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Comparison of protein synthesis and degradation in incubated and perfused muscle

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Rates of muscle protein synthesis and degradation measured in the perfused hindquarter were compared with those in incubated epitrochlearis muscles. With fed or starved mature rats, results without insulin treatment were identical. With insulin treatment, protein synthesis in perfused hindquarters was greater, though protein degradation was the same. Thus rates of muscle protein degradation estimated by these two methods *in vitro* correspond closely.

The isolated perfused hindquarter of rats has been used by several investigators to measure rates of muscle protein synthesis and degradation under different physiological conditions (Jefferson *et al.*, 1977; Goodman *et al.*, 1981; Flugel-Link *et al.*, 1982). However, the validity of this model has been challenged by Preedy & Garlick (1981), who concluded that it probably does not reflect the rate of muscle protein degradation because of protein turnover occurring in other tissues of the hindquarter. Indeed, the proportion by weight of other tissues being perfused in this preparation has been estimated to be 23–44% (Ruderman *et al.*, 1971; Jefferson, 1975; Mitch, 1981; Preedy & Garlick, 1981). It would be important to determine whether muscle protein degradation can be estimated with the perfused hindquarter, because its advantages for measuring substrate metabolism, hormonal effects and protein turnover have made it a popular preparation for studying muscle metabolism (Berger *et al.*, 1976; Jefferson *et al.*, 1977; Brady *et al.*, 1981).

The present studies were undertaken to compare rates of muscle protein synthesis and degradation measured in the perfused hindquarter with those measured in an incubated mixed-fibre muscle. An incubated muscle was used so that the measured rates of protein turnover *in vitro* would not be affected by metabolic processes occurring in other tissues. Because starvation can change muscle protein turnover, we also contrasted results from fed rats with those from rats starved for 48h to determine if any changes induced could be detected equally well in perfused muscle.

Experimental

Animals

Male Sprague–Dawley rats weighing 200–220g (Charles River Laboratory) were kept in a 12h-light/12h-dark cycle for at least 3 days before being studied. They were allowed free access to water and were fed RMH 1000 chow (Agway Country Foods) *ad libitum*. During starvation, rats were deprived of food and water and were housed in individual wire-bottomed cages to prevent coprophagia. Before being studied, rats were anaesthetized with pentobarbitol (5mg/100g body wt.). Mature rats were used in all studies because of the variable response of muscle protein degradation to starvation in immature rats (Li *et al.*, 1979; Goodman *et al.*, 1981).

Materials

[U-^{14}C]Tyrosine and [U-^{14}C]phenylalanine were obtained from Schwarz/Mann, and reagent-grade chemicals from Fisher. Lactate dehydrogenase was obtained from Sigma, and crystalline bovine insulin from Eli Lilly. Bovine serum albumin obtained from Miles Laboratories was dissolved in Krebs–Henseleit bicarbonate buffer (Krebs & Henseleit, 1932) and dialysed against a 10-fold excess of the buffer for 24h. The dialysed albumin solution was then stored at −20°C until used in hindquarter perfusions.

Hindquarter perfusions

The perfusion procedure was similar to that used previously (Mitch & Chan, 1979; Mitch, 1981), which is based on the technique of Ruderman *et al.*

(1971). Perfusates (150ml each) were prepared freshly before each experiment and gassed with O_2/CO_2 (19:1). The media consisted of aged washed human erythrocytes (haematocrit, 25%) in Krebs–Henseleit bicarbonate buffer, albumin (final concn. 0.3g/l), 10mM-glucose, 0.5mM-phenylalanine and other amino acids at concentrations found in rat plasma. Thus this preparation was similar to that used previously to assess muscle protein turnover (Jefferson *et al.*, 1977; Li *et al.*, 1979; Goodman *et al.*, 1981; Preedy & Garlick, 1981). In experiments assessing the effects of insulin, 500 μunits of insulin/ml were added after the equilibration period (Jefferson *et al.*, 1977; Richter *et al.*, 1982).

