Amino acid infusion increases the sensitivity of muscle protein synthesis *in vivo* to insulin

Effect of branched-chain amino acids

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Rates of muscle protein synthesis were measured *in vivo* in tissues of post-absorptive young rats that were given intravenous infusions of various combinations of insulin and amino acids. In the absence of amino acid infusion, there was a steady rise in muscle protein synthesis with plasma insulin concentration up to 158 μ units/ml, but when a complete amino acid mixture was included maximal rates were obtained at 20 μ units/ml. The effect of the complete mixture could be reproduced by a mixture of essential amino acids or of branched-chain amino acids, but not by a non-essential mixture, alanine, methionine or glutamine. It is concluded that amino acids, particularly the branched-chain ones, increase the sensitivity of muscle protein synthesis to insulin.

INTRODUCTION

The control of muscle protein synthesis by insulin and amino acids has been studied extensively in isolated and perfused preparations. Results have indicated that an increased concentration in the medium of insulin, a complete mixture of amino acids or only the branched-chain amino acids results in an increase in protein synthesis (see, e.g., Fulks *et al.*, 1975; Buse & Reid, 1975; Li & Jefferson, 1978). We have been investigating the role of these effects, observed *in vitro*, in mediating the stimulation of muscle protein synthesis *in vivo* by feeding. We showed that intravenous infusion of insulin into post-absorptive rats had little effect on muscle protein synthesis when the plasma concentration was similar to that in fed animals, although at higher concentrations there was an increase, suggesting that another factor was involved (Garlick *et al.*, 1983). Similarly, infusion of a mixture of amino acids did not stimulate protein synthesis, but, when given together with glucose, which stimulated insulin production, an increase in synthesis occurred (Preedy & Garlick, 1986). It was therefore suggested that the sensitivity of the muscle to insulin might be stimulated by amino acids and that the increase in protein synthesis after feeding might depend on the simultaneous presence of both these factors.

The present work was designed to test the hypothesis that amino acids increase the sensitivity of muscle protein synthesis to insulin and to investigate the specificity of this effect for individual or groups of amino acids.

MATERIALS AND METHODS

Animals

Male hooded Lister rats of the Rowett strain were individually caged from 80 g body wt. (29 days of age) in a temperature-controlled room (21 °C) with a 12 h-light/12 h-dark cycle (lights on at 06:00 h) until the day of the experiment, when they weighed about 100 g. In each experiment animals were allocated to groups of six, with equal mean body weight. Food was removed from the

cages at 23:00 h on the night before the experiment. Between 09:30 and 13:30 h animals were given intravenous (tail-vein) infusions of mixtures of insulin, glucose and/or amino acids at a rate of 1.5 ml/h for 1 h periods, as described previously (Garlick *et al.*, 1983). Exactly 10 min before death each rat was injected with 30 μ Ci of [2,6-³H]phenylalanine (300 μ Ci/mmol; Amersham International, Amersham, Bucks., U.K.) into a tail vein for measurement of protein synthesis. The procedures for injection, killing and sampling of tissues and blood have been described previously (Garlick *et al.*, 1980, 1983).

Infusion solutions

Infusion solutions were made by mixing 1 ml of insulin solution (containing 1.4 mg of sodium acetate, 7 mg of NaCl and 1 mg of methyl *p*-hydroxybenzoate as vehicle) or glucose solution (1.11 M in sterile water) with 5 ml of amino acid solution or insulin vehicle. In Expt. 1 the amino acid solution consisted of Synthamin 17 (Travenol Laboratories, Thetford, Norfolk, U.K.), which is referred to as the 'complete' mixture. This contained 16.5 g of N/1 and the following amino acids (mM): alanine 233; glycine 137; serine 48; proline 59; arginine 66; tyrosine 2; leucine 56; isoleucine 46; valine 50; phenylalanine 34; histidine 31; methionine 27; lysine hydrochloride 32; threonine 35; tryptophan 9. In Expt. 2 the 'complete mixture' and the other mixtures and single amino acids were made from individual amino acids at the same concentrations as those in Synthamin 17. Glutamine was not present in any of the mixtures, but when infused alone a solution of 256 mM was used.

