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Effects of insulin *in vitro* on protein turnover in rat epitrochlearis muscle

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Rates of protein synthesis and degradation were measured in the isolated rat epitrochlearis muscle by radiotracer techniques, by using the specific radioactivity of tRNA-bound amino acid as precursor for protein synthesis. The tissue maintained linear rates of protein synthesis for 3h of incubation in the presence of amino acids and glucose and in the absence of insulin. Under these conditions, however, the muscles were in negative nitrogen balance, with rates of protein degradation exceeding rates of protein synthesis. Under steady-state conditions of labelling, the specific radioactivities of tRNA-bound leucine, phenylalanine and valine were significantly less than their respective values in the incubation medium, at concentrations in the medium varying from 1 to 10 times those in normal rat serum. Insulin caused a dose- and time-dependent increase in tRNA-based protein synthesis rates, more than doubling rates at 5 and 50ng of insulin/ml. At the lower, physiological, concentration of insulin, the stimulation of protein synthesis was not observed until the third hour of incubation with the hormone, whereas the rate of protein synthesis at the higher concentration was elevated during the second hour. There were no delays in the stimulation by insulin of glucose conversion into glycogen. The delayed stimulatory effects of insulin on the rate of protein synthesis brought the tissue to a nitrogen balance near zero. The presence of the hormone also prevented the increase in the rate of protein degradation seen in the third hour of incubation in the absence of the hormone. These studies demonstrate the viability of the incubated rat epitrochlearis muscle with respect to protein metabolism and sensitivity to the protein anabolic effects of physiological concentrations of insulin, and indicate that the preparation is a suitable experimental model for the study of the control of protein metabolism in fast-twitch skeletal muscle.

Insulin plays a major role in the maintenance of nitrogen balance in skeletal muscle (Rannels *et al.*, 1977; Waterlow *et al.*, 1978). Studies of a variety of animal models have demonstrated wasting of skeletal muscle to be a prominent feature of diabetes mellitus reversible by the administration of insulin, at least *in vivo*. This clearly implies effects of the hormone on protein turnover.

The complicated nature of the expression of the disease and its reversal by insulin, as well as the difficulty of design and interpretation of experiments *in vivo*, have led to the study of both isolated skeletal muscles incubated *in vitro* (Manchester, 1970; Fulks *et al.*, 1975; Frayn & Maycock, 1979) and the perfused rat hemicorpus (Jefferson *et al.*, 1972) in order to assess the direct effects of the hormone on specific parameters of protein metabolism. The accumulated evidence suggests that a principal acute effect of the hormone is to modulate the rate of muscle protein synthesis by increasing the translational efficiency of ribosomes, and long-term effects also involve increases in the capacity for protein synthesis by increasing the concentration of ribosomes (Wool, 1975; Jefferson, 1980). However, results from many published studies have been difficult to interpret, for a number of reasons. In studies of isolated muscles, insulin has been shown to stimulate the incorporation of radioactive amino acids into protein of the tissue. Reliance on tracer methodology as a measure of the rate of protein synthesis in the absence of direct measurements of the precursor specific radioactivity has necessitated controversial assumptions of the relationship between measured free amino acid pools and the aminoacyl-tRNA precursor (Rannels *et al.*, 1977), as well as the constancy of the relationship in the presence and absence of the hormone (Wool, 1975). In addition, the effects of supraphysiological

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concentrations of insulin used in many studies, together with the often ill-defined state of nitrogen balance of isolated muscles, may misrepresent or obscure those effects most relevant to the role of the hormone *in vivo*.

These considerations have guided the design of the present study, where we have examined the suitability of the isolated epitrochlearis muscle of the rat for studies of the effects of insulin on protein turnover. The epitrochlearis, a foreleg muscle composed of 85% fast fibres, has proved to be a useful model *in vitro* for studies of mechanical performance (Nesher *et al.*, 1980) and intermediary metabolism (Garber *et al.*, 1976). In the present paper the steady-state relationships between tRNA-bound amino acids and free amino acids in the medium have been documented and used as a basis for studying the time course of the effects of physiological concentrations of insulin to modulate the rates of protein synthesis and degradation. We have found the predominant effect of physiological concentrations of insulin to be on protein synthesis, with effects on degradation of lesser magnitude and only observed at long incubation times. In addition, there appears to be a dose-dependent delay in the onset of the effect of insulin on synthesis.

