Effect of a protein-free diet on muscle protein turnover and nitrogen conservation in euthyroid and hyperthyroid rats

William J. CARTER, Wieke S. van der Weijden BENJAMIN and Fred H. FAAS

Veterans Administration Medical Center and Department of Medicine, University of Arkansas for Medical Sciences, Little Rock, AR 72206, U.S.A.

(Received 8 July 1983/Accepted 20 September 1983)

Although protein turnover in skeletal muscle is increased in hyperthyroidism and decreased in hypothyroidism, a deficient protein intake tends to increase serum T_3 (tri-iodothyronine) while decreasing muscle protein turnover. To determine whether this diet-induced decrease in protein turnover can occur independent of thyroid status, we have examined muscle protein turnover and nitrogen conservation in hyperthyroid rats fed on a protein-free diet. After inducing hyperthyroidism by giving $20\mu\text{g}$ of $T_3/100\text{g}$ body wt. daily for 7 days, groups of euthyroid and hyperthyroid animals were divided into subgroups fed on basal and protein-free diets. Muscle protein turnover was measured by N^m -methylhistidine excretion and [^{14}C]tyrosine infusion. Urinary nitrogen output of euthyroid and hyperthyroid animals fed on the protein-free diet was also measured. Although hyperthyroidism increased the baseline rates of muscle protein synthesis and degradation, it did not prevent a decrease in these values in response to protein depletion. Furthermore, hyperthyroid rats showed greatly decreased nitrogen excretion in response to the protein-free diet, although not to values for euthyroid rats. These findings suggest that protein depletion made the experimental animals less responsive to the protein-catabolic effects of T_3 .

Since protein turnover in skeletal muscle is increased in hyperthyroidism (Goldberg *et al.*, 1980; Carter *et al.*, 1980, 1982) and decreased in hypothyroidism (Millward *et al.*, 1980; Flaim *et al.*, 1978), thyroid hormone appears to have an important influence on muscle protein turnover. However, the role of thyroid hormone in causing the decrease in muscle protein turnover in response to deficient protein intake is not clear. On the one hand, starvation decreases serum T_3 in man and animals (Vagenakis *et al.*, 1975; Azizi, 1978). Furthermore, restoration of postabsorptive T_3 concentration during starvation increased urea excretion, suggesting that decreased T_3 during starvation had a protein-sparing effect (Gardner *et al.*, 1979; Vignati *et al.*, 1978). On the other hand, diets low in protein but adequate in energy content increased serum T_3 (Tulp *et al.*, 1979; Edozien *et al.*, 1978), even though muscle protein turnover is decreased in this circumstance (Garlick *et al.*, 1975; Haverberg *et al.*, 1975). Therefore, factors other than thyroid hormone appear to be important in

decreasing muscle protein turnover in response to a low protein intake. To determine whether this diet-induced decrease in protein turnover can occur independent of thyroid status, we have examined muscle protein turnover and nitrogen conservation in hyperthyroid rats fed on a protein-free diet. The present study shows that hyperthyroidism increased the baseline rates of skeletal-muscle protein synthesis and degradation, but did not prevent a decrease in these values in response to protein depletion. Furthermore, hyperthyroid rats decreased their nitrogen excretion in response to the protein-free diet, although the response was somewhat delayed and smaller than that of euthyroid controls.

Materials and methods

Materials

L-Tyrosine decarboxylase (EC 4.1.1.25), L-tyrosine, L-tyramine, T_3 and Urea Assay Kit no. 640 were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. L-[U- ^{14}C]Tyrosine (sp. radioactivity 400Ci/mol) was obtained from ICN Chemical and Radioisotope Division, Irvine, CA,

Abbreviation used: T_3 , tri-iodothyronine.

U.S.A. All other chemicals used were reagent grade. Male Sprague-Dawley rats weighing 135–165 g were purchased from Charles River Laboratories, Wilmington, MA, U.S.A. Purified basal diet no. 5755 was purchased from Ralston Purina Co., St. Louis, MO, U.S.A. This diet contains (w/w): Solka floc, 3.0%; casein, 21.0%; sucrose, 15.0%; corn oil, 5.0%; lard, 5.0%; dextrin, 43.6%; choline chloride, 0.2%; RP vitamin mixture, 2.0%; RP mineral mix no. 11, 5.0%; DL-methionine, 0.15%. This diet provides 17.1 kJ (4.1 kcal)/g, with 20% of the energy provided by protein, 58% by carbohydrate and 22% by fat. Protein-free diet no. 5765 was also purchased from this company. This diet contains (w/w) Solka floc, 3.0%; sucrose, 33.1%; corn oil, 5.0%; lard, 5.0%; choline chloride, 0.2%; dextrin, 46.7%; RP vitamin mixture 2.0%; RP mineral mixture no. 11, 5%. This diet provides 17.1 kJ (4.1 kcal)/g, with 78% of the energy provided by carbohydrate and 22% by fat.

