Contrasting response of protein degradation to starvation and insulin as measured by release of *N*⁷-methylhistidine or phenylalanine from the perfused rat heart

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An isotope-dilution method is described for the measurement of *N*⁷-methylhistidine release from the perfused rat heart. We argue that release of *N*⁷-methylhistidine is indicative of cardiac actin degradation. *N*⁷-Methylhistidine release is compared with phenylalanine release in the presence of cycloheximide (phenylalanine release being a measure of degradation of mixed proteins). In hearts perfused with glucose plus acetate, the rate of actin degradation was increased by starvation and was not inhibited by insulin. In contrast, the rate of mixed-protein degradation was decreased by starvation and was inhibited by insulin. The fractional rate of degradation of mixed proteins in hearts from fed or starved rats was greater than that for actin. It is suggested that there are at least two pools of intracellular protein, the degradation rates of which differ in terms of their response to insulin and starvation.

INTRODUCTION

Phenylalanine is not appreciably metabolized by the heart (Williams *et al.*, 1981). Thus release of this amino acid from hearts perfused *in vitro* in the presence of cycloheximide (to prevent re-incorporation of phenylalanine into protein) has frequently been used to measure degradation of proteins to completion (Morgan *et al.*, 1979; Sugden, 1984). The obvious disadvantage of this approach is that it measures the resultant degradation rate of a population of mixed proteins of differing turnover rates, and hence lacks specificity.

In all muscle tissues investigated, actin is post-translationally methylated at the *N*⁷ position of histidine-73 (Vandekerckhove & Weber, 1984). In fast-twitch (white) fibres, a histidine residue in the myosin heavy chain is also specifically *N*⁷-methylated (for a review, see Young & Munro, 1978). After intracellular degradation of myofibrillar proteins, *N*⁷-methylhistidine (3MH) is not re-incorporated into protein. In man and rat, 3MH (or its hepatically acetylated derivative) is quantitatively excreted. Since skeletal muscle contains the major proportion of protein-bound 3MH (Haverberg *et al.*, 1975; Young & Munro, 1978), 3MH excretion *in vivo* has frequently been used as an index of skeletal-muscle breakdown.

Although there are objections to the use of 3MH to monitor skeletal-muscle degradation *in vivo* (Millward *et al.*, 1980; Rennie & Millward, 1983) in that much of the excreted 3MH may be derived from the rapidly turning-over actomyosin pool in the gut (Li & Wassner, 1982), 3MH release is potentially useful to monitor actomyosin degradation in isolated muscle preparations. Because in the heart there is much evidence that actin is the only protein containing 3MH (Kuehl & Adelstein, 1970; Huszar & Elzinga, 1972; Haverberg *et al.*, 1974), 3MH release from this organ represents specifically the intracellular degradation of actin to completion. In the

present paper, we report the use of an isotope-dilution method to measure 3MH release from perfused hearts from fed and starved rats and compare 3MH release with that of phenylalanine.

EXPERIMENTAL

Materials and animals

[*Me*-¹⁴C]3MH was from Amersham International, Amersham, Bucks., U.K. Ion-exchange resin (analytical grade) was from Bio-Rad, Watford, Herts., U.K., and was precycled in 3M-NaOH, water, 3M-HCl and water before use. All other materials were from the sources given in Preedy *et al.* (1984). Rats (225–275 g on arrival) were kept for at least 3 days before use, as described by Preedy *et al.* (1984). When required, rats were starved for 3 days in grid-bottomed cages.

Measurement of protein degradation rates in perfused hearts

Hearts from rats weighing approx. 300 g were perfused anterogradely for 2 h by the method of Taegtmeier *et al.* (1980) as described in detail previously (Sugden & Smith, 1982b; Preedy *et al.*, 1984) at a filling pressure of 0.5 kPa and an aortic pressure of 7 kPa. The perfusate (volume 100 ml) was Krebs & Henseleit (1932) bicarbonate-buffered saline containing 10 mM-glucose, 20 mM-sodium acetate (prepared as in Sugden & Smith, 1982a) and 0.02 mM-cycloheximide. [The NaCl concentration in the perfusate was lowered appropriately to keep the Na⁺ concentration as specified by Krebs & Henseleit (1932).] When present, insulin (50 munits/ml) was added to both the retrograde pre-perfusion and the anterograde perfusion. Although insulin concentrations used were grossly hyperphysiological, we have previously shown effects of physiological concentrations of insulin on protein degradation as measured by phenylalanine