Analytical procedures

Preparation of the tissues for analysis and measurement of specific radioactivity of free and protein-bound phenylalanine have been described previously (Garlick *et al.*, 1980; McNurlan *et al.*, 1982). Plasma glucose was measured as described by Trinder (1969), and insulin by radioimmunoassay (Basset & Thorburn, 1971). The rate of protein synthesis in muscle and heart was calculated from the specific radioactivity of free and

Table 1. Effect of infusion of a mixture of amino acids on the rate of muscle protein synthesis in rats infused with insulin at different rates

Rats were infused for 1 h with the 'complete' mixture of amino acids (AA) plus a variable amount of insulin, and rates of protein synthesis were measured during the last 10 min. Plasma glucose and insulin were measured on samples taken at death. Statistical significance of differences: * $P < 0.01$, ** $P < 0.001$ from 'Vehicle' group; † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$ from 'Vehicle + AA' group; and ‡ $P < 0.02$, ‡‡ $P < 0.001$ from the corresponding '+ AA' group.

Infusion	Insulin infusion (munits/h)	Plasma insulin (μ units/ml)	Plasma glucose (mM)	Protein synthesis (%/day)
Vehicle	0	1.6 ± 2.1	5.1 ± 0.2	9.92 ± 0.55
Vehicle + AA	0	4.9 ± 1.4	7.2 ± 0.2	11.36 ± 1.04
Insulin 1	30	4.6 ± 1.5	5.1 ± 0.2	10.93 ± 0.24 †‡
Insulin 1 + AA	30	19.5 ± 9.8	5.6 ± 0.9	14.68 ± 0.63 **†††
Insulin 2	47	40.7 ± 11.5	3.2 ± 0.3	12.74 ± 0.60 †‡
Insulin 2 + AA	47	38.9 ± 9.0	3.5 ± 0.7	14.92 ± 0.65 **†††
Insulin 3	75	88.6 ± 11.5	2.6 ± 0.2	13.33 ± 0.63 **†
Insulin + AA	75	89.2 ± 8.3	2.3 ± 0.2	14.36 ± 0.37 **††
Insulin 4	105	158.5 ± 32.2	2.4 ± 0.1	14.04 ± 0.55 **††

protein-bound phenylalanine as described by McNurlan *et al.* (1982). Amino acid concentrations in plasma taken at death were measured in HClO_4 supernatants by ion-exchange chromatography (Chromaspek; Hilger Analytical).

Results are present as mean values $\pm$ S.E.M. for groups of five or six animals, and means were compared by two-tailed t tests by using the pooled estimate of variance. Log transformation of values was performed when indicated.

RESULTS

The effect of varying the rates of insulin infusion either with or without simultaneous infusion of the complete amino acid mixture (Expt. 1) is shown in Table 1. In the absence of amino acid infusion, increasing the rate of insulin infusion increased the plasma concentration of the hormone and decreased the plasma glucose concentration. The rate of protein synthesis in gastrocnemius muscle showed an increase that was significant with an insulin concentration of $40.7 \mu\text{units/ml}$ and was progressive up to the highest insulin concentration, $158.5 \mu\text{units/ml}$. The dose-response curves for insulin concentration and muscle protein synthesis are illustrated in Fig. 1. In the presence of amino acids the pattern was rather different. Infusion of amino acids without insulin caused small increases in plasma glucose and insulin, but had no significant effect on protein synthesis, as was observed previously (Preedy & Garlick, 1986). Amino acid infusion only affected plasma insulin at the lowest rate of insulin infusion, when a pronounced increase in concentration was observed. This probably resulted from different degrees of binding of insulin to the infusion apparatus in the presence of amino acids, which was more of a problem at low infusion rates. Infusion of amino acids caused the rise in muscle protein synthesis with increasing insulin concentration to be more rapid (Fig. 1), so that the rate was almost maximal at a plasma concentration of only $20 \mu\text{units/ml}$, and similar to the value given at the highest insulin concentration ($159 \mu\text{units/ml}$) without amino acids. The difference resulting from amino acid infusion was particularly

Fig. 1. Relationship between plasma insulin concentration and muscle protein synthesis rate in rats infused with various amounts of insulin either with (○) or without (●) an amino acid mixture

Data are taken from Table 1.

pronounced (and statistically significant) at insulin infusion rates of 30 and $47 \mu\text{units/h}$ (Table 1).

