Effects of Dietary Protein Restriction on the Fractional Rates of Protein Synthesis in Perfused Rat Hindlimb

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Summary The effects of dietary protein restriction on protein synthesis were investigated in perfused rat hindlimb. The fractional rate of protein synthesis was measured with $[^3]$H phenylalanine in young adult (7-week-old) rats fed a low protein (5% casein) diet and a protein-free diet for 3 weeks. The low protein diet (LPD) allowed a moderate gain in body weight. The fractional rate of protein synthesis fell to 70% of the control value in LPD group and further fell to less than a half in the protein-free diet (PFD) group. Thus, the protein synthesis rate decreased as the dietary protein content was reduced. The fall of protein synthesis was mainly accompanied by the reduction of RNA activity (mg protein/mg RNA/day) rather than RNA concentration (RNA/protein). The rate of protein breakdown was calculated by subtracting growth rate from protein synthesis rate. The breakdown rate was decreased in LPD group and increased slightly in PFD group. From the low rates of protein synthesis and breakdown, it appears that dietary protein restriction, at least allowing a gain in body weight, makes the turnover rate slow down. The overall changes in protein synthesis obtained in the perfused hindlimb are consistent with the reported results in vivo.

Key Words perfused rat hindlimb, protein synthesis, protein breakdown, skeletal muscle, low protein diet, protein-free diet, RNA activity, RNA concentration

There is an evident correlation between body weight and muscle mass in

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animals of different sizes, indicating that the muscle mass increases with increasing body size (1). As skeletal muscle in man and rat comprises a large portion (40–45%) of whole body weight, the muscle protein would be expected to make a significant contribution to the whole body protein turnover rate. In muscle, as well as in other tissues, the protein mass is determined by the balance of the rates between protein synthesis and breakdown. For example, gain in muscle protein during the growth period or work-induced hypertrophy is a result of the rate of protein synthesis exceeding that of breakdown (2,3). Muscle protein turnover is affected by nutrition (4), hormonal status (5, 6), and muscular activity (2, 6). Young and his coworkers (4) have shown that the rate of protein synthesis is reduced rapidly by dietary protein depletion but is enhanced above that for well-nourished control by refeeding an adequate diet. Insulin is known to enhance protein synthesis and suppress protein degradation in muscle (5). Treatment of hypophysectomized rats with growth hormone leads to a gain in muscle and this effect is due to an increase in protein synthesis (6). Rapid compensatory growth of the rat soleus and plantaris muscles is induced by tenotomy of the synergistic gastrocnemius (2,3). During such muscle growth, protein synthesis clearly increases in response to increased muscular work. However, these observations of hypertrophy occur when rats are fed a normal diet in which there is an abundant amino acid supply for protein synthesis. If, therefore, dietary protein is withheld, the effects of hormones and muscular activity would be questioned.

On the other hand, the rates of protein synthesis in skeletal muscle have been obtained in the whole animal by constant infusion (7,8) or a large dose of labeled amino acid in vivo (3,9) and in isolated muscles by incubation in medium with labeled amino acid in vitro (3,10). In contrast to the number of the reports in vivo and in vitro, there have been few in the perfused rat hindlimb preparation (11,12). The measurements in the perfused rat hindlimb (11,13,14) are expected to be more reliable in several aspects: 1) Nutrients are carried to the tissues through normal and intact capillaries in the preparation. 2) The oxygen consumption keeps the tissues in good physiological state during perfusion. 3) The hindlimb perfusion could avoid numerous and complex regulatory factors from other organs in vivo, because the perfusion medium passes mainly through muscle tissues.

Although dietary protein is an important regulator of muscle protein synthesis, investigations in the perfused hindlimb during protein deficiency have never been done. We have therefore used the hindlimb perfusion technique and investigated adaptive changes in rates of protein synthesis and breakdown to low protein and protein-free diets.

