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Inability to stimulate skeletal muscle or whole body protein synthesis in Type 1 (insulin-dependent) diabetic patients by insulin-plus-glucose during amino acid infusion: studies of incorporation and turnover of tracer L-[1-^{13}C]leucine

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Summary. Despite its anabolic effects on protein balance, acute administration of insulin has been reported to have no effect on skeletal muscle or whole body protein synthesis in man. However, insulin also reduces plasma and intramuscular amino acid availability, which may limit protein synthesis. We have therefore measured the acute effects of insulin on skeletal muscle (anterior tibialis) protein synthesis and whole body leucine turnover in eight insulin-withdrawn Type 1 (insulin-dependent) diabetic patients. They were studied initially when insulin deficient, but during infusion of mixed amino acids at a rate sufficient to raise plasma amino acids by 30% i.e. to 4 mmol/l in total; measurements were continued when insulin was infused together with an increased rate of amino acids to maintain insulinopoenic plasma amino acid concentrations. Using ^{13}C-α-ketoisocaproate in plasma as an index of the intracellular precursor labelling, incorporation of [1-^{13}C]leucine into skeletal muscle protein was 0.068 ± 0.007%/h during insulin withdrawal and was unaltered during insulin infusion. The value is higher than observed in muscle of healthy man, possibly because of a stimulatory effect of endogenous intramuscular amino acids. Also, calculated on the basis of α-ketoisocaproate labelling, non-oxidised whole body leucine disappearance (i.e. whole body protein synthesis) was 110 ± 4 μmol · kg^{-1} · h^{-1} during insulin withdrawal; this also was unchanged during insulin infusion. Despite stable or increased plasma concentrations of most amino acids, the intramuscular concentrations of a number of amino acids decreased during insulin infusion. This may have limited any anabolic effect of insulin on protein synthesis. Alternatively, pre-existing high intramuscular amino acids may have maximally stimulated muscle protein synthesis, so that the further elevation was obscured, especially with the tendency to depletion of precursor amino acids.

Key words: Protein turnover, insulin, amino acids, muscle, Type 1 (insulin-dependent) diabetes mellitus.

Insulin has a protein anabolic effect in mammals. Insulin deficiency in immature streptozotocin-diabetic rats depresses skeletal muscle protein synthesis, both in vitro and in vivo, which is corrected by replacement of insulin [1–4]. However insulin stimulation of protein synthesis is difficult to reproduce in non-diabetic postabsorptive adult rats [5] and lambs [6]. Muscle protein synthesis in postabsorptive insulin-deficient Type 1 (insulin-dependent) diabetic patients is reported as neither below that in healthy subjects nor responsive to insulin [7]. Furthermore, although close arterial infusion of insulin (which caused no systemic fall in plasma amino acids) increased the net protein balance of the forearm in postabsorptive healthy men [8], local protein synthesis was unaffected; the anabolism appeared to be entirely the effect of insulin in reducing forearm protein breakdown.

Studies in man suggest that the availability of amino acids, which is reduced by insulin in plasma [9–11] and in muscle [11], is a major factor modulating rates of whole body [9, 10, 12] and muscle [12, 13] protein synthesis. Other factors which reduce (3-hydroxybutyrate [14] or increase (e.g. glucagon [15, 16]) amino acid oxidation may indirectly regulate protein synthesis by changing amino acid availability. Infusion of insulin plus glucose, together with amino acids sufficient to maintain plasma concentrations, had no effect on whole body protein synthesis but appeared to reduce whole body protein breakdown [9, 10] thereby improving protein balance; these results raise doubts about any physiologically important role in man for insulin in controlling whole body protein synthesis. Nevertheless, hyperaminoacidaemia during hyperinsulinaemia was accompanied by a rise in whole body protein synthesis [10].

In extrapolating from these results to muscle, caution needs to be exercised: if insulin stimulation of human protein synthesis occurred principally in skeletal muscle this might only be disclosed by direct measurement of muscle protein synthesis, which accounts for 30–50% of the whole body value [12, 17]; such a demonstration might also require that insulin-induced muscle amino acid deficiency, by inhibition of protein breakdown, did not constrain

muscle protein synthesis. The present work was undertaken to investigate the acute effects of insulin infusion on skeletal muscle protein synthesis in Type 1 diabetic subjects under conditions in which any constraint on protein synthesis from insufficient amino acid availability was minimized by continuous infusion of a proprietary mixed amino acid solution at a rate which was increased during the insulin administration.

Subjects and methods

Subjects

We studied eight Type 1 diabetic male patients, in good health and free of major diabetic complications despite diabetes for 14 ± 3 years; biochemical tests indicated good diabetic control (HbA_1 $8.6 \pm 0.7\%$, normal $< 8.5\%$) and normal renal, hepatic and thyroid function. Five were completely, and three partially, deficient in C-peptide (0.13, 0.21 and 0.43 nmol/l during hyperglycaemia and ketosis); their total daily insulin was 52 ± 6 IU. Three additional age-matched subjects were studied without tracer to check for effects of the metabolic interventions on baseline $^{13}CO_2$ enrichments. After a full explanation, all subjects gave informed consent for the study, which was approved by the Tayside Health Board ethical committee.

Materials

L-[1-^{13}C]Leucine and sodium [^{13}C]bicarbonate (both 99% ^{13}C) were purchased from Tracer Technologies Inc. (Somerville, Mass, USA). Synthamin 14 mixed amino acid solution was obtained from Baxter Healthcare (Egham, UK). Potato-starch glucose (with a low ^{13}C abundance [18]) was obtained from Tunnel Refineries Ltd. (Greenwich, UK) and was prepared for infusion.

