A comparison of rates of protein turnover in rat diaphragm *in vivo* and *in vitro*

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Protein synthesis and degradation rates in diaphragms from fed or starved rats were compared *in vivo* and *in vitro*. For fed rats, synthesis rates *in vivo* were approximately twice those *in vitro*, but for starved rats rates were similar. Degradation rates were less *in vivo* than *in vitro* in diaphragms from either fed or starved rats.

INTRODUCTION

As pointed out in an Anonymous (1983) article, incubated muscle preparations poorly simulate the situation *in vivo*, since, even when taken from fed animals, they are often in gross negative nitrogen balance. The causes of this imbalance are frequently unknown, but they must be a consequence of incubation inducing unequal changes in protein synthesis and degradation rates. Furthermore, it is rarely known whether rates of protein turnover *in vitro* approximate to those *in vivo*. To our knowledge, there has been no detailed study of these aspects of the incubated diaphragm preparation (Goldberg *et al.*, 1975), although, because its simplicity, it is probably the preparation most commonly used in studied *in vitro*.

EXPERIMENTAL

Sources of materials and rats have been given previously (Preedy *et al.*, 1984). Male rats (50–60 g on arrival) were housed as in Preedy *et al.* (1984) and allowed free access to food and water until they reached about 90 g. This size of rat is recommended for incubated-diaphragm preparations (Goldberg *et al.*, 1975). When required, rats were starved for 48 h in grid-bottomed cages.

For protein-synthesis experiments *in vitro*, rats were killed by a blow to the head and diaphragms were removed quickly into ice-cold Krebs & Henseleit (1932) bicarbonate-buffered saline equilibrated with O₂/CO₂ (19:1). After removal of the lumbar section, they were divided into right and left halves and blotted lightly. Each hemidiaphragm was placed in a 25 ml Erlenmeyer flask containing 5 ml of Krebs & Henseleit (1932) saline equilibrated at 37 °C with O₂/CO₂ (19:1) supplemented with 5 mM-glucose, 1 mM-phenylalanine, 0.04 mM-cysteine and the remaining amino acids at their concentrations in plasma of fed rats (Williams *et al.*, 1980). Media were re-equilibrated with O₂/CO₂ before the flasks were sealed. After a 30 min preincubation at 37 °C in a shaking water bath, hemidiaphragms were lightly blotted and transferred into flasks containing fresh medium (5 ml) containing 5 mM-glucose, 5 mM-[4-³H]phenylalanine (sp. radioactivity 1 Ci/mol), 0.2 mM-cysteine, and the remaining amino

acids were present at 5 times their concentrations in plasma of fed rats (Williams *et al.*, 1980). Each hemidiaphragm acted as a control for its contralateral half (three right and three left hemidiaphragms in each group). Insulin, when present, was added to both the preincubation and the test incubation at a concentration of 0.1 unit/ml. The flasks were incubated for a further 2 h at 37 °C, after which hemidiaphragms were immersed in ice-cold Krebs–Henseleit medium and divided laterally. In three right posterior and three left anterior quarters, [4-³H]phenylalanine incorporation into protein was determined as described previously, as was S_E (Smith & Sugden, 1983*a,b*). Protein-synthesis rates in the remaining right anterior and left posterior diaphragm quarters were determined by the method of Garlick *et al.* (1980) as described in Preedy *et al.* (1984).

For protein-degradation experiments, incubations were similar to those for protein-synthesis experiments, except that quarter diaphragms (systematically randomized between incubations) in 3 ml of medium (supplemented with 5 mM-glucose/0.5 mM-cycloheximide) were used. Preincubations (30 min) and incubations (2 h) were carried out either in the absence of amino acids or in their presence (phenylalanine excluded) at 5 times their plasma concentration as above. At the end of the incubation, media were heated at 100 °C and phenylalanine was assayed by the method of Rubin & Goldstein (1970) as modified by Sugden & Smith (1982). Standard curves were set up in the appropriate incubation media.

For experiments *in vivo*, protein synthesis was measured by the method of Garlick *et al.* (1980) as described in Preedy *et al.* (1984). The specific radioactivity of the 150 mM-[4-³H]phenylalanine injected was 1 Ci/mol. After removal from the rat, the diaphragm was dissected into approximately equal quarters. Data from protein-synthesis experiments *in vivo* and *in vitro* were directly comparable because we used the same batch of rats, and incubations and sample processing were carried out simultaneously.

