Action of Insulin and Growth Hormone on Protein Synthesis in Muscle from Non-Hypophysectomized Rabbits

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The separate effects of insulin and growth hormone on the uptake and incorporation of five amino acids into diaphragm muscle from non-hypophysectomized rabbits has been examined. Both growth hormone and insulin, when present in the medium separately, stimulated the incorporation into protein of the amino acids, leucine, arginine, valine, lysine and histidine. Insulin also stimulated amino acid uptake, but growth hormone did not. When insulin and growth hormone were present in the incubation medium together, the uptake and incorporation of valine, the only amino acid studied under these conditions, tended to be greater than the sum of the separate effects of the two hormones.

Protein synthesis is impaired in tissues from hypophysectomized animals but is restored to normal by administering growth hormone to the animal before death, or by the inclusion of growth hormone in the incubation medium *in vitro*. This has been demonstrated with liver (Simpson, Evans & Li, 1949; Korner, 1965) and muscle tissue (Kostyo & Knobil, 1959; Manchester & Young, 1961). It has also been shown that the accumulation of the non-metabolized amino acid α -aminoisobutyric acid in muscle from hypophysectomized rats is stimulated *in vitro* by the presence of growth hormone in the medium (Kostyo, Hotchkiss & Knobil, 1959; Hjarlmasen, 1968; Kostyo, 1968) and that this effect can be blocked by inhibitors of protein synthesis such as puromycin (Hjarlmasen, 1968) and cycloheximide (Kostyo, 1968). The stimulation of protein synthesis in muscle from hypophysectomized rats by growth hormone was not altered by blocking membrane transport (Kostyo, 1964). However, despite the clear-cut effects of growth hormone on protein synthesis and amino acid transport in muscle from hypophysectomized animals *in vitro*, such effects on muscle obtained from intact animals *in vitro* have not been reported.

The plasma concentration of growth hormone fluctuates in relation to feeding, and is known to be high after prolonged starvation and low after feeding (Garcia & Geschwind, 1968). In the present experiments, therefore, diaphragm muscle for investigation *in vitro* was taken from intact rabbits 2 h after feeding, when the plasma growth hormone

concentration was low. At this stage the plasma insulin concentration is similar to that observed after starvation for 17 h (Turner, Reeds & Munday, 1971a).

In order to reproduce as nearly as possible the situation *in vivo* it would be desirable to incubate tissues *in vitro* in a medium containing all the naturally occurring amino acids. This approach was adopted by Clemens & Korner (1970) who showed a stimulation by growth hormone *in vitro* of protein synthesis in liver from intact rats, in the presence of increased concentrations of extracellular amino acids. In the work described below the uptake and incorporation of amino acids was measured in diaphragm muscle in a medium containing five radioactively labelled amino acids at physiological concentrations. A stimulation of protein synthesis by growth hormone in the absence of any effect on amino acid uptake was demonstrated for all five amino acids by using this experimental approach. A preliminary report of part of this work has been published (Reeds, Munday & Turner, 1971a).

EXPERIMENTAL

Animals. Hybrid rabbits of the F.1. generation derived from Dutch females and semi-Lop males were weaned on to a stock diet (SGI, obtained from Rank, Hovis, MacDougall Ltd., Southampton, U.K.) and used at 3 kg body weight. Diaphragm muscle was taken from rabbits killed 2 h after they had been fed. Blood samples were obtained from ear veins of rabbits familiar with the bleeding procedure. Heparin (25 units/ml of blood) was used as an anticoagulant.

Materials

Pig growth hormone (lot P-522A of potency 2i.u./mg) was a gift from the National Institutes of Health, Bethesda, Md., U.S.A. Ox insulin (six-times recrystallized: potency 23i.u./mg) was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

Unlabelled amino acids were from a chromatographic set supplied by BDH Chemicals Ltd. The labelled amino acids, which were obtained from The Radiochemical Centre, Amersham, Bucks., U.K., were L-[4,5-³H]leucine, L-[4,5-³H]lysine, L-[5-³H]arginine, L-[2,5-³H]histidine and L-[U-¹⁴C]valine. These were added to the unlabelled amino acids to give a final specific radioactivity for each amino acid of 0.2 μ Ci/ μ mol. The scintillant used for the measurement of radioactivity was 5-(4-biphenyl)-2-(4-tert.-butylphenyl)-1-oxa-3,4-diazole from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.

Methods

Amino acid. Plasma amino acids were separated by two-dimensional t.l.c. and estimated colorimetrically with ninhydrin, as described by Reeds, Munday & Turner (1971b).