Protocol

Each subject was studied over 240 min after overnight insulin withdrawal and then for a further 240 min during insulin and glucose infusion. During the period prior to the study all subjects were prescribed high-fibre diets in which carbohydrate supplied over 50% and fat 30–40% of total energy. They were given half of their usual dose of short-acting insulin, with no intermediate or long-acting insulin, at 18.00 hours prior to the study day; they then fasted until the end of the study.

At 09.00 hours a cannula was inserted retrogradely into a dorsal hand vein, and maintained patent by slow infusion of 150 mmol/l NaCl; the hand was inserted into a thermostatically controlled chamber (75°C) for 15 min prior to collection of arterialized venous blood samples [19]. Priming doses of L-[1-^{13}C]leucine (6.84 ± 0.25 μmol/kg) and sodium [^{13}C]bicarbonate (2.83 ± 0.33 μmol/kg) were then administered (time 0 min) and a continuous infusion of L-[1-^{13}C]leucine (7.59 ± 0.32 μmol · kg^{-1} · h^{-1}) commenced via a contralateral forearm vein cannula. At the same time a priming dose of Synthamin 14 mixed amino acid solution (29 μmol leucine/kg, 52 mg amino acids/kg) was given over 10 min; Synthamin 14 was then continuously infused at a rate providing leucine 25 μmol · kg^{-1} · h^{-1} until the end of the period of insulin withdrawal (time 270 min). A priming dose [20] of Humulin S neutral human insulin (Eli Lilly and Co. Ltd., Basingstoke, UK) was then given followed by a continuous infusion at 40 mU · m^{-2} · min^{-1} and the rate of Synthamin 14 was increased to provide leucine at 93 μmol · kg^{-1} · h^{-1} until time 510 min. Glucose was infused when the plasma glucose concentration fell to 5 mmol/l.

Thirty min after priming with L-[1-^{13}C]leucine and beginning the mixed amino acid infusion (i. e. elapsed time 30 min), a percutaneous muscle biopsy was obtained from the tibialis anterior [21] at a depth of 10 mm below the fascia using 6.5 mm Tilley Henckel ethmoid punches (S. Murray and Co, Sheffield, UK), via a 10 mm long skin incision made 2–4 min after 5 ml of lignocaine 2% s.c. A second muscle biopsy was taken from the same or the contralateral leg 240 min later at the end of the insulin withdrawal period, and a third biopsy from the leg contralateral to that first biopsied at time 510 min, the end of the insulin infusion period. The muscle samples were immediately frozen, and stored in, liquid nitrogen until analysis.

Blood samples were obtained before tracer administration, at the time of the first muscle biopsy (30 min), and every 60 min thereafter. Coincident samples of expired air were collected into evacuated 20 ml glass tubes (Vacutainer, Becton Dickinson, NJ, USA) for subsequent determination of $^{13}CO_2$ enrichment. Total carbon dioxide production (by infrared analysis) and oxygen consumption (by paramagnetic analysis) were determined for 11 min every 60 min with a ventilated-hood indirect calorimeter, as previously described [22]. Energy expenditure was calculated from Weir's formula [23].

In all subjects we avoided interference with the estimation of [^{13}C]leucine oxidation from oxidation of maize-derived dextrose, which has a high natural abundance of ^{13}C (-9.85 ($\delta^{13}C_{PDB}$‰)), by the use of potato starch glucose, which does not (-20.180 ($\delta^{13}C_{PDB}$‰)) [18]. In the three control subjects who did not receive ^{13}C tracer, breath samples were obtained to measure possible background changes, including the effects of increased amino acid oxidation, in the ^{13}C enrichment of expired CO_2.

^{13}C analysis

Plasma α-ketoisocaproate, plasma leucine and intramuscular free leucine ^{13}C enrichments were measured by gas chromatography mass spectrometry as described previously [12]; plasma α-ketoisocaproate concentrations were determined using α-ketovalerate as internal standard. The true intramuscular free leucine enrichment was calculated from that measured on the basis that the sample contained both intracellular and extracellular fluid in the assumed ratio of 13:87 [24]. An isotope ratio mass spectrometer was used to determine both expired CO_2 ^{13}C enrichment [25] and protein bound leucine ^{13}C enrichment in ninhydrin-cleaved carboxyl-CO_2 [26] of leucine, purified by preparative-gas chromatography [27], from acid-hydrolysed muscle protein [12].

Metabolite and hormone concentration assays

An automated analyser (LC5000, Biotronic GmbH, Munich, FRG) was used to measure concentrations of amino acids from plasma and intramuscular water [12], the latter calculated on the assumption that 13% of the muscle biopsy water was extracellular [27]. Plasma glucose was determined with a Glucose Analyzer 2 (Beckman Instruments, Irvine, Calif, USA). At 210, 270, 450 and 510 min blood D-(-)-3-hydroxybutyrate concentration was measured by an NAD-linked enzyme assay [28], and plasma free insulin, glucagon, cortisol and insulin-like growth factor 1 were assayed using commercial radioimmunoassay kits [12]: free insulin was polyethylene glycol separated at the bedside [29] before assay and insulin-like growth factor 1 was measured with a double antibody kit from Nicols Institute Diagnostics (San Juan Capistrano, Calif, USA). C-peptide concentration, measured only once, initially, was determined with a double antibody kit (Ire-Medgenix Sa, Brussels, Belgium).

Mixed amino acid infusion

Each litre of Synthamin 14 contained the following L-amino acids: alanine 17.6 g, arginine 9.78 g, glycine 8.76 g, histidine 4.08 g, isoleucine 5.10 g, leucine 6.20 g, lysine 4.93 g, methionine 3.40 g, phenylalanine 4.76 g, proline 5.78 g, serine 4.25 g, threonine 3.57 g, tryptophan 1.53 g, tyrosine 0.34 g, valine 4.93 g, total amino acids 85.0 g.

