Responses of Metabolic Hormones to Prolonged Intraduodenal Infusion of Amino Acids in Sheep

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Abstract Glycine (Gly), L-leucine (Leu) or L-aspartic acid (Asp) were continuously infused into the duodenum of six castrated male sheep fitted with a chronically indwelling catheter in the duodenum to investigate the effects of these amino acids on GH, IGF-I, insulin and glucagon responses. Amino acids were infused at doses of 3.6, 7.2 and 14.4 mmol/kg/day for 96 h. The effects on plasma concentrations of glucagon, insulin, growth hormone (GH) and insulin-like growth factor-I (IGF-I) were studied during the last 24 h of infusion. Gly administration brought about a significant and dose-related increase in plasma glucagon and insulin concentrations, the molar ratio of insulin to glucagon (I/G ratio) being significantly reduced. Leu and Asp administration significantly increased the concentrations of insulin, glucagon and IGF-I. However, none of the amino acids used caused a significant increase in the GH concentration at these infusion rates. The present results suggest that it would be possible to modify plasma concentrations of glucagon, insulin and IGF-I by intraduodenal infusion of Leu and Asp, and that Gly might be used to reduce the I/G ratio in sheep.

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It has been proposed that hormones such as glucagon, insulin and growth hormone (GH) are important regulators of the partition and utilization of energy-yielding substrates and nutrients, thereby influencing animal performance and production. Glucagon is known to stimulate both glycogenolysis and gluconeogenesis, resulting in glucose release from the liver³⁾ and an increased supply of glucose that is required for milk synthesis in lactating animals²⁾. Insulin is one of the most important factors enhancing protein synthesis¹²⁾ and glu-

cose uptake by peripheral tissues. GH possesses both galactopoietic and growth promoting actions, which are partially mediated by insulin-like growth factor-I (IGF-I) : its physiological significance has been reviewed with particular emphasis to animal production¹¹⁾.

Intravenous arginine (Arg) administration, the most widely used amino acid infusion, elevated the plasma concentrations of GH and prolactin and increased milk yield in dairy cows⁴⁾. Administration of amino acids and proteins into the small intestine has also been

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reported to improve milk production in cows⁵⁾. From these results, it has been proposed that changes in the hormonal status induced by amino acid administration might be one of several strategies that could be adopted to improve animal production²⁵⁾.

In our previous report¹⁷⁾ in which seventeen amino acids were intravenously administered to sheep, we found that each amino acid exerted a different stimulating effect on the arterial concentrations of GH, glucagon and insulin. Among these amino acids, glycine (Gly), alanine and serine were most effective in increasing the concentration of glucagon, while L-leucine (Leu) was the most potent stimulator for insulin. On the other hand, L-aspartic acid (Asp) was the most effective amino acid in stimulating GH secretion. Arg was only a moderate secretagogue for the secretion of these hormones.

The aim of present experiment, on the basis of our previous findings, was to investigate the effects of continuous infusions of Gly, Leu and Asp into the duodenum for 4 days on the plasma concentrations of GH, glucagon, insulin and IGF-I in sheep. It was shown that intra-duodenal administration on these amino acids modified the plasma levels of these hormones responsible for nutrient metabolism.

Materials and Methods

Animal: Six cross-bred castrated male sheep aged 5–7 years and weighing 48–53 kg were used. They were housed in metabolic cages and offered lucerne (*Medicago sativa*) hay cubes (46.4 (DM) g/kg body-weight^{0.75}) once daily at 17:00 hours. Feed was withdrawn at 18:30 hours and uneaten food was weighed. Water was available continuously.

Animals had their left common carotid artery surgically placed in a loop of skin. A chronic indwelling catheter was placed in the duodenum at least 3 weeks before the experiment began, approximately 10 cm distal to the pylorus. The catheter extended 3 cm into the

duodenum in a distal direction. Animals were anesthetised with sodium pentobarbitone (25 mg/kg) for both surgical procedures. Catheters were flushed and filled with distilled sterile water. During the post-operative recovery period, animals were trained to become accustomed to the experimental procedure and surroundings.

Experimental procedure: Gly and Asp were dissolved in warm distilled sterile water and the solution was adjusted to pH 7.0 with sodium hydroxide before use. Because of the low solubility of Leu at pH 7.0, the Leu solution was adjusted to pH 2.5 with hydrochloric acid, at the same concentration as the other solutions.

