (Drewes *et al.* 1997, de Precigout *et al.* 2000) or completely lacking. In continuation and extension of a promising pilot study we describe hormonal changes as anabolic or catabolic, with two different control groups being involved. One group ingested an iso-caloric drink, the other remained fasted. Finally, based on the findings of our study, and on data from the literature, we sought to develop a model of endocrine responses to oral AA ingestion in healthy subjects.

Materials and Methods

Subjects, AA dose, and sample preparation

Twenty-eight healthy male adults (aged 18–40 yrs, BMI 18.0–24.5 kg/m²) participated in the study. The proposed study was fully explained to each subject and informed consent was obtained in advance. The protocol was approved by the local ethics committee.

All subjects reported to our laboratory at 0800 h having fasted for 10–12 h. A permanent catheter (Vasocan, Braun, Melsungen, Germany) was inserted into an antecubital vein and the first blood sample was collected to obtain basal values. Immediately thereafter, 13 volunteers ingested the test mixture of essential AA (SHS International, Heilbronn, Germany) together with 200 ml water within 1 min. Mean load consisted of 24 g AA mixture (Val, Leu, Ile, Phe, Tyr, Try, Lys, Met, Cys, Thr, His, minerals, trace elements and vitamins), normalised to 0.35 g AA per kg body weight. Fifteen subjects served as controls without AA uptake, nine ingested an iso-caloric, skimmed milk powder solution (Naturaflor, Töpfer, Dietmannsried, Germany), containing (per 100 g powder) 36 g protein, 52 g carbohydrates and 1 g fat. The last six controls remained fasted during the entire study.

During the test, volunteers were rested and allowed to drink water *ad libitum*. No adverse effects, such as gastrointestinal discomfort, were observed after ingestion of the test powder.

Blood samples were drawn at 10 min intervals until the end of the experiment ($t_{\text{end}} = 300$ min), with the exception of the fasting controls, where intervals of 30 min were chosen.

Blood was collected in sterile tubes without anticoagulants. After clotting (10 min), the tubes were centrifuged at 1000 g for 5 min at room temperature and serum aliquots were kept frozen at -80°C until analysis.

Analysis of AA, glucose, and free fatty acids

AA measurement was carried out on an LC-3000 analyser (Eppendorf, Hamburg, Germany) after deproteinisation with 5-sulfosalicylic acid. Free fatty acids (FFA) were analysed enzymatically (Wako, Neuss, Germany) using a Hitachi 904 (Roche, Mannheim, Germany). For glucose

analysis membrane-bound glucose oxidase was used (Care diagnostica, Voerde, Germany).

Hormone measurements

Radiochemicals and RIAs for the analysis of leptin, ghrelin and cortisol were purchased from Mediagnost (Reutlingen, Germany), Phoenix (Belmont, California, USA), and DSL (Sinsheim, Germany). ELISA assays for the measurement of insulin and hGH were purchased from DSL.

All immunological systems have been evaluated in our laboratory, where insufficient data on pre-analytic influences on the assay were provided by the manufacturers (Gröschl *et al.* 2000, Gröschl *et al.* 2002). Sensitivities and intra-assay coefficients of variation of the assays were 32 ng/l for leptin (7.3%), 2 ng/l for ghrelin (4.0%), 3.0 nmol/l for cortisol (5.2%), 0.01 µg/l for hGH (5.3%) and 0.26 IU/l for insulin (6.2%).

Statistics and calculations

Data are expressed as means $\pm$ S.E.M., if not otherwise indicated. For graphical presentation, variations were expressed as percentages relative to the basal (t_0) value rather than in concentrations. The area under curve (AUC) for serum concentration versus time for each AA and hormone was calculated using Prism software (GraphPad, San Diego, USA). Statistical analysis was performed using ANOVA with Bonferroni's *t*-test and differences were considered to be significant when $P < 0.05$. Linear regression analysis was performed for hormone values, percentual variations, and AUC results.