Materials and methods

Female rats (Sprague–Dawley, 110 ± 10 g) obtained from Charles River (Montreal, Quebec, Canada) were housed on a 12 h-light/12 h-dark cycle and provided with Purina laboratory chow and water *ad libitum*. Animals were killed by cervical dislocation, and the lumbar vertebral column was crushed to prevent muscle spasm. The epitrochlearis muscles from each foreleg were removed intact and transferred to oxygenated (O_2/CO_2, 19:1) Krebs–Ringer bicarbonate buffer, pH 7.4 (with Ca^{2+} decreased to 1.2 mM; Harter *et al.*, 1979), containing 15 mM-glucose, 0.01 mM-phenylalanine and all other amino acids at normal plasma concentrations (Morgan *et al.*, 1980), except where noted in the text. The muscles were trimmed of any inherent fat under a dissecting microscope while immersed in the above solution at room temperature and then transferred to plastic tubes containing 700 μl of the same buffer containing, in addition, 5 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer, pH 7.4, 0.2% (w/v) gelatin (pig skin; type I; catalogue no. G-2500; Sigma, St. Louis, MO, U.S.A.) and insulin (Iletin; Eli Lilly and Co., Indianapolis, IN, U.S.A.) at the stated concentration. Insulin was diluted on the day of the experiment with Krebs–Ringer bicarbonate buffer containing 0.2% (w/v) gelatin. The muscles were first preincubated for 15 min in this medium with continuous exposure to a stream of O_2/CO_2 (19:1) in a shaking water bath (70 oscillations/min). The muscles were then transferred to an identical medium containing ^{3}H-labelled amino acids as specified in the text, with final specific radioactivities of 800–2200 d.p.m./pmol, or containing [^{14}C]glucose (500 d.p.m./nmol). All radioactive reagents and substrates were purchased from Amersham Corp. (Arlington Heights, IL, U.S.A.). At specified times, muscles were removed and rinsed quickly (<5 s) at 37°C in Krebs–Ringer bicarbonate buffer to remove gelatin, the presence of which compromised the accurate determination of the specific radioactivity of amino acids in protein hydrolysates. The muscles were then frozen in isopentane cooled to the temperature of liquid nitrogen and stored at −80°C until analysis. Preliminary experiments established that the specific radioactivity of tRNA-bound amino acids was unchanged by the brief washing procedure from values determined for muscles frozen immediately between aluminium blocks precooled to the temperature of liquid nitrogen.

Sample analysis

A measured volume of the incubation medium was added to an equal volume of 20% (w/v) trichloroacetic acid for subsequent determination of the concentration of phenylalanine and the specific radioactivity of amino acids by methods described by Airhart *et al.* (1979). Frozen muscles were transferred to 15 ml Corex glass round-bottomed centrifuge tubes containing 2.0 ml of 0.05 M-sodium cacodylate buffer, pH 6.0, and 1% (w/v) sodium dodecyl sulphate and homogenized immediately with a Polytron tissue homogenizer for 1 min at a low setting to minimize foaming. The homogenizer probe was rinsed with 1.5 ml of the same solution, the rinse solution was pooled with the homogenate, thoroughly mixed, and the mixture was then centrifuged for 1 min at 10000 ***g*** to remove foam. The insoluble fibrous fraction of the tissue homogenate pelleted during this procedure was found to contain less than 2% of the total trichloroacetic acid-precipitable radioactivity of the muscle and no detectable amounts of tRNA-bound amino acids, and therefore was excluded from subsequent analyses.

Samples (100 μl) from each homogenate were transferred to silicone-treated disposable glass test tubes, 1 ml of 10% trichloroacetic acid was added to each, and after mixing they were stored at 0°C until analysed for total radioactivity in protein and specific radioactivity of hydrolysed amino acids by methods described below. The remainder of the homogenate was transferred to 30 ml round-bottomed centrifuge tubes and an equal volume of freshly distilled phenol equilibrated with the homogenizing buffer was added. The samples were mixed intermittently at room temperature on a Vortex

