Rates of protein turnover *in vivo* and *in vitro* in ventricular muscle of hearts from fed and starved rats

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Starvation of 300 g rats for 3 days decreased ventricular-muscle total protein content and total RNA content by 15 and 22% respectively. Loss of body weight was about 15%. In glucose-perfused working rat hearts *in vitro*, 3 days of starvation inhibited rates of protein synthesis in ventricles by about 40–50% compared with fed controls. Although the RNA/protein ratio was decreased by about 10%, the major effect of starvation was to decrease the efficiency of protein synthesis (rate of protein synthesis relative to RNA). Insulin stimulated protein synthesis in ventricles of perfused hearts from fed rats by increasing the efficiency of protein synthesis. *In vivo*, protein-synthesis rates and efficiencies in ventricles from 3-day-starved rats were decreased by about 40% compared with fed controls. Protein-synthesis rates and efficiencies in ventricles from fed rats *in vivo* were similar to values *in vitro* when insulin was present in perfusates. *In vivo*, starvation increased the rate of protein degradation, but decreased it in the glucose-perfused heart *in vitro*. This contradiction can be rationalized when the effects of insulin are considered. Rates of protein degradation are similar in hearts of fed animals *in vivo* and in glucose/insulin-perfused hearts. Degradation rates are similar in hearts of starved animals *in vivo* and in hearts perfused with glucose alone. We conclude that the rates of protein turnover in the anterogradely perfused rat heart *in vitro* closely approximate to the rates *in vivo* in absolute terms, and that the effects of starvation *in vivo* are mirrored *in vitro*.

The perfused rat heart *in vitro* has been used extensively in studies of protein synthesis and degradation. It is not known how closely rates of these processes *in vitro* match those *in vivo*. It is important before extrapolating from results obtained *in vitro* to the heart *in vivo* that rates of protein turnover should be comparable. Studies on other tissues have given conflicting results. In rabbit forelimb muscles, protein-synthesis rates *in vitro* were 22–38% of rates *in vivo*, depending on whether muscles were stretched (Palmer *et al.*, 1981). In rat soleus muscle, synthesis rates *in vitro* were only 50–70% of those *in vivo*, whereas degradation rates *in vitro* were 16–112% greater than those *in vivo*, depending on pretreatment (overloading, denervation) of the muscle *in vivo* (Goldspink *et al.*, 1983). The preparation *in vitro* was always in negative nitrogen balance, a

situation common in preparations *in vitro*. Studies in two other systems have shown increases in protein synthesis *in vitro*. In the perfused hemi-corpus, protein-synthesis rates in the soleus were elevated as much as 2-fold over those *in vivo*, but in the gastrocnemius, rates of protein synthesis were similar *in vivo* and *in vitro* (Preedy & Garlick, 1983). In rabbit alveolar macrophages protein synthesis was 5 times more rapid *in vitro* than *in vivo* (Oliver *et al.*, 1984).

It is also important to know to what extent interventions *in vivo* are reflected *in vitro*, and vice versa. There has been considerable confusion concerning the effects of starvation on cardiac protein synthesis. *In vivo*, some experiments have suggested that cardiac protein is relatively slowly lost after 2 days of starvation compared with, for example, liver (Garlick *et al.*, 1975; Goodman & Ruderman, 1980), whereas others indicated a large loss of cardiac mass on more prolonged starvation (Li & Goldberg, 1976; Griffin & Wildenthal, 1978). *In vivo*, 2 days of starvation decreased

Abbreviations used: k_s , fractional rate of protein synthesis; k_d , fractional rate of protein degradation; S_p , tissue specific radioactivity of free [4-³H]phenylalanine.

protein synthesis by about 50% (Garlick *et al.*, 1975) in rats of about 100g body wt. However, in perfused hearts taken from 250–350g rats, protein-synthesis rates were depressed by only 16% compared with fed rats, whereas in skeletal muscle (gastrocnemius) rates were decreased by 46% (Rannels *et al.*, 1978). Inhibition of heart protein synthesis was entirely attributable to loss of RNA (Rannels *et al.*, 1978). Some of the confusion may have resulted because different workers have used rats of different ages, periods of starvation have varied and animal groups may not have been accurately weight-matched. Thus there has been no single study on loss of heart protein on starvation and protein turnover rates *in vivo* and *in vitro*.