Table 1. Effect of insulin on plasma hormone, glucose and blood D-(-)-3-hydroxybutyrate concentrations

	Insulin withdrawn 210–270 min	Insulin infused 450–510 min
Free insulin (mU/l)	4.6 (2.0, 10.5)	62.6 (56.2, 69.6)[b]
Glucagon (pmol/l)	53 (30, 95)	69 (47, 100)
Cortisol (nmol/l)	300 (145, 624)	306 (170, 548)
Insulin-like growth factor 1 (mU/ml)	560 (330, 970)	500 (320, 790)
Glucose (mmol/l)	12.3 ± 2.0	6.2 ± 0.9[a]
D(-)3-hydroxybutyrate (mmol/l)	0.53 (0.16, 1.74)	0.09 (0.03–0.29)[a]

Data given as means, with 95% confidence limits in parenthesis [a] $p < 0.01$, [b] $p < 0.001$

Calculations

The average rate of mixed skeletal muscle protein synthesis (k_s) during the 240 min periods between biopsies was calculated as:
$k_s(\%/h) = (\Delta E_m \times 100)/(E_p \times \Delta t)$
where ΔE_m = increase in protein bound leucine enrichment between biopsies and E_p = mean plasma α-ketoisocaproate enrichment during the elapsed period (Δt) between biopsies [12]. Whole body leucine oxidation was calculated from the mean ^{13}C enrichment of plasma α-ketoisocaproate (the immmediate precursor of leucine oxidation), the mean ^{13}C enrichment of expired CO_2 (assuming a bicarbonate recovery fraction of 0.81) and the total CO_2 production rate during the periods 90–270 min and 330–510 min [30]. Plasma α-ketoisocaproate ^{13}C enrichment was used to calculate whole body leucine rates of appearance (Ra) and of disappearance (Rd) with non-steady state equations [31]; the whole body leucine pool size was calculated from the sum of plasma leucine and α-ketoisocaproate concentrations and a distribution volume of 0.6 l/kg body weight assuming that plasma leucine concentration reflects that within tissue cells during both amino acid infusion [12] and during insulin infusion [11]. If a distribution volume of 0.125 l/kg was used the actual values obtained were different but there were no important relative differences (results not shown) and we have adopted the larger volume as likely to be more physiological. Endogenous leucine appearance (i.e. whole body protein breakdown) was calculated by subtracting the infusion rate of Synthamin 14 leucine from Ra, and non-oxidised leucine disappearance (i.e. whole body protein synthesis) was calculated by subtracting leucine oxidation from Rd. Mean leucine kinetics over the periods 90–270 min and 330–510 min were used as the basis of assessment of the effects of insulin and glucose upon whole body protein turnover. Plasma α-ketoisocaproate ^{13}C enrichment was taken as the most reliable index upon which to calculate [12, 32, 33] whole body leucine kinetics and muscle k_s. Nevertheless, we have calculated muscle synthesis using intracellular leucine labelling and whole body turnover using plasma leucine labelling, and the resulting data are presented where appropriate.

Statistical analysis

Mean ± SEM values are presented; for D-(-)-3-hydroxybutyrate and hormones, which showed log-normal distributions, the values were transformed to their natural logarithms before analysis; the means and 95% confidence intervals presented are the antilog$_e$ transformations. Significance of differences for each phase of the study were compared by Student's two-tailed t-test for paired data. A value of $p < 0.05$ was considered significant. A stepwise regression analysis (Microstat, Ecosoft Inc, Indianapolis, Ind, USA) was performed to compare changes in the rates of skeletal muscle protein synthesis with changes in blood D-(-)-3-hydroxybutyrate concentration and respiratory quotient and with the rate of glucose infusion.

Table 2. Plasma amino acid concentrations (μmol/l) before and during amino acid (AA) infusion, during insulin withdrawal and during insulin infusion

Amino acid	Before AA infusion –15 min	Insulin withdrawn 30–270 min	Insulin infused 330–510 min
Alanine	266 ± 24	423 ± 92[c]	791 ± 190[d]
Arginine	133 ± 21	203 ± 26[d]	304 ± 39[d]
Asparagine	44 ± 3	47 ± 3[b]	29 ± 2[d]
Glutamic acid	61 ± 8	60 ± 25	48 ± 23[c]
Glutamine	632 ± 45	695 ± 140[c]	612 ± 94[b]
Glycine	229 ± 23	369 ± 40[d]	588 ± 59[d]
Histidine	99 ± 5	141 ± 8[d]	200 ± 15[d]
Isoleucine	110 ± 15	183 ± 19[d]	274 ± 25[d]
Leucine	211 ± 24	333 ± 32[d]	407 ± 39[c]
Lysine	202 ± 12	257 ± 18[c]	328 ± 32[b]
Methionine	27 ± 2	52 ± 6[d]	103 ± 12[d]
Phenylalanine	64 ± 2	96 ± 4[d]	161 ± 7[d]
Serine	131 ± 17	191 ± 28[c]	251 ± 34[d]
Taurine	66 ± 6	64 ± 5	47 ± 3[d]
Threonine	145 ± 18	198 ± 28[c]	247 ± 37
Tryptophan	48 ± 4	60 ± 3	96 ± 6[d]
Tyrosine	57 ± 3	54 ± 4[a]	41 ± 3[d]
Valine	320 ± 32	431 ± 37[d]	588 ± 60[c]
Total amino acids	2950 ± 150	4000 ± 240[d]	5120 ± 350[d]

Comparisons are 30–270 min vs −15 min and 330–510 min vs 30–270 min; [a] $p < 0.05$, [b] $p < 0.02$, [c] $p < 0.01$, [d] $p < 0.001$; values are means ± SEM

Results

Indirect calorimetry

During insulin whithdrawal carbon dioxide production was 204 ± 7 ml/min and increased by 11% during insulin infusion ($p < 0.01$); respiratory quotient was 0.72 ± 0.01 and increased to 0.75 ± 0.01 ($p < 0.01$); calculated heat production was 5.69 ± 0.26 kJ/min and increased by 5% to 6.00 ± 0.26 kJ/min during insulin replacement ($p < 0.01$).