Insulin. Plasma insulin was determined by the double antibody radioimmunoassay (Hales & Randle, 1963) with kits supplied by The Radiochemical Centre.

Growth hormone. Plasma growth hormone was determined by a double antibody radioimmunoassay technique with antibodies raised in guinea pigs to pig growth hormone [Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.], pig growth hormone standards (National Institutes of Health: lot P-522A) and ¹²⁵I-labelled pig growth hormone (National Institutes of Health: lot P-522A) iodinated by the method of Hunter, Greenwood & Glover (1963), but by using Na¹²⁵I (IMS3; The Radiochemical Centre). The specific radioactivity of the labelled hormone was 190–220 μ Ci/ μ g. The behaviour of labelled and unlabelled growth hormone was similar on gel filtration (Sephadex G-75) and also on cellulose acetate and polyacrylamide-gel electrophoresis.

The antibody to pig growth hormone was raised in guinea pigs by five injections of growth hormone (1 mg) in 1 ml of Freund's complete adjuvant (Difco Laboratories, Detroit, Mich., U.S.A.)-iso-osmotic saline (1:1, v/v) at 2-week intervals followed by two injections of 1 mg of growth hormone in 1 ml of iso-osmotic saline at 1-week intervals. The animals were bled 10 days after the last injection and the sera were pooled and stored at –20°C.

For the assay 0.1 ml of the anti-(growth hormone) serum diluted 1:3000 with an iso-osmotic saline–0.05 M-sodium phosphate buffer, pH 7.4, containing 0.5% bovine serum albumin (Fraction V, grade b: Calbiochem, Los Angeles, Calif., U.S.A.) and 0.025% sodium thiomersalate (BDH Chemicals Ltd.) was added to a test tube containing rabbit plasma (0.1 ml) or to 0.1 ml of a standard solution containing 1–50 ng of growth hormone/ml. The tubes were incubated for 8 h at 4°C and 0.1 ml of ¹²⁵I-labelled pig growth hormone solution (containing 250 pg of hormone in 0.05 M-sodium phosphate buffer, pH 7.4, containing 0.5% bovine serum albumin and 0.025% sodium thiomersalate) was added; the incubation was continued for a further 24 h at 4°C. To each tube was added 0.1 ml of rabbit serum

containing antibodies to guinea-pig plasma proteins (Burroughs Wellcome and Co., Beckenham, Kent, U.K.), diluted 1:4 with the saline-phosphate buffer described above, and the incubation continued for a further 24 h at 4°C.

The contents of each tube were filtered through batch-tested 2.5 cm Oxoid cellulose acetate filters (The Radiochemical Centre) then washed through twice with 1 ml of the phosphate buffer described above but diluted 1:1 (v/v) with Horse Serum no. 2 (Burroughs Wellcome and Co.). The filter discs were placed in glass vials, then oven-dried for 10 min at 110°C before 5 ml of 0.8% scintillant in sulphur-free toluene was added. The radioactivity was measured in a Beckman Scintillation Spectrometer by using the full spectrum counting module.

The assay gave parallel curves for pig growth hormone and rabbit plasma, and was unaffected by human serum. The absolute values for plasma growth hormone in starved rabbits determined by this procedure against pig growth hormone standards were similar to those obtained by Garcia & Geschwind (1968), who used rabbit growth hormone standards, and antibodies raised against rabbit growth hormone.

Incubation procedure. Rabbits were starved for 2 h then anaesthetized with Nembutal (60 mg/kg body weight, intravenously). The diaphragm was removed rapidly (within 1 min), washed in ice-cold, oxygenated 0.9% saline, then sliced along the axis of the muscle fibres into strips approx. 1–2 mm wide $\times$ 10 mm long, each weighing approx. 20 mg.

Diaphragm slices obtained from individual rabbits were mixed randomly, blotted and transferred to 25 ml Ehrlenmeyer flasks (10 strips/flask) containing 5 ml of Krebs bicarbonate buffer, pH 7.4 (Krebs & Henseleit, 1932), gassed with O₂ + CO₂ (95:5) and containing glucose (2 mg/ml) and the amino acid mixture [³H]leucine (0.16 mM), [³H]arginine (0.15 mM), [³H]lysine (0.18 mM), [³H]histidine (0.05 mM) and [¹⁴C]valine (0.12 mM) all at a specific radioactivity of 0.2 μ Ci/ μ mol. The concentrations of amino acid used were approximately twice those found in the peripheral plasma of rabbits that had been starved for 17 h. Experimental flasks also contained growth hormone (0.5 μ g/ml), insulin (0.1 unit/ml) or a mixture of growth hormone (0.1 μ g/ml) and insulin (0.01 unit/ml).