Abbreviations used: 3MH, *N*⁷-methylhistidine; [*Me*-¹⁴C]3MH, *N*⁷-[*Me*-¹⁴C]methylhistidine; 1MH, *N*⁷-methylhistidine; *k*_d, fractional rate of protein degradation.

release (Sugden & Smith, 1982a); high concentrations were used in the present experiments to ensure maximal responses. After switching to the antegrade perfusion, 25 μ l of 20 μ M-[Me - ^{14}C]3MH (about 15000 d.p.m.) was added to the perfusate, which was recirculated for 2 h. Samples of perfusate (0.5 ml) were removed every 20 min throughout the perfusion and were treated as in Sugden & Smith (1982a) for subsequent measurement of phenylalanine release.

At the end of the perfusion period, the heart was removed from the cannula, weighed and homogenized in 4 ml of 0.125 μ M-[Me - ^{14}C]3MH (sp. radioactivity about 30 d.p.m./pmol) with a Polytron homogenizer. Then 0.05 vol. of 12.5 M-HClO₄ was added and the homogenate re-homogenized. Precipitated protein was removed by centrifugation in a bench centrifuge, and the supernatant was neutralized by using 5 M-KOH and B.D.H. Universal indicator. After standing on ice for several minutes, precipitated KClO₄ was removed by centrifugation and 0.02 vol. of 5 M-pyridine was added to the supernatant, which was stored at -20 °C.

To the perfusate, conc. HCl (0.83 ml) was added (final concn. about 0.1 M) and the acidified perfusate was recirculated through the apparatus. It was collected, degassed under vacuum and stored at -20 °C. The perfusate volume was calculated from the dilution of [Me - ^{14}C]3MH radioactivity. There was no loss of 3MH through binding to glassware.

Purification of 3MH

Perfusates were desalted on columns (1 cm $\times$ 26 cm) of Bio-Rad AG50W-X8 (200–400 mesh, H⁺ form) in water, essentially as described by Tomas *et al.* (1979). All of the perfusate recovered after each 2 h perfusion was desalted. After application of perfusates, the columns were washed with 200 ml of 2 M-HCl, followed by 4 M-HCl. The 4 M-HCl fraction eluted between 15 and 80 ml (which contained 3MH) was collected and rotary-evaporated. The residues were taken up in 0.1 M-pyridine (3 ml) and applied to columns (0.8 cm $\times$ 4 cm) of Bio-Rad AG50W-X8 as above. Columns were washed with 0.2 M-pyridine (34 ml), followed by 1 M-pyridine, and 2 ml fractions were collected (Haverberg *et al.*, 1974). 3MH was eluted at 32–40 ml, i.e. just at the volume where the pyridine concentration was increased. The pooled fractions were rotary-evaporated and taken up in 0.5 ml of 0.18 M-trilithium citrate/10 μ M-1MH containing 0.02 vol. of 25% (w/w) thiodiglycol and 0.001 vol. of liquified phenol adjusted to pH 2.0 with HCl (sample buffer) for amino acid analysis. Recovery, as calculated from recovery of perfusate [Me - ^{14}C]3MH radioactivity, of 3MH was about 70%.

[Me - ^{14}C]3MH in supernatants of heart homogenates was similarly purified by cationic ion-exchange chromatography with pyridine elution, except that 1 cm $\times$ 4 cm columns were used and the volume of pyridine used for elution was proportionally increased. Residues were taken up in 0.5 ml of sample buffer. Recovery of 3MH, calculated from recovery of [Me - ^{14}C]3MH radioactivity, was about 50%.

In all column steps, a 'control' column to which 40 nmol of [Me - ^{14}C]3MH (sp. radioactivity 30 d.p.m./pmol) was added (in the appropriate volume of medium) was run simultaneously. This allowed 3MH elution to be monitored and the [Me - ^{14}C]3MH specific radioactivity to be determined empirically.

Determination of cardiac protein-bound 3MH and phenylalanine contents

Heart protein (10 mg, purified as described by Haverberg *et al.*, 1975) was hydrolysed under N₂ for 48 h in 6 M-HCl at 110 °C. Under these conditions, amino acid release was maximal (Smith & Sugden, 1983b). 3MH and phenylalanine were stable to this treatment. After hydrolysis, 0.5 nmol of [Me - ^{14}C]3MH (15000 d.p.m.) was added, HCl was removed over P₂O₅/solid NaOH *in vacuo*, and the residues were taken up in 0.1 M-pyridine (3MH) or sample buffer (phenylalanine). [Me - ^{14}C]3MH was purified by cationic ion-exchange/pyridine-elution chromatography (1 cm $\times$ 4 cm columns) as described above.