Hormone and substrate concentrations (Table 1)

Blood bicarbonate before infusion was 23.1 ± 0.5 μmol/l and remained in the range 22–25 throughout. Plasma hormone concentrations were stable within each phase of the study. During insulin infusion, plasma free insulin concentration increased from low (4.6 mU/l) to high physiological values (62.6 mU/l). Plasma glucagon, cortisol and insulin-like growth factor 1 were unchanged. Plasma glucose and blood D-(-)-3-hydroxybutyrate were stable during insulin withdrawal and decreased during insulin infusion. During the final 60 min of insulin infusion the rate of glucose infusion was 2.17 ± 0.64 $mg \cdot kg^{-1} \cdot min^{-1}$.

Amino and keto acid concentrations (Tables 2 and 3)

Mean plasma leucine concentration (Fig. 1) was 211 ± 24 μmol/l before the start of the primed amino acid infusion. The plasma leucine concentration increased by 13% throughout the period when leucine kinetics were calculated during insulin withdrawal ($p < 0.02$); mean plasma leucine was 337 ± 33 μmol/l during this period. The plasma leucine concentration decreased by 18% throughout the period when leucine kinetics were calcu-

Table 3. Effect of insulin on intramuscular free amino acid concentrations (μmol/l) during amino acid infusion

Amino acid	Insulin withdrawn		Insulin infused 510 min
	30 min	270 min	
Alanine	1920 ± 140	2090 ± 180	3340 ± 160[d]
Arginine	608 ± 104	598 ± 112	607 ± 113
Asparagine	897 ± 44	914 ± 71	850 ± 67
Glutamic acid	4750 ± 300	4820 ± 460	3050 ± 210[c]
Glutamine	13400 ± 800	13200 ± 1900	11600 ± 900[a]
Glycine	1510 ± 100	1570 ± 90	1640 ± 110
Histidine	597 ± 72	652 ± 95	562 ± 85[a]
Isoleucine	149 ± 17	175 ± 31	173 ± 33
Leucine	298 ± 32	341 ± 9	282 ± 50[c]
Lysine	1808 ± 257	2214 ± 286	1376 ± 344
Phenylalanine	95 ± 8	105 ± 8	145 ± 9[d]
Serine	690 ± 75	670 ± 86	650 ± 84
Taurine	17600 ± 900	17700 ± 1300	16100 ± 1100
Threonine	769 ± 56	729 ± 68	705 ± 68
Tyrosine	89 ± 6	81 ± 4[b]	21 ± 7[d]
Valine	448 ± 79	444 ± 79	492 ± 100
Total amino acids	45200 ± 1300	45000 ± 2100	41200 ± 170[a]

Comparisons are 30 min vs 270 min and 270 min vs 510 min [a] $p < 0.05$, [b] $p < 0.02$, [c] $p < 0.01$, [d] $p < 0.001$; values given are means ± SEM

Table 4. Enrichments (Atoms % excess, APE) of plasma and intramuscular free leucine and α-ketoisocaproate, and their ratios, during [1-^{13}C]leucine infusion

	Insulin withdrawn 90–270 min	Insulin infused 330–510 min
Plasma leucine (APE)	5.01 ± 0.21	4.19 ± 0.18[b]
Plasma α-ketoisocaproate (APE)	4.70 ± 0.31	4.19 ± 0.23[a]
Intramuscular leucine (APE)[d]	3.82 ± 0.27[c]	3.33 ± 0.32[c]
Plasma α-ketoisocaproate/plasma leucine	0.94 ± 0.05	0.99 ± 0.03
Intramuscular leucine/plasma leucine	0.76 ± 0.04	0.80 ± 0.07
Intramuscular leucine/plasma α-ketoisocaproate	0.82 ± 0.05	0.80 ± 0.06

Comparisons are 330–510 vs 90–270 min [a] $p < 0.02$; [b] $p < 0.001$; [c] Intramuscular leucine enrichment was significantly different ($p < 0.02$) from plasma leucine and plasma α-ketoisocaproate at 90–270 and 330–510 min; [d] Intramuscular leucine was measured at 270 min and 510 min

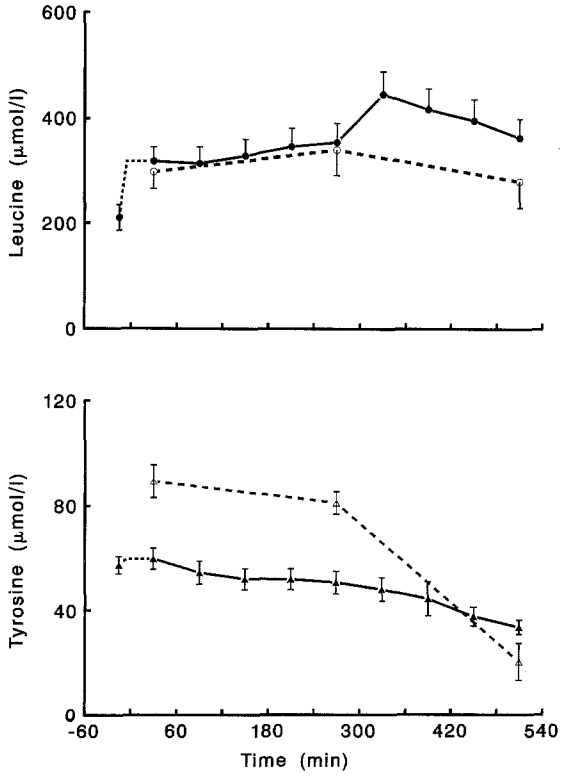

Fig. 1. Upper panel: plasma (–●–) and intramuscular (–○–) leucine concentrations were not different during insulin withdrawal but the intramuscular leucine was lower ($p < 0.01$) during insulin infusion. Lower panel: plasma (–▲–) and intramuscular (–△–) tyrosine concentrations decreased ($p < 0.001$ in each case) to low levels during insulin infusion; values are means ± SEM