**MATERIALS AND METHODS**

*Animals and diets.* Male Sprague-Dawley rats, initially weighing 230–250 g, were used throughout. All rats were housed individually in stainless steel cages at 22–24°C, with a 12 h light–12 h dark cycle. They were allotted randomly into three
dietary groups: 20, 5, and 0% casein diets, and were fed *ad libitum* each diet and water for 3 weeks. The 5 and 0% casein diets correspond to protein deficiency and protein depletion, respectively, while the 20% casein diet served as the normal growth control. The composition of the 20% casein diet was: 20% milk casein, 53% cornstarch, 5% sugar, 8% cellulose, 6% safflower oil, 6% mineral mixture, 2% vitamin mixture. The mineral and vitamin mixtures were purchased from Oriental Yeast Co., Tokyo, Japan. The 5 and 0% casein diets were similar to the control diet except that casein was replaced with cornstarch. Animals were not fasted at the time of sacrifice.

**Perfusion technique.** After rats were anesthetized with pentobarbital (5 mg/100 g B.W.), the surgical preparation for the perfusion was performed as described by Ruderman *et al.* (11). A midline abdominal wall was incised and the superficial epigastric vessels and inferior epigastric vessels were ligated. The intestines, colon, spleen, and testes were excised after the ligation of their great relative vessels. The iliolombar vessels were also ligated. The aorta and vena cava were carefully separated and were quickly ligated just above the right renal vessels. Another ligature for fixation of cannulas was placed above the iliolombar vessels. The cannulation was completed and then the rat was sacrificed with intracardiac pentobarbital. The hemicorpus was moved into the perfusion chamber (kept at 37°C) and the cannulas were immediately connected to the perfusion system. The starting volume of 150 ml was flowed for 15 min. After the preliminary period of perfusion, L-[4-3H] phenylalanine (Phe) (Amersham Japan) was added to a fresh perfusion medium for measurements of protein synthesis rates. The flow rate was adjusted to 12 ml/min. The perfusion was single-pass perfusion, although the starting perfusate was recirculated. All perfusates were continuously gassed with 95% oxygen and 5% carbon dioxide and carried out at 37°C. At the end of the perfusion (15–30 min), the quadriceps muscle from the left leg was excised and frozen in liquid nitrogen.

**Perfusion medium.** The perfusion medium contained a modified Krebs-Henseleit solution (12), 4% bovine albumin (Fraction V, Seikagaku Fine Chemicals), 6.0 mM glucose, 0.15 mM pyruvate, rejuvenated aged human erythrocytes and amino acids at normal plasma concentration (8), except phenylalanine, which was 20 times normal plasma concentration. The erythrocytes had been rejuvenated by the method of Valeri (15). To determine the rate of protein synthesis, [3H]phenylalanine was added to give a specific radioactivity of 0.35 μCi/μmol Phe with unlabeled phenylalanine, as described above. The buffer and bovine albumin solution were all passed through filters of pore size 0.45 μm previously.

**Rate of protein synthesis.** The frozen muscles were weighed and homogenized in cold 2% perchloric acid (PCA) with a Polytron homogenizer. The homogenate was centrifuged at 2,800 × g for 15 min. The PCA was removed from the supernate by adding saturated tripotassium citrate. The amount of free phenylalanine was determined fluorometrically after enzymic conversion into β-phenylethylamine and subsequent solvent extraction (9,16). The extract was also used for the specific
radioactivity of free phenylalanine. The protein pellet was washed three times with 2% PCA and was solubilized in 0.3 N NaOH at 37°C. After reprecipitating and washing in PCA, the protein was hydrolyzed in 6 N HCl at 110°C for 24 h. HCl in hydrolysate was removed completely by evaporation and the specific radioactivity of phenylalanine was measured as described previously in the supernate. The fractional rates of protein synthesis were calculated from specific radioactivities of free ($S_A$) and protein-bound ($S_B$) phenylalanine in muscle by the equation (9):

$$K_S = S_B \times 100/S_A t,$$

where $K_S$ is the fractional rates of protein synthesis (%/day) and $t$ is the time of incorporation of [3H]phenylalanine. All measurements of radioactivity were made in a Packard Liquid Scintillation System (TRI-CARB 460C) in a commercial scintillant (ACSII Amersham Co.).

