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The effect of insulin and intermittent mechanical stretching on rates of protein synthesis and degradation in isolated rabbit muscle

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Tyrosine balance and protein synthesis were studied during the same incubation in isolated rabbit forelimb muscles. From these measurements, protein degradation was calculated. Isolated muscles were usually in a state of negative amino acid balance, principally as a result of the 75% decrease in protein synthesis. Muscles from rabbits starved for 18h had lower rates of both protein synthesis and degradation compared with muscles from normally fed rabbits. Intermittent mechanical stretching and the addition of insulin at 100μunits/ml increased rates of both protein synthesis and degradation. Increases in the rate of protein synthesis were proportionately greater in the muscles from starved animals. In muscles from both fed and starved donors, increases in protein-synthesis rates owing to intermittent stretching and insulin were proportionately greater than the increases in degradation rates. For example, insulin increased the rate of protein synthesis in the muscles from starved donors by 111% and the rate of degradation by 31%. Insulin also increased the rate of protein synthesis when added at a higher concentration (100munits/ml); at this concentration, however, the rate of protein degradation was not increased. The suppressive effect of insulin on high rates of protein degradation in other skeletal-muscle preparations may reflect a non-physiological action of the hormone.

Protein synthesis and degradation are both of importance in determining whether a muscle grows or atrophies (Waterlow *et al.*, 1978), but protein degradation has proved more difficult to measure. Protein degradation *in vivo* is often calculated from the difference between the rate of protein synthesis and the rate of accretion of muscle protein (Goldspink *et al.*, 1983). In an analogous manner, protein degradation can be measured in an isolated muscle, from the rate of release into the medium of a non-metabolized amino acid such as tyrosine and a measurement of the rate of protein synthesis. Degradation may be measured separately from synthesis in incubations in which synthesis is blocked with cycloheximide (Fulks *et al.*, 1975). The alternative approach is to calculate the algebraic sum of protein synthesis and tyrosine release measured during the same incubation (Tischler *et al.*, 1982; Stirewalt & Low, 1983).

The effect on protein synthesis of factors such as starvation and insulin is well established both *in vivo* (Millward *et al.*, 1975; Garlick *et al.*, 1983; Pain *et al.*, 1983) and *in vitro* (Fulks *et al.*, 1975; Stirewalt & Low, 1983; Reeds & Palmer, 1983). The effects of such factors on degradation remain more controversial, partly owing to differences in the experimental conditions studied, such as the duration of starvation (Millward & Waterlow, 1978), or the concentration of insulin used *in vitro* (Jefferson *et al.*, 1977; Frayn & Maycock, 1979).

In the present paper we have attempted to compare rates of protein synthesis and degradation *in vitro* and *in vivo* and to examine the effects of a variety of conditions (intermittent mechanical stretching, insulin and starvation) which are known to have a marked effect on rates of protein synthesis.

Experimental

Materials

L-[2,6-^{3}H]Phenylalanine and L-[U-^{14}C]phenylalanine were purchased from Amersham International (Amersham, Bucks., U.K.). Insulin (Actrapid; monocomponent, pig) was obtained from Farillon (Romford, Essex, U.K.). All other chemicals, including those used in the preparation of the

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medium, were purchased from B.D.H. or Sigma Chemical Co. (both Poole, Dorset, U.K.).

Animals

Male rabbits weighing 600–1000g were used in all experiments. These were either allowed free access to food and water or starved for 18h before the removal of the muscles. In some of the normally fed animals, rates of protein synthesis were measured both *in vivo* and *in vitro*. In these animals the fractional growth rate was calculated from the daily weight gain over a 6-day period before the synthesis measurement.

Protein synthesis in vivo

Measurements of the rate of protein synthesis were based on the incorporation of phenylalanine under 'flooding' conditions (Garlick *et al.*, 1980). Phenylalanine (200mM; 1ml/150g body wt.) containing 50μCi (1.85MBq) of L-[2,6-^{3}H]phenylalanine was injected into a peripheral ear vein. After 3 or 10min the rabbits were killed, digit extensor muscles from one forelimb were immediately frozen in liquid N_2, and the same muscles from the other limb were incubated as described below.