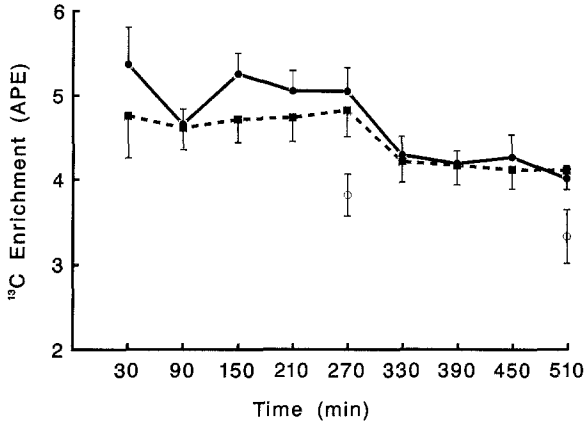

Fig. 2. Plateaux of plasma α-ketoisocaproate (–■–) and leucine (–●–) ^{13}C enrichment (Atoms % excess, APE) were observed during each phase of study. Intramuscular leucine enrichment (○) did not change; values are means ± SEM

lated during insulin infusion ($p < 0.01$); the mean concentration during this period (407 ± 39 μmol/l) was 21% higher than during insulin withdrawal ($p < 0.01$). Plasma α-ketoisocaproate concentration was 53 ± 9 μmol/l during insulin withdrawal and was not significantly different during insulin infusion (43 ± 7 μmol/l). The total concentration and concentrations of most individual amino acids in plasma (Table 2) were increased by the amino acid infusion given at a faster rate during insulin administration. Exceptions were asparagine, glutamic acid, glutamine, tyrosine (Fig. 1) and taurine which all decreased. The concentration of free leucine in intramuscular water (Fig. 1, Table 3) was not different from the plasma concentration before insulin, i.e. at 30 or at 270 min. During insulin infusion the intramuscular leucine concentration decreased (when sampled at the end, i.e. at 510 min) and was actually lower than both the plasma value at the same time ($p < 0.01$) and the preceding intramuscular value ($p < 0.01$). Despite the general increase in plasma amino acid concentrations when insulin plus additional amino acids were infused a reduction was found in the concentration of total amino acids in intramuscular water. The biggest change occurred for tyrosine which decreased from

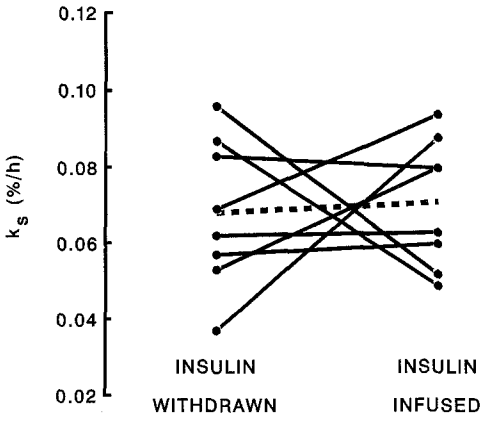

Fig. 3. Mean skeletal muscle protein synthesis rate did not change during insulin infusion; individual (—) and mean values (-----) are shown

81 ± 4 μmol/l to a mean of only 21 ± 7 μmol/l; it was reduced below detectable values in three subjects (Fig. 1).

Enrichment of possible precursors for muscle protein synthesis (Table 4)

For both plasma α-ketoisocaproate and for plasma leucine plateaux of ^{13}C enrichment were observed during insulin withdrawal and significantly reduced plateaux were found during insulin replacement (Table 4, Fig. 2). However, leucine enrichment in muscle was unchanged. No differences occurred in the ratios of enrichment of free leucine and its metabolite in plasma or muscle or between them as a result of insulin administration.

Table 5. Anterior tibialis muscle protein synthetic rates (k_s, %/h) calculated on the basis of enrichment of various possible precursors

Possible precursor	Insulin withdrawn	Insulin infused
Plasma leucine	0.063 ± 0.007	0.071 ± 0.007
Plasma α-ketoisocaproate	0.068 ± 0.007	0.071 ± 0.006
Intramuscular leucine	0.084 ± 0.010[a]	0.091 ± 0.007[a]

[a] $p < 0.05$ at least in comparison with plasma leucine or α-ketoisocaproate based synthetic rates

Table 6. Effect of insulin on leucine kinetics during amino acid infusion

	Insulin withdrawal (μmol·kg^{-1}·h^{-1})	Insulin infusion (μmol·kg^{-1}·h^{-1})
Total flux	150 ± 5	176 ± 5[a]
Infusion	25	93
Breakdown (endogenous Ra)	125 ± 5	83 ± 5[b]
Total Rd	145 ± 5	185 ± 4[b]
Synthesis (Non-Ox Rd)	110 ± 4	117 ± 4
Oxidation	35 ± 3	68 ± 4[b]

[a] $p < 0.01$, [b] $p < 0.001$; values given are means ± SEM; Ra = rate of appearance and Rd = rate of disappearance