Experimental

Materials

Radiochemicals were from Amersham International, Amersham, Bucks., U.K. Chemicals were from BDH Chemicals, Dagenham, Essex, U.K. Biochemicals were from Sigma Chemical Co., Poole, Dorset, U.K., except for tRNA, which was from BCL, Lewes, East Sussex, U.K. Novo Industries Actrapid mono-component pig insulin (100 units/ml) was from the National Heart Hospital pharmacy.

Animals

Male Sprague-Dawley rats were from Bantin and Kingman, Hull, Humberside, U.K. They were kept in the animal house at 22–24°C for at least 1 week before use, during which time they had free access to water and food (Diet 86A; E. Dixon and Sons, Ware, Herts., U.K.). Animal-house lighting was set for a 12h-light/12h-dark cycle, with the light phase starting at 08:00h. All rats used in this study were about 300g body wt. (see the Table legends for precise weights). Rats were starved in grid-bottomed cages.

Measurement of protein synthesis *in vitro* and *in vivo*

For measurements *in vitro*, hearts were perfused as anterograde (working) preparations by the method of Taegtmeier *et al.* (1980) as described in detail previously (Sugden & Smith, 1982b). Hearts were initially perfused retrogradely at 10kPa pressure as non-recirculating preparations with Krebs & Henseleit (1932) bicarbonate-buffered saline containing 5mM-glucose and equilibrated with O₂/CO₂ (19:1). After cannulation of the left atrium (which was done as quickly as possible), the preparation was switched to an anterogradely perfused preparation in which medium was recirculated. The filling pressure was 0.5kPa and the aortic pressure was 7kPa. The medium was the same as in the retrograde perfusion, but contained

additionally 0.4mM-[4-³H]phenylalanine (sp. radioactivity 0.75Ci/mol) and the remaining plasma amino acids each at a concentration of 0.2mM. Insulin, when present, was added to media used for both the retrograde and anterograde perfusions at a concentration of 50munits/ml (0.35µM). After 2h of perfusion (or earlier when the time course of protein synthesis was studied), hearts were removed from cannulae, dissected, frozen in liquid N₂ and stored at –80°C. The *k_s* values were determined as in Garlick *et al.* (1980), with HClO₄ (final concn. 0.625M) as the protein precipitant. Hearts from fed animals were perfused in the morning starting at 09:30h, and from starved animals in the afternoon starting at 14:00h. Preliminary work had established, however, that there was no significant difference between protein-synthesis rates of hearts from fed animals perfused in the morning or afternoon.

For measurements of protein synthesis *in vivo*, the 'flooding dose' method of Garlick *et al.* (1980) was used. [4-³H]Phenylalanine (150mM; sp. radioactivity 0.7Ci/mol) was administered via a lateral tail vein at a dose of 1ml/100g body wt. Injections of fed animals were started at 08:45h and finished by 10:00h. Starved animals were injected subsequently, finishing by 11:00h. Animals were beheaded at 2 or 10min after injection, blood was collected and hearts were removed into an ice/water mixture as quickly as possible (about 45s after decapitation) for subsequent dissection. Ventricles were frozen in liquid N₂ and stored at –80°C. The *k_s* values were determined as in Garlick *et al.* (1980). Plasma specific radioactivities were also measured, and in all cases *S_i* values were equilibrated with plasma specific radioactivities at 2 and 10min.

For experiments *in vitro* only, *k_s* was also calculated from the incorporation of phenylalanine in terms of pmol/mg of protein determined as in Smith & Sugden (1983b,c) and the phenylalanine content (0.276±0.020µmol/mg of protein; 19 observations) of protein [purified as in Smith & Sugden (1983b)] hydrolysed in 6M-HCl for 48h (which produced maximal phenylalanine release). Perfusate [4-³H]phenylalanine specific radioactivity was measured as in Smith & Sugden (1983b,c). In all these determinations phenylalanine was assayed as in Rubin & Goldstein (1970) as modified by Sugden & Smith (1982a).

Measurement of protein degradation *in vitro* and *in vivo*

Hearts were perfused essentially as in protein-synthesis experiments, except that amino acids were omitted from perfusates and 0.02mM-cycloheximide was included. Methodology has been described in detail previously (Sugden & Smith,

1982a). Values of k_d were calculated from the rate of release of phenylalanine into the perfusate (in units of pmol/h per g dry wt.), the heart protein/dry wt. ratio (0.8) and the phenylalanine content of heart protein. *In vivo*, k_d values were calculated from the difference between k_s and the fractional rate of protein deposition.