Results

Peak serum AA concentrations were reached at 30 min for Met and at 60 min for Val, Leu, Phe, Try, Lys, Thr and Ile (Fig. 1). For Tyr, peak serum values were reached at 150 min and for Cys at 180 min. Serum concentrations increased, in relation to the t_0 values, up to 353% for Ile, 301% for Val, 294% for Met, 361% for Leu, 262% for Try, 274% for Lys, 225% for Thr and 221% for Phe ($P < 0.0001$). The correlation between the dose and the AUC values for a particular AA was significant in most cases. Additionally, a significant correlation was found between the AUC values of Leu and Ile ($r^2 = 0.863$, $P < 0.01$), Met and Val ($r^2 = 0.870$, $P < 0.01$), Met and Ile ($r^2 = 0.799$, $P < 0.01$), Leu and Val ($r^2 = 0.725$, $P < 0.05$), Met and Leu ($r^2 = 0.634$, $P < 0.05$), Leu and Thr ($r^2 = 0.634$, $P < 0.05$) and Ile and Thr ($r^2 = 0.571$, $P < 0.05$).

Interestingly Leu, with the highest concentration in the test mixture, did not show the highest serum concentration or greatest AUC value, nor did Try at the lowest concentration in the mixture show the flattest serum peak

Figure 1 Serum kinetics of essential AA after oral ingestion, eight of the 11 components of the mixture shown as examples (mean $\pm$ S.E.M.). Compared with t_0 , values up to 353% for Ile, 301% for Val, 294% for Met, 361% for Leu, 262% for Try, 274% for Lys, and 225% for Thr and 221% for Phe were reached ($P < 0.0001$).

or smallest AUC value. In the iso-caloric control group, however, no changes in circulating AA could be observed, despite the fact that the milk proteins during digestion are also cleaved into lower molecular AA before gradual absorption through the gastric mucosa.

In all three groups serum levels for blood glucose remained constant throughout the study period. The individual mean values differed only slightly from 4.96 ± 0.38 mmol/l to 5.35 ± 0.09 mmol/l.

FFA (mmol/l) increased gradually irrespective of whether the volunteers ingested either AA (from 0.32 ± 0.06 to 0.61 ± 0.10 , mean increase 215%) or skimmed milk powder (from 0.32 ± 0.08 to 0.52 ± 0.05 , mean increase 217%) or remained fasted (from 0.45 ± 0.1 to 0.69 ± 0.05 , mean increase 178%). These changes were not significant in any of the groups ($P > 0.05$).

Endocrine parameters showed diverse responses to the ingestion of the AA mixture. The most dramatic responses were found for insulin concentrations (Fig. 2). These increased significantly ($P < 0.0001$) within 20 min with an early return to basal values by 150 min. Starting with basal values of 4.3 ± 0.6 IU/l a sharply defined maximum of 19.5 ± 4 IU/l (500% of t_0) was reached. Immediately after this peak insulin values declined exponentially, reaching basal values at around 150 min. From then on, insulin decreased further and reached final concentrations of 2.7 ± 0.4 IU/l, representing 32% of the initial values. In the iso-caloric controls, an identical pattern could be observed basal values of 4.2 ± 0.6 IU/l rapidly increased to a peak at t_{20} (14.5 ± 3 IU/l, 400% of t_0) followed by an exponential decline to basal values. This lowest point was reached earlier than in the AA group (t_{90}). In fasting control subjects however, insulin remained unchanged throughout the entire study period.

Cortisol levels declined constantly ($P < 0.01$) from 519 ± 36 nmol/l beginning immediately after AA uptake

Figure 2 Serum insulin after ingestion of a single physiological dose of essential AA $\blacklozenge$, in the iso-caloric $\circ$ and in the fasting control group $\bullet$ compared with initial values in %. Data are presented as mean $\pm$ S.E.M. The increase in the AA and iso-caloric control group was significant ($P < 0.0001$).