Incubation conditions

Forelimb digit extensor muscles (Palmer *et al.*, 1981) were removed intact and with tendons attached. These muscles were 2–2.5cm long, 2–2.5mm in diameter and weighed 35–60mg. They were incubated under a constant tension of 10g or with intermittent mechanical stretching for 100ms every 3s. The tyrosine content of the incubation medium was 40μM, otherwise the medium (Reeds *et al.*, 1980) and incubation conditions (Smith *et al.*, 1983) were as described previously. The medium was sterilized before use, by filtration through a 0.2μm Acrodisc (Gelman, Northampton, U.K.).

Stock solutions of insulin in medium were added to the appropriate incubation vessels (capacity approx. 2ml) to achieve a nominal concentration of either 500μunits/ml or 100munits/ml. In practice the concentration attained at the lower dose was 100μunits/ml, as determined at the end of a preliminary series of incubations. Typically incubations lasted 5½h, and, when added, insulin was present throughout this time. The medium was replaced after 1h and again after 5h. During the final 30min (i.e. 5–5½h) the medium contained in addition 1.5mM-phenylalanine and L-[2,6-^{3}H]phenylalanine (final sp. radioactivity in medium 1500d.p.m./nmol). Muscles from animals which had been injected with [^{3}H]phenylalanine were incubated for the final 30min in the same concentration of phenylalanine but containing L-[U-^{14}C]phenylalanine (sp. radioactivity 500d.p.m./nmol). At the end of the incubation period muscles were treated as described previously (Palmer *et al.*, 1981). The phenylalanine content of the media and muscle protein was measured by fluorescence assay after conversion into β-phenethylamine (Garlick *et al.*, 1980). Radioactivity in the β-phenethylamine extracts was measured in a Packard 460 CD liquid-scintillation counter with NE265 scintillant (A. and J. Beveridge, Edinburgh, Scotland, U.K.) as described previously (Smith *et al.*, 1983). Muscles removed from the animals for the measurement of protein-synthesis rates *in vivo* were treated in an identical manner, and the specific radioactivity of the tissue homogenate pool of free phenylalanine was also determined in those tissues by analysis of the neutralized $HClO_4$ supernatants.

Tyrosine release was determined by a fluorimetric assay (Waalkes & Udenfriend, 1957). Portions (0–10nmol) of a standard L-tyrosine solution were added to 1ml samples of incubation medium (containing 40nmol of tyrosine/ml). Thus the tyrosine content of the standards was 40–50nmol/ml. Readings were made in an Aminco–Bowman spectrophotofluorimeter on which the zero setting was adjusted so that the 40nmol standard gave a reading of 0–10 and the amplification of the photomultiplier output was increased so that the 50nmol standard gave a reading of 90–100. In this way changes of 0.5nmol in the tyrosine content of the medium could be measured reliably in the presence of the high initial tyrosine content.

Intracellular phenylalanine specific radioactivity in vivo

The specific radioactivity of the muscle free phenylalanine was determined in muscles 3 and 10min after injection, by using the $HClO_4$ homogenate supernatant. At 3min after the injection, the free phenylalanine had attained a specific radioactivity 85% of that of the injection. The value was unchanged 10min after the injection, and the fractional rate of protein synthesis *in vivo* was calculated as described previously (Garlick *et al.*, 1980; Smith *et al.*, 1983) by using the intracellular free phenylalanine specific radioactivities of the individual rabbits.

Stability of intracellular tyrosine pools in vitro

The integrity of the intracellular free tyrosine is of great importance in measurements of protein degradation that involve tyrosine release into the medium. In preliminary experiments the free tyrosine content was determined in homogenates of freshly dissected muscles and of muscles incubated for 5h in a medium containing no tyrosine (a condition that is widely used in investigations *in vitro*) or 40nmol of tyrosine/ml.

Freshly dissected muscle had a free tyrosine content of 75.6 ± 3.8 nmol/g ($n = 14$). After incubation in tyrosine-free medium, the free tyrosine content of the muscles had fallen to 42 nmol/g, i.e. to $56 \pm 5.8\%$ ($n = 18$) of the value in fresh muscle. However, incubation in a medium containing 40 μM-tyrosine did not result in a decrease in the homogenate pool; after 5 h incubation the tyrosine content was 73.8 ± 2.8 nmol/g ($n = 12$). Addition of insulin or the application of mechanical stimulation had no significant effect on the concentration of free tyrosine in the muscle homogenate pool.