Treatment of animals, and [^{14}C]tyrosine-infusion procedure

Age and weight-matched rats were divided into control and T_3 -treated groups containing 24 animals each. All animals were housed in individual plastic metabolism cages (Nalge Co., Rochester, NY, U.S.A.) and fed on the purified basal diet *ad lib*. The animals and the diet consumed were weighed daily at 09:00 h. The animal room was kept at a temperature of $21 \pm 1^\circ\text{C}$, with light from 06:00 to 18:00 h and darkness from 18:00 to 06:00 h. Hormone-treated rats were given $20\mu\text{g}$ of T_3 /100 g body wt. daily. The T_3 was dissolved in 5 mM-NaOH and injected intraperitoneally in a volume of 0.10 ml/100 g. Control animals were given an equivalent volume of 5 mM-NaOH. After induction of hyperthyroidism by 7 days of T_3 treatment, euthyroid and hyperthyroid rats were divided into subgroups, which were fed on the basal and protein-free diets *ad lib*. for an additional 14 days. Urine was collected for 24 h periods from all animals on days 3, 7, 10 and 14 of the protein-free diet. Urine samples were preserved during the collection period by adding 2–3 ml of toluene to collection cups. At the end of the collection periods, cage funnels were washed with distilled water, the volumes of urine and washes measured, and samples frozen until time of assay. At the end of the 3-week treatment period, animals were immobilized by wrapping them in a plastic mesh, and a fine polyethylene catheter was inserted into a tail vein. L-[U- ^{14}C]tyrosine (12.5 Ci/mol) was dissolved in 0.9% NaCl at a concentration of $2.5\mu\text{Ci/ml}$ and infused at $1.2\mu\text{Ci/h}$ for 6 h. All [^{14}C]tyrosine infusions were begun between 08:00 and 09:00 h. At the end of the infusion, animals

were rapidly killed by decapitation and blood was collected. The heart, kidneys and gastrocnemius muscles were immediately removed, weighed, and frozen in liquid N_2 . Before being weighed, the heart was opened, washed free of blood with ice-cold 0.9% NaCl, and blotted. Skeletal-muscle tissue was stored at -70°C and serum at -20°C until processed.

Measurement of the fractional rate of skeletal-muscle protein synthesis, growth, and degradation by [^{14}C]tyrosine infusion

Protein and protein-free supernatant fractions were prepared from muscle tissue as described (Carter *et al.*, 1982). The specific radioactivity of L-tyrosine in protein hydrolysates and protein-free supernatant fractions was measured by the procedure of Garlick & Marshall (1972). Muscle protein content was measured by the method of Hartree (1972). The fractional rate of muscle protein synthesis was calculated from the ratio of the specific radioactivities of protein-bound and cellular free L-tyrosine by the formula of Garlick *et al.* (1973). The ratio of protein-bound to free tyrosine in muscle was found to be 405 in the euthyroid basal-diet subgroup, 750 in the euthyroid protein-free-diet subgroup, 380 in the hyperthyroid basal-diet subgroup, and 350 in the hyperthyroid protein-free-diet subgroup.

To calculate the rate of muscle protein degradation, it is necessary to estimate the rate of muscle protein growth at the time of the [^{14}C]tyrosine infusion. Since the ratio of the protein content of the gastrocnemius muscle to body weight did not change during the 3-day period before the death of the animals, the daily percentage change in body weight during this interval was used to estimate the fractional rate of muscle protein growth or loss at the time of death. The fractional rate of muscle protein degradation was calculated by subtracting the fractional growth rate from the fractional synthesis rate.

Measurement of N^α -methylhistidine excretion

After urine samples were hydrolysed in 6 M-HCl to convert acetylated N^α -methylhistidine into the free form, the samples were desalted (Burini *et al.*, 1981). The N^α -methylhistidine was then isolated by the pyridine elution technique described by Carter *et al.* (1981). The amino acid peaks were identified and quantified by the ninhydrin reaction described by Rosen (1957).

Determination of urinary nitrogen compounds

Total urinary nitrogen was measured by a micro-Kjeldahl technique (Munro & Fleck, 1969) in which ammonia was determined by the phenol-

hypochlorite reaction (Mann, 1963). Urinary urea and preformed ammonia were determined with the Sigma Urea Assay Kit no. 640. Creatinine and creatine were measured by a modification of the Jaffe reaction (Bonsnes & Taussky, 1945). Urinary α -amino nitrogen was measured by the ninhydrin reaction (Rosen, 1957), after urea and ammonia were removed by an ion-exchange-resin procedure (Matthews *et al.*, 1964).