The ability of various amino acids and amino acid mixtures to stimulate muscle protein synthesis in the presence of insulin (Expt. 2) is shown in Table 2. In this experiment the plasma insulin concentration was raised by infusion of glucose rather than by direct infusion of the hormone, to avoid adsorption of the insulin on to the infusion apparatus, which we have found to be a problem when attempting to achieve concentrations below $40 \mu\text{units/ml}$ (e.g. Table 1). The actual concentrations in this experiment are not available, but previous experience with the same rates of infusion has shown it to be $20\text{--}30 \mu\text{units/ml}$ (Preedy & Garlick, 1986). We have also shown previously that the effect of insulin is the same whether accompanied by hyper- or hypo-glycaemia (Garlick *et al.*, 1983). In the absence of amino acid infusion, an increase in plasma insulin resulting from glucose infusion caused a small increase in protein synthesis in gastrocnemius muscle, whereas infusion of glucose plus a complete mixture of amino acids caused a significant further rise in synthesis. A similar increase was brought about by infusion of a mixture of essential amino acids or only the branched-chain amino acids, but the non-essential amino acids, methionine or alanine had little effect. Glutamine was infused both with and without

Table 2. Effect of infusion of glucose plus various mixtures of amino acids on the rate of protein synthesis in gastrocnemius, plantaris and cardiac muscles

Rats were infused for 1 h with various mixtures of amino acids with or without glucose, and rates of protein synthesis were measured during the last 10 min. Plasma glucose was measured on samples taken at death. Statistical significance of differences: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ from 'control' group; † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$ from glucose-only group.

Amino acid infused	Glucose infusion	Plasma glucose (mm)	Protein synthesis (%/day)		
			Gastrocnemius	Plantaris	Heart
None (control)	—	5.8±0.1	7.58±0.44	8.45±0.48	15.60±0.45
None (glucose)	+	9.6±0.3	8.03±0.31	9.26±0.34	16.98±0.38*
Complete mixture	+	10.2±0.2	10.48±0.08***†††	12.92±0.46***†††	18.34±0.68***†
Essentials	+	9.8±0.3	9.94±0.49***†††	12.09±0.64***†††	18.67±0.40***†
Non-essentials	+	10.0±0.3	8.56±0.43	9.79±0.52	18.01±0.26***
Branched-chain	+	9.1±0.4	9.92±0.34***†††	11.81±0.76***††	17.81±0.20**
Methionine	+	10.4±0.6	7.46±0.32	9.10±0.55	17.74±0.21**
Alanine	+	10.1±0.4	7.45±0.11	9.15±0.51	17.19±0.61*
Glutamine	+	9.2±0.4	7.15±0.38	8.85±0.48	16.40±0.26
Glutamine	—	5.9±0.1	7.32±0.39	7.82±0.39	15.97±0.69

Table 3. Amino acid concentrations (μM) in plasma after 1 h of infusion with glucose plus various amino acid mixtures

Significance of differences: * $P < 0.05$, ** $P < 0.01$ from control; † $P < 0.05$, †† $P < 0.01$ from glucose alone.