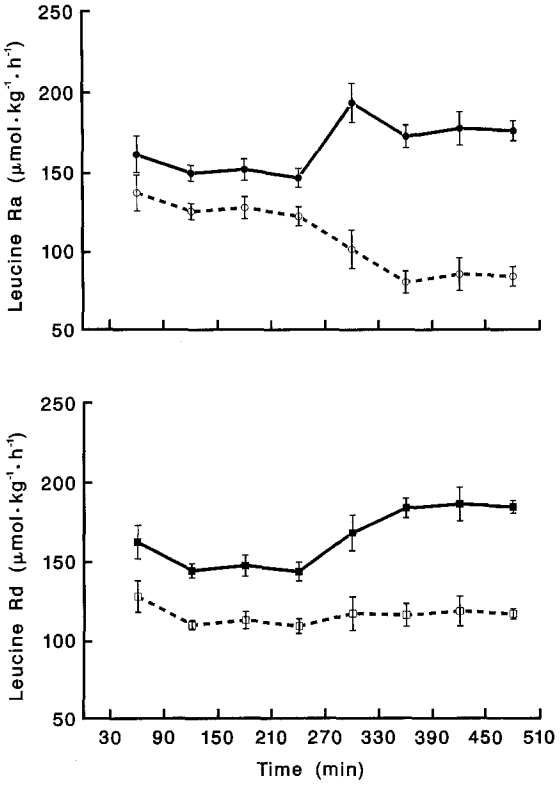

Fig. 4. Upper panel: whole body total leucine appearance (–●–) increased ($p < 0.01$), on account of the increased rate of exogenous infusion, and endogenous leucine appearance (--○--) decreased ($p < 0.01$) during insulin infusion. Lower panel: total leucine disappearance (–■–) increased ($p < 0.01$), due to increased leucine oxidation ($p < 0.001$), and leucine disappearance to protein (--□--) was unchanged. Ra = rate of appearance; Rd = rate of disappearance

Skeletal muscle mixed protein synthesis

Mixed skeletal muscle protein synthesis (k_s) was 0.068 ± 0.007%/h during insulin withdrawal (Fig. 3) and was unchanged (0.071 ± 0.006%/h) during insulin replacement. If muscle protein synthesis rates were calculated on the basis of the enrichment of plasma or intracellular leucine (Table 5), they tended to be lower or were higher than on the basis of the plasma α-ketoisocaproate labelling, respectively, but no effects of insulin-plus-glucose were seen. From the present α-ketoisocaproate based results it can be calculated that there was a 83% likelihood of detecting an increase of 30% in skeletal muscle protein synthesis during insulin infusion ($\alpha = 0.05$, two-tailed test); thus, the chance of a type II error was only 17%. Individual subjects showed different changes in k_s during insulin infusion but on stepwise regression analysis no correlation was found between the changes in k_s and changes in the concentration of D-(-)-3-hydroxybutyrate, changes in the respiratory quotient or the absolute glucose infusion rate during hyperinsulinaemia.

Whole body leucine kinetics (Table 6)

Expired CO_2 ^{13}C enrichment was 0.0188 ± 0.0013 atoms % excess (APE) during insulin withdrawal and was higher during insulin infusion (0.0298 ± 0.0012 APE, $p < 0.001$). The corresponding mean values were 0.0002 ± 0.0001 and

0.0009 ± 0.0005 APE in three subjects not infused with ^{13}C tracer leucine, suggesting that leucine oxidation may have been overestimated by only 3% during the latter period due to $^{13}CO_2$ released from oxidation of the infused glucose.

Leucine oxidation was 35 ± 3 μmol·kg^{-1}·h^{-1} during insulin withdrawal and increased by 84% during insulin replacement ($p < 0.001$). Whole body leucine turnover was stable during the final 180 min of each phase of the study. Total leucine appearance (Fig. 4) was 150 ± 5 μmol kg^{-1}·h^{-1} during insulin withdrawal and increased 18% during insulin replacement ($p < 0.01$); endogenous leucine appearance (protein breakdown) was 125 ± 5 μmol·kg^{-1}·h^{-1} and decreased by 33% ($p < 0.01$). On the basis of plasma α-ketoisocaproate labelling, total leucine disappearance (Fig. 4) was 145 ± 5 μmol·kg^{-1}·h^{-1} during insulin withdrawal and increased by 28% during insulin replacement ($p < 0.01$); the non-oxidised component of Rd (whole body protein synthesis) was 110 ± 4 μmol·kg^{-1}·h^{-1} and was not significantly different during insulin replacement (117 ± 4 μmol·kg^{-1}·h^{-1}). However, if the plasma leucine labelling was used instead of α-ketoisocaproate and the non-steady equations were applied, then whole body protein synthesis increased from 96.3 ± 5.4 to 119.1 ± 7.1 μmol·kg^{-1}·h^{-1} ($p < 0.01$). On this basis there was also a reduction in whole body protein breakdown which was highly significant ($p < 0.001$) but it was only by 23% of the pre-insulin value (results not shown).

Discussion

In the investigations described here plasma free insulin was increased by insulin infusion from low to high physiological concentrations over 4 h. During insulin infusion, increased infusion of amino acids resulted in indispensable amino acids being maintained at, or slightly above, plasma concentrations seen before insulin. Despite the increased supply of amino acids no significant increase occurred in skeletal muscle protein synthesis during insulin replacement, irrespective of the precursor used as the basis of the calculations.

A number of possibilities might explain this negative result. Although it is possible that in the adult human body the capacity for modulation of muscle protein synthesis by insulin is absent, this seems unlikely, especially in view of the extensive evidence from animal studies [1–4]. Alternatively, insulin may have been ineffective on account of a post-receptor defect of protein metabolism in the diabetic patients (whose glucose metabolism was markedly insulin resistant, as indicated by the low respiratory quotients and glucose disposal rates), but the possible nature of such an effect is presently unknown.