Stability of tyrosine in the medium

The quantity of tyrosine released by the muscles during 5 h incubation is very small; for example a 50 mg muscle in which the rate of protein degradation typically exceeds the rate of synthesis by 3%/day would release a total of 10 nmol of tyrosine in 5 h. Changes in the tyrosine concentration of the medium owing to bacterial growth, tyrosine oxidation or even evaporation during the incubation could be sources of considerable error. To minimize changes in the volume of medium, all vessels were covered with Parafilm during the incubations, so that any loss by evaporation condensed on the cover and could be replaced at the end of the incubation. Under these conditions no change in the volume of the medium could be detected. Covering the vessels also served to minimize bacterial contamination and, as the medium was sterilized, the principal source of bacterial contamination was the tissue itself. To minimize this, forelimbs were swabbed with 70% (v/v) ethanol before being skinned before dissection. When this precaution was taken, the addition to the medium of penicillin (100 μunits/ml) and streptomycin (100 μg/ml) had no effect on the amount of tyrosine measured at the end of the incubation, and therefore no antibiotics were used in the main series of experiments. However, sterilized medium, incubated alone for 5 h, had a slightly lower tyrosine content than did unincubated medium. This loss of tyrosine, possibly owing to oxidation, represented only about 1 nmol (i.e. 2% of the total tyrosine), but would nevertheless result in an underestimation of the rate of tyrosine release. In all incubations an additional vessel containing 2 ml of sterilized medium but no muscle was included, and any difference between its tyrosine content and that of the same batch of unincubated medium used in the preparation of the standards was allowed for in calculations of the tyrosine release.

Calculation of rates of synthesis and degradation

Rates of protein synthesis were calculated as described previously (Smith *et al.*, 1983). Rates of protein degradation were calculated as the algebraic sum of the rate of protein synthesis and the rate of tyrosine release. The rate of tyrosine release was converted into protein equivalents by using a tyrosine content of 35 ± 2 ($n = 8$) μmol/g fresh wt. of muscle (R. M. Palmer, unpublished work).

Results

Rates of protein synthesis *in vivo* were in the range 7–11%/day (Table 1) and were decreased by isolation of the muscles to a mean of 26% of the rate *in vivo*. This decrease appeared to be the principal cause of the net catabolic state of the isolated muscles, since the calculated rate of degradation was not greatly affected by isolation of the muscles.

In the main experiments, rates of tyrosine release and protein synthesis were measured sequentially in the same muscles, tyrosine release was measured during the first 5 h of the incubation and protein synthesis for the final 30 min. To assess the validity of the approach, protein synthesis was measured over 30 min periods after various periods

Table 1. *Rates of protein synthesis and calculated rates of protein degradation in vivo and in vitro in the forelimb digit extensor muscles of rabbits*

Rates of protein synthesis *in vivo* were determined over a period of 10 min after the injection of a high concentration of [^{3}H]phenylalanine as described in the text. Rates of protein synthesis *in vitro* were determined in similar muscles from the opposite forelimb by incubating the muscles under a constant tension for 30 min in [^{14}C]phenylalanine after a preliminary incubation of 5 h during which tyrosine release was measured. The release of tyrosine is expressed as % of protein-bound tyrosine released/day, assuming that the protein-bound tyrosine of muscle is 35 μmol/g fresh wt. k_d was calculated *in vivo* from the difference between k_s and k_g and *in vitro* as the algebraic sum of k_s and tyrosine release.

	In vivo			*In vitro*		
Wt. of animal (g)	Synthesis (k_s) (%/day)	Growth rate (k_g) (%/day)	Degradation (k_d) (%/day)	Synthesis (%/day)	Tyrosine release (%/day)	Degradation (%/day)
590	11.22	5.45	5.77	2.69	3.08	5.77
774	9.24	3.31	5.93	2.09	3.94	6.03
790	6.81	2.71	4.10	2.32	0.83	3.15
808	7.92	5.20	2.72	1.91	1.92	3.83

of preincubation in unlabelled medium. The results (Table 2) show that protein synthesis was not influenced by decreasing or increasing the preincubation period. In fact it has been shown that isolated rabbit muscles incubated under these conditions maintain a constant rate of protein synthesis and an ability to respond to intermittent stretching for up to 21 h (results not shown). Tyrosine release was also measured at hourly intervals over a 5 h preincubation period (Table 3), and no significant changes in tyrosine release were found during this period.