Amino acid	Infusion . . .	Control	Glucose	Glucose + complete mixture	Glucose + essential amino acids	Glucose + non-essential amino acids	Glucose + branched-chain amino acids
Ser		340±18	319±21	474±18***††	290±14	555±18***††	325±18
Gly		449±7	522±85	907±108***††	404±24	855±122***††	394±21
Ala		391±20	664±64**	1604±241***††	658±44**	1259±252***††	558±47*
Arg		116±11	100±16	259±30***††	123±20	410±30***††	90±5
Pro		150±5	148±9	474±59***††	127±17	458±79***††	119±13
Tyr		364±9	323±43	251±15***†	235±15***††	323±13	210±20***††
Asp		62±4	57±14	64±8	83±26	76±13	45±6
Glu		337±36	392±95	392±19	346±39	383±58	268±29
Gln		283±45	334±36	278±42	289±40	255±48	222±23
His		86±3	98±4	168±11***††	190±13***††	90±3	63±5***††
Lys		480±19	414±53	475±56	490±43	376±26	287±20***†
Met		10±1	11±1	74±8***††	125±14***††	—	—
Thr		354±11	330±25	570±23***††	581±19***††	350±12	254±39***†
Leu		153±8	98±10**	217±8***††	246±18***††	84±2**	247±14***††
Ile		103±5	57±3**	184±7***††	210±12***††	63±4**	192±11***††
Val		198±7	147±12	366±15***††	418±31***††	152±6	365±24*††

glucose, but did not stimulate protein synthesis in either case.

Results for plantaris muscle and heart are also shown in Table 2. The response of plantaris to insulin plus amino acids was very similar to that of gastrocnemius muscle, except that the increase in synthesis was greater, indicating a higher sensitivity to these factors. In heart muscle the rate of protein synthesis was 2-fold higher than in the skeletal muscles. Infusion of glucose plus the complete or essential amino acid mixture caused a significant increase in synthesis by comparison with glucose only, but none of the other mixtures or single amino acids had a significant effect. In particular, the stimulation by branched-chain amino acids was smaller

than that produced by non-essential amino acids, and was not significant.

The concentrations of amino acids in plasma in Expt. 2 are shown in Table 3. By comparison with the group given neither glucose nor amino acids (control), the group given glucose alone showed little change in most amino acids except the branched-chain ones, which decreased, and alanine, which increased. When glucose plus the complete mixture of amino acids was infused, most amino acids increased in concentration relative to the control group, except that there was a fall in tyrosine and no change in aspartate, glutamate, glutamine and lysine. Infusion of the essential amino acids plus glucose increased all the essential amino acids except lysine.

There was no change in the non-essential ones, except for an increase in alanine and a decrease in tyrosine. The effect of infusing non-essential amino acids plus glucose on the concentrations of the essential ones was similar to the effect of infusion of glucose alone, whereas the effect on the concentrations of non-essential amino acids was to cause either an increase or no change (aspartate, glutamate, glutamine and tyrosine). Infusion of the branched-chain amino acids had the same effect on concentrations of non-essential and branched-chain amino acids as infusion of essential ones, but concentrations of essential amino acids other than the three branched-chain ones fell.

Overall the concentrations of aspartate, glutamate, glutamine, tyrosine and lysine were relatively unresponsive to treatments, whereas the branched-chain amino acids methionine, alanine, proline and arginine were the most responsive. However, of those that were responsive, only the branched-chain amino acids exhibited any relationship between plasma concentration and the rate of muscle protein synthesis.

DISCUSSION

Both insulin and amino acids have previously been shown to cause an increase in protein synthesis when added to the medium of incubated or perfused muscle (Fulks *et al.*, 1975; Buse & Reid, 1975; Li & Jefferson, 1978; Li *et al.*, 1978). However, quite large changes in concentration of these substances were used. Insulin has usually been added at concentrations in excess of 1 munit/ml (see, e.g., Fulks *et al.*, 1975; Lundholm & Schersten, 1977; Li & Jefferson, 1978; Preedy & Garlick, 1983), although concentrations of about 100 μ units/ml have also been used (Frayn & Maycock, 1979; Stirewalt & Low, 1983; Palmer *et al.*, 1985). Amino acids have typically been added at 5 or 10 times normal plasma concentrations (see, e.g., Li & Jefferson, 1978; Lundholm & Schersten, 1977), but addition of normal concentrations compared with none has also been shown to be effective (Fulks *et al.*, 1975). The physiological significance of such changes can be questioned. When measurements were made in post-absorptive rats *in vivo*, we showed that insulin infusion would stimulate muscle protein synthesis at a concentration of 70 μ units/ml (Garlick *et al.*, 1983), although this was still in excess of the concentration found in fed animals. Infusion of a mixture of amino acids did not stimulate protein synthesis *in vivo* unless glucose was also infused, leading us to hypothesize that the amino acids increased the sensitivity of muscle to insulin, allowing it to stimulate protein synthesis at a normal physiological concentration (Preedy & Garlick, 1986). This mechanism has now been confirmed (Fig. 1). In the absence of amino acid infusion, insulin had only a small effect on protein synthesis in the normal range of plasma insulin concentration (0–40 μ units/ml), and the rate had not apparently reached a maximum at a concentration of 158 μ units/ml. By contrast, when amino acids were also infused, the stimulation of protein synthesis appeared to be almost maximal at an insulin concentration of 20 μ units/ml. Studies in isolated muscle have not shown this synergism between insulin and amino acids, but relatively higher concentrations were used (Fulks *et al.*, 1975; Lundholm & Schersten, 1977).