mixer for 15 min and then centrifuged at 4°C for 15 min at 10000 *g* in a Sorvall centrifuge. The aqueous supernatant was carefully removed and adjusted to contain 1 mM-EDTA, 10 mM-$MgCl_2$ and a total of 2 A_{260} units of yeast tRNA (Sigma), which had been previously deacylated as described by von Ehrenstein & Lipmann (1961). Control experiments indicated that residual amino acids from yeast tRNA contributed less than 3% of the total leucine, valine or phenylalanine released from tRNA prepared from one rat epitrochlearis muscle. The sample was applied to a column (1.2 cm × 2.0 cm) of DEAE-cellulose (Whatman DE-52, preswollen) which had been pre-equilibrated in 1 mM-EDTA/10 mM-$MgCl_2$/0.05 M-sodium cacodylate buffer, pH 6.0. Free amino acids, rRNA and residual protein were eluted from the column with 6 ml of the equilibration buffer, followed by 6 ml of the same buffer containing 0.25 M-NaCl. Aminoacyl-tRNA was eluted from the column with 5 ml of the starting buffer containing 0.7 M-NaCl and precipitated at −20°C after addition of 0.1 vol. of 20% (w/v) potassium acetate, pH 5.0, and 2.5 vol. of cold 95% (v/v) ethanol.

Conditions for deacylation of aminoacyl-tRNA and acid hydrolysis of tissue protein were as described by Hildebran *et al.* (1981). Analysis of the specific radioactivity of tRNA-bound amino acids, amino acids from hydrolysed protein and free amino acids in the tissue incubation medium was performed by application of the ultramicro-method described by Airhart *et al.* (1979). For determination of total radioactivity in protein, the trichloroacetic acid precipitates from samples of the tissue homogenate were collected on glass-fibre filters (Whatman Laboratory Products, Clifton, NJ, U.S.A.), washed three times with ice-cold 5% trichloroacetic acid, transferred to scintillation vials containing 0.5 ml of NCS tissue solubilizer (Amersham Corp.), heated to 55°C for 1 h and cooled to room temperature, after which 10 ml of Econofluor (New England Nuclear, Boston, MA, U.S.A.) was added to each vial. Radioactivity was determined by liquid-scintillation spectrometry. The radioactivity-counting efficiency, as determined by using an external standard, averaged 40%.

In experiments monitoring the incorporation of [^{14}C]glucose into glycogen, a portion of the tissue homogenate was added to an equal volume of 6 M-KOH and, after heating for 2 h at 95°C, was cooled in an ice bath and glycogen was precipitated by the addition of 2.5 vol. of ice-cold ethanol. After centrifugation for 5 min at 3000 *g*, the pellet was washed three times with 95% ethanol, dried with a stream of N_2, dissolved in 1 ml of water and added to 10 ml of Aquasol II (New England Nuclear). The counting efficiency for ^{14}C radioactivity averaged 52%.

Protein content of the epitrochlearis was determined directly by the method of Lowry *et al.* (1951) and was found to be 160 ± 4 (S.E.M.) mg/g wet wt. of the muscle. Alternatively, for experiments with labelled leucine, the leucine content of the muscle was calculated from the incorporation data (total d.p.m. incorporated into protein divided by the specific radioactivity of leucine in the protein hydrolysate) and an equivalent wet weight of the muscle calculated by dividing the leucine content by 109 μmol of leucine/g of tissue (Waterlow *et al.*, 1978).

Protein synthesis

Over 98% of the total radioactivity in protein was in leucine, as determined by chromatographic analysis of the amino acid products of acid hydrolysis. Therefore, protein synthesis was calculated in two ways:

$$\text{Leucine incorporated (pmol)/h per g} = \frac{\text{d.p.m. in protein/h per g}}{\text{d.p.m./pmol of tRNA-bound leucine}}$$

$$\text{Leucine replacement (\%/day)} = \frac{\text{d.p.m./pmol of amino acid from protein hydrolysate per h}}{\text{d.p.m./pmol of tRNA-bound leucine}} \times \frac{24\text{ h}}{\text{day}}$$