Rate of growth and other measurements. Another experiment in each group was done for the rate of growth and for measurements of muscle RNA. Rates of growth were measured as the net protein accumulation in the quadriceps muscle from left leg for 3 days at a point near the time when the rates of synthesis were measured. Protein content was determined by the method of Lowry et al. (17). The fractional rates of growth ($K_g$) were expressed as the percent change in muscle protein content per day and the means were taken (18). Estimations of the rate of protein breakdown ($K_b$) were made by subtracting growth rate from synthesis rate: $K_b = K_s - K_g$. Muscle RNA was measured in the quadriceps muscle from right leg by the method of Kerr et al. (19), following extraction (20).

RESULTS

Growth and muscle composition

Growth curve is shown in Fig. 1. Body weight of LPD group fed 5% casein diet was increased by 14% during 3 weeks experimental period, while that of control group fed 20% casein diet was increased by 64%, indicating normal growth. Although the 5% casein diet thus allowed a moderate gain in the body weight, the protein-free diet did not allow growth. Over 3-weeks feeding, PFD group lost 19% of their initial body weights. The respective final body weights in LPD and PFD were 70 and 50% of control value. The reduced ratio to control value in the body weight was very similar to the parameters in the tissue weight and the protein mass of quadriceps muscle, as shown in Table 1. The muscle protein mass of PFD group fell to almost half the control value, while the corresponding value of LPD group was 70% of control value. Therefore, the loss of body weight probably reflects that of muscle protein in LPD and in PFD groups.

Rate of protein synthesis

Hindlimb perfusion system has been used to determine the rate of protein synthesis. Time course for the change in specific radioactivity of [3H]phenylalanine
Fig. 1. Growth curves of three dietary groups. Each point is the mean±SD of 5 rats. ○, rats fed 20% casein diet; ▲, rats fed 5% casein diet; ◆, rats fed protein-free diet. The animals were fed each diet ad lib. for 3 weeks.

Table 1. Changes in weight and composition of quadriceps muscles with dietary protein content.

| Dietary group | Final body weight (g) | Tissue weight (g/muscle) | Tissue protein (mg/muscle) | RNA (mg/muscle) |
|---------------|-----------------------|--------------------------|---------------------------|----------------|
| Control       | 381±13                | 2.68±0.33                | 490±60                    | 6.26±1.13      |
| LPD           | 265±7*                | 2.02±0.11*               | 357±35*                   | 4.06±0.24*     |
| PFD           | 195±4*↑               | 1.53±0.04*↑             | 258±25*↑                  | 2.79±0.28*↑    |

Control: rats fed 20% casein diet. LPD: rats fed 5% casein diet. PFD: rats fed protein-free diet. Values are means±SD of 5 rats. Comparisons between groups were made using Student's t-test. *p<0.01 when compared with control group. ↑p< 0.01 when compared with LPD group. As described in MATERIALS AND METHODS, male rats were allotted into three groups and were fed 20%, 5% casein and protein-free diets. After 3 weeks of feeding the experimental diets, animals were killed for analysis of muscle composition.