For the dose-response curve the amino acid mixture

that was infused was a solution intended for intravenous feeding of hospital patients. Although we have termed this the 'complete' mixture, not all of the amino acids were included. Glutamate and aspartate and their amides, as well as cysteine, were absent, and tyrosine was only present in very small amounts. Previous work had shown this solution to be effective in stimulating protein synthesis in the presence of insulin (Preedy & Garlick, 1986), so we used it for the present experiments, and as the basis for the mixtures and individual amino acids that were studied. Table 2 shows that most of the effect of the 'complete' mixture could be attributed to the essential amino acids. The non-essential ones had a small effect, but this was not significant. The individual amino acids were selected because of suggestions in the literature that they might have special roles, except alanine, which was chosen as a control for the other single amino acids, to supply non-essential nitrogen and energy. Methionine, as well as occupying a unique position in peptide-chain initiation, has been shown to inhibit urinary nitrogen loss in protein-deprived animals (Yokogoshi & Yoshida, 1976). It did not, however, affect muscle protein synthesis in the present experiments. Glutamine was selected because of a report that its concentration correlates with muscle protein synthesis in the perfused rat hindlimb (Rennie *et al.*, 1986). As this amino acid was not included in the 'complete' mixture and its plasma concentration did not change when the various mixtures were infused (Table 3), it could not have been involved in the observed stimulation of protein synthesis. It was therefore infused separately, both with and without glucose. This raised the plasma glutamine concentration in the latter group to 440 ± 41 μ M, compared with 283 ± 45 μ M in saline controls. However, either alone or with glucose glutamine did not affect protein synthesis.

There have been numerous reports of a stimulation of muscle protein synthesis by branched-chain amino acids (see below). Table 2 shows that in skeletal muscle all of the effect of the essential amino acids, and most of the effect of the 'complete' mixture, can be brought about by infusion of these three amino acids only.

Measurement of free amino acids in plasma was necessary to confirm that infusion of amino acids did indeed increase their concentrations. In almost all cases concentrations followed a path that was predictable from three parameters: the increase in muscle protein synthesis, the rate of infusion of that amino acid, and its pool size. Thus concentrations of aspartate, glutamate and glutamine were insensitive to treatment because they were not present in the infusion mixtures, that of lysine because of its relatively large pool size and that of tyrosine because it was given in relatively small amounts. Tyrosine concentration was also increased above normal by synthesis from phenylalanine given as a large dose to measure the rate of protein synthesis: for this reason values for phenylalanine itself are not given. Concentrations of the branched-chain amino acids, methionine and arginine were sensitive to treatments because they have small pool sizes, and that of alanine as a result of its synthesis from glucose via pyruvate (the glucose-alanine cycle), as well as its high concentration in the infusion mixtures. With these points in mind, the effect of the glucose-only infusion was to decrease the concentrations of the branched-chain amino acids, with smaller decreases in several others, because of the change

in the balance between protein synthesis and degradation, represented here by the small increase in synthesis. With infusion of glucose plus total, essential, non-essential and branched-chain amino acids there were increases in concentration of those that were infused, relative to infusion of glucose alone. In addition concentrations were lower when the rate of protein synthesis was elevated. For example, with infusion of glucose plus branched-chain amino acids there were substantial increases in the concentration of the three branched-chain amino acids, relative to infusion of glucose alone, but most of the others fell, as a result of the higher rate of protein synthesis.

