Effects of Insulin, Amino Acids and Fasting on Myofibrillar Protein Degradation in Perfused Hindquarters of Rats

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(Received March 13, 1985)

Summary The rate of 3-methylhistidine (3-MH) release from rat perfused hindquarters was measured to investigate the effects of insulin, amino acids and fasting on myofibrillar protein degradation. Since release of 3-MH into the perfusate increased linearly and the pool of free 3-MH in the perfused muscle did not change significantly during 2 h of perfusion, it was concluded that 3-MH release reflected the rate of myofibrillar protein degradation. Tyrosine release in the presence of cycloheximide represented the degradation rate of total muscle protein. Insulin suppressed the net release of tyrosine in normal rats, but did not affect the rate of release of 3-MH and tyrosine in the presence of cycloheximide. 3-MH release was not influenced by perfusate amino acid concentrations at zero to 5 times the normal plasma levels. When rats were fasted for one and two days, 3-MH release increased 1.7 and 2.6 times, respectively, compared with the fed rats, which showed that the rate of degradation of myofibrillar protein in skeletal muscle rose just after the beginning of fasting.

Key Words 3-methylhistidine, myofibril, skeletal muscle, protein degradation, insulin, plasma amino acids, fasting, hindquarter perfusion

The urinary excretion of 3-methylhistidine (N-methylhistidine, 3-MH) is used as an index of muscle protein degradation in vivo (1, 2). 3-MH is derived from actin and myosin, is not reutilized for protein synthesis, and is excreted quantitatively into the urine. Thus, it has been proposed that this reflects the rate of myofibrillar protein degradation in muscle. However, Millward et al. claimed that this amino acid is released not only from skeletal muscle but also from the gastrointestinal tract and skin, and that the former contributes only 38–52% of total urinary 3-MH excretion (3). The hindquarter perfusion system is of great advantage for measuring the rate of release of 3-MH from skeletal muscle because the contribution of the gastrointestinal tract can be excluded. Skin is included even in this system, but its
The rate of release of tyrosine or phenylalanine from incubated or perfused skeletal muscle has been employed to measure the rate of total protein degradation in muscle in vitro (5–8). Though these amino acids are not metabolized in muscle, they are reutilized for protein synthesis, and therefore inhibitors of protein synthesis must be added to the medium in order to measure the true rate of protein degradation. However, some reports show that these kinds of inhibitors, e.g., cycloheximide, affect not only protein synthesis but also degradation (9,10). Therefore, the release of 3-MH from perfused muscle seems to be a simpler, more accurate and more specific method than that of tyrosine or phenylalanine.

In the present study, by employing the measurement of the rate of release of 3-MH from perfused hindquarters, we elucidated the effects of insulin, amino acids and fasting on the rate of myofibrillar protein degradation in muscle. Comparison with the rate of tyrosine release was also made.

MATERIALS AND METHODS

Animals. Male Wistar rats were obtained from Shizuoka Agricultural Cooperative Associations for Laboratory Animals (Hamamatsu, Japan), maintained on a 12 h-light/12 h-dark cycle, and given a commercial stock diet and water ad libitum. In the fasting study, animals were trained to take all of the daily feed within 4 h (10:00–14:00). Their hindquarters were perfused just after the meal, and one and two days after the last meal.

Hindquarter perfusion. Rat hindquarters were perfused according to Ruderman et al. (11) as described previously (12). The perfusate consisted of Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 33% fresh bovine erythrocytes at a hematocrit of approx. 25.3% bovine serum albumin (Cohn Fraction V, Sigma Chemical Co.), and 10 mM glucose. In one experiment, amino acids at normal plasma levels (×1) or 5 times the normal levels (×5) were included in the perfusate as described earlier (12). In order to prevent the appearance of edema in the hindquarters, we improved the preparation of the perfusate. The buffer solution including serum albumin was dialyzed for two days with two changes of the same buffer. Erythrocytes were added just before the start of perfusion. Bovine insulin (Sigma Chemical Co.) and cycloheximide (Nakarai Chemical Co.) were added to the perfusate after dialysis. The initial 30 ml of perfusate was discarded and the remaining 100 ml was recirculated for 2 h at a flow rate of 8 ml/min.

Analyses. After perfusion, samples of perfusate and muscle were taken and treated as described previously (12). 3-MH in the perfusate 'plasma' and muscle free pool was analyzed according to Wassner et al. (13) using C_{18} reversed phase high performance liquid chromatography (ODS-SSC-372, Senshu Scientific Co., Japan). The mobile phase was composed of 10 mM Na-phosphate buffer (pH 7.5) with 27% acetonitrile. Tyrosine was determined fluorometrically according to Waalkes and...
Udenfriend (14).