Other methods

RNA was measured by the method of Munro & Fleck (1969) and protein by the method of Gornall *et al.* (1949), by using the standardization procedure described by Smith & Sugden (1983b).

Statistical methods

Results are expressed as means $\pm$ S.E.M. with the numbers of observations in parentheses where applicable. Statistical significance was determined by a two-tailed unpaired Student's *t* test. Values of $P < 0.05$ were taken as being statistically significant.

Results

Effects of starvation on ventricular weight, protein content and RNA content

After 3 days of starvation, rats had lost 22% of their body weight and 19% of their ventricular wet weight (Table 1). Cardiac protein concentration did not alter significantly. The decrease in total ventricular protein content was less than the decrease in body weight, resulting in a significant increase in the ventricular-protein-content/body-weight ratio. Thus the heart loses protein less rapidly than the whole body loses weight during starvation. Starvation decreased the ventricular RNA/protein ratio and RNA content, as shown previously (Garlick *et al.*, 1975; Rannels *et al.*, 1978; Goodman & Ruderman, 1980). From these results, starvation should cause alterations in rates of both protein and RNA turnover.

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

Rates of protein synthesis in ventricles of hearts perfused *in vitro* from fed or 3-day-starved rats were linear with time for at least 2 h of perfusion (results not shown). After 2 h of perfusion, free tissue [4-³H]phenylalanine specific radioactivities (measured as in Garlick *et al.*, 1980) were 97% of perfusate values (measured as in Smith & Sugden, 1983b,c). Effects of starvation and insulin on ventricular protein-synthesis rates *in vitro* are shown in Table 2(a). Measurement of k_s in the same ventricles by the method of Smith & Sugden (1983b,c) and protein phenylalanine content (see the Experimental section) gave values of $5.28 \pm 0.22\%$ /day for fed rats, $2.81 \pm 0.19\%$ /day for starved rats and $8.04 \pm 0.43\%$ /day for perfusions in the presence of insulin ($P < 0.001$ for fed group versus starved or insulin-perfused groups), confirming that the experimentally determined value for the phenylalanine content of ventricular protein is correct. Insulin (50 munits/ml) stimulated protein synthesis *in vitro* by 52–70%, whereas starvation decreased it by 38–47%. Although a decrease in the RNA/protein ratio (i.e. a loss of protein-synthesis capacity) caused a 15% inhibition of protein synthesis during starvation, 63% of the inhibition was attributable to a decrease in efficiency of protein synthesis. Rannels *et al.* (1978) reported that the 16% inhibition of cardiac protein synthesis induced by 2 days of starvation was wholly attributable to a decrease in the RNA/protein ratio and not to any change in the efficiency of protein synthesis. Our results contradict the conclusions of Rannels *et al.* (1978). Variations in experimental design or technique could have caused these differences. Rannels *et al.* (1978) perfused hearts retrogradely with 15 mM-glucose, apparently in the absence of insulin, whereas we perfused anterogradely with 5 mM-glucose. Rat weights were similar. We used rats starved for 3 days rather than 2 days, although we have shown that the efficiency of ventricular

Table 1. *Body weights, ventricular weights, protein and RNA contents of fed and 3-day-starved rats*

The number of rats used in the fed group was 13 and in the 3-day-starved group was 6. Statistical significance of values versus those for fed rats: * $P < 0.01$; ** $P < 0.001$.

	Fed	3-day-starved	Change (%)
Initial body wt. (g)	—	283 ± 3	—
Body wt. on decapitation (g)	286 ± 2	$222 \pm 3^{**}$	–22
Ventricular wet wt. (g)	893 ± 14	$723 \pm 19^{**}$	–19
Total ventricular protein content (mg)	142 ± 2	$120 \pm 4^{**}$	–15
Total ventricular protein content/body wt. (mg/g)	0.497 ± 0.008	$0.544 \pm 0.016^*$	+9
Ventricular RNA/protein ratio ($\mu\text{g}/\text{mg}$)	6.85 ± 0.13	$5.86 \pm 0.16^{**}$	–15
Total ventricular RNA content (μg)	975 ± 25	$705 \pm 13^{**}$	–28

Table 2. *Ventricular protein-synthesis rates in vitro and in vivo*

Hearts were taken from fed or 3-day-starved rats and processed by the method of Garlick *et al.* (1980) as described in the Experimental section. For the groups *in vitro*, body weights were 287 ± 3 g for the fed group (eight rats), 286 ± 3 g for the fed + insulin group (five rats) and as in Table 1 for the 3-day-starved group. For each of the groups *in vivo*, seven rats (body wts.: fed rats, 304 ± 5 g; starved rats, 288 ± 5 g initially, 229 ± 4 g after 3 days starvation) were used. S_i was measured at 120 min for experiments *in vitro* and at 10 min for experiments *in vivo*. Statistically significance of values versus those for fed rats: * $P < 0.05$; ** $P < 0.01$ (statistical analysis was not done on S_i values).