Measurement of phenylalanine released into the perfusate by the heart

Phenylalanine was measured by the isotope-dilution-tRNA-binding assay of Rubin & Goldstein (1970) as modified by Sugden & Smith (1982a). Standard curves for phenylalanine were set up in perfusion medium. The rate of phenylalanine release was determined from the slope of a linear regression line for phenylalanine released versus time of perfusion. In some cases, phenylalanine concentrations in perfusates were checked by using an amino acid analyser (see below). Both methods gave identical results.

Amino acid analysis

This was done with a Biotronik (Munich, West Germany) LC-2000 amino acid analyser equipped with fluorescence detection and with a Trivector (Sandy, Beds., U.K.) Trilab 2 integrator and computer graphics. The manufacturer's recommended physiological-fluids programme was used. The automatic sampler was modified to accept sample volumes of 150 μ l. The detecting agent was 1 M-potassium borate, pH 10.2, containing 0.5 g of *o*-phthalaldehyde/l, 30 mM-2-mercaptoethanol, 0.002 vol. of 30% (v/v) Brij-35 and 0.025 vol. of 37% (v/v) formaldehyde. Addition of formaldehyde at this concentration (Emery & Rennie, 1982) to the detecting reagent had three effects: (i) although it decreased the 3MH fluorescence yield by 40%, it decreased interfering histidine fluorescence yield by 90%; (ii) it greatly improved baseline stability towards the end of the chromatogram, partly because of quenching of NH₄⁺ fluorescence; (iii) it improved the stability of the detecting solution, which could be used successfully for at least 4 weeks. For the determination of phenylalanine (elution time 125 min), norleucine (elution time 115 min) was used as an internal standard. For the determination of 3MH (elution time 176 min), 1MH (elution time 168 min) was used as an internal standard (preliminary experiments showed that no 1MH was present in the samples examined by amino acid analysis). Physiological-fluids amino acids standards (10 μ M) were used to standardize the analyser daily. By using this system of analysis, 3MH was well separated from all other amino acids, and as little as 150 pmol could be determined with ease.

Determination of [Me - ^{14}C]3MH specific radioactivity

A volume (50 μ l) of [Me - ^{14}C]3MH in sample buffer was diluted with 0.95 ml of water, and radioactivity therein determined by liquid-scintillation spectrometry in

Table 1. Degradation of actin and mixed protein in the perfused heart

Perfusions and analyses were carried out as described in the Experimental section. Statistical significance: ^a*P* < 0.05, ^b*P* < 0.02, ^c*P* < 0.01, ^d*P* < 0.001 for perfusions using hearts from starved rats versus perfusions using hearts from fed rats; ^e*P* < 0.01 for perfusions in the presence of insulin using hearts from starved rats versus perfusions in the absence of insulin using hearts from starved rats.

	Fed	Starved	Starved, insulin-perfused
Heart content of free 3MH (nmol/g wet wt.) after:			
0 h perfusion	2.79 ± 0.22 (7)	3.91 ± 0.23 (11) ^c	—
2 h perfusion	1.28 ± 0.08 (13)	2.24 ± 0.18 (13) ^d	2.49 ± 0.17 (5) ^d
Protein-bound 3MH content (nmol/mg of protein)	1.55 ± 0.02 (6)	1.59 ± 0.02 (6)	—
Release of 3MH derived from protein degradation (nmol/2 h per g wet wt.)	1.34 ± 0.13 (10)	1.92 ± 0.17 (10) ^b	2.14 ± 0.17 (5) ^c
Actin <i>k_d</i> (%/day)	6.52 ± 0.64 (10)	8.70 ± 0.77 (10) ^a	9.70 ± 1.22 (5) ^c
Release of phenylalanine derived from protein degradation (nmol/2 h per g wet wt.)	614 ± 22 (5)	502 ± 38 (5) ^a	278 ± 36 (5) ^{d,e}
Mixed-protein <i>k_d</i> (%/day)	15.66 ± 0.56 (5)	12.19 ± 0.92 (5) ^a	6.75 ± 0.87 (5) ^{d,e}

10 ml of PCS scintillant. Quenching was determined by the external-standard method. Collection of the eluate from amino acid analysis showed that there was only a single peak of radioactivity in [*Me*-¹⁴C]3MH purified by cationic ion-exchange/pyridine-elution chromatography. [*Me*-¹⁴C]3MH concentration was determined by amino acid analysis as described above.