The perfusate was recirculated in the perfusion chambers for 15min or more while it was gassed and heated to 37°C. The pH was then checked and if necessary, adjusted to pH7.4. After placement of the aortic and vena caval cannulae, the perfusion was begun and the spinal cord was severed to avoid high perfusion pressures occurring as the animal died. Throughout the perfusion, the flow rate (Gilmont Flow Meter) was maintained at 12–14ml/min. The initial 30ml of media was discarded and the remainder was recirculated continuously. After an equilibration period of 30min, measurements were made over a 60min period. Experiments were discarded when there was evidence of an inadequate perfusion, e.g. a flow rate of less than 10ml/min, a rising perfusion pressure or lactate release exceeding 8μmol/h per g (Ruderman *et al.*, 1971).

Samples of perfusate were obtained at 30 and 90min for subsequent determination of lactate, tyrosine and phenylalanine concentrations. Rates of uptake or release of these compounds were calculated from the changes in their concentrations and the volume of the perfusate. They were expressed per g of muscle perfused, by using the relationships between body weight and muscle 'perfused' determined previously (Mitch, 1981).

Epitrochlearis-muscle incubation

The epitrochlearis muscle was used to study muscle protein synthesis and degradation because its weight (28–32mg) in adult rats is comparable with that of the soleus and extensor digitorum longus muscles of immature rats. Additionally, the dimensions of the muscle provide a large surface relative to its weight (Nesher *et al.*, 1980), and therefore, presumably, more effective diffusion of substrates (Goldberg *et al.*, 1975). The epitrochlearis contains approx. 10–15% slow-twitch, red, 20% fast-twitch, red, and 65% fast-twitch, white, muscle fibres (Nesher *et al.*, 1980).

The muscles were weighed and placed in individual flasks containing 3ml of Krebs–Henseleit bicarbonate buffer, 10mM-glucose and 0.5mM-phenylalanine (with or without 0.05μCi of [U-^{14}C]phenylalanine/ml, as indicated). Preparations in which the muscle weight exceeded 40mg were discarded. The flasks were stoppered, gassed for 3min with O_2/CO_2 (19:1) and placed in a rotating (60 cycles/min) bath maintained at 37°C. After 30min of preincubation, the muscles were removed, blotted and transferred to flasks containing 3ml of the same media, with or without 1munit of insulin/ml, as indicated, and incubated for an additional 2h period. This procedure is similar to that used previously to study muscle protein synthesis and degradation in incubated muscles (Li *et al.*, 1973; Fulks *et al.*, 1975).

This concentration of insulin was studied because it has been shown to cause an increment in glucose uptake by the incubated muscle comparable with that produced by 0.5 munit of insulin/ml in the perfused hindquarter preparation (Brady *et al.*, 1981).

Protein synthesis

As shown by Li *et al.* (1973), muscle protein synthesis measured with radiolabelled amino acids will vary depending on changes in the intracellular specific radioactivity of the amino acid. Therefore we measured the intracellular specific radioactivity of the amino acid being studied (phenylalanine and tyrosine) at both the beginning and the end of the experimental periods and compared them with the extracellular specific radioactivity in perfused and incubated muscle at the same times. Phenylalanine and tyrosine were chosen as the radiolabelled amino acids because muscle neither synthesizes nor degrades these amino acids. To calculate the intracellular specific radioactivity (Jefferson *et al.*, 1977; Li *et al.*, 1973), previously determined values (Mitch, 1981) for water content and extracellular volume of perfused muscle were used; the water content of the epitrochlearis muscle was measured by drying to constant weight and found to be 69.5 ± 1.4% (mean ± S.E.M., $n = 6$). Its extracellular volume was measured with [^{14}C]inulin and found to be 23.6 ± 2.7% of weight ($n = 5$). As shown in Fig. 1, the intracellular and extracellular specific radioactivities of phenylalanine in muscles from starved rats were virtually identical after 30min of either perfusion or incubation; there was no significant change in the ratio during the experimental period. Similar results were obtained in muscle from fed rats (results not shown). Determining that this equilibration occurred rapidly permitted us to calculate synthesis based on the extracellular specific radioactivity of phenylalanine, which decreased the number of analyses substantially.