Incubations were performed for 1 h at 37°C with shaking at 80 oscillations/min, after which the diaphragm slices were blotted, weighed, transferred to 20 ml of ice-cold ethanol, in which they were minced and then homogenized at a temperature of between 0°C and 4°C.

Tissue analysis. The precipitated tissue protein was separated by centrifugation at 800g and the supernatant stored at 0°C–2°C. The protein was washed for 15 min each in 3 ml of ice-cold 3% (w/v) trichloroacetic acid, 3% (w/v) trichloroacetic acid at 90°C, chloroform-methanol (1:1, v/v), chloroform-methanol (2:1, v/v) and finally two changes of sodium-dried ether. These protein samples were dried at 40°C, then weighed portions were suspended in 6 M-HCl, sealed under N₂ and hydrolysed overnight at 110°C. The hydrolysed samples were again centrifuged at 800g, then evaporated to dryness under N₂ and dissolved in aq. 90% methanol.

Samples of the incubation media, ethanol supernatants and hydrolysed protein were examined by two-dimensional t.l.c. The radioactivity associated with each amino

acid was determined by scintillation counting with 0.6% scintillant in methanol-sulphur-free toluene (1:4, v/v) in an Intertechnique ABAC SL40 liquid-scintillation spectrometer with external quench correction calculated from a ^3H - ^{14}C quench curve for the scintillation fluid used.

Expression of results. 1. The tissue/medium isotope distribution ratio is expressed as d.p.m./ml of intracellular water and d.p.m./ml of medium after correction for radioactivity in the extracellular fluid estimated by the method of Hjarlmasen (1968).

2. Amino acid concentrations are expressed as nmol calculated by relating the measured radioactivity to the specific radioactivity of the amino acid used.

3. Statistical analysis: the paired-sample analysis for Student's *t* test has been used throughout.

RESULTS

Rabbits were fed then killed 2 h later, and their diaphragm muscle was removed, at which time the plasma growth hormone concentration was low (2 h-starved, 0.8 ng/ml; 17 h-starved, 8.5 ng/ml) and the plasma insulin concentration was similar to that after overnight starvation (2 h-starved, 36.1×10^{-6} unit/ml; 17 h-starved, 26.9×10^{-6} unit/ml; $P < 0.2$) (Table 1).

The sucrose space can be taken as equivalent to the volume of extracellular water, and this was shown to be similar in both the sliced muscle and hemi-diaphragms of intact rabbits (Table 2). Incubation of the diaphragm muscle slices in a medium containing added growth hormone (0.5 $\mu\text{g}/\text{ml}$) together with the amino acids valine, leucine, arginine, lysine and histidine, at concentrations approximately twice those found in rabbit plasma after 17 h of starvation, resulted in a significant increase in the incorporation of all of the amino acids into the tissue protein, but had no effect on the rate of amino acid transport into the cell (Table 3). These results suggest that under the influence of growth hormone, amino acid was being incorporated into muscle protein at the expense of

the intracellular pool of amino acids and this effect was reflected in the tissue/medium isotope distribution ratio, which was significantly decreased for each of the amino acids (Table 3).

On the other hand, in the presence of insulin (0.1 unit/ml) in the incubation medium, the rate of amino acid uptake by the diaphragm muscle was increased so that the tissue/medium isotope distribution ratio was significantly increased for each amino acid and this occurred despite a significant increase in incorporation of amino acid into protein (Table 4).

When growth hormone (0.1 ng/ml) and insulin (0.01 unit/ml) were present together in the incubation medium there was a significant increase in the tissue/medium isotope distribution ratio for valine, the only amino acid that was measured in this experiment. However, the rate of incorporation of valine into protein was significantly greater than with either growth hormone or insulin separately, and tended to be greater than the sum of the separate effects of the hormones ($0.1 > P > 0.05$) (Table 5).

DISCUSSION

Our results show that growth hormone stimulated the incorporation of amino acids into protein without stimulating amino acid uptake, in muscle from intact rabbits incubated *in vitro* in a medium containing a mixture of amino acids at physiological concentrations. This is in contrast with observations on muscle from hypophysectomized animals in which both protein synthesis and amino acid uptake were found to be stimulated by growth hormone *in vivo* (Riggs & Walker, 1960) as well as *in vitro* (Snipes & Kostyo, 1962; Hjarlmasen & Ahren, 1965; Martin & Young, 1965). When intact

Table 1. *Growth hormone and insulin in the plasma of rabbits 2 or 17 h after being fed*

Values are the mean from five animals $\pm$ s.e.m. *** $P < 0.001$. Hormone concentrations were determined by double antibody radioimmunoassay, by the method of Hales & Randle (1963) for insulin and that described in the text for growth hormone.