Figure 3 Serum cortisol after ingestion of a single physiological dose of essential AA $\blacklozenge$, in the iso-caloric $\circ$ and in the fasting control group $\bullet$ compared with initial values in %. Data are presented as mean $\pm$ S.E.M. The decrease following AA ingestion was significant ($P < 0.01$).

to 265 ± 19 nmol/l (51% of t_0) at the end of the study. The course of cortisol in relation to basal values is shown in Fig. 3. Cortisol levels in both control groups also tended to decline during the study. This decline was not significant in fasting controls ($P > 0.05$). In iso-caloric controls however, initial values ($t_0 = 326 \pm 30$ nmol/l) were almost regained at the end of the study period ($t_{300} = 295 \pm 25$ nmol/l) after an initial decline to 229 ± 14 nmol/l (t_{90}).

As with insulin, hGH was also dramatically affected by AA administration in all of our subjects. However, the

Figure 4 Course of hGH in serum after ingestion of a single physiological dose of essential AA $\blacklozenge$, in the iso-caloric $\circ$ and in the fasting control group $\bullet$ compared with initial values in percentages. Data are presented as means $\pm$ S.E.M. Increases in the AA group were significant ($P < 0.0001$). Please consider the log scale for this figure.

individual response was not as uniform, and the mean values formed a broad plateau-like peak (Fig. 4). The concentrations rose up to $5.1 \pm 2.2 \mu\text{g/l}$ ($2100 \pm 1013\%$ of t_0) after 40 min ($P < 0.0001$) beginning with low basal values of $0.3 \pm 0.05 \mu\text{g/l}$. The results forming the plateau presented in Fig. 4 should be seen in a differentiated way. Only eight of the 13 volunteers showed a plateau in hGH concentrations lasting for a minimum of 30 min; the remaining AA-subjects showed either one ($n=3$) or two ($n=2$) peaks at 60, 150, and/or 180 min, respectively. In contrast, iso-caloric control subjects showed no significant alterations in the hGH values during the study, and in fasting subjects hGH levels tended to increase (not significant) towards the end of the study to up to $148 \pm 50\%$ of the initial values.

The effects shown by leptin during the first hour of the study varied between groups (Fig. 5). In the AA group, a decline down to 60% of the t_0 values occurred. In contrast, the fasting controls showed leptin values increasing to up to 121% of the t_0 values. However, neither these temporal changes nor the changes during the entire study period for any of the three groups were significant.

Interesting changes were found for the gastric peptide hormone ghrelin. In the AA group concentrations increased slightly up until t_{180} ($P < 0.05$). After that, a significant rise was observed until the end of the study period ($P < 0.001$). The minimum ghrelin concentration ($286 \pm 39 \text{ ng/l}$) was noted at the initial sampling. At the end of the study period, maximum values of $787 \pm 139 \text{ ng/l}$ were found. This corresponds to a 2.75-fold increase, as presented in Fig. 6. Interestingly, in fasting control subjects the increase of ghrelin was less marked ($t_0 = 181 \pm 23 \text{ ng/l}$ up to $t_{300} = 299 \pm 63 \text{ ng/l}$, 1.7-fold

Figure 5 Serum leptin after ingestion of a single physiological dose of essential AA $\blacklozenge$, in the iso-caloric $\circ$ and in the fasting control group $\bullet$ compared with initial values in percentages. Data are presented as means $\pm$ S.E.M.

Figure 6 Serum ghrelin after ingestion of a single physiological dose of essential AA $\blacklozenge$, in the iso-caloric $\circ$ and in the fasting control group $\bullet$ compared with initial values in percentages. Data are presented as mean $\pm$ S.E.M. Ghrelin levels differed significantly between the two groups from 240 min onwards ($P < 0.01$).

increase) but still significant ($P < 0.05$). In the iso-caloric controls, however, no significant changes were found at all. Starting with initial values of $188 \pm 16 \text{ ng/l}$, values of $207 \pm 15 \text{ ng/l}$ were found at the end of the study period.

Performing linear regression analysis, we could not find any significant correlation between either the hormone concentrations or the calculated percent changes in relation to the basal t_0 value. Interestingly, there was no significant correlation between the AUC values of the hormones after baseline correction.

Figure 7 Simplified compartmental model of the endocrine responses to oral AA ingestion based on the findings of this study and data from the literature. Detailed explanation is provided in the discussion. Included are tissues with depository (muscle, liver, kidney), and endocrine (stomach, pancreas, adrenal glands, central nervous system, adipose tissue) function. Arrows symbolise interrelationships. +, positive regulation; -, negative regulation.