The specific radioactivity of the [*Me*-¹⁴C]3MH that was added to the perfusates at zero time or to the tissue homogenates (i.e. *S_A*; see below) was determined in triplicate each time perfusate or tissue samples were analysed. For the four sets of amino acid analyses from which data in Table 1 were derived, the initial specific radioactivity of [*Me*-¹⁴C]3MH purified by desalting and pyridine-elution chromatography was 33.12 ± 0.38 d.p.m./pmol (mean ± S.E.M.). The radiochemical purity of the [*Me*-¹⁴C]3MH before purification was 93%. These values were similar to those given on the data sheet from Amersham International, which were 31.07 d.p.m./pmol and 95% respectively.

Calculation of results

3MH release was estimated by isotope dilution according to the equation:

$$3\text{MH release} = \left[\frac{S_A}{S_B} - 1 \right] \frac{A}{x \cdot t}$$

where *S_A* and *S_B* are the specific radioactivities of [*Me*-¹⁴C]3MH added to the perfusate at zero time and in the perfusate at the end of the perfusion respectively, *A* is the amount of [*Me*-¹⁴C]3MH added to the perfusate at zero time, *x* is the heart weight and *t* is the perfusion time. An analogous equation can be used to calculate protein-bound and tissue unbound 3MH contents. From a knowledge of the rate of release of phenylalanine or 3MH derived by intracellular protein degradation relative to heart wet weight, the amino acid composition of heart protein and the heart protein concentration in mg/g wet wt, the fractional rate of protein degradation (*k_d*) can be calculated.

Other methods

Protein was determined by the method of Gornall *et al.* (1949). Results are presented as means ± S.E.M. with the numbers of observations (*n*) in parentheses. Statistical significance was determined by a two-tailed Student's *t*-test for unpaired data, with *P* < 0.05 taken as being statistically significant.

RESULTS

Free 3MH contents in hearts

Release of phenylalanine from the perfused heart in the presence of cycloheximide is taken as measuring intracellular protein degradation, since tissue contents remain constant over 2 h of perfusion (Sugden & Smith, 1982a) and in any case are small compared with the total release of phenylalanine. The situation for 3MH is different. Tissue contents decreased during the perfusion and contributed significantly to 3MH release. Thus, in order to calculate the amount of 3MH produced by intracellular protein degradation during the perfusion, the content of free 3MH in individual hearts after 2 h of perfusion was measured and subtracted from the content at 0 h. This allowed correction for release of pre-existing free intracellular 3MH during the perfusion.

Starvation for 3 days increased heart free 3MH contents (Table 1). Insulin in the perfusate did not affect the contents in hearts from starved rats after 2 h of perfusion. The increased free 3MH contents after starvation may reflect an increased rate of actin degradation under these conditions (see below).

Rates of intracellular protein degradation

Initial experiments showed that release of 3MH from perfused hearts of fed rats was linear for 4 h. As measured by release of 3MH (after correction for release of pre-existing free 3MH in the heart), actin degradation was increased by 3 days of starvation and was not inhibited by insulin (Table 1). In contrast, in starved animals, protein degradation as measured by phenylalanine

release was decreased by 18% by starvation (see also Crie *et al.*, 1980; Curfman *et al.*, 1980; Preedy *et al.*, 1984) and was further decreased (by 45%) by insulin. By using a wet-weight/dry-weight ratio of 5.30 ± 0.01 ($n = 5$) for hearts perfused for 2 h, rates of phenylalanine release can be shown to be similar to those reported previously (Sugden & Smith, 1982b; Smith & Sugden, 1983a).

Values of k_d based on release of 3MH or phenylalanine were calculated from the protein-bound 3MH content (Table 1), the protein-bound phenylalanine content [296 ± 4 nmol/mg of protein ($n = 7$)] and the protein content for hearts perfused for 2 h [159 ± 2 mg/g wet wt. ($n = 13$) for fed rats, 167 ± 7 mg/g wet wt. ($n = 6$) for starved rats]. In fed rats, the k_d based on phenylalanine release was significantly greater ($P < 0.001$) than that based on 3MH. This was also true in starved rats ($P < 0.05$).