Intermittent mechanical stretching and insulin (100 μunits/ml) both increased the rate of protein synthesis in the muscles from fed donors by more than 60%, to a value approx. 45% of the rate measured *in vivo* (Table 4). The rate of protein degradation was also increased. Starving the donor animals for 18 h decreased both the rates of protein synthesis and degradation, and, as in the fed animals, insulin and intermittent mechanical stretching increased the rates of synthesis and degradation. The response of protein synthesis in muscles from starved animals to both stimuli was

Table 2. *Effect of different periods of preincubation on the rate of protein synthesis in isolated muscles*
Protein synthesis was measured during a 30 min period, as described in the text, after incubation of the muscles in unlabelled medium for 1.5, 5 or 7.5 h. Values are means ± S.E.M. for a minimum of four experiments: ND, not determined.

Animals ...	k_s (%/day)			
	Fed		Starved	
Time of measurement of k_s (h)	Constant tension	Mechanically stretched	Constant tension	+ Insulin (100 μunits/ml)
1.5–2	2.25 ± 0.11	4.98 ± 0.11	1.01 ± 0.07	1.82 ± 0.09
5–5.5	2.49 ± 0.24	4.26 ± 0.10	0.84 ± 0.08	1.77 ± 0.21
7.5–8	2.40 ± 0.23	5.01 ± 0.23	ND	ND

Table 3. *Release of tyrosine measured at hourly intervals during the incubation of muscles*
Muscles were incubated for 5 h as described in the text. Every hour the medium was replaced and the release of tyrosine during that hour was measured. Values are means ± S.E.M. for five experiments.

Time (h) ...	Tyrosine release (%/day)				
	0–1	1–2	2–3	3–4	4–5
Constant tension	2.98 ± 1.2	4.02 ± 0.6	3.43 ± 0.9	3.26 ± 1.0	3.26 ± 0.9
Mechanically stretched	3.63 ± 1.1	2.53 ± 0.4	4.11 ± 1.0	4.11 ± 1.2	3.68 ± 0.7

Table 4. *Effect of insulin (100 μunits/ml) and intermittent stretching (MS) on rates of protein synthesis and degradation in muscles from rabbits fed ad libitum or starved for 18 h*
Muscles were incubated for a total of 5½ h as described in the text. Tyrosine release was measured during the first 5 h and protein synthesis during the final 30 min of the incubation. Values are means ± S.E.M. for a minimum of six experiments. The effects of insulin or MS are expressed as % of the control value (constant tension) and the combined effect of insulin and MS is expressed as % of the value obtained with MS alone. Significance of differences: by paired *t* test: (*a*) significantly different from the corresponding (fed or starved) constant tension, *$P<0.05$, **$P<0.025$, ***$P<0.01$; (*b*) significantly different from corresponding value with MS alone, †$P<0.05$; and by unpaired *t* test: (*c*) significantly different from corresponding value in muscles from fed rabbits, ‡$P<0.05$, ‡‡$P<0.025$, ‡‡‡$P<0.01$.

		Control (%/day)	Change compared with control (%)		Change compared with MS (%)
			Control + insulin	MS	MS + insulin
Fed	Protein synthesis (k_s)	2.49 ± 0.24	+64 ± 25*	+71 ± 7***	+26 ± 17
	Tyrosine release	3.02 ± 0.61	−23 ± 24	+18 ± 17	−17 ± 17
	Protein degradation (k_d)	5.51 ± 0.46	+15 ± 11	+58 ± 6**	+8 ± 16
Starved	Protein synthesis (k_s)	0.84 ± 0.08‡‡‡	+111 ± 14**	+158 ± 27***	+46 ± 15†
	Tyrosine release	2.75 ± 0.60	−5 ± 6	+29 ± 18	+26 ± 18
	Protein degradation (k_d)	3.43 ± 1.01‡	+31 ± 18‡	+60 ± 25	+33 ± 19

Table 5. *Effect of a high concentration of insulin (100 munits/ml) on rates of protein synthesis and degradation in muscles from starved rabbits held under a constant tension*

Protein-synthesis rates and tyrosine release were measured as described in the text. Values are means ±S.E.M. for six experiments; *by paired *t* test the effect of insulin on rates of protein synthesis was significant ($P<0.05$).