Protein degradation

The initial concentration of phenylalanine in the incubation medium was adjusted to 0.01 mM, approximately one-eighth that of normal rat serum, to facilitate measurements of changes in phenylalanine concentration in the medium as the basis for assessing the balance between the rates of protein synthesis and degradation (Morgan *et al.*, 1980). Preliminary experiments established that the rates of leucine incorporation into protein of muscles incubated in the presence of this concentration of phenylalanine were unchanged relative to the rates measured at serum concentrations of the amino acid (results not shown). Rates of protein degradation were estimated from the sum of the rate of phenylalanine incorporation into protein and the net change with time of the amount of phenylalanine in the medium and tissue. The validity of this approach depended on the absence of any appreciable metabolism of phenylalanine by the tissue. To assess this,

muscles were incubated for 2h with [2,4-6-^{3}H]-phenylalanine. Over 95% of the initial radioactivity was recovered as phenylalanine, as determined by the measured amount and specific radioactivity of the amino acid in the tissue and medium. The phenylalanine not accounted for by this procedure represented less than 10nmol/g of muscle, an amount that would not change significantly the rates of protein degradation calculated in this study.

The content of free phenylalanine in the tissue after the 15min preincubation period was measured after 0, 1 and 3h of incubation in the absence of insulin and after 3h with insulin (50ng/ml) and were found to be 18.2 ± 3.0, 16.7 ± 5.3, 13.1 ± 5.3 and 10.3 ± 2.4 nmol/g wt wt. respectively (means $\pm$ S.D., $n = 4$). Since the observed differences between the values were insignificant relative to their contribution in calculating rates of protein degradation, changes in the concentration of phenylalanine in the incubation medium were used as a basis for the calculations.

Protein degradation rates were estimated simultaneously with protein synthesis rates from the sum of the measured net change in the amount of phenylalanine in the medium over 1h time intervals and the amount of phenylalanine incorporated into protein over the same interval. The latter was calculated from the measured incorporation of radioactive leucine into protein, by using a conversion factor of 0.44mol of phenylalanine incorporation into muscle protein per mol of leucine. This ratio was determined by comparing the measured rates of incorporation of the two amino acids into protein. For this purpose radiolabelled leucine and phenylalanine were added together in the incubation medium. The percentage replacement rate for each amino acid, based on the specific radioactivity of tRNA-bound amino acids, was found to be the same (results not shown). Therefore the ratio of the rates of incorporation of leucine and phenylalanine is equal to the molar ratio of the amino acids in the protein of the tissue as determined by analyses of protein hydrolysates. A similar approach has been described for simultaneous measurement of protein synthesis and degradation in single pieces of isolated rat diaphragm muscle (Tischler *et al.*, 1982).

Results

Conditions for measurements of protein synthesis

The early time course of incorporation of radiolabelled leucine into protein and labelling of tRNA-bound leucine was examined to establish conditions under which protein synthesis could be monitored during steady-state conditions (Fig. 1). The increment in the specific radioactivity of leucine in protein was nearly linear with time by 15min, a time at which tRNA-bound leucine specific radioactivity had reached 100% of its steady-state value, at the physiological leucine concentration in the medium of 0.166mM (Scharff & Wool, 1964). In the absence of insulin, the protein-synthesis rate of the incubated muscles was constant for at least 3h (Fig. 2). Thus equilibration of leucine in the medium with leucyl-tRNA was rapid in this system, as others have found for this amino acid in the heart after labelling *in vivo* (Everett *et al.*, 1981). Therefore calculated rates of protein synthesis over the first hour of incubation based on the specific radioactivity of protein at the end of the hour divided by the precursor specific radioactivity at steady state were underestimated by no more than 10%.

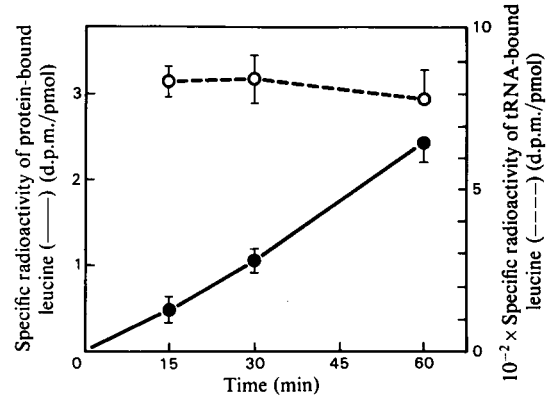

Fig. 1. *Specific radioactivity of tRNA-bound leucine* (○) *and protein leucine* (●) *in rat epitrochlearis muscle incubated in vitro with* [^{3}*H*]*leucine*

Epitrochlearis muscles were incubated in medium without label for 15min and then transferred to medium containing L-[4,5-^{3}H]leucine at a final leucine concentration of 0.166mM. At each time point muscles were removed and the tissue was processed as described in the Materials and methods section. Results are shown as means $\pm$ S.D.; $n = 4$ for each determination.