(Phe) in muscle protein and perfusate is shown in Fig. 2. During perfusion the specific radioactivity in the perfusate remains constant because single-pass perfusion is used. The [³H]Phe specific radioactivity in muscle protein was increased almost linearly with the time during the period up to 30 min. We calculated the fractional rate of synthesis from the [³H]Phe specific radioactivity in muscle but not in the perfusate. As shown in Table 2, the fractional rates of protein synthesis in LPD and PFD were decreased by 30% and by 50% compared with control,
respectively, indicating an adaptive response to protein deficiency. When growth rates were determined as the daily change in the protein mass, the growth rates declined with the decrease in dietary protein content as demonstrated in earlier studies (4, 21). The breakdown rate was calculated as the difference between growth rate and synthesis rate. The breakdown rate in LPD was decreased by about 50% compared with control. Thus, both breakdown and synthesis rates in LPD were decreased more or less equally, that is the low protein diet feeding makes the rates of protein turnover slow down. In contrast, the breakdown rate in PFD was expected to be decreased but rather increased slightly, while the synthesis rate was decreased. Therefore, when the rate of protein breakdown exceeds that of protein synthesis, it appears to result in loss of body weight with the reduction of muscle protein mass in PFD.

The effects of dietary protein content on concentration and activity of RNA in muscle are shown in Table 2. When the concentration of RNA was expressed relative to protein content, the RNA concentration was decreased by 10% in both LPD and PFD. The RNA concentration of LPD was significantly ($p < 0.02$) less than that of control, while there was no significant difference between PFD and control, due to a large standard deviation in PFD value. The activity of RNA may be assessed as the amount of protein synthesized per RNA. The RNA activity was
Table 2. Effect of dietary protein content on the amount of RNA in muscle and on rate of protein synthesis in the perfused hindlimb.

| Dietary group | RNA/prot (mg/g) | $K_s$ (%/day) | RNA activity (mg prot/mg RNA/day) | $K_g$ (%/day) | $K_b$ (%/day) |
|---------------|----------------|--------------|----------------------------------|--------------|-------------|
| Control       | 12.71±0.76     | 3.13±0.10    | 2.47±0.15                        | 1.96         | 1.17        |
| LPD           | 11.40±0.52*    | 2.17±0.16*   | 1.91±0.09*                       | 1.57         | 0.60        |
| PFD           | 10.92±1.77     | 1.45±0.13*   | 1.35±0.24*                       | −0.04        | 1.49        |

Values are means±SD of 5 observations in each group. *p<0.02 when compared with control group. †p<0.01 when compared with LPD group. The fractional rate of protein breakdown ($K_b$) was calculated by subtracting the mean growth rate ($K_g$) from the mean fractional rate of protein synthesis ($K_s$) which was measured for the quadriceps muscle in the perfused hindlimb. The growth rates were measured over 3 days for each group and expressed as the percentage changes in the protein mass per day.

decreased by 20% in LPD and by 50% in PFD, respectively, following a similar pattern to the changes in the fractional rates of protein synthesis during protein deficiency. The reduction of RNA activity was greater than that of RNA concentration. Thus, the fall in protein synthesis rate appeared to be accompanied to a great extent by the reduction of RNA activity and to a lesser extent by the reduction of RNA concentration.

DISCUSSION

The perfusion system attempts to mimic physiologically normal status in vivo, because the substances and the oxygen are supplied to the tissues through intact capillaries. Preedy et al. (8) compared rates of synthesis measured in the perfused hindlimb with those in vivo simultaneously using [14C]tyrosine as tracer. They observed that the estimates were very similar in gastrocnemius muscle but not in soleus muscle in the two techniques, indicating large variations among the muscles. However, there does not seem to be a large difference between those measurements in quadriceps muscle from which turnover rates of skeletal muscle protein have been obtained in our study. In the perfused hindlimb, the rate of protein synthesis fell to 70% of control value after 3 weeks on the low protein diet. The rate was further reduced to 50% in rats on the protein-free diet. Similarly, Garlick et al. (21) obtained that the rate of protein synthesis in vivo was reduced to 20% after 3 weeks on protein-free diet. The more rapid fall in the synthesis would be probably due to younger age of rats used in vivo, suggesting that the age of the animal may influence the response of skeletal muscle to protein deficiency. Similar suppressions of muscle protein synthesis have been observed in vivo in protein-deficient rats (4, 7, 21). It is clear that the overall changes in rates of protein synthesis obtained in the perfused hindlimb are consistent with those measurements in vivo.