Addition ...	None	Insulin (100 munits/ml)
k_s (%/day)	1.01±0.09	1.81±0.08*
Tyrosine release (%/day)	3.11±0.61	2.15±0.07
k_d (%/day)	4.12±0.58	3.96±0.53

proportionately larger than the response of muscles from fed animals. For example, insulin and intermittent mechanical stretching increased protein synthesis by 110% and 158% respectively in the muscles from starved animals, but by only 64% and 71% when the donor animals were in a fed state before death. It was interesting that the response to insulin in muscles from fed animals was less consistent than in the muscles from starved donors. This may have reflected differences in the nutritional status of the animals, as it was observed that the response to insulin was greatest in those muscles in which the protein-synthesis rates of the controls were below the mean, whereas in the muscles in which the control rate of protein synthesis was highest there was no response to insulin.

Very high concentrations of insulin (100 munits/ml) produced no further increase in the rate of protein synthesis (Table 5), but at this concentration the rate of protein degradation was not increased.

Discussion

Direct isotopic measurements of protein-degradation rates *in vivo* are problematical because of isotope reutilization and heterogeneous turnover of tissue proteins (Garlick & Millward, 1972; Garlick *et al.*, 1976). In the present work we have attempted to obtain some estimate of the rate of degradation of muscle protein *in vivo* by measuring the fractional growth rate of the animal and the fractional rate of protein synthesis (Table 1). Although this approach has been used previously, it is open to criticism, as it is assumed that the growth rate of the whole body equals that of the forelimb muscles and that the rates of growth measured over a period of days can be equated with rates of synthesis measured over a much shorter period, taking no account of diurnal variation. However, although these criticisms of the technique are valid, our purpose in attempting to calculate an approximate rate of muscle protein degradation in the donor animals was to investigate whether this was greatly increased in isolated muscles. In the event it appeared that the principal reason for the negative amino acid balance of this preparation was the fall in the rate of protein synthesis (Table 1).

The rates of protein synthesis and degradation *in vitro* were measured during the same incubation by using a balance technique in which degradation was calculated as the algebraic sum of tyrosine release (expressed as a fraction of the protein-bound tyrosine) and protein synthesis (expressed as a fractional rate), in preference to the independent measurement of degradation when synthesis was blocked by cycloheximide (Fulks *et al.*, 1975; Flaim *et al.*, 1978). A similar approach has been used previously (Tischler *et al.*, 1982; Stirewalt & Low, 1983), but this previous work utilized media which contained none of the amino acid (tyrosine or phenylalanine) whose release was to be studied. In our experience this leads to a significant decrease in tissue free tyrosine concentration, and, although previous reports (e.g. Stirewalt & Low, 1983) have not found this, it is to be noted that the tissue free tyrosine concentration in their studies was much lower than we have observed in rabbit muscles and others have found in freshly dissected rat muscles (Waterlow *et al.*, 1978). This decrease in tyrosine concentration complicates the interpretation of the release of tyrosine; we have therefore used a medium in which the concentrations of all the amino acids (including tyrosine) are within the physiological range. A further advantage of this approach is that muscles incubated *in vitro* are in general in a state of negative amino acid balance and, since tyrosine and phenylalanine are neither synthesized nor degraded by muscle, either of these amino acids is a potential limitation of protein deposition.