The specific radioactivity of tRNA-bound leucine at steady state was less than half that of free leucine in the incubation medium and was not appreciably increased when the leucine concentration in the medium was raised to 2.0mM (Table 1). Hence a significant fraction of leucine bound to tRNA came from a non-radioactive source under these conditions. The lack of isotopic equilibrium with the precursor was also observed for two other amino acids. Radiolabelled valine and phenylalanine, included in the medium at 1 and 5 times normal serum concentration respectively, resulted in steady-state specific radioactivities of the tRNA-bound amino acids which were only 30% of those in the medium (Table 1). The result obtained for phenylalanine was different from that obtained for perfused rat hearts (McKee *et al.*, 1978), where phenylalanine added to

the perfusion medium at a concentration above 0.2 mM (approx. twice normal serum values) effected isotopic equilibrium between tRNA-bound phenylalanine and that in the medium.

Effects of insulin on protein synthesis

The addition of insulin to the incubation medium at a concentration of 50 ng/ml did not affect the incorporation of labelled leucine into protein over the 1 h of incubation with radioisotope. Incorporation was more than doubled, however, in the second hour (Table 2, Fig. 2). Significantly, the steady-state relationship between the specific radioactivity of tRNA-bound leucine and leucine in the medium was not affected by this or any concentration of the hormone tested. Hence, the increased incorporation of radioactivity into protein in the presence of the hormone was a true reflection of an effect on the rate of protein synthesis and was not secondary to an effect of the hormone to deliver radiolabelled leucine to the leucyl-tRNA precursor. Confirmation of the absence of an insulin effect in the first hour of incubation and the magnitude of the response in the second hour was obtained in separate experiments based on rates of protein synthesis determined from the incorporation of [^{3}H]phenylalanine and [^{3}H]-valine (results not shown).

In order to distinguish between a general insulin resistance in the first hour and lack of responsiveness of protein metabolism to the hormone, the effect of the hormone (50 ng/ml) on the incorporation of radioactivity from [^{14}C]glucose into muscle glycogen was measured simultaneously with measurements of protein synthesis. A highly significant stimulation of the transfer of glucose to glycogen was observed during the first hour of incubation with the hormone, whereas in the same muscles the rate of protein synthesis was unchanged until the second hour (Table 3).

The effects of lower, physiological, concentrations of insulin on the rate of protein synthesis were examined as a function of time (Fig. 2); 50 ng of insulin/ml gave the expected response during the second hour of incubation (cf. Table 2) and the rate of protein synthesis remained elevated relative to controls in the third hour. Insulin was also effective at the physiological concentration of 5.0 ng/ml (Flaim *et al.*, 1980), though, surprisingly, the onset of the response was delayed until the third hour. The hormone was without effect at 0.5 ng/ml over the time course of the experiment.

Effects of insulin on protein degradation

A substantial decrease in net phenylalanine release from muscles was observed during time periods when insulin was observed to increase the rate of protein synthesis, most of which could be accounted for by the effects of the hormone on protein synthesis (Table 4). Thus, with the excep-

Table 1. *Effects of insulin and amino acid concentration on the ratio of tRNA-bound specific radioactivity to that in the medium in rat epitrochlearis muscle in vitro*

Muscles were incubated for periods of 1–3 h in medium containing the radioactively labelled amino acid at the final concentration shown in parentheses. No significant differences were observed at different times of incubation in any one group: therefore, some means were calculated from values obtained at different times of incubation. Results are means ± S.E.M. (*n*).

	Ratio	
Insulin (ng/ml) ...	0	50
Amino acid (mM)		
Leucine (0.166)	0.43 ± 0.02 (11)	0.45 ± 0.02 (6)
Leucine (2.00)	—	0.53 ± 0.03 (6)
Valine (0.100)	0.30 ± 0.04 (4)	0.31 ± 0.04 (4)
Phenylalanine (0.4)	0.32 ± 0.03 (4)	0.34 ± 0.03 (4)

Table 2. *Effects of insulin on the specific radioactivity of tRNA-bound leucine and the rate of protein synthesis in rat epitrochlearis muscle in vitro*

Muscles were incubated for the times shown with the addition of L-[4,5-^{3}H]leucine at a final leucine concentration of 0.166 mM in the absence (control) and presence of insulin (50 ng/ml). Rates of protein synthesis were calculated from the total radioactivity incorporated during 1 h intervals divided by the specific radioactivity of tRNA-bound leucine in muscles at the end of the time period. Results are means ± S.E.M. (*n*). Significance of difference from control during same interval (unpaired *t* test): $^*P < 0.001$.