DISCUSSION

In this paper, we describe for the first time release of 3MH from the perfused heart. Although release of 3MH from the perfused hind-quarter (Nagasawa *et al.*, 1982) and hemicorpus (Li & Wassner, 1984; Bylund-Fellenius *et al.*, 1984) has been described, it should be noted that these preparations are less homogeneous than the heart in terms of tissue type, in that they contain bone, skin etc. (Preedy & Garlick, 1981). In the Introduction, we argue that 3MH release measures the degradation of cardiac actin to completion. Our measurements of cardiac protein-bound 3MH contents agree well with those of Haverberg *et al.* (1975) and reasonably well with those calculated from data in Watkins & Morgan (1979). However, before 3MH release is used to measure actin degradation in molar terms, it would be necessary to establish that all of the histidine residues at position 73 in actin are methylated. It is unclear whether this is true. Haverberg *et al.* (1974) suggest about 80% of skeletal-muscle actin is methylated. If cardiac actin is fully methylated, our data show that the actin content of cardiac muscle is 10.75 mg/g wet wt. Assuming that the stoichiometry of the myofibril in heart is similar to that in skeletal muscle, data from Yates & Greaser (1983) show that, if cardiac actin is fully methylated, the myofibrillar protein content of the heart should be about 0.29 g/g of total protein. This is very close to the value of 0.304 ± 0.014 g/g (15 observations) that we obtained by independent methods (Smith & Sugden, 1985). Thus most (> 95%) of the cardiac actin is methylated, and 3MH release from the heart probably represents degradation of actin in molar terms. If methylation of actin were to decrease the rate of actin degradation significantly, it remains possible that there could be a small pool of non-methylated actin which contributes disproportionately to the overall degradation of actin.

In the heart, myocytes constitute about 80% of total protein. Since the heart contains cells which are not cardiomyocytes, but which presumably contain actin, it remains possible that actin turnover rates may be dissimilar throughout the heart. Again, a small, rapidly turning-over, non-cardiomyocytic pool of actin could contribute disproportionately to 3MH release.

Values of k_d calculated from 3MH release differ from those calculated from phenylalanine release and respond differently to starvation or insulin. *In vitro*, in hearts from fed or starved rats, mixed protein is degraded more

rapidly than actin. Mixed-protein degradation is inhibited by insulin, but actin degradation is not. It should be noted that, in hearts from fed rats *in vivo* or in hearts from fed rats perfused with insulin *in vitro*, the k_d values for actin and mixed protein are likely to be closer, since insulin partially inhibits mixed-protein degradation by 33–38% (Sugden & Smith, 1982a), but will presumably not inhibit actin degradation. These findings suggest that there are two pools of protein: one that turns over more rapidly than actin in the absence of insulin *in vitro* and whose degradation is insulin-sensitive, and a second, including actin, which turns over more slowly and whose degradation is not insulin-sensitive. The latter pool could include other myofibrillar proteins. The degradation of the rapidly turning-over pool of protein is decreased by starvation (possibly because of depletion of the protein substrates for proteolytic activity after 3 days of starvation), whereas degradation of the slowly turning-over pool is increased. Measurement of phenylalanine release is thus not representative of the degradation of all cell proteins. It should be noted that, in the rat heart *in vivo*, the fractional rate of actin synthesis is less than that of mixed protein (Moalic *et al.*, 1984). Furthermore, the k_d for actin calculated from our data in the fed rat corresponds to a half-life of 10 days. Zak *et al.* (1977) found a half-life *in vivo* of 8 days for actin in the hearts of 200–250 g female rats, by using a double-isotope method.

Our results largely agree with those of Li & Wassner (1984), who used the perfused hemicorpus. They showed that actomyosin degradation was slower than mixed-protein degradation. It was not inhibited by insulin, in contrast with mixed-protein degradation. The rates of both actomyosin and mixed-protein degradation were increased by starvation. In contrast, Nagasawa *et al.* (1982) showed in the perfused hind-limb that streptozotocin-diabetes increased actomyosin degradation, an effect that could be reversed by insulin. Insulin was without effect in fed controls.

We thus conclude that insulin primarily affects degradation of proteins of shorter half-life than actin and does not affect actin degradation. Actin degradation in the heart is increased by starvation.

Since this paper was submitted, Lowell *et al.* (1986) have reported that release of protein-bound 3MH from the perfused skinned rat hindquarter is increased by starvation and is not inhibited by insulin. In contrast, degradation of mixed proteins as measured by tyrosine release in the presence of cycloheximide was susceptible to inhibition by insulin. These results are very similar to those reported here and those obtained by Li & Wassner (1984) in the perfused hemicorpus.

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