	S_i (d.p.m./nmol)	k_s (%/day)	RNA/protein ratio (μ g/mg)	Efficiency of protein synthesis (g of protein synthesized/day per g of RNA)
<i>(a) In vitro</i>				
Fed	1458 ± 11	5.05 ± 0.41	6.81 ± 0.22	7.48 ± 0.67
3-day-starved	1408 ± 13	$3.12 \pm 0.26^{**}$	$5.86 \pm 0.16^*$	$5.32 \pm 0.41^*$
Fed + insulin	1428 ± 8	$8.56 \pm 1.27^{**}$	6.92 ± 0.06	$12.33 \pm 1.88^*$
<i>(b) In vivo</i>				
Fed	1358 ± 25	8.95 ± 0.74	6.23 ± 0.20	14.54 ± 1.01
Starved	1487 ± 6	$5.35 \pm 0.76^{**}$	5.84 ± 0.17	$8.93 \pm 1.33^{**}$

protein synthesis is decreased *in vitro* by 43% in 2-day-starved rats (D. M. Smith & P. H. Sugden, unpublished work). We suggest that cardiac protein synthesis is much more sensitive to starvation than was suggested by Rannels *et al.* (1978).

In vivo, preliminary experiments established that there was only a 3–5% decline in plasma [4- 3 H]phenylalanine specific radioactivity between 2 and 10 min after injection of radiolabel. The mean decrease in ventricular free [4- 3 H]phenylalanine specific radioactivity between 2 and 10 min was $5.0 \pm 1.4\%$ (14 observations). Starvation for 3 days inhibited protein synthesis (Table 2b). *In vivo*, efficiencies of protein synthesis were significantly decreased by starvation. We conclude that cardiac protein synthesis is inhibited by 3 days of starvation, as was suggested by experiments *in vitro* (Table 2a).

Rates of protein synthesis and the effects of starvation *in vitro* and *in vivo* may be compared. The k_s values in ventricles of rats perfused *in vitro* with media containing insulin were 96% of those for fed rats *in vivo*. The efficiency of protein synthesis *in vitro* was 85% of values *in vivo*, reflecting the contribution of blood to the total heart protein *in vivo*. Rates of protein synthesis in the perfused heart are thus very similar to rates *in vivo*. The comparison between insulin-perfused hearts and hearts from fed rats *in vivo* is justified, since in these post-prandial rats insulin concentrations are likely to be high. Infusion of insulin into fed rats *in vivo* to very high plasma concentrations ($> 2500 \mu$ units/ml) did not stimulate skeletal-muscle protein synthesis, although much lower concentrations (about 70μ units/ml) stimulated protein synthesis in post-absorptive animals (Garlick *et al.*,

1983). To our knowledge, analogous experiments have not been reported for the heart. Goldspink *et al.* (1983) also included insulin in media for their experiments *in vitro*. The k_s values and efficiencies of protein synthesis *in vivo* in ventricles of 3-day-starved rats were similar to rates *in vitro* for glucose-perfused hearts taken from fed animals, and were greater ($P < 0.05$) than rates *in vitro* for glucose-perfused hearts taken from 3-day-starved animals. This finding may indicate the importance of non-carbohydrate fuels (Rannels *et al.*, 1974) or other factors in the partial maintenance of cardiac protein-synthesis rates during starvation. These results also show a good qualitative correlation of the effects of starvation between experiments *in vitro* and *in vivo*.