To determine protein-synthesis rates in the hindquarter experiments, 0.05μCi of [U-^{14}C]phenylalanine/ml was added to the perfusate and samples of perfusate and gastrocnemius muscle were ob-

tained after 30 and 90min of perfusion. The gastrocnemius was used because it, like the epitrochlearis, is composed of both red and white muscle fibres. The perfusate was deproteinized with an equal volume of 10% (v/v) trichloroacetic acid, and the extracellular specific radioactivity of phenylalanine was determined. The muscle sample was weighed and immediately homogenized in 3ml of 10% trichloroacetic acid, washed successively with 10% trichloroacetic acid and ethanol/diethyl ether (1:1, v/v) and dissolved in Soluene (Packard) for scintillation counting, with correction for quenching by using an external standard. The difference in the radioactivity incorporated into muscle protein between 30 and 90min divided by the average extracellular specific radioactivity was used to estimate the rate of protein synthesis.

To determine the rate of protein synthesis in experiments with the epitrochlearis muscle, 0.05μCi of [U-^{14}C]phenylalanine/ml was added to the media and one muscle was harvested, washed and dissolved in Soluene, as described above, for determination of radioactivity, after 30min of incubation; the contralateral muscle was treated similarly at the end of the experimental period. As with the perfusion experiments, the difference in incorporated radioactivity was then divided by the extracellular specific radioactivity of phenylalanine and expressed per g of muscle wt. to calculate the rate of synthesis. This procedure permitted the correction for variable rates of [^{14}C]phenylalanine incorporation during the period when the intracellular specific radioactivity of phenylalanine was rising.

To assess the effect of insulin on protein synthesis in the epitrochlearis muscle, the [^{14}C]phenylalaine incorporated into protein was determined without added insulin in eight muscles during the initial 30min. This initial amount of [^{14}C]phenylalanine incorporated was subtracted from that occurring during paired experiments in which one muscle was incubated without insulin and the contralateral muscle with insulin for the experimental period.

Protein degradation

In the perfused-hindquarter experiments, the rates of total protein degradation were measured from the change in the perfusate content of tyrosine in the presence of 0.5mM-cycloheximide (Goodman *et al.*, 1981). In the experiments with incubated epitrochlearis muscle, total protein degradation was measured by incubating with 0.5mM-cycloheximide and calculating the rate of release of tyrosine into the incubation media during the 2h experimental period.

Analyses

Phenylalanine and tyrosine were measured fluorimetrically (Andrews *et al.*, 1973; Waalkes & Udenfriend, 1957), and lactate was determined enzymically (Hohorst, 1963). The results are expressed as means ± S.E.M. and differences were tested by analysis of variance, except for the changes induced by insulin in protein synthesis and degradation in incubated muscles. In these experiments, the results were compared by using a paired *t* test.

Results and discussion

As shown in Fig. 1, when there was a high concentration of phenylalanine in either the incubation media or perfusate, there was no significant difference between the extracellular and intracellular specific radioactivities of phenylalanine after 30min. This rapid equilibration has been found previously in studies of other perfused organs (Jefferson *et al.*, 1977; Rannels *et al.*, 1982), but has not been shown to occur with the incubated epitrochlearis muscle. In the epitrochlearis muscle incubated in 0.5mM-tyrosine, we also found that this ratio attained a value insignificantly different from that at 30min and did not change over the next 2h (results not shown). Thus there was rapid equilibration of the amino acid being used to measure protein synthesis in both preparations, and the specific radioactivities remained equal during the experimental period. This property permitted calculation of the rate of muscle protein synthesis from the extracellular specific radioactivity and thereby decreased the number of analyses required (Rannels *et al.*, 1982).