That the rates of anterior tibialis muscle protein synthesis found in the present study during insulin withdrawal tended to be faster ($p = 0.12$) than those in seven healthy postabsorptive subjects studied by identical means [12] may offer a further possibility. It seems that at low concentrations of insulin muscle protein synthesis was not pathologically reduced in these diabetic subjects and may even have been elevated, a result in accordance with the results from Halliday's group [7]: thus, skeletal muscle protein synthesis may have been already maximally stimulated in the diabetic patients and a further increase by insulin may not have been possible. This would be in marked contrast to the situation in immature rats in which muscle protein synthesis is reduced at low insulin concentrations [1–4].

There is also the slight possibility that if insulin were initially totally absent, instead of present at very low concentrations (see Table 1), it might have been possible to observe an impairment of basal skeletal muscle protein synthesis in our patients, since in the rat at least, sensitivity of protein synthesis to insulin is already maximal over 0–10 mU/l [34]. Evidence against this comes from our findings that two subjects in whom insulin was not detectable by our assay had muscle protein synthetic rates comparable with those of the other subjects in whom it was. Nevertheless, this argument cannot be conclusive since the range of insulin sensitivity of protein synthesis in adult man is unknown and may differ markedly from that in young rats and other animals.

The possibility of limitation of intracellular amino acid availability remains to be considered. Amino acid infusion at an increased rate did not prevent an insulin-induced reduction in the muscle water concentration of certain amino acids, most markedly for tyrosine. This reduction may have acted as a constraint on muscle protein synthesis (even though the affinity of tRNA for amino acids is very high in vitro [35]) or the normal stimulatory effect [12] of amino acids on muscle protein synthesis may have been reduced. The amino acid mixture used contained only a small quantity of tyrosine, whose relative insolubility limits its delivery by infusion. Although not indispensable in the whole body in adults, usually being produced in the liver from phenylalanine, it is essential in muscle which cannot synthesize it; the ratio between protein-bound and free tyrosine is high, so that the "safety factor" for tyrosine availability is small [36]. Decreases in the plasma tyrosine may have limited uptake of tyrosine into the limb since its net uptake depends upon the plasma concentrations [37]. Its uptake, by the insulin-independent L-system transporter [38, 39], may also have been competitively inhibited by the relatively high concentrations of the branched-chain amino acids, phenylalanine and tryptophan; furthermore, these amino acids may have depleted muscle tyrosine by trans-stimulation [40] of the L-system.

In comparison to healthy postabsorptive subjects [12], the patients with diabetes we studied had markedly raised plasma amino acid concentrations during insulin withdrawal, partly, presumably, as a result of increased protein breakdown as well as amino acid infusion. It seems likely that elevated amino acid availability initially may have contributed to the elevated muscle protein synthetic rates we measured in the insulin withdrawn state, since we have shown previously that muscle protein synthesis in healthy postabsorptive men increased by an average of 35% during infusion of mixed amino acids alone [12], and we have recently confirmed the effect by arterio-venous tracer amino acid flux measurements in a separate group of subjects [13]. It is difficult to choose which of two possible effects, i.e. stimulation by an initially high intramuscular amino acid concentration or limitation by removal of muscle essential amino acids was most important for the

lack of a further stimulation by insulin, but both could conceivably have been involved. In any event, our present and previous findings are consistent with the proposition that, in man, availability of amino acids rather than of insulin, is important in maintaining rates of skeletal muscle protein synthesis, and that insulin is less so.

A number of other substrates which could have influenced muscle protein synthesis changed in response to insulin infusion. 3-Hydroxybutyrate apparently stimulates muscle protein synthesis, but by only 10% as its concentration increases over a fourfold range from 0.5 mmol/l [14]: we found that insulin caused a small decrease in 3-hydroxybutyrate, making it unlikely that such a change would have relieved a previously stimulatory effect on skeletal muscle protein synthesis. In rat diaphragm and heart muscle in vitro, glucose increases protein synthesis [41] and deficiency of glucose results in increased leucine oxidation [42]. Plasma glucose was elevated during insulin withdrawal but muscle was unlikely to have been able to utilize it efficiently because of lack of insulin and little sparing of amino acid catabolism could have occurred. Other factors not otherwise considered in our patients include elevations of fatty acids and medium-chain triglycerides, the latter being reported to improve forearm balance of amino acids [43].

In addition a number of hormones may have altered in concentration during insulin infusion. Growth hormone (not measured) may have been initially elevated but the patients were in good diabetic control, and plasma insulin-like growth factor 1 was in the normal range making this unlikely. Paracrine and systemic growth factors other than insulin-like growth factor 1 may influence protein synthesis in ways not accessible to our analyses. During insulin infusion plasma free insulin increased into the high physiological range but nevertheless remained beneath the threshold necessary to stimulate insulin-like growth hormone receptors. However, insulin-like growth factor 1 was measured in plasma only and any local muscle cell changes would not have been apparent. We suggest that this is unlikely not only because of a lack of any anabolic effect, but also because increases in insulin-like growth factor 1 normally require considerable exposure to insulin [44].

Marked changes in the rates of muscle protein synthesis were evident between patients; increases occurred in three, but in two patients there were decreases. Stepwise regression analysis showed these changes to correlate with neither indices of insulin sensitivity nor changes in the plasma 3-hydroxybutyrate. Although it is not possible with the present study design to identify specific factors which induced increases in protein synthesis in particular individuals during insulin infusion, the occurrence of the increases suggests that there may have been some stimulation of protein synthesis by a combination of insulin and some unidentified factor.