Rates of protein degradation in vivo and in vitro

In the rats used, body weight increased by 2.5%/day for the week before experimentation. Because rats of 180–350 g body wt. have a constant ventricular-wet-weight/body-weight ratio and ventricular protein concentration (P. H. Sugden & D. M. Smith, unpublished work), the k_d value in fed rats *in vivo* is 6.45%/day. These calculations may not be entirely reliable, since k_s was measured over 10 min, and thus does not allow for variations occurring during the day. During starvation, the situation is more complex, since rates of protein synthesis and degradation are not in a steady state. From Tables 1 and 2, the average k_d value is 10.35%/day over the 3 days of starvation. Starvation thus increases cardiac protein degradation *in vivo*. However, for glucose-perfused hearts taken from fed and 2- and 3-day-starved rats (initial body wt. about 300 g), k_d values were 17.9 ± 0.7 (5), 15.5 ± 0.3 (7) and 13.1 ± 0.6 (7) %/day respectively

($P < 0.01$ for starved versus fed animals). Crie *et al.* (1980) and Curfman *et al.* (1980) have also shown inhibition of cardiac protein degradation *in vitro* by starvation. This discrepancy can be explained since the inhibition caused by starvation is not as great as that produced by saturating insulin concentrations at physiological work-loads, when the rate of protein degradation can be calculated to be $7.7 \pm 0.4\%$ /day (Sugden & Smith, 1982a; Smith & Sugden, 1983a). Cardiac protein-degradation rates *in vivo* during starvation could thus still be increased above those for fed rats, even though a stable inhibition of protein degradation by starvation is observed *in vitro*.

Discussion

General methodological considerations

The flooding-dose technique (Henshaw *et al.*, 1971; McNurlan *et al.*, 1979; Garlick *et al.*, 1980) minimizes many problems previously associated with measurement of protein-synthesis rates *in vivo*. Of the previous reports in which protein-synthesis rates *in vitro* and *in vivo* were compared, this technique has been used only more recently (Goldspink *et al.*, 1983; Oliver *et al.*, 1984). Other workers have used infusion techniques *in vivo* where problems of interpretation have arisen because of differing extracellular and intracellular specific radioactivities of amino acid precursor (Palmer *et al.*, 1981; Preedy & Garlick, 1983). Furthermore, in some studies *in vitro*, the tissue and perfusate amino acid specific radioactivities were not equilibrated after 1 h in the hemicorpus (Preedy & Garlick, 1983) or 6 h in rabbit skeletal muscles (Palmer *et al.*, 1981). In the anterogradely perfused working rat heart used in our experiments, the perfusate and tissue specific radioactivities of $[4\text{-}^3\text{H}]$ phenylalanine were equilibrated at the end of 2 h of perfusion, and experiments of other workers (McKee *et al.*, 1978) suggest equilibration to be complete in 5–10 min. Our results show that, when the flooding-dose technique and a working-heart perfusion model are used, protein-synthesis rates *in vitro* closely approximate to rates *in vivo*, and changes in synthesis rates caused by starvation are accurately qualitatively reflected *in vitro*. Efficiency of protein synthesis may be 15% lower *in vitro* than *in vivo*. One possible reason for this is that depletion of cysteine from the perfusate by oxidation may be responsible, and this may be prevented by including mercaptodextran in the medium (Chua *et al.*, 1983). Mercaptodextran was not available to us. In comparing k_s values *in vivo* and *in vitro*, it should be noted that in fresh rinsed hearts blood comprises about 15% of the total protein.

Insulin and cardiac nitrogen balance

The inclusion of insulin in perfusates containing glucose and amino acids improves cardiac nitrogen balance *in vitro*. Physiological concentrations of insulin inhibit cardiac protein degradation *in vitro* (Rannels *et al.*, 1975; Sugden & Smith, 1982a; Smith & Sugden, 1983a). Thus, at physiological work-loads, rates of cardiac protein degradation (Smith & Sugden, 1983a) are $15.7 \pm 0.6\%$ /day (13) in the absence of insulin and $7.7 \pm 0.4\%$ /day (13) at saturating insulin concentrations. From Table 2(a), hearts in the absence of insulin will be in negative nitrogen balance, but inclusion of insulin induces positive nitrogen balance. If these findings can be extrapolated to the situation *in vivo*, insulin may be important in regulating cardiac nitrogen balance. Furthermore, cardiac nitrogen balance may be sensitive to variations in plasma insulin concentrations occurring during the day in normal animals. We do not suggest that insulin is the only factor involved: others, such as non-carbohydrate fuels and amino acids, could be important. Although the insulin concentrations used in this study were hyperphysiological, others have shown that physiological insulin concentrations stimulate protein synthesis in the retrogradely perfused heart (Flaim *et al.*, 1983), in the diaphragm (Manchester & Young, 1959; Manchester *et al.*, 1959) and in skeletal muscles (Frayn & Maycock, 1979).

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