Table 1 shows the calculated rates of muscle protein synthesis and degradation in incubated epitrochlearis muscles and in the perfused hind-

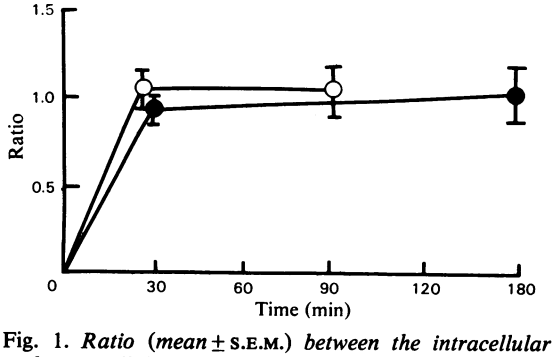

Fig. 1. *Ratio (mean ± S.E.M.) between the intracellular and extracellular specific radioactivity of phenylalanine in muscle during hindquarter perfusion* (○, $n = 6$) *and epitrochlearis incubation* (●, $n = 8$) *experiments*
The intracellular specific radioactivity after 30min and at the end of the experiments were calculated as described by Jefferson *et al.* (1977) and Li *et al.* (1973). The results shown are from starved rats and did not differ significantly from those for fed rats.

Table 1. *Components of protein turnover measured in the incubated epitrochlearis muscle and perfused hindquarter preparations*

The rate of protein synthesis was measured in perfused and incubated muscle as described in the text; the rate of total protein degradation was measured as tyrosine released when 0.5 mM-cycloheximide was present in perfusion and incubation media. Results are presented as means ± S.E.M. for six to eight experiments. By analysis of variance, there was a significant (*$P < 0.025$) decrease in basal protein synthesis induced by starvation. The rate of protein synthesis in muscle perfused with 0.5 munit of insulin/ml was significantly greater (†$P < 0.05$) than that in muscle incubated with 1 munit of insulin/ml.

Protein synthesis (nmol of phenylalanine/h per g)	Normal fed			Starved 48 h		
	Basal	+ Insulin	% Change	Basal	+ Insulin	% Change
Epitrochlearis	45 ± 2	65 ± 5	+47	34 ± 2*	56 ± 4	+65
Hindquarter	51 ± 5	82 ± 5†	+61	38 ± 3*	72 ± 5†	+89
Protein degradation (nmol of tyrosine/h per g)	**Normal fed**			**Starved 48 h**		
	Basal	+ Insulin	% Change	Basal	+ Insulin	% Change
Epitrochlearis	142 ± 6	114 ± 7	−20	140 ± 5	112 ± 3	−20
Hindquarter	138 ± 6	103 ± 4	−25	149 ± 5	119 ± 6	−20

quarters of fed and 48 h-starved adult rats. By analysis of variance, it was determined that there was no significant difference in the basal rate of protein synthesis in fed rats measured by either technique. The two techniques also gave comparable results for starved rats. Moreover, the significant ($P < 0.025$) decrease in the basal rate of protein synthesis induced by starvation was the same when measured by either technique (−24%, epitrochlearis incubation; −25%, hindquarter perfusion) and similar to that in perfused hindquarters reported previously (Li *et al.*, 1979). Similarly, basal rates of protein degradation measured in both preparations were comparable. As expected (Li *et al.*, 1979; Goodman *et al.*, 1981), starvation of mature rats for 48 h did not cause an increase in muscle protein degradation in either incubated muscle or the perfused hindquarter. The effect of perfusion on the intracellular concentration of tyrosine was assessed by comparing values measured in different types of hindlimb muscles of fed and starved rats with values obtained in similarly treated rats at the end of a perfusion. In the gastrocnemius, soleus and extensor digitorum longus muscles of fed rats, the intracellular tyrosine concentrations (means ± S.E.M.) were 171 ± 12, 168 ± 11 and 181 ± 13 μM respectively; for starved rats, the values were 191 ± 17, 198 ± 18 and 183 ± 7 μM respectively. After perfusion, values for the intracellular tyrosine concentrations of gastrocnemius and extensor digitorum longus muscles of both fed ($n = 6$) and starved ($n = 6$) rats were slightly (<10%), but insignificantly, higher. In soleus muscle of fed rats also, intracellular tyrosine concentration was not changed significantly by perfusion, though it was 16% higher in perfused solei of starved rats ($P < 0.05$). There also was no change in intracellular tyrosine concentration during incubation. As noted by others, this suggests that tyrosine release reflects the rate of muscle protein degradation (Jefferson *et al.*, 1977; Goodman *et al.*, 1981).