Calculations. The rates of release of 3-MH and tyrosine were calculated as follows:

\[
\text{Rate of release of 3-MH or Tyr (nmol/h/g muscle)} = \frac{\Delta 3\text{-MH or } \Delta \text{Tyr (nmol/ml) \times 100 (ml) \times (1-0.25) \times \frac{1}{2(h)} \times \frac{1}{\text{B.W.} \times 0.216 (g)}}}{100 (ml) \times (1-0.25) \times \frac{1}{2(h)} \times \frac{1}{\text{B.W.} \times 0.216 (g)}}
\]

where \( \Delta 3\text{-MH or } \Delta \text{Tyr} \) is the difference between perfusate plasma levels at 0 and 2 h, 100 (ml) is the mean perfusate volume, 0.25 is the hematocrit, and 0.216 is the proportion of the mass of the perfused muscle (12). The fractional degradation rate (FDR) of myofibrillar protein was calculated from the rate of release of 3-MH divided by 3-MH content in muscle protein (0.7 \( \mu \)mol/g muscle) (15). FDR of total muscle protein was calculated from the rate of release of tyrosine divided by tyrosine content in muscle protein (27 \( \mu \)mol/g muscle) (16, 17) multiplied by 0.69, since some tyrosine is released from non-muscle tissues. The contribution of non-muscle tissues to total tyrosine release was tentatively reckoned to be 31% on the assumption that non-muscle tissues share 31% by weight of the total perfused tissues (12).

Statistical significance was tested by Student's \( t \)-test (18).

RESULTS

Release of 3-MH and tyrosine from perfused hindquarters

The time-course of the release of 3-MH from perfused hindquarters was measured in order to choose the optimal conditions for the estimation of myofibrillar protein degradation (Fig. 1a). When the perfusate was recycled for 2 h without passage through rat hindquarters, the concentration of 3-MH in the perfusate plasma ran at a very low level. This means that the perfusate itself, including erythrocytes, does not produce free 3-MH. During 2 h of perfusion, the concentration of 3-MH in the perfusate plasma increased linearly. It has been reported that there is an initial rapid release of 3-MH for 30 min, presumably due to the 'wash out' effect from muscle (4, 19). In our experiments, this was not observed. Free 3-MH content in muscle did not change significantly during the perfusion (9.13 \( \pm \) 2.86 and 6.03 \( \pm \) 0.50 nmol/g muscle, at 0 and 2 h, respectively). Therefore, it was considered that the rate of release of 3-MH from hindquarters reflected the rate of myofibrillar protein degradation. The rate of degradation of myofibrillar protein was then calculated from the difference at 0 and 2 h between the concentrations of 3-MH in the perfusate plasma.

The rate of tyrosine release from perfused hindquarters was measured at the same time (Fig. 1b). Tyrosine was also released linearly during 2 h of perfusion with cycloheximide in the perfusate. Tyrosine concentration in the perfusate plasma did not increase when the perfusate was recycled through the apparatus without passing
Fig. 1. Time-courses of (a) 3-methylhistidine (3-MH) and (b) tyrosine (Tyr) release from perfused hindquarters. Rat hindquarters were perfused as described in MATERIALS AND METHODS in the presence of cycloheximide (100 μM). The figures show control perfusion for 2 h (●) and recycling of the perfusate without hindquarters (○).

through the hindquarters. Free tyrosine content in muscle did not change significantly (0.27 ± 0.06 and 0.21 ± 0.01 μmol/g muscle, at 0 and 2 h, respectively). Therefore, the rate of total muscle protein degradation was calculated from the rate of release of tyrosine from hindquarters as in the case of 3-MH release.