Discussion

In this study we investigated the simultaneous responses of a number of important metabolic hormones, including leptin and ghrelin, to a single oral bolus of essential AA. In order to differentiate whether the hormonal changes were anabolic or catabolic, we included two different control groups. One was iso-caloric in order to analyse the anabolic pathways, and one was fasted in order to resolve the catabolic pathways after AA ingestion. Furthermore, changes in glucose and FFA were documented. The comparison of these parameters, particularly in the AA

group and the skimmed milk group, proves that the caloric load in these groups was in fact iso-caloric.

After oral ingestion and transport through the gastrointestinal epithelia the AA circulate within the bloodstream until transported into peripheral tissues. The intestinal uptake is mediated by several hormones, some of which we investigated in their time courses and interactions.

Based on the results of this study and data from the literature, we have summarised the hormonal responses to the ingestion of a physiological AA mixture in a simplified compartment model, presented in Fig. 7.

In both the AA group and the iso-caloric controls, insulin was secreted rapidly within the first minute of the experiment reaching a maximum after 20 min. Consequently, the organism reacts to ingestion by increasing the permeability of the cell membranes to valuable essential AA. It has been shown that insulin facilitates this uptake into different tissues rapidly after its release (Sugahara *et al.* 1987), as indicated in Fig. 7 (Prior & Smith 1983). It is interesting to see that the effect of insulin is dose dependent (Tessari 1994) allowing individual clearance of serum AA according to the metabolic needs of the subject. It is notable that the insulin release after the intake of the milk powder solution mimics the course of the AA group. Caloric load, therefore, seems to be the main determinant for insulin secretion, rather than the composition of the ingested meal.

After a certain time delay after insulin release, hGH serum concentrations increased rapidly. It has been shown that the release of hGH by the pituitary is supported by the initial insulin peak (Muggeo *et al.* 1975), as has been considered in our model. In contrast to the insulin peak, which was very homogenous in the volunteers of the AA group, the hGH release showed a higher individual variability. This indicates that personal response may be based on factors such as age, individual food preferences or training status, as described by Chromiak & Antonio (2002). However, a highly significant hGH release was observed in all of the members of the AA group, which correlated well with previously published data (Muggeo *et al.* 1975, Cameron *et al.* 1988, Lundeberg *et al.* 1991). This indicates a shift towards anabolic processes (Miers & Barrett 1998) and is supported by the data from the control groups. No changes were found in the iso-caloric controls, but a slight, significant increase could be observed in the fasting group. In our view, this finding suggests that the insulin peak alone is not responsible for the hGH secretion. Possibly a supply of essential AA causes both insulin and hGH release, the latter of which is facilitated by the preceding insulin peak.

The significant decline of serum cortisol within 30 min after AA ingestion underlines the general view that AA also stimulate catabolic pathways (Chaussain *et al.* 1976, Kerr *et al.* 1978). This also explains the constancy of blood glucose levels. Glucocorticoids are responsible for the transcription and activation of gluconeogenic enzymes (Khani & Tayek 2001). It is possible that the supply of AA enables the organism to reduce this enzymatic activity until additional fuels are cleared from the serum. Moreover, this effect may also be mediated by insulin (Fig. 7), which acts antagonistically to cortisol (Louard *et al.* 1994). The negative influence of glucocorticoids on AA uptake by various tissues is also indicated in our model. The reduced serum cortisol found in the study would therefore be a prerequisite for an optimal AA uptake similar to the uptake of glucose into peripheral tissues (Munck 1971). This is supported by our finding that a slight, but

insignificant decrease in cortisol levels was also observed in the iso-caloric control group.