The phenylalanine and tyrosine contents of rat diaphragm protein hydrolysed for 48 h as described previously (Smith & Sugden, 1983*a*) were determined with a Biotronik LC2000 amino acid analyser with elution with lithium buffers. Norleucine was used as an internal standard. Phenylalanine and tyrosine contents

Abbreviations used: k_s , fractional rate of protein synthesis; k_d , fractional rate of protein degradation; k_{RNA} , rate of protein synthesis relative to total RNA; S_p , S_E , S_i and S_B , specific radioactivities of [4-³H]phenylalanine unbound in plasma, unbound in the medium in incubations *in vitro*, unbound in tissue and bound in tissue protein respectively.

Table 1. Protein-synthesis rates in the rat diaphragm *in vitro* and *in vivo*

Protein-synthesis rates were measured by the method of Garlick *et al.* (1980) as described in the Experimental section. For the experiments *in vivo* there were eight rats in the fed group and seven rats in the starved group. For the experiments *in vitro* there were six rats in each group. The body wt. of the fed rats was 90 ± 1 g and that of the group destined for starvation was 87 ± 1 g (not significantly different). After 2 days starvation, the body wt. of the latter group decreased by 26%, to 64 ± 1 g. S_p in the fed group was 2089 ± 18 d.p.m./nmol and in the starved group it was 2044 ± 9 d.p.m./nmol. The S_E value at the end of the 2 h incubation in the fed group was 2369 ± 10 d.p.m./nmol, in the fed + insulin group it was 2403 ± 28 d.p.m./nmol, in the starved group it was 2344 ± 19 d.p.m./nmol and in the starved + insulin group it was 2369 ± 12 d.p.m./nmol. Values of k_s were calculated from S_i , although there was no material difference when they were calculated from S_p or S_E . Statistical significance for fed *in vitro* versus fed *in vivo*: * $P < 0.001$.

Quarter analysed		S_i (d.p.m./nmol)	k_s (%/day)	k_{RNA} (g of protein/ day per g of RNA)
(a) <i>In vivo</i>				
Fed	Right anterior	2086 ± 23	15.58 ± 0.62	14.58 ± 0.49
	Right posterior	2111 ± 15	14.50 ± 0.45	14.11 ± 0.39
	Left posterior	2082 ± 24	14.10 ± 0.49	14.68 ± 0.28
	Left anterior	2110 ± 9	14.48 ± 0.63	14.63 ± 0.44
Starved	Right anterior	1962 ± 41	5.55 ± 0.56	9.01 ± 0.68
	Right posterior	1927 ± 40	5.81 ± 0.74	9.55 ± 0.91
	Left posterior	1993 ± 46	5.58 ± 0.72	9.70 ± 1.00
	Left anterior	2048 ± 45	5.37 ± 0.55	9.95 ± 1.22
(b) <i>In vitro</i>				
Fed	See the	2232 ± 20	$7.36 \pm 0.32^*$	$7.27 \pm 0.37^*$
Fed + insulin	Experimental	2216 ± 16	$7.90 \pm 0.72^*$	$8.42 \pm 0.58^*$
Starved	section	2227 ± 21	4.84 ± 0.23	8.12 ± 0.36
Starved + insulin		2180 ± 21	6.59 ± 0.14	10.59 ± 0.40

were 0.277 ± 0.004 and 0.220 ± 0.003 $\mu\text{mol/mg}$ of protein respectively (means $\pm$ S.E.M.; $n = 4$; tyrosine/phenylalanine = 0.794 ± 0.003).

Protein and RNA were measured by the methods of Lowry *et al.* (1951) and Munro & Fleck (1969) respectively. For protein-synthesis experiments, results were calculated directly as fractional rates (k_s) when tissue was processed by the method of Garlick *et al.* (1980). When tissue was processed as in Smith & Sugden (1983a,b), synthesis rates in terms of pmol of phenylalanine incorporated/2 h per mg wet wt. were converted into k_s by using the phenylalanine content of diaphragm protein and the diaphragm protein concentration. Analogously, k_d was converted into phenylalanine release.

Results are presented as means $\pm$ S.E.M. When appropriate, the number of observations (n) is given in parentheses. Statistical significance was determined mainly by an unpaired two-tailed Student's t test, with $P < 0.05$ being taken as being significant. In some cases (indicated), a paired t test was used.