Gly, Asp or Leu (Wako Pure Chemical Industries, Osaka, Japan) were infused into the duodenum through the catheter for 96 h at 9-days intervals and each infusion began at 12:00 hours.

The infusion rates for each amino acid were 0 (saline), 3.6, 7.2 and 14.4 mmol/kg/day with the exception of Leu which was not infused at the highest rate, because of the low solubility of Leu at the highest dose. The infusion volume was constant (1 ml/min), while the order of amino acid administration and the infusion rate used were randomized.

Blood sampling was carried out during the last 24 h of the infusion at 2-h intervals from the exteriorized carotid artery through a catheter. The indwelling catheter was inserted by puncturing the artery at least 1 h before blood sampling, and was filled with 3.8% trisodium citrate solution.

Arterial blood collected into heparinized syringes was immediately transferred into polyethylene test tubes cooled in ice water and centrifuged at 4°C. Each tube contained 0.05 mmol of benzamidine per ml of blood. A portion of plasma was deproteinized with trichloroacetic acid (0.3 M) and the supernatant was stored at –20°C until used for glucose determination by the glucose oxidase method¹⁴⁾

and α -amino nitrogen estimation by the method of LEE and TAKAHASHI¹⁹. A further aliquot of plasma was stored at -20°C for glucagon, insulin, GH and IGF-I assays. Plasma glucagon was assayed²² using dextran-coated charcoal and antiserum G-42 E which was highly specific for the C-terminal portion of glucagon²³. The assay revealed a minimal detectable concentration of 8 pg/ml. Plasma insulin was assayed by the method of SASAKI and TAKAHASHI²⁷. A highly purified bovine GH used for iodination and as standard was prepared from bovine pituitaries by the method of SPITSBERG³² in this laboratory as described previously¹⁷. Plasma GH was assayed by the double antibody method using antiserum NIDDK-anti-oGH-2 (AFP-C0123080). The assay revealed a minimal detectable concentration of 0.2 ng/ml. Plasma IGF-I was assayed by the double antibody method using NIDDK-anti-IGF-I rabbit serum (UBK 487). Plasma samples were extracted by mixing with an acid-ethanol solution to dissociate IGF-I from binding proteins before assay⁷. The assay revealed a minimal detectable concentration of 7 pg/ml.

Calculation and Statistics: The results are shown as the mean and S.E.M. in the text and tables, but only the means are shown in the figures. The mean plasma concentrations of hormones and metabolites were calculated from all the values recorded over 24 h. The molar hormone concentration ratio of insulin to glucagon (I/G ratio) was calculated from the mean concentrations of plasma insulin and glucagon. The General Linear Model of the SAS program package (SAS Institute, Cary, NC, USA) was used for statistical analysis. Significant differences in plasma hormone and metabolite concentrations between doses of amino acids within each amino acid used and between amino acids within each dose were determined by single-factor analysis of variance (ANOVA). When the P value was less than 0.05 by ANOVA, Duncan's multiple range

test was employed to compare the responses with the control value.

Results

Effects of Gly administration (Fig. 1): The intraduodenal Gly infusion caused a reduction of feed intake by 14% of the control at a dose of 14.4 mmol/kg/day.

Mean plasma concentrations of glucagon and insulin were 28 ± 7 pg/ml and 12.4 ± 1.6 $\mu\text{U}/\text{ml}$, respectively, in the control (saline-infused) animals. These values were significantly ($P < 0.01$) increased by the Gly infusion at all doses for glucagon and at the highest Gly dose for insulin. The maximum mean concentrations of glucagon and insulin were 148 ± 20 pg/ml at a dose of 7.2 mmol/kg/day and 26.2 ± 3.4 $\mu\text{U}/\text{ml}$ at a dose of 14.4 mmol/kg/day, respectively. The mean plasma GH concentration was 1.9 ± 0.2 ng/ml in the control animals, and was only significantly ($P < 0.05$) increased, to 2.5 ± 0.4 ng/ml, by infusion at the dose of 14.4 mmol/kg/day. The mean plasma IGF-I concentration was 51.0 ± 6.8 ng/ml in the control animals, and was not altered by the infusion. Mean plasma glucose and α -amino nitrogen concentrations were 51.9 ± 2 mg/100 ml and 4.8 ± 0.1 mgN/100 ml, respectively, in the control animals. These values were significantly ($P < 0.01$) increased by the Gly infusion at 14.4 mmol/kg/day for glucose and at 7.2 and 14.4 mmol/kg/day for α -amino nitrogen.