**Effect of insulin in normal rats**

We reported previously that insulin restores the accelerated release of 3-MH from perfused hindquarters in diabetic rats, but does not affect the release of 3-MH in normal rats (20). However, the net release of tyrosine was suppressed significantly by insulin both in diabetic and normal rats. There are two possible explanations for these results. One is that protein synthesis increased in the presence of insulin and the reutilization of tyrosine was enhanced, resulting in the decrease of release of this amino acid from muscle. The other is that the degradation of myofibrillar protein and total muscle protein was differently regulated and insulin suppressed the latter and not the former. To determine which, we added cycloheximide to the perfusate to inhibit protein synthesis. The results are shown in Table 1. As reported previously, addition of insulin to the perfusate (25 mU/ml) did not affect the rate of release of 3-MH significantly, but decreased the rate of release of tyrosine to 60% that of the control. When cycloheximide was added (100 μM), 3-MH release did not change significantly, but tyrosine release increased to 125% that of the control. This means that cycloheximide did not influence myofibrillar protein degradation, and that approximately 20% of tyrosine released by the degradation of muscle protein is reutilized for protein synthesis under the present experimental conditions. When
Table 1. Effects of insulin and cycloheximide on the release of 3-methylhistidine and tyrosine from perfused hindquarters in normal rats.
Insulin was added at 25 mU/ml and cycloheximide at 100 μM. Fractional degradation rate (FDR) of myofibrillar protein was calculated from the release of 3-MH, and that of total muscle protein from the release of tyrosine as described in MATERIALS AND METHODS. Data are means ± SE. The number of observations is in parentheses.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>3-MH</th>
<th></th>
<th>Tyre</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Release (nmol/h/g)</td>
<td>FDR (%/day)</td>
<td>Release (nmol/h/g)</td>
<td>FDR (%/day)</td>
</tr>
<tr>
<td>− − (5)</td>
<td>1.37 ± 0.05</td>
<td>4.70 ± 0.17</td>
<td>48.0 ± 2.9</td>
<td>−</td>
</tr>
<tr>
<td>+ − (5)</td>
<td>1.11 ± 0.12</td>
<td>3.81 ± 0.41</td>
<td>28.1 ± 3.8**</td>
<td>−</td>
</tr>
<tr>
<td>− + (3)</td>
<td>1.40 ± 0.06</td>
<td>4.80 ± 0.21</td>
<td>60.3 ± 3.4*</td>
<td>3.71 ± 0.21</td>
</tr>
<tr>
<td>+ + (4)</td>
<td>1.21 ± 0.14</td>
<td>4.15 ± 0.48</td>
<td>53.7 ± 8.1</td>
<td>3.30 ± 0.50</td>
</tr>
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* p<0.05 vs -insulin and -cycloheximide. ** p<0.01 vs -insulin and -cycloheximide.

insulin was added to the perfusate in the presence of cycloheximide, neither 3-MH nor tyrosine release decreased significantly. Therefore, it was concluded that both myofibrillar and total muscle protein degradation were not influenced by insulin in normal rats, and that the apparent suppressive effect of insulin on net tyrosine release was due to stimulated reutilization of tyrosine for protein synthesis. The fractional degradation rates (FDR) of myofibrillar and total muscle protein were calculated to be 4-5% and 3-4%, respectively. These degradation rates are very close to those obtained in studies carried out in vivo (15).

Effect of amino acid levels
Rats, which were fasted overnight, were perfused for 2 h with no amino acids (×0), with amino acids at normal plasma levels (×1) or with those at 5 times normal levels (×5). The rate of 3-MH release did not change significantly among the 3 groups (Table 2), suggesting that plasma amino acid concentrations do not affect the rate of degradation of myofibrillar protein.

Effect of fasting
To investigate the changes in myofibrillar protein degradation during fasting, rats were meal-fed for 4 h, and were subjected to perfusion study just after feeding and one and two days after food deprivation. As shown in Table 3, the rate of release of 3-MH from perfused hindquarters increased 1.7 and 2.6 times after fasting for one and two days, respectively. These results clearly showed that the rate of myofibrillar protein degradation in skeletal muscle accelerated from the beginning of fasting.
Table 2. Effect of perfusate amino acid levels on release of 3-methylhistidine from perfused hindquarters. Amino acids were added to the perfusate at normal plasma level (×1) or at 5 times the normal level (×5) as described previously (12). Data are means ± SE. The number of observations is in parentheses.

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Release (nmol/h/g)</th>
<th>FDR (%/day)</th>
</tr>
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<tbody>
<tr>
<td>Control [×0] (5)</td>
<td>1.98 ± 0.27</td>
<td>6.79 ± 0.93</td>
</tr>
<tr>
<td>Normal [×1] (4)</td>
<td>2.26 ± 0.15</td>
<td>7.75 ± 0.51</td>
</tr>
<tr>
<td>Excess [×5] (5)</td>
<td>2.27 ± 0.25</td>
<td>7.78 ± 0.86</td>
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</table>

Table 3. Effect of fasting on release of 3-methylhistidine from perfused hindquarters. Neither amino acids nor cycloheximide was added to the perfusate. Data are means ± SE (n = 3).

<table>
<thead>
<tr>
<th>3-MH</th>
<th>Release (nmol/h/g)</th>
<th>FDR (%/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fed</td>
<td>1.15 ± 0.18</td>
<td>3.94 ± 0.62</td>
</tr>
<tr>
<td>Fasted, 1 day</td>
<td>1.95 ± 0.21*</td>
<td>6.69 ± 0.72*</td>
</tr>
<tr>
<td>Fasted, 2 days</td>
<td>2.98 ± 0.42**</td>
<td>10.22 ± 1.44**</td>
</tr>
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*p<0.05 vs. fed. **p<0.01 vs. fed.