Duration of starvation (h)	Hormone concentration in plasma	
	Growth hormone (ng/ml)	Insulin ($10^6 \times$ unit/ml)
2	0.8 ± 0.2	36.1 ± 5.0
17	*** 8.5 ± 1.1	26.9 ± 7.6

Table 2. *Extracellular water in rabbit diaphragm muscle with and without growth hormone or insulin treatment*

Values are the mean of the number of determinations shown in parentheses $\pm$ s.e.m. Extracellular water was determined by the sucrose method of Hjarlmasen (1968). Diaphragms were incubated for 1 h at 37°C in Krebs bicarbonate buffer, pH 7.4, containing D-glucose (2 mg/ml) and [^{14}C]sucrose (0.1 $\mu\text{Ci}/\text{ml}$).

Tissue	Addition	Extracellular water (ml/100 g wet wt. of liver)
Hemi-diaphragm	0	22.4 ± 1.5 (6)
Diaphragm slices	0	22.5 ± 0.8 (4)
Diaphragm slices	Growth hormone (0.5 ng/ml)	23.3 ± 1.0 (4)
Diaphragm slices	Insulin (0.1 unit/ml)	21.7 ± 0.7 (4)

Table 3. *Amino acid accumulation and incorporation into protein in vitro in diaphragm muscle taken from intact rabbits, in the presence or absence of growth hormone*

Values are the means of five observations $\pm$ s.e.m. Diaphragm slices were incubated for 1 h at 37°C in Krebs bicarbonate buffer, pH 7.4, containing D-glucose (2 mg/ml), L-leucine (0.16 mM), L-arginine (0.15 mM), L-lysine (0.18 mM), L-valine (0.12 mM) and L-histidine (0.05 mM) in the presence or absence of growth hormone (0.5 μ g/ml). The differences between the values for untreated and treated muscle are given in parentheses. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Amino acid	Addition of growth hormone to medium	Amino acid transported	Amino acid incorporated	Tissue/medium isotope distribution ratio
Leucine	—	158	53.2	2.29
	+	150 (-7.2 ± 9.7)	68.0 (*** 15.4 ± 0.9)	1.53 (** -0.71 ± 0.19)
Arginine	—	102	38.0	1.31
	+	105 (2.5 ± 6.4)	49.3 (** 11.0 ± 1.8)	0.91 (0.32 ± 0.15)
Valine	—	80	23.8	1.43
	+	80 (0.6 ± 6.7)	42.2 (** 17.6 ± 2.4)	0.98 (-0.52 ± 0.15)
Lysine	—	48	17.1	1.25
	+	51 (2.8 ± 2.3)	29.9 (** 12.6 ± 2.5)	0.78 (** -0.52 ± 0.10)
Histidine	—	32	14.1	2.31
	+	32 (-0.8 ± 2.9)	19.0 (** 5.0 ± 1.4)	1.74 (** -0.48 ± 0.10)

Table 4. *Amino acid accumulation and incorporation in vitro into protein in diaphragm muscle from intact rabbits in the presence or absence of insulin*

Values are the means of five observations $\pm$ s.e.m. Diaphragm slices were incubated for 1 h at 37°C in Krebs bicarbonate buffer, pH 7.4, containing D-glucose (2 mg/ml), L-leucine (0.16 mM), L-arginine (0.15 mM), L-lysine (0.18 mM), L-valine (0.12 mM) and L-histidine (0.5 mM) in the presence or absence of insulin (0.01 unit/ml). The differences between the values for treated and untreated muscle are given in parentheses. * $P < 0.05$, ** $P < 0.01$.

Amino acid	Addition of insulin to medium	Amino acid transported (nmol/h per g wet tissue)	Amino acid incorporated (nmol/h per g wet tissue)	Tissue/medium isotope distribution ratio
Leucine	—	131	47.4	2.22
	+	201 (** 70.4 ± 19.10)	69.0 (** 21.6 ± 9.41)	4.13 (** 1.90 ± 0.66)
Arginine	—	80	28.7	1.42
	+	126 (** 45.9 ± 8.0)	49.9 (** 21.9 ± 6.7)	2.30 (** 0.90 ± 0.27)
Valine	—	69	17.1	1.54
	+	101 (** 31.4 ± 7.8)	34.1 (** 16.6 ± 5.2)	2.43 (** 0.88 ± 0.20)
Lysine	—	34	12.5	1.21
	+	56 (** 20.1 ± 4.9)	20.8 (** 8.6 ± 2.3)	1.69 (** 0.50 ± 0.10)
Histidine	—	25	8.2	1.90
	+	38 (** 12.2 ± 2.9)	15.4 (** 6.5 ± 1.9)	2.83 (** 0.93 ± 0.18)