The concentrations of some essential amino acids in plasma are reduced in rats
on a low protein diet (22), suggesting that amino acid concentration may be limiting the rate of protein synthesis. On the other hand, Li et al. (14) observed that the addition of amino acids at normal rat plasma concentration did not significantly alter the rates of protein synthesis in perfused skeletal muscle by fasted rats. In the present experiment, perfusate used in protein-deficient rats contained the same concentrations of amino acids as those of the control. The addition of amino acids at normal plasma level did not seem to restore rate of protein synthesis during protein deficiency (Table 2).

The concentration of RNA provides an index of RNA capacity for protein synthesis, while the amount of protein synthesized per RNA may provide an index of the activity of RNA for protein synthesis. Both feeding low protein and protein-free diets reduced the concentration of RNA in rat skeletal muscle by 10% (Table 2). The reduction of RNA concentration presumably might originate from a fall in ribosomal RNA, since most of muscle RNA is ribosomal and this proportion was maintained during protein deficiency (23). There was a much more rapid fall in the activity of RNA with from 20 to 50% (Table 2). Although RNA concentration is very sensitive to protein deficiency in growing rats (21, 24), the present study suggests that the change of RNA concentration with protein deficiency may be small in adult rats. Nevertheless, it appears that both the capacity and the activity of RNA reduced the rate of the protein synthesis.

In rats fed a low protein diet, the rate of protein breakdown was decreased, while in rats fed a protein-free diet, the rate of breakdown was increased slightly. Therefore, the low protein diet feeding made the turnover of protein metabolism slow as shown both in reduction of protein synthesis and breakdown rates. On the other hand, the protein-free diet decreased protein synthesis and increased breakdown. Thus, a pattern where the rate of breakdown parallels that of synthesis and changes in both rates are in the same direction does not exist. The increased rate of protein breakdown during protein depletion is in agreement with the earlier finding of Millward with \([^{14}\text{C}]\)carbonate (25) that feeding a protein-free diet for 3 days decreased the rate of synthesis and increased the rate of breakdown of myofibrillar proteins. A similar alteration in breakdown was observed with \([^{14}\text{C}]\)-aspartate (4). However, there is a technical problem of reutilization of labeled amino acid, as the estimations of breakdown were determined from the decay rate of labeled amino acid (26). On the other hand, the estimations of breakdown is calculated as the difference between growth rate and synthesis rate. As the calculated estimations are assessed as those excluded complicated error of recycling of label (26, 27), we used the indirect method for protein breakdown. The indirect measurements of Garlick et al. are not in agreement with the results obtained in the present experiment, showing that the breakdown rates of muscle protein in young rats were decreased after feeding a protein-free diet (21). Recent findings of breakdown in the perfused hindlimb have been reported by Kadowaki et al. (28). They showed that tyrosine release in rats fed a protein-free diet for 1 week was decreased to almost half the control value, while 3-methylhistidine release did not
change. The direct measurements would suggest that myofibrillar protein breakdown might not be decreased by a protein-free diet. Further experiments in the perfusion system are required to investigate the discrepancy between our results and those of Garlick et al. (21).

In conclusion, the results in the present study using the hindlimb perfusion method to measure the synthesis rates in rat skeletal muscle confirm that the rate of protein synthesis declines as dietary protein decreases. The low protein intake reduced protein synthesis and protein breakdown. The reduction of protein synthesis seems to be due, in part, to decrease of the RNA concentration and activity. Hence the findings suggest that turnover rate slows down as an adaptive response to protein deficiency to conserve protein in rats fed low protein diet at least. When a protein-free diet is given, protein synthesis is further reduced and protein breakdown may be elevated to perhaps mobilize amino acids which could be used in the synthesis of tissue protein other than skeletal muscle. The overall changes in protein synthesis obtained in the perfused hindlimb are consistent with those measurements in vivo.

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