		Specific radioactivity (d.p.m./pmol)		Protein synthesis		
	Incubation time (min)	Leucine in protein	tRNA-bound leucine	Incubation interval (min)	(nmol of leucine/g of muscle in 1 h)	(%/day)
Control	60	1.58 ± 0.20 (4)	763 ± 37 (3)	0–60	243 ± 9	5.1 ± 0.6
	120	3.23 ± 0.28 (4)	734 ± 43 (3)	60–120	253 ± 44	5.2 ± 0.9
Insulin	60	1.65 ± 0.08 (4)	765 ± 77 (3)	0–60	245 ± 23	5.2 ± 0.6
	120	5.52 ± 0.36 (4)	802 ± 36 (4)	60–120	566 ± 92*	11.5 ± 0.7*

tion of the third hour of incubation, the rate of protein degradation was unchanged by the hormone. In the absence of insulin, the rate of protein degradation in the third hour was significantly increased above that observed in the first 2h, although rates of protein synthesis remained constant during the same interval. Insulin at 50ng/ml prevented the increase in protein degradation ($P < 0.05$), and the data were suggestive that insulin at 5ng/ml may have similarly attenuated the increase ($P < 0.15$). Variability in the calculated degradation rates of control muscles in the third hour may have precluded a definitive conclusion in the latter case.

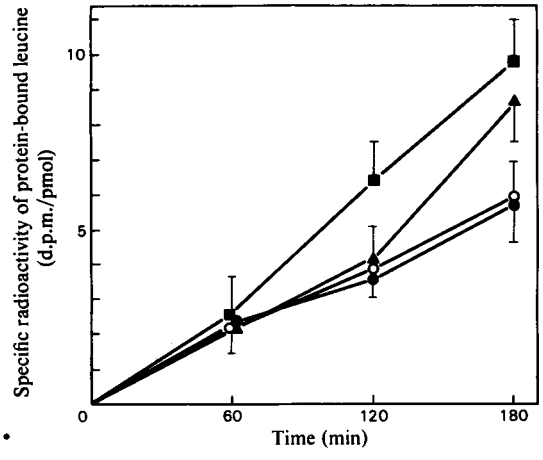

Fig. 2. *Effects of insulin on the specific radioactivity of protein leucine in rat epitrochlearis muscle in vitro*
Muscles were preincubated for 15min in medium without label but with insulin at the concentrations specified [(ng/ml): ○, 0; ●, 0.5; ▲, 5.0; ■, 50] and then transferred to an identical medium containing L-[4,5-^{3}H]leucine at a concentration of 0.166mM. The specific radioactivity of tRNA-bound leucine (d.p.m./pmol) was the same for each group. Therefore the relative slopes of the lines drawn reflect the relative rates of protein synthesis over the 1h time intervals measured. Results are shown as means ± S.E.M.; $n = 4$ for each group.

Discussion

The present study defines conditions under which the rates of protein synthesis and protein degrada-

Table 3. *Effects of insulin on the rate of protein synthesis and the incorporation of [^{14}C]glucose into glycogen*
Muscles were incubated for 1h and 2h with L-[4,5-^{3}H]leucine at a final leucine concentration of 0.166mM and D-[U-^{14}C]glucose at a final glucose concentration of 15mM. Rates of protein synthesis were based on the specific radioactivity of tRNA-bound leucine. Rates of glucose incorporation into glycogen were based on the specific radioactivity of glucose in the medium. Rates are expressed as means ± S.E.M.; $n = 4$ for each value shown. Significance of difference from control during same interval (unpaired t test) $^*P < 0.01$, $^{**}P < 0.001$.