Leptin, a peptide hormone produced primarily in adipocytes (Friedman & Halaas 1998) and the gastrointestinal tract (Bado *et al.* 1998, Gröschl *et al.* 2001), is responsible for satiety and decreases food intake through its interaction with feeding centres in the hypothalamus (Schwartz 2001). This is indicated in our model by a negative interaction between adipose tissue and the CNS. Additionally, insulin up-regulates the release of leptin after food intake in healthy rodents and humans (Boden *et al.* 1997, Askari *et al.* 2000), as leptin causes a negative feedback in the pancreas (Ahren & Havel 1999), as shown in Fig. 7. Furthermore leptin facilitates AA uptake by the activation of specific transporters, as reported for human placenta (Jansson *et al.* 2003).

In our study however, serum leptin did not change significantly. Slight but insignificant changes as observed during our study may also be based on the rhythmic secretion of this hormone (Nishiyama *et al.* 2000). This finding may indicate that a single physiological dose of AA is not a sufficient stimulus for a neuroendocrine response leading to significantly decreased leptin levels, despite the marked insulin secretion. Not only the supply of metabolic fuel but also mechanoreceptive stimuli in the stomach play an important role in inducing satiety. These stimuli are additionally responsible for an increased release of leptin (Gage *et al.* 2002). The lack of gastric stimulation before and during the study may therefore explain the more or less constant leptin values. This may also explain why leptin levels remained unchanged in the iso-caloric control group. Surprisingly, leptin values remained constant in fasting controls too. In this group we expected decreasing values in response to hunger. Perhaps the constancy of glucose levels during the study inhibited a significant decrease of this peptide. Additionally it must be mentioned that leptin has been described as a rather long-term regulator of energy expenditure (Wilding 2002). Significant changes in leptin values after intake of AA or other kinds of food may therefore not occur within the 5 h of the present study.

Data from the literature indicate that there is a negative interaction between hGH and leptin (Rauch *et al.* 1998), whereas glucocorticoids have a stimulatory effect on leptin release (Kiess *et al.* 1996, Trayhurn & Rayner 1996). In our model, increased hGH and decreased cortisol levels after AA ingestion have an inhibiting effect on the synthesis of leptin.

This explanation is additionally supported by the continuously rising ghrelin levels in each of our subjects. This gastric peptide has been described as inducing hunger, driving the organism to increase food intake (Nakazato *et al.* 2001). The slight increase shown in the control subjects can be understood simply as a response to the fasting state. In contrast, the iso-caloric load in the control group ingesting milk powder seems to be sufficient to

suppress this signal of starvation. Ghrelin also has a growth hormone releasing function (Date *et al.* 2000), which is further evidence for the induction of anabolic pathways by oral AA ingestion. This point is also considered in our model, although in our study no further hGH release was observed in response to the rising ghrelin levels. It is possible however, that the fasting state of the subjects lasting more than 12 h before starting the experiment, had already led to high ghrelin t_0 values, which then facilitated the hGH release after the AA uptake. Another possible explanation for this finding is that the strong stimulatory effect of ghrelin on the release of hGH may partially be equalised by the inhibitory influence of the slight, insignificant increase of FFA levels on hGH (Broglia *et al.* 2002). Furthermore, recently published data indicate that ghrelin release is not suppressed by insulin (Caixas *et al.* 2002); moreover insulin secretion seems to be additionally stimulated by ghrelin, as has been shown in rodents (Adeghate & Ponery 2002).

The frequently described negative relationship between leptin and ghrelin (Bagnasco *et al.* 2002, Beck *et al.* 2002, Kalra *et al.* 2003) is considered in our model although we could not find any significant correlation of the hormone values measured. We would have expected at least a weak negative correlation between leptin and ghrelin, but this was absent due to the unexpected constancy of leptin. Other studies describe a rather strong negative correlation between the two hormones in study groups with a wide spread of body mass indices. A further explanation for the lack of correlation here could be our relatively homogeneous study group regarding fat content, age and health. These findings will be considered in further studies planned to investigate endocrine parameters in volunteers with more diverse auxological conditions.

In conclusion, this study provides data on the endocrine responses to a physiological dose of essential AA. In fact, it is the first study to consider the newly discovered peptides leptin and ghrelin and to show the course of the observed hormones in relation to each other. Further investigations with regard to other kinds of food supply are necessary for a better understanding of the relations of hormones in a metabolic context.

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