RESULTS

Changes in diaphragm wet weight, protein content and RNA content

On starvation, the wet weight of the whole diaphragm used in protein-synthesis experiments decreased by 19%, from 182 ± 4 mg ($n = 14$) to 148 ± 5 mg ($n = 13$; $P < 0.001$), but the total protein content decreased by only 9%, from 23.07 ± 1.7 mg to 20.94 ± 0.61 mg (not significantly different). Protein concentration increased by 12%, from 126.3 ± 1.7 to 141.5 ± 1.7 mg/g wet wt. ($P < 0.001$) on starvation. Although this increase may in part result from the loss of glycogen and triacylglycerol

(3 and 2 mg/g wet wt. respectively in fed rat muscle), it should be noted that, in 300 g rats, water intake falls from 36 ± 1 ml/day ($n = 11$) in the fed group to 13 ± 1 ml/day ($n = 6$; $P < 0.001$) in the 3-day-starved group. Part of the starvation-induced increase in protein concentration may thus result from dehydration. Total RNA content decreased by 42%, from 241 ± 4 to 140 ± 6 $\mu\text{g/diaphragm}$ ($P < 0.001$), and RNA/protein ratio decreased by 35%, from 10.45 ± 0.19 to 6.75 ± 0.34 $\mu\text{g/mg}$ ($P < 0.001$). Thus starvation affects RNA content to a greater extent than protein content, suggesting that large changes in RNA turnover must occur.

Rates of protein turnover *in vivo* and *in vitro*

In vivo, starvation decreased diaphragm k_s values on average by 62% (Table 1; $P < 0.001$). This decrease was attributable to a combination of decreases of 35% in RNA/protein ratio (see above) and 34% in k_{RNA} ($P < 0.01$). In diaphragms of fed rats incubated *in vitro* with insulin, k_s and k_{RNA} were only 54% and 58% of values *in vivo*, respectively. However, in diaphragms from starved rats (no insulin), k_s and k_{RNA} *in vitro* were about 85% of the values *in vivo*. Thus protein-synthesis rates *in vitro* resemble values *in vivo* only when diaphragms are from starved rats. Insulin significantly stimulated k_s *in vitro* ($P < 0.01$) only in diaphragms from starved rats (Table 1). The magnitude of the decrease in k_s which occurs *in vivo* on starvation is underestimated *in vitro*, the latter being only 39% ($P < 0.001$). Furthermore, starvation did not decrease the k_{RNA} *in vitro*, contrasting with the situation *in vivo*.

Protein-synthesis rates *in vitro* in diaphragm processed as in Smith & Sugden (1983a,b) could be converted into k_s values as described in the Experimental section (results not shown). There was good agreement with results

Table 2. Protein-degradation rates in the rat-diaphragm *in vitro*

Degradation was measured as in the Experimental section with six rats in each group. Amino acid and insulin concentrations are given in the Experimental section. The body wt. of the fed rats was 85 ± 1 g and that of the group destined for starvation was 85 ± 2 g. After 2 days of starvation, the body wt. of the latter group decreased by 29%, to 61 ± 2 g. Diaphragm wet wt. decreased by 21%, from 158 ± 4 to 125 ± 5 mg. Starvation significantly increased degradation in the absence of amino acids at $P < 0.01$ and in the presence of amino acids at $P < 0.05$. Amino acids decreased degradation in all cases at $P < 0.01$. Insulin significantly decreased degradation only in the absence of amino acids ($P < 0.01$).

Additions to the incubation medium	Protein degradation (pmol of phenylalanine released/h per mg wet wt.)	
	Fed	Starved
None	313 ± 18	436 ± 30
Insulin	227 ± 13	306 ± 21
Amino acids (except phenylalanine)	192 ± 15	258 ± 16
Amino acids (except phenylalanine) + insulin	164 ± 15	218 ± 17

obtained by processing analogous samples by the method of Garlick *et al.* (1980).

Rates of protein synthesis *in vitro* obtained by others (e.g. Fulks *et al.*, 1975; Hedden & Buse, 1982; Rodemann & Goldberg, 1982; Tischler *et al.*, 1982) can, after conversion into phenylalanine equivalents, be compared with our data. It is not possible to convert these rates accurately into k_s values because protein concentration is not known. (Because of changes in protein concentration on starvation, it is preferable to express protein-turnover rates relative to the protein content rather than the more frequently used wet weight.) Most rates of protein synthesis *in vitro* agree with our rates determined *in vitro* in diaphragms processed as in Smith & Sugden (1983a,b), which were (in pmol of phenylalanine incorporated/h per mg wet wt. for six observations): fed, 87 ± 3 ; fed + insulin, 99 ± 8 ; starved, 63 ± 3 ; starved + insulin, 87 ± 9 . Results of Fulks *et al.* (1975) seem exceptionally high, probably because, at the low tyrosine concentration used (0.05 mM), the intracellular specific radioactivity from which rates were calculated may be lower than in tyrosyl-tRNA (McKee *et al.*, 1978).