Feeding caused complex changes in the concentrations of all hormones and metabolites except for α -amino nitrogen which maintained a steady level over 24 h.

Effects of Leu administration (Fig. 2): Plasma concentrations of glucagon and insulin were significantly ($P < 0.01$) increased by Leu infusion. The maximum mean concentrations were 109 ± 9 pg/ml and 41.8 ± 2.3 $\mu\text{U}/\text{ml}$, respectively, at a dose of 7.2 mmol/kg/day. In contrast, plasma GH was significantly ($P < 0.01$) reduced to 1.2 ± 0.2 ng/ml by the infusion at the same dose. The plasma IGF-I concentration

Fig. 1. The effects of intraduodenal continuous infusion of Gly at doses of 0 (●, saline), 3.6 (□), 7.2 (△), and 14.4 (○) mmol/kg/day on the plasma concentrations of glucagon, insulin, GH, IGF-I, glucose and α -amino nitrogen in sheep. All infusions were continued for 96h. Plasma hormones and metabolites were determined every two hours from 72h to 96h after starting the infusion. Animals were fed at 17:00 hours as indicated by an arrow. The results are represented as the mean of six sheep.

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Fig. 2. The effects of intraduodenal continuous infusion of Leu at doses of 0 (●), 3.6 (□) and 7.2 (△) mmol/kg/day on the plasma concentrations of glucagon, insulin, GH, IGF-I, glucose and α -amino nitrogen in sheep. Other details are given in the legend to Fig. 1.

was dose-dependently and significantly ($P < 0.01$) increased from 43.6 ± 2.1 ng/ml in the control to 51.0 ± 3.1 ng/ml at a dose of 7.2

mmol/kg/day.

The plasma glucose concentration was significantly ($P < 0.01$) reduced to 47.5 ± 1.7 mg/

Fig. 3. The effects of intraduodenal continuous infusion of Asp at doses of 0 (●), 3.6 (□), 7.2 (△), and 14.4 (○) mmol/kg/day on the plasma concentrations of glucagon, insulin, GH, IGF-I, glucose and α -amino nitrogen in sheep. Other details are given in the legend to Fig. 1.

100 ml at a dose of 7.2 mmol/kg/day, while that of α -amino nitrogen was significantly ($P < 0.01$) increased to the maximum value of 6.1 ± 0.1 mgN/100 ml at a dose of 7.2 mmol/kg/day.

Feeding tended to increase the plasma concentrations of glucagon, insulin, IGF-I and glucose, but decreased the GH concentration.

Table 1. The molar ratio of insulin to glucagon during intraduodenal continuous infusions of Gly, Leu and Asp at doses of 3.6, 7.2 and 14.4 mmol/kg/day in sheep

Amino acid	Infusion rates (mmol/kg/day)		
	3.6	7.2	14.4
	(%)	(%)	(%)
Gly	53.1±8.3 ^a	35.5±9.8 ^a	40.6±7.9 ^a
Leu	89.1±9.1 ^b	107.0±9.9 ^b	—
Asp	95.9±5.8 ^b	87.7±8.0 ^b	95.5±7.6 ^b

Values are given as percentages of the control (saline infusion), and shown as the mean±S.E.M. of six sheep. Values with different superscripts (a, b) within each infusion rate differ significantly (Duncan's test after ANOVA).

The α -amino nitrogen concentration was maintained constant throughout 24 h as seen with the Gly infusion.

Effects of Asp administration (Fig. 3): The mean plasma glucagon and insulin concentrations were significantly ($P<0.01$) increased by Asp infusion at doses of 7.2 and 14.4 mmol/kg/day and at 14.4 mmol/kg/day, respectively. The maximum mean values for glucagon and insulin were 139 ± 15 pg/ml and 33.2 ± 2.6 μ U/ml, respectively, at a dose of 14.4 mmol/kg/day. However, the GH concentration was not influenced by Asp administration even at the maximum dose. The IGF-I concentration was significantly ($P<0.05$) increased at doses of 7.2 and 14.4 mmol/kg/day. The maximum mean value was 55.4 ± 2.6 ng/ml at a dose of 14.4 mmol/kg/day.

The Asp infusion caused a significant increase in glucose ($P<0.05$) and α -amino nitrogen ($P<0.01$) concentrations to 55 ± 1.5 mg/100 ml and 6.4 ± 0.5 mgN/100 ml, respectively, at the maximum dose.