DISCUSSION

The validity of the urinary 3-MH excretion as an index of muscle protein degradation in vivo depends on the contribution of non-skeletal muscle tissues, mainly gut and skin, which are also the origin of 3-MH release. Thus, some investigators have employed the isolated muscle perfusion system to eliminate the contribution of other organs to the rate of 3-MH release (4, 16, 19–22). The perfused hindquarter preparation is separated from the gastrointestinal tract, but still contains skin and bone. Preedy and Garlick claimed that their perfused hemicorpus preparation contained 39% by weight of non-muscle tissues such as skin and bone (23). These tissues were more active in protein synthesis than muscle, and therefore they concluded that the contribution of non-muscle tissues to the total turnover rate of protein in perfused tissues should be carefully evaluated. Our hindquarter preparation contained less non-muscle tissue (approximately 31% by weight) than theirs (12), in which skin still occupies the major portion (approximately 20%). However, it was reported that the contribution of skin to the total

rate of release of 3-MH into the perfusate was negligible (4). Tyrosine release from non-muscle tissues should be taken into account in estimating the rate of total muscle protein degradation. Recently, Clark and Mitch compared the rates of protein synthesis and degradation in perfused hindquarters with those in incubated epitrochlearis muscle (8). They obtained similar results for the two preparations and concluded that the hindquarter preparation can best be used to determine the protein metabolism of skeletal muscle.

In a previous paper, we reported that insulin suppresses myofibrillar protein degradation in diabetic rats, but not in normal rats (20). On the other hand, simultaneous net release of tyrosine was suppressed by insulin even in normal rats. In the present study, tyrosine release was not altered by insulin in the presence of cycloheximide in normal rats (Table 1). Insulin restored the accelerated rate of degradation of myofibrillar protein, but did not suppress the basal degradation rate. These results are inconsistent with those of several other investigators (5, 7, 8, 24), who reported that insulin suppresses muscle protein degradation both in fed and fasted rats. The reason for this discrepancy is not clear at present, but it may be due to variations in the degrees of the catabolic state among experimental animals, or to the contribution of pre-existing insulin in tissue.

Plasma amino acids are thought to be other major factors influencing muscle protein degradation. Branched-chain amino acids, especially leucine, have been reported to suppress the rate of protein degradation in incubated muscle (5, 7, 25) and perfused hemicorpus (26) or heart (27). These results were obtained by measuring the rate of release of tyrosine or phenylalanine from the tissues. Contrary to these findings, McNurlan et al. showed that leucine did not affect the rate of protein synthesis or degradation in vivo (28). We investigated the effect of a mixture of all amino acids on the rate of release of 3-MH from perfused hindquarters and showed that there was no effect on myofibrillar protein degradation even at 5 times normal plasma concentrations (Table 2). Li and Jefferson suggested that the age and nutritional state of the animal may influence the muscle protein degradation response to altered amino acid levels (26). Muscle protein degradation of fasted young rats (80 g) was affected by a mixture of branched-chain amino acids, but that of fed young (130 g) and fasted older (200 g) rats was not. It is not clear whether these conflicting results are due to differences between the methods used to measure the rate of muscle protein degradation such as the tyrosine or 3-MH release methods, or are due to differences among the physiological states of the animals or tissues used.

There have been many conflicting results on the effect of fasting, especially of short duration, on muscle protein degradation. Goldberg and his colleagues reported that starvation enhanced protein degradation in diaphragm (5), soleus and extensor digitorum longus muscles (6), using a method to measure the rate of tyrosine release in the presence of cycloheximide. Similar results were shown in vivo from urinary 3-MH excretion (29–31). On the other hand, some investigators showed that starvation did not change the rate of protein degradation in perfused
skeletal muscle (8, 24). Some others showed a decreased rate of protein degradation in vivo (32). Millward et al. measured the rate of muscle protein degradation by the constant infusion method and concluded that the rate of protein degradation decreased after fasting for 2 days but increased after 4 days (33). Cotellessa et al. showed that muscle protein degradation did not change during the first 3 days of fasting, although the total 3-MH production rate increased gradually with fasting (34). They presumed that the initial increase in 3-MH production was derived not from skeletal muscle but from smooth muscle in the gut (35). However, our results on the release of 3-MH from perfused rat hindquarters showed clearly that myofibrillar protein degradation increased gradually up to 2 days of fasting.

In the present paper, we calculated fractional degradation rates of myofibrillar and total muscle protein. Both rates were about 3–8% per day in normal rats. Recently, Li and Wassner reported that fractional degradation rates of actomyosin and total protein were 1.5 and 13.2%/day, respectively, in fed rats (16). However, many lines of evidence suggest that the rate of turnover of myofibrillar protein in vivo is similar to, or somewhat slower than, that of total muscle protein (15). The results reported in this paper are close to those of in vivo studies. The present method will prove useful for elucidating the factors regulating muscle protein degradation.

REFERENCES


Vol. 31, No. 4, 1985