In the presence of insulin, the rate of protein synthesis in perfused muscle from fed or starved rats was greater than that in incubated muscle from comparably treated animals (Table 1). This is reminiscent of the greater sensitivity of insulin-stimulated glucose uptake by the perfused hindquarter (Brady *et al.*, 1981). Although the amino acids contained in the perfusion media could have contributed to the higher rate of protein synthesis that we observed (Table 1), it seems unlikely that they were the sole reason. Amino acids have been shown to stimulate muscle protein synthesis measured *in vitro* (Morgan *et al.*, 1971), but their effect does not augment the increase in protein synthesis caused by the addition of glucose and insulin (Fulks *et al.*, 1975).

When starved rats were studied, it was found that the rate of muscle protein synthesis was lower than that of fed rats in both the presence and the absence of insulin (Table 1). The lower rate of protein synthesis probably was caused by a lower RNA content of muscle, which would limit the capacity for protein synthesis (Millward *et al.*, 1974; Li *et al.*, 1979). However, responsiveness to insulin as indicated by the percentage increase in the rate of protein synthesis was still present in starved rats. Regardless of the differences in rates of protein synthesis, in both fed and starved rats, rates of tyrosine release measured in the two preparations were similar in the basal state and were depressed by insulin by a similar amount. This indicates that the effects of insulin on protein degradation were similar when measured by either the perfusion or incubation technique.

The similarity of these results obtained by using

two methods is presumably related to the following factors. Firstly, the gastrocnemius muscle used to estimate muscle protein synthesis in the perfusion experiment and the epitrochlearis muscle are both 'mixed fibre' muscles (Jefferson, 1975; Nesher *et al.*, 1980). Secondly, the large surface area of the epitrochlearis muscle relative to its weight decreases the variability in rates of protein synthesis and degradation that is caused by unequal diffusion of oxygen, glucose and other substrates (Goldberg *et al.*, 1975). Thirdly, the experiments were performed on rats of similar age, which avoids the differing sensitivities of muscles from mature and immature rats to physiological stimuli such as starvation (Li *et al.*, 1979; Goodman *et al.*, 1981). Although these characteristics of the epitrochlearis permitted a comparison between incubated and perfused muscle of rats of similar age, they do not explain fully the correspondence between measured rates of tyrosine release. Protein synthesis occurring in non-muscle tissues of the perfused hindquarters (Preedy & Garlick, 1981) should lead to a greater rate of tyrosine release by the hindquarter. The finding that this was not the case suggests that the rate of protein degradation in the incubated muscle was higher than in perfused muscle. This could be a consequence of differences in protein metabolism by these muscles, differences in the composition of the incubation and perfusion media, and/or differences in the rates of protein degradation between perfused and incubated muscle. This last possibility should be considered because of the metabolic changes induced by these procedures *in vitro*, i.e. the balance between protein synthesis and degradation is invariably negative (Goldberg *et al.*, 1975; Jefferson *et al.*, 1977; Li *et al.*, 1979; Goodman *et al.*, 1981; Table 1), and insulin-responsiveness of incubated and perfused muscle differs (Brady *et al.*, 1981; Table 1). An alternative explanation is that the effects of protein metabolism in perfused non-muscle tissues may not be detectable by these methods.

It can be concluded that, under these conditions, the basal rate of tyrosine release during perfusion of the hindquarters of fed or starved mature rats is quite similar to that released by incubated epitrochlearis muscles taken from comparably treated rats of the same age. Moreover, the response of muscle protein degradation to insulin is similar. Although these results are consistent with the view that tyrosine release by the perfused hindquarter reflects that of 'mixed-fibre' muscles, it has not been established that the rates also would be similar in other conditions (e.g. diabetes), in which protein turnover in perfused non-muscle tissues might obscure assessment of protein degradation in muscle.

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