Values for k_d for fed rats *in vivo* were calculated to be 4.8%/day (convertible into 70 pmol of phenylalanine released/h per mg wet wt.) by subtraction of the growth rate ($9.85 \pm 0.36\%$ /day; $n = 31$) from k_s , assuming that for lean, growing, rats the rate of accumulation of diaphragm protein is the same as the growth rate. From the rate of loss of diaphragm protein on starvation (4.62%/day), the mean k_d can be calculated to be 10.2%/day (giving 167 pmol of phenylalanine released/h per mg wet wt.).

Rates of protein degradation *in vitro* are given in Table 2. Starvation increased degradation (but note that part of this effect is attributable to an increase in diaphragm protein concentration). Amino acids decreased degrad-

ation. Insulin significantly decreased degradation only in the absence of amino acids (although there was a trend towards a decrease in their presence). These rates of degradation and findings are largely similar to those of others (e.g. Fulks *et al.*, 1975; Sugden, 1980; Tischler *et al.*, 1982). In diaphragms from fed rats, the minimum degradation rate *in vitro* is about twice that *in vivo*. Rates *in vitro* in starved rats are greater than *in vivo*, although comparisons are more difficult because the rate *in vivo* is unlikely to be in a steady state and is averaged over the whole period of starvation.

Regional variation of protein-synthesis rates in the diaphragm

In fed rats, k_s in the right anterior quarter was significantly greater ($P < 0.5$; paired *t* test) than that in the left posterior quarter (Table 1). The respective RNA/protein ratios were 10.75 ± 0.48 and 9.58 ± 0.17 $\mu\text{g}/\text{mg}$ of protein ($P < 0.05$). Although these differences are small, unless diaphragm quarters are systematically randomized, misleading results could be obtained.

DISCUSSION

In spite of the extensive use of muscle preparations *in vitro*, scant attention has been paid to how well protein turnover rates simulate the situation *in vivo*. When skeletal-muscle protein turnover *in vivo* and *in vitro* has been compared, k_s in isolated muscle preparations has generally been lower and k_d higher than *in vivo* (Palmer *et al.*, 1981, 1985; Goldspink *et al.*, 1983). In the perfused hemicorpus, the k_s for gastrocnemius compared well with values *in vivo*, but the k_s for soleus was twice that *in vivo* (Preedy & Garlick, 1983). In the perfused working rat heart, k_s was close to values *in vivo* (Preedy *et al.*, 1984, 1985). Possibly, delivery of O_2 and nutrients through an intact circulatory system could be of relevance.

Lower k_s values *in vitro* could result from slow or incomplete equilibration of S_E with S_i (see Morgan *et al.*, 1979; Rannels *et al.*, 1982). In the perfused heart, S_i and the specific radioactivity of the phenylalanyl group of phenylalanyl-tRNA rapidly equilibrate with the S_E of 0.4 mM- ^{14}C phenylalanine, but at 0.01 mM- ^{14}C phenylalanine the specific radioactivity of ^{14}C phenylalanyl-tRNA is greater than S_i but less than S_E (McKee *et al.*, 1978). In the rat incubated epitrochlearis muscle, the ratio of the specific radioactivity of aminoacyl-tRNA to S_E was 0.30–0.53 for 0.1 mM-valine, 0.166 mM- or 2 mM-leucine, or 0.4 mM-phenylalanine (Stirewalt & Low, 1983), although aminoacyl-tRNA specific radioactivity was constant over 1–3 h. Presumably there was dilution of S_i from proteolysis. We used high (5 mM) concentrations of ^{14}C phenylalanine. We showed that, after 2 h of incubation *in vitro*, the S_i/S_E ratio was 0.973 ± 0.006 (24). There was no significant difference between fed and starved groups. If dilution of S_i by protein degradation had been a problem, it should have been greater in the starved group. If equilibration of radiolabel was a problem, it is difficult to see why k_s values *in vivo* and *in vitro* should differ in the fed animal, yet not in the starved. Perhaps an increase in the rate of transport of amino acids could occur in the fed-to-starved transition, but this seems unlikely. Conversely, since rates of protein synthesis in the fed animal are relatively high, amino acid transport *in vitro* could be rate-limiting. However,

measurements of S_1 did not show any difference between phenylalanine concentrations in diaphragms from fed and starved rats. It is unlikely that the inhibition of protein synthesis in diaphragms of fed rats *in vitro* could result from trauma associated with excision or cutting (see Seider *et al.*, 1980) of the muscle or from removal of the stimulation of protein synthesis by stretch (Goldspink, 1977, 1978; Laurent *et al.*, 1978; Palmer *et al.*, 1981, 1985), since decreases might also be expected in starved rats. The discrepancy between k_s values *in vivo* and *in vitro* in fed animals remains a mystery. There may be a factor (not insulin) in the serum of fed rats which stimulates protein synthesis.

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