The muscle biopsy technique used, although invasive, enables measurement of muscle protein synthesis with few inherent assumptions. The plasma enrichment of α-ketoisocaproate during infusion of leucine tracer is assumed to represent the labelling of leucyl-tRNA within cells: we have recently obtained good evidence that for pig muscle, with and without infusion of amino acids, it comes closer to the leucyl-tRNA than do a number of other possible indices such as plasma or intracellular leucine [44]. Only circumstantial evidence concerning this question is currently available in man, but it is consistent with our approach [12, 33, 34]. Given the present study conditions, in which plasma leucine concentration was high throughout both phases of investigation, tracer exchange between plasma and the intracellular fluid was likely to be rapid; certainly, we observed ratios of the enrichment of plasma α-ketoisocaproate/leucine, intramuscular leucine/plasma leucine and intramuscular leucine/α-ketoisocaproate much closer to unity in the diabetic patients than in healthy subjects studied without infusion of amino acid [12]. There is, therefore, less variation in the magnitudes of rates of muscle protein synthesis in the diabetic patients than in healthy subjects [12] when different precursor pools are used to calculate the muscle protein synthesis rate.

The rates of whole body protein leucine kinetics are in agreement with values published by other workers [46–48]. The apparent lack of stimulation by insulin of whole body protein synthesis in the diabetic patients is consistent with results from healthy subjects showing that rates of protein synthesis correlated only with plasma leucine [9, 10]. In the present study the observed lack of insulin stimulation of skeletal muscle is consistent with a lack of change in whole body protein synthesis. That leucine oxidation increased with insulin infusion is also in agreement with previous work in which plasma leucine was maintained during insulin infusion [9]. Although insulin deficiency increases leucine oxidation in perfused hind-limbs, from fasted or fed rats, its replacement reduces leucine oxidation in the fed preparation but markedly increases it in the fasted preparation [49]. Also, insulin has been shown to increases leucine oxidation in adipose tissue in vitro [50]. It appears likely that the increased leucine oxidation we observed during insulin infusion occurred in muscle and adipose tissue. The extent of leucine oxidation in different human tissues in vivo is not well documented although the distribution of the α-keto acid dehydrogenase [51] suggests muscle to be a major site, and our own arterio-venous measurements suggest that it must be the major one in healthy man [13, 52].

The measurement of muscle protein synthesis uses fewer uncheckable assumptions than the assessment of whole body protein synthesis with the tracer dilution technique. In addition to uncertainties of precursor pool labelling [12], changes in insulin action result in alterations of plasma and intramuscular leucine concentration with the result that whole body protein synthesis, calculated with steady state equations, tends to be overestimated during insulin withdrawal with the opposite effect during insulin replacement. Although not independently validated the equations that we used take account of the changing tracer concentrations. A further source of error which may have caused underestimation of protein synthesis during the present study conditions is recycling of tracer, which may lead to increased plasma tracer enrichment and underestimation of both flux and protein synthesis despite coincident underestimation of leucine oxidation; however, this is usually only a problem in more prolonged infusions [53]. In the present investigations a bicarbonate recovery value of

0.81 was used, derived from i. v. tracer-bicarbonate infusion in healthy postabsorptive subjects. As determined from the appearance of tracer-carbon labelling in glucose by incorporation of the carbons during gluconeogenesis, the proportion of bicarbonate fixed during infusions of leucine tracer from oxidation of leucine carboxyl-carbon within mitochondria is greater than occurs during infusion of bicarbonate for the same degree of plasma bicarbonate labelling. Therefore, general use of a bicarbonate recovery value of 0.81 may lead to additional underestimation of leucine oxidation and thus, overestimation of protein synthesis. During provision of glucose, which stimulates insulin secretion and inhibits gluconeogenesis, fixation of tracer-carbon from bicarbonate by gluconeogenesis is reduced [54] and under these conditions the figure of 0.81 may markedly overestimate leucine oxidation exacerbating the underestimation of protein synthesis. Changes also occur in the whole body bicarbonate pool during insulin withdrawal and insulin replacement inducing additional errors in estimates of bicarbonate fixation and leucine oxidation. We probably avoided one source of error in glucose infusion: if maize-derived glucose had been infused, $^{13}CO_2$ released from maize glucose oxidation would have led to an overestimation of leucine oxidation and a further corresponding underestimation of whole body protein synthesis during hyperinsulinaemia. Such factors collectively result in underestimation of whole body protein synthesis by the [1-^{13}C]leucine dilution technique during hyperinsulinaemic conditions and may induce qualitative errors in the interpretation of the effects of insulin action on whole body protein synthesis, both in our work and that of others [9, 10, 46].

Comparison of the present whole body leucine metabolism results with those from studies in non-diabetic men, in which whole body protein synthesis was not stimulated by insulin [9, 10], are consistent with a lack of any fundamental difference in sensitivity of amino acid metabolism to insulin between diabetic and non-diabetic subjects [47]. Similar conclusions might be drawn by comparing the skeletal muscle leucine incorporation results reported here for Type 1 diabetic subjects with the reported lack of effect of insulin on forearm arterio-venous uptake of phenylalanine in healthy man [8]; however, the different methods used in the two investigations, and the differences in the subjects studied may make comparisons invalid.

To conclude, in the present study we could not show any acute anabolic effect of systemic insulin infusion on skeletal muscle protein synthesis in insulin-withdrawn adult diabetic patients. It appears possible that reduced intracellular concentrations for a number of amino acids during insulin infusion, in particular tyrosine, may have constrained muscle protein synthesis. The hypothesis that such a deficiency of amino acids, particularly tyrosine, is the reason why protein synthesis is not stimulated by insulin needs to be further investigated using amino acid mixtures including tyrosine dipeptides [55] to increase its availability.

In the meantime the likelihood that increased total amino acid availability maintains muscle protein synthesis in diabetic subjects and that acute treatment with insulin does not stimulate it further must weaken the perception that the general anabolic effect of insulin includes stimulation of muscle protein synthesis. It may be, however, that in normal healthy subjects or in diabetic patients with physiological amino acid concentrations, insulin does stimulate muscle protein synthesis, if sufficient substrate is supplied; the fact that Type 1 diabetic children may grow normally suggests that this is so. We must now design appropriate investigations to test these ideas.

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