Feeding caused a marked increase in the glucose concentration.

I/G ratio (Table 1): The mean I/G ratio for the control animal was 9.4 ± 2.3 . Gly administration significantly ($P<0.05$) reduced the I/G ratio even at the minimum dose, when

compared with Asp or Leu administration.

Discussion

The present results show that intraduodenal infusions of Gly, Leu and Asp significantly increased the plasma concentration of glucagon, insulin and IGF-I (with the exception of Gly), and that the I/G ratio was significantly reduced by the Gly infusion. However, neither Asp nor the other two amino acids was able to increase GH concentration even at 14.4 mmol/kg/day.

In our previous paper, we examined the effects of intravenous infusions of seventeen amino acids (each at a dose of 3 mmol/kg over 30 min) on the secretion of glucagon, insulin, GH and IGF-I in castrated male sheep¹⁷. Gly increased both glucagon and insulin secretion, as did serine and alanine. Leu was the most effective amino acid in stimulating insulin secretion without influencing glucagon and GH secretion. Asp was the most effective in increasing GH secretion. However, no amino acid was able to stimulate glucagon secretion alone without also increasing insulin and GH secretion. IGF-I concentration was not changed by any of the amino acids used. Thus, our interest in the present experiment was to know the effects of continuous intraduodenal infusions of Gly, Leu and Asp lasting for 4 days on the secretion of these hormones in sheep.

The I/G ratio has been used as an index of the relative balance between the actions of glucagon and insulin³⁴. The significant reduction in the I/G ratio induced by Gly infusion suggests that the secretion rate of glucagon exceeds that of insulin, implying a dominant action of glucagon on glycogenolysis and gluconeogenesis. Therefore, when intraduodenally infused at appropriate doses, Gly might be a useful amino acid to enhance catabolism, allowing a large hepatic output of glucose, such as is required in lactation². Administration of a large amount of Gly, however, might not be advisable, since it suppressed feed intake at

a dose of 14.4 mmol/kg/day. On the other hand, Leu and Asp infusion did not change the I/G ratio since they enhanced both glucagon and insulin secretion to a similar extent.

Leu appears to be one of most effective branched-chain amino acids in promoting protein anabolism by sparing amino acid catabolism in protein wasting conditions²⁴, as well as being effective in enhancing glucose utilization in peripheral tissues. In our previous paper¹⁷, however, intravenous Leu infusion decreased glucagon secretion. This discrepancy might be explained by the suggestion that the enhanced plasma glucagon level induced by an intraduodenal Leu infusion was due to other factors such as hypoglycemia induced by an enhanced insulin secretion. SASAKI *et al.*³⁰ reported that glucagon secretion induced by intravenous arginine infusion was sustained for a longer period than was insulin secretion.

Asp, when infused intravenously at 3 mmol/kg over 30 min, caused a marked increase in GH secretion¹⁷. This response was not reproduced in the present intraduodenal infusion experiment, where the maximum infusion rate was 14.4 mmol/kg/day. Although further detailed study is required to account for the discrepancy, one possible explanation is that the α -amino nitrogen concentration, which reflects the net absorption of Asp from the intestine into the blood, was not increased to a high enough level to stimulate GH secretion. A rapid increase in plasma amino acid concentrations seems to be necessary to stimulate GH secretion, because the GH secretion induced by intravenous Arg administration at a low infusion rate over a long period was reported to be smaller than that induced by a high rate of infusion over a short period even when the same total dose was given¹³. It is known that in the ovine small intestine the Gly transport system is not saturated, although it shows a lower affinity and lower transport rate than for other amino acids¹⁵. Thus, the large increase in plasma α -amino nitrogen concentra-

tion during Gly infusion in the present study might be due to the large capacity of Gly transport systems. On the other hand, Asp infused into the duodenum might be converted to other metabolites by the intestinal epithelium or might be consumed by bacteria in the small intestine before being absorbed, resulting in a small increase in α -amino nitrogen concentration and consequently a very limited increase in GH secretion.

In the present study, the plasma IGF-I level was increased by Leu and Asp infusions, but seemed to be independent of plasma GH levels. The plasma IGF-I level in humans is known to be increased more rapidly when essential amino acids are added to a nitrogen deficient diet than when non-essential amino acids are used⁶. Dietary protein might be the primary nutritional determinant of basal IGF-I levels with undernutrition also attenuating the IGF-I response to GH in cattle¹⁰. IGF-I release was stimulated by insulin administration¹⁶, but IGF-I was a potent inhibitor of insulin secretion itself¹⁸. Furthermore, IGF-I exerted a suppressive action on GRF-induced GH secretion by a negative feed-back system in ovine pituitary cells *in vitro*³¹. These findings indicate the complex relationships existing between GH and IGF-I.