Incubation interval (min)	Insulin (ng/ml)	Protein synthesis (nmol of luecine/g of muscle in 1h)	Glucose incorporation into glycogen (μmol/g of muscle in 1h)
0–60	0	202 ± 52	0.84 ± 0.11
	50	245 ± 63	2.53 ± 0.33**
60–120	0	192 ± 31	1.33 ± 0.24
	50	465 ± 72*	4.72 ± 0.90**

Table 4. *Effects of insulin on the fractional rates of protein synthesis and protein degradation in rat epitrochlearis muscle in vitro*
Muscles were incubated for intervals up to 3h with [^{3}H]leucine at a leucine concentration of 0.166mM and phenylalanine at an initial concentration of 0.01mM. Fractional rates of protein synthesis were based on the increment in the specific radioactivity of protein leucine and the steady-state specific radioactivity of tRNA-bound leucine. Protein-degradation rates in the same muscles were calculated from the rate of protein synthesis and the net change of phenylalanine concentration in the medium during the intervals shown (see the Materials and methods section). Values are means ± S.E.M.; $n = 4$ for 0–60min and $n = 8$ for 60–120min and 120–180min. Significance of differences from no-insulin controls during the same interval (unpaired t test): $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$.

Incubation interval (min)	Insulin (ng/ml)	Protein synthesis (nmol of leucine/g of muscle in 1h)	Net phenylalanine release (nmol/g of muscle in 1h)	Fractional rate (%/day)	
				Synthesis	Degradation
0–60	0	290 ± 46	150 ± 35	6.1 ± 1.0	13.2 ± 1.7
	5	274 ± 31	121 ± 40	5.8 ± 0.7	11.5 ± 0.8
	50	333 ± 52	103 ± 27	7.0 ± 1.1	11.9 ± 1.7
60–120	0	219 ± 26	162 ± 11	4.6 ± 0.5	12.3 ± 1.1
	5	257 ± 27	194 ± 9	5.4 ± 0.6	14.7 ± 1.1
	50	509 ± 31	1 ± 7	10.7 ± 0.6***	10.7 ± 0.6
120–180	0	276 ± 28	248 ± 21	5.8 ± 0.6	17.6 ± 2.2
	5	609 ± 55	22 ± 8	12.8 ± 1.1***	13.9 ± 1.1
	50	490 ± 35	81 ± 9	10.3 ± 0.7**	12.9 ± 0.7*

tion can be measured accurately in the isolated rat epitrochlearis muscle. It establishes the suitability of the preparation for investigation of the modulation of protein metabolism in fast-twitch muscle. A principle contribution of the study is the demonstration for the first time in skeletal muscle of the stimulation by insulin of the transfer of radioactivity from tRNA-bound amino acids to protein, thereby providing more direct evidence for an effect of the hormone on the rate of protein synthesis than heretofore has been presented. The results are in agreement with the evidence obtained in radioisotopic tracer studies of other models *in vitro* of individual skeletal muscles (Manchester, 1970; Fulks *et al.*, 1975; Frayn & Maycock, 1979; Odessey & Parr, 1982) as well as of the rat hemicorpus preparation (Jefferson *et al.*, 1974). Similarly, the results are consistent with reported effects *in vivo* of insulin on skeletal muscle in diabetic rats (Stirewalt *et al.*, 1967; Pain & Garlick, 1974; Odedra *et al.*, 1982).

Previous studies of protein turnover of striated muscle both *in vivo* and *in vitro* have involved use of the specific radioactivity of the intracellular or extracellular free pool of a labelled amino acid as the direct precursor for protein synthesis (reviewed by Rannels *et al.*, 1977). It was not the purpose of the present study to evaluate the validity of these approaches; however, we found that tRNA-bound leucine, valine and phenylalanine were not at isotopic equilibrium with their respective free amino acids in the extracellular medium, even when the concentration of two of them, leucine and phenylalanine, was raised substantially. The situation is somewhat different in the perfused heart, where high concentrations of phenylalanine in the perfusate effect isotopic equilibrium between extracellular, intracellular and tRNA-bound phenylalanine (McKee *et al.*, 1978). Caution must therefore be exercised in the interpretation of absolute protein-synthesis rate measurements in skeletal muscle which are based on analyses of free amino acids without knowledge of the relationship of the free pools to the obligatory precursor, tRNA-bound amino acid. It should be pointed out that the magnitude of the effect of insulin is the same in the present experiments regardless of whether the specific radioactivity of amino acid in the extracellular medium or that bound to tRNA is used to calculate synthesis rates. At the same time, estimates of nitrogen balance and protein-degradation rates are very much different, depending on which pool of amino acid is used as radioisotope precursor. The methodologies described in the present paper should facilitate examination of these relationships in other systems where calculation of accurate rates of protein synthesis are required to assess the effects of hormones on that process, or to calculate protein-degradation rates indirectly from synthesis rates and protein content.