Although there is general agreement that rates of protein synthesis are increased by insulin both *in vivo* (Garlick *et al.*, 1983; Reeds *et al.*, 1985) and *in vitro* (Jefferson *et al.*, 1977; Goldspink *et al.*, 1983), evidence concerning the effect of insulin on degradation is less conclusive. In the present paper insulin at a concentration of 100 μunits/ml increased the rates of synthesis and degradation, and at a concentration of 100 munits/ml increased only the rate of synthesis. Others have reported that the insulin-stimulated rise in synthesis rates is accompanied by a decrease in the rate of degradation (Fulks *et al.*, 1975; Frayn & Maycock, 1979; Goldspink *et al.*, 1983). Two factors may account for these contradictory reports. Firstly, the most reliable responses of muscle protein degradation to insulin have been achieved with isolated muscles exhibiting very high rates of protein degradation.

For example, in a rat diaphragm-muscle preparation the fall in protein degradation induced by insulin was from 29 to 20%/day (Fulks *et al.*, 1975); the rates of protein degradation reported in these and other preparations of rat (Goldspink *et al.*, 1983) and mouse muscle (Frayn & Maycock, 1979) even in the presence of insulin remained above the rate of protein degradation that would have been expected *in vivo*. Given the generally normal rate of protein degradation in isolated rabbit muscles, it seems possible that insulin (even at pharmacological concentrations) may inhibit only pathologically high rates of protein degradation and have little effect on true 'basal' rates, but we cannot of course exclude a species difference in this respect.

Secondly, many experiments which have demonstrated decreased rates of degradation in the presence of insulin have used concentrations of the hormone which are well above the physiological range (> 1 munit/ml) (Rannels *et al.*, 1975; Clark & Mitch, 1983). Where, as in the present experiments, concentrations of insulin closer to the physiological range have been used, data on degradation rates are less consistent. For example, insulin (120 μunits/ml) did not immediately decrease degradation rates, although it blocked the rise in degradation which occurred in the later stages of incubation of rat epitrochlearis (Stirewalt & Low, 1983). Perhaps the most striking demonstration of this dose-related effect was the report by Jefferson *et al.* (1977) on their work with the perfused rat hemicorpus. In this preparation, in which the rate of protein degradation was lower than in most isolated rat muscles (approx. 8%/day), insulin at all concentrations above 100 μunits/ml stimulated protein synthesis, but a concentration of at least 500 μunits/ml was required to achieve a decrease in protein degradation.

Within the present paper we found a consistent positive relationship between the rates of protein synthesis and degradation. Intermittent stretching and insulin (100 μunits/ml), which increased rates of protein synthesis, also increased degradation rates, and muscles from starved rabbits showed decreases in both synthesis and degradation. Despite these usually concordant changes in protein synthesis and degradation, the magnitude of the effects on the two rates was not necessarily the same. For instance, insulin had a greater effect on synthesis than on degradation in muscles from both fed and starved donors, resulting in a decrease in net protein breakdown. These findings also appear to conform to observations *in vivo* where rates of protein synthesis and degradation rise and fall together in response to a variety of stimuli including chronic diabetes (Pain *et al.*, 1983). Millward & Waterlow (1978) have commented on the fact that only in extreme circumstances, such as prolonged starvation (Millward *et al.*, 1976) or acute streptozotocin-induced diabetes (Pain *et al.*, 1983), is a fall in the rate of synthesis accompanied by a rise in the rate of degradation, a combination of events which results in a pathological loss of muscle protein. Many isolated muscle preparations from small rodents demonstrate similar characteristics, and it is possible that insulin decreases only pathologically elevated rates of protein degradation.

In contrast with many observations with rat and mouse muscle, the rate of degradation in our isolated rabbit muscles was not greatly elevated above that calculated *in vivo*. The reason for the lower rate of protein degradation in our preparation remains obscure. It may be due in part to the incubation conditions or the composition of the medium, specifically the near-physiological concentration of all the amino acids, including glutamine. Equally it may be due to the different species used, since work by Harris *et al.* (1985) has demonstrated that intact muscles from rats and mice develop a central core of α-glucan phosphorylase and glycogen-depleted fibres, indicating central anoxia, more rapidly than do muscles from rabbits. This difference is compatible with the proposition made some years ago (Trowell, 1959) that the high metabolic rate and hence oxygen requirement of very small species may make them unsuitable as donors of isolated muscles and that muscles from a larger species (such as the rabbit) may, despite their larger size, be more suitable for metabolic studies *in vitro*.

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