Feeding behavior is known to alter the status of hormones and metabolites in plasma. The plasma concentrations of glucagon²¹ and insulin¹¹ were slightly increased, but glucose-induced insulin secretion was decreased²⁹, after feeding in sheep. Furthermore, both plasma GH concentration and GRF-induced GH secretion were reduced after feeding in sheep^{1,33}. The enhanced release of GH shown at the maximum dose of Gly infusion (Fig. 1) might result from the suppression of feed intake induced by the infusion, because it has been reported that the removal of food for 10 h promptly increased both the size and frequency of GH secretion in sheep⁹, and that arginine-induced GH secretion was greater in fasted

than in fed heifers²⁰⁾. When Asp was infused into the duodenum at 14.4 mmol/kg/day the suppression of GH secretion after feeding tended to be overcome. As pointed out earlier, the intravenous infusion of Asp was the most effective amino acid in stimulating GH secretion. Therefore, the slight and significant increase in the plasma α -amino nitrogen, Asp may relate to the attenuation of the reduced GH secretion after feeding during the highest Asp infusion. Feeding is known to cause a transient decrease followed by a sustained increase in the glucose concentration, while it caused a transient increase in the α -amino nitrogen concentration in sheep^{1,21,28)}.

In lactating cattle, OLDHAM *et al.*²⁶⁾ reported that an increased supplementation of amino acids into the intestine by feeding protein supplements enhanced the blood GH concentration about two-fold, but did not influence milk production. In contrast, plasma GH levels were elevated five- to ten-fold when milk yield was increased by GH injection³⁾. Therefore, it may require a large increase in the plasma GH level to enhance milk production by a galactopoietic response to GH when attempts are made to preferentially increase plasma GH secretion by feeding dietary protein and/or amino acids.

In conclusion, intraduodenal infusions of amino acids (Gly, Leu, Asp) significantly increased the plasma concentrations of glucagon, insulin and IGF-I (with the exception of Gly), and the I/G ratio was significantly reduced by the Gly infusion. The present results suggest that it would be possible to modify plasma concentrations of glucagon, insulin and IGF-I by intraduodenal infusion of some amino acids.

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ヒツジの代謝性ホルモン分泌に及ぼす 十二指腸内アミノ酸投与の影響

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反芻家畜へのアミノ酸投与は、各種の代謝性ホルモンの分泌を介して生産性に寄与すると考えられる。本研究では、グリシン、ロイシン、アスパラギン酸をそれぞれ4日間(96時間)、十二指腸内に連続投与した場合の血漿ホルモン濃度について検討した。予め麻酔下に十二指腸カテーテルおよび頸動脈ループを装着した去勢雄成ヒツジ6頭を供試し、各アミノ酸連続投与4日目に、頸動脈より経時的に採血し、GH、インスリン、グルカゴン、IGF-Iの血漿濃度を測定した。アミノ酸の注入量は、0, 3.6, 7.2, 14.4 mmol/kg/dayとした。グリシン投与により、血漿インスリンおよびグルカゴン濃度は、投与量に依存して上昇した。最も高い1日平均血漿インスリン濃度は、グリシン14.4 mmol/kg/day注入時の $26.2 \pm 3.4 \mu\text{U/ml}$ (対照 $12.4 \pm 1.6 \mu\text{U/ml}$)であり、血漿グルカゴン濃度は、グリシン7.2 mmol/kg/day注入時の $148 \pm 20 \text{ pg/ml}$ (対照 $27.9 \pm 7.1 \text{ pg/ml}$)が最大であった。また、グリシン注入によりI/G比(インスリン/グルカゴン:モル比)は、他のアミノ酸注入に比べ、有意に低下した。グリシンは、血漿GH、IGF-I濃度に明かな変動を与えなかった。一方、ロイシン、アスパラギン酸の投与により、血漿インスリン、グルカゴン、IGF-I濃度が各々有意に上昇したが、血漿GH濃度にはほとんど影響を与えなかった。以上の結果から、特定アミノ酸を十二指腸に注入することで、ヒツジの血漿インスリン、グルカゴンおよびIGF-I濃度を調節できることが示唆された。

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