It is important to note that rates of protein synthesis based on the specific radioactivity of the total tissue tRNA-bound amino acids, as in the present study, still may be in error if the aminoacyl-tRNA pool were functionally heterogeneous and acylation of tRNA occurred from two or more pools of free amino acids with different specific radioactivities (see Zak *et al.*, 1979). This possibility remains to be tested.

The relationship of the measured rates of protein synthesis and degradation *in vitro* to the rates *in vivo* are difficult to assess in the absence of measurements of the same parameters for epitrochlearis muscle *in vivo*. However, the fractional rate of synthesis of 12%/day measured in the presence of insulin is close to the rate of 13.9%/day measured for the rat plantaris muscle *in vivo* (Odedra *et al.*, 1982), a muscle of similar fibre type to the epitrochlearis. Since the muscles of the young rats used in the present study were growing at a rate of approx. 5%/day, the rate of protein synthesis must necessarily be higher or the rate of protein degradation lower *in vivo* than the rates measured in the isolated muscle. Given the dependence of skeletal-muscle growth on activity (Goldberg, 1972; Goldspink, 1977) and stretch (Goldspink, 1978; Reeds *et al.*, 1980), it is perhaps reasonable to expect protein turnover to have been altered in the absence of the stimuli *in vitro*.

The reason for the observed delay in the effect of insulin (50ng/ml) on protein synthesis is not clear, but certainly warrants further attention. Insensitivity of the psoas muscle to insulin *in vitro* in the first hour of perfusion has been observed by Jefferson *et al.* (1974) in the rat hemicorpus preparation. An explanation proposed for this initial insensitivity is that endogenous insulin is present in the tissue and it becomes metabolized or diluted during incubation. This explanation appears reasonable for the rat hemicorpus preparation, where, in the absence of insulin, the rate of protein synthesis declines after the first hour of perfusion and the decline is prevented by the addition of insulin to the perfusate. However, in the present experiments the rate of protein synthesis in control muscles was constant for 3h (Table 4 and Fig. 2). Furthermore, we found that insulin has an immediate, stimulatory effect on the conversion of glucose into glycogen. We also found that the dose-dependence of the delayed effect on synthesis was in a relation opposite to that expected if endogenous insulin was a factor. Finally, there appeared to be no delay in the effect of insulin to prevent the increase in protein degradation that occurred in the third hour of incubation.

In experiments most closely resembling the conditions used *in vitro* in this study, Frayn &

Maycock (1979) found no substantial delay in the effect of insulin (45 ng/ml) on the incorporation of [^{3}H]tyrosine into protein of soleus muscle from mice and observed a significant stimulation measured at 1 h after transfer of the muscle to medium containing label. However, they preincubated muscles for 30 min with hormone, compared with 15 min in the present experiments, and this may have obscured a delay. In the same study, the effects of insulin on protein synthesis in extensor digitorum longus and soleus muscles were measured at the end of a 3 h incubation. The small effects of the hormone (+25%) on the fast-twitch extensor digitorum longus muscle from fed animals over that time period may actually have been greater had delays in the response of the tissue occurred. If we had measured the average rate of protein synthesis of epitrochlearis muscle over the 3 h incubation time, we would have computed very different synthesis rates for doses of insulin of 5 and 50 ng/ml. If measurements were made only at the end of 2 h, no effect of 5 ng of insulin/ml would have been observed.

The nutritional or hormonal status of the donor animal may influence the apparent acute insulin resistance, as the delay in the insulin stimulation of protein synthesis in the perfused hemicorpus of fed rats was observed to disappear if animals were starved before being killed (Jefferson *et al.*, 1977; Li *et al.*, 1979). We have found the same for epitrochlearis in preliminary experiments. These complexities may complicate attempts to assess *in vitro* the relative sensitivities of skeletal muscle to insulin in terms of the modulation of protein turnover. At the same time, the identification of the factors responsible for the delay may contribute substantially to understanding the mechanism by which the hormone stimulates protein synthesis in muscle.

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