rabbits were fed on a protein-free diet for 5 days, growth hormone was shown to stimulate both protein synthesis and amino acid uptake in muscle *in vitro* (Turner, Reeds & Munday, 1971b). These observations imply that in muscle from normal

intact animals, growth hormone will stimulate only protein synthesis, but when protein-synthetic activity is decreased, for example by hypophysectomy or by protein deficiency, growth hormone will then stimulate both protein synthesis and amino

Table 5. *Accumulation and incorporation of valine in muscle from intact rabbits in the presence and absence of insulin and growth hormone separately or together*

Values are the means of five observations $\pm$ S.E.M. Diaphragm slices were incubated for 1 h at 37°C in Krebs bicarbonate buffer, pH 7.4, containing D-glucose (2 mg/ml) and L-leucine (0.16 mM), L-arginine (0.15 mM), L-lysine (0.18 mM), L-valine (0.12 mM) and L-histidine (0.05 mM) in the presence or absence of insulin (0.01 unit/ml), and growth hormone (0.1 μ g/ml) separately or together. N.S., not significant.

Addition to medium	Valine transported (nmol/h per g wet tissue)	Valine incorporated (nmol/h per g wet tissue)	Tissue/medium isotope distribution ratio
Control	68.9 $\pm$ 9.2	16.7 $\pm$ 2.4	1.25 $\pm$ 0.15
Insulin	88.5 $\pm$ 11.3	21.6 $\pm$ 2.6	1.57 $\pm$ 0.21
Growth hormone	66.9 $\pm$ 9.5	22.3 $\pm$ 2.8	1.03 $\pm$ 0.06
Insulin + growth hormone	91.6 $\pm$ 9.6	30.1 $\pm$ 2.8	1.46 $\pm$ 0.20
Differences from control			
Insulin	19.6 $\pm$ 2.6 $P < 0.01$	5.0 $\pm$ 1.7 $P < 0.05$	0.29 $\pm$ 0.08 $P < 0.05$
Growth hormone	-1.4 $\pm$ 1.2 N.S.	5.7 $\pm$ 0.9 $P < 0.01$	-0.23 $\pm$ 0.10 N.S.
Insulin + growth hormone	22.6 $\pm$ 6.0 $P < 0.05$	14.0 $\pm$ 1.0 $P < 0.001$	0.22 $\pm$ 0.05 $P < 0.01$
Differences from insulin			
Growth hormone	-21.6 $\pm$ 2.5 $P < 0.01$	0.8 $\pm$ 1.0 N.S.	-0.54 $\pm$ 0.16 $P < 0.05$
Insulin + growth hormone	3.1 $\pm$ 5.5 N.S.	8.4 $\pm$ 1.0 $P < 0.01$	-0.11 $\pm$ 0.03 $P < 0.05$
Differences from growth hormone			
Insulin + growth hormone	24.6 $\pm$ 6.2 $P < 0.05$	7.7 $\pm$ 0.9 $P < 0.01$	0.47 $\pm$ 0.14 $P < 0.05$

acid uptake. The mechanism of this membrane effect is not known.

If responses to growth hormone similar to those observed *in vitro* were to occur in the intact animal *in vivo*, the role of insulin in stimulating amino acid uptake in muscle would be important for the full action of growth hormone on protein synthesis. Our results suggest that these two hormones tend to act synergistically on protein synthesis.

The increased accumulation observed in rabbit muscle of the amino acids leucine, valine, arginine, lysine and histidine, as a result of insulin action differs from the results of earlier observations on rat hemi-diaphragm in which of the amino acids listed above only histidine was shown to accumulate more rapidly in the presence of insulin (Akedo & Christensen, 1962; Manchester & Wool, 1963; Wool, 1964; Manchester, 1970). We have also found that the accumulation of leucine is not stimulated by insulin in rat hemi-diaphragm (P. J. Reeds, K. A. Munday & M. R. Turner, unpublished work) and this may suggest a species difference.

Since growth hormone can stimulate protein synthesis in muscle without stimulating amino acid uptake it appears that the effect of insulin on amino acid uptake could, in the whole animal, be most important for the full action of growth hormone on protein synthesis, which may only proceed at an optimum rate in the presence of both insulin and growth hormone.

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