The important conclusions from the amino acid concentrations are, first, that for most amino acids the concentration appears to depend on, rather than control, the rate of protein synthesis. Secondly, the only amino acids that could be regulating protein synthesis, and hence show increases in concentration when muscle protein synthesis is elevated, are the three branched-chain ones, further supporting the conclusion that it is these amino acids that modify the sensitivity of muscle protein synthesis to insulin.

We have no information on how the branched-chain amino acids have their effect on insulin-sensitivity, but there have been a number of studies of their influence on protein metabolism (see reviews by Adibi, 1980; Walser, 1984). In incubated and perfused muscle preparations, stimulation of protein synthesis and inhibition of breakdown by complete amino acid mixtures has been shown to result mainly from an effect of branched-chain amino acids (e.g. Fulks *et al.*, 1975; Buse & Reid, 1975; Li & Jefferson, 1978). Leucine has been shown to be particularly effective, but isoleucine and valine might also be active (Fulks *et al.*, 1975). There have also been many reports of improvements in nitrogen balance in patients given branched-chain amino acids or enriched mixtures (Adibi, 1980; Walser, 1984). However, attempts to show an effect of branched-chain amino acids on muscle protein metabolism *in vivo* have not always been successful. McNurlan *et al.* (1982) injected leucine into young rats that were fed, fasted or protein-deprived, and observed no stimulation of muscle protein synthesis, and when leucine was infused into fasting human subjects the release of aromatic amino acids from the leg was not altered (Hagenfeldt *et al.*, 1980). By contrast, in septic and injured rats there was an increase in muscle protein synthesis in response to branched-chain amino acid infusion (Sakamoto *et al.*, 1979; Moldawer *et al.*, 1981). In none of these experiments was the combined effect of insulin and branched-chain amino acids studied. Buse *et al.* (1979) injected leucine into rats 1–2 h before they were killed and demonstrated an increase in the proportion of ribosomes in polysomes of muscle from starved, but not fed, animals. In particular, these rats were injected with glucose and insulin (so that they would not depend on leucine for energy) and might therefore be comparable with those in the present experiments. Additionally, Buse & Reid (1975) noted that the effect of branched-chain amino acids in isolated muscle was most reproducible when insulin was added to the incubation medium. Furthermore, an interaction between insulin and amino acids in suppressing protein degradation in perfused liver has also been reported (Mortimore *et al.*, 1987). The need for a combined stimulus from insulin and amino acids might explain why

effects of branched-chain amino acids have not been consistently observed *in vivo*.

The effects that we have observed were similar in the two skeletal muscles (Table 2), with plantaris being somewhat more responsive than gastrocnemius. Similar differences in responsiveness to insulin between these two muscles have been reported previously (e.g. Preedy & Garlick, 1983). In heart the results show an increase in protein synthesis with glucose plus 'complete' or essential amino acids, but not with branched-chain ones, which had less effect than non-essential ones. This is contrary to observations in perfused heart, when leucine, but not valine or isoleucine, has been shown to stimulate protein synthesis and inhibit breakdown (Chua *et al.*, 1979). However, although the effects of the 'complete' or essential amino acids were statistically significant, they were proportionately much smaller than those seen with the two skeletal muscles. This lower responsiveness of heart makes it more difficult to obtain significant differences between groups, and in particular to distinguish whether the effect of branched-chain amino acids is really smaller than that of the complete mixture.

The effect on protein metabolism of insulin and amino acids is important in determining the mechanism of the response to food intake. After meals both insulin and amino acid concentrations in systemic blood rise, but there are other circumstances when the two might increase independently (e.g. insulin after a carbohydrate meal and amino acids in starvation; Waterlow *et al.*, 1978). The increase in the sensitivity of muscle to insulin brought about by amino acids, and in particular the branched-chain amino acids, might therefore facilitate more sensitive control of muscle protein synthesis.

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