Results

Growth curves of euthyroid and hyperthyroid animals fed on basal and protein-free diets

Fig. 1 shows the body weights of euthyroid and hyperthyroid animals fed on the basal and protein-free diets. While on the protein-free diet, the body weight of hyperthyroid animals decreased by $23.2 \pm 0.82\%$ (mean $\pm$ S.E.M.). The corresponding decrease in control animals was $19.8 \pm 0.26\%$, indicating that hyperthyroid animals lost a slightly greater fraction of body weight during protein depletion. However, the rate of weight loss during protein depletion was the same in euthyroid and hyperthyroid subgroups.

Amount of diet consumed by experimental animals

During the final 2 weeks of the experimental period, the average daily diet consumption was as follows (g/100g body wt.): euthyroid basal-diet subgroup, 7.5 ± 0.2 ; euthyroid protein-free-diet

subgroup, 5.6 ± 0.3 ; hyperthyroid basal-diet subgroup, 10.0 ± 0.2 ; hyperthyroid protein-free-diet subgroup, 8.7 ± 0.3 (means $\pm$ S.E.M., $n = 12$ in each group). Both basal and protein-free diets provided 17.2 kJ (4.1 kcal)/g. Diet consumption increased by 33% in hyperthyroid animals fed on the basal diet and by 55% in hyperthyroid animals fed on the protein-free diet compared with corresponding euthyroid controls.

Serum T_3 concentrations and organ/body-weight ratios in experimental animals

As indicated in Table 1, euthyroid animals fed on the protein-free diet had higher serum T_3 concentrations than did euthyroid animals fed on the basal diet. An increase in T_3 in euthyroid rats fed on a protein-deficient diet has previously been observed (Tulp *et al.*, 1979; Edozien *et al.*, 1978). On the other hand, the T_3 dosage used produced similar hormone concentrations and degree of cardiac and renal hypertrophy in hyperthyroid animals fed on the basal and protein-free diets. This suggests that a comparable degree of hyperthyroidism had been induced in both dietary subgroups. The ratio of the protein content of the gastrocnemius muscle to body weight was similar in all subgroups.

Estimation of muscle protein degradation by N^3 -methylhistidine excretion

Table 2 shows N^3 -methylhistidine excretion per 100g body wt., which has been used as an index of muscle protein degradation (Young & Munro, 1978). In euthyroid animals, the N^3 -methylhistidine excretion of the protein-depleted subgroup fell below that of the basal-diet subgroup on day 7 of the protein-free diet. On day 14 of protein depletion, the N^3 -methylhistidine output of the protein-depleted subgroup was 38% below that of the basal-diet subgroup. In hyperthyroid animals, the N^3 -methylhistidine excretion of the protein-depleted subgroup did not fall below that of the basal-diet subgroup until day 10 of the protein-free diet. On day 14 of protein depletion, the N^3 -methylhistidine output of the protein-depleted subgroup was 14% below that of the basal-diet subgroup. Therefore hyperthyroid animals decreased N^3 -methylhistidine excretion in response to protein depletion, but the response was less rapid and smaller than that of euthyroid animals.

Estimation of muscle protein turnover by use of the $[^{14}C]$ tyrosine-infusion technique

As shown in Table 3, protein depletion in euthyroid animals decreased the fractional rate of muscle protein synthesis by 56% and the fractional rate of muscle protein degradation by 20%. Likewise, protein depletion in hyperthyroid animals de-

Fig. 1. Growth curves of euthyroid and hyperthyroid rats fed on basal and protein-free diets

●—●, euthyroid, basal diet; ●—●, hyperthyroid, basal diet; ○—○, euthyroid, protein-free diet; ○—○, hyperthyroid, protein-free diet. On day 0, half of the animals were started on T_3 at a dose of $20 \mu\text{g}/100\text{g}$ body wt. daily. All animals were fed on the basal diet *ad lib.* for the first week. On day 7, one euthyroid and one hyperthyroid subgroup were started on the protein-free diet *ad lib.* Each data point is the mean for 12 animals.

Table 1. Serum T_3 concentrations and organ/body-weight ratios in euthyroid and hyperthyroid rats fed on basal and protein-free diets

As described in the Materials and methods section, groups of euthyroid and hyperthyroid rats were divided into subgroups, which were fed on basal or protein-free diets *ad lib*. Serum T_3 measurements were performed 8 h after the last T_3 injection. Values presented in the Table are means $\pm$ S.E.M. Serum T_3 measurements were performed on six animals in each subgroup. All other measurements were performed on 12 animals in each subgroup. Heart and kidney weights are wet weights. * $P < 0.01$ compared with euthyroid basal group, and ** $P < 0.001$ compared with corresponding euthyroid group, by Student's t test.

Parameter	Diet ...	Euthyroid rats		Hyperthyroid rats	
		Basal	Protein-free	Basal	Protein-free
Serum T_3 (ng/100ml)		125 $\pm$ 7.4	184 $\pm$ 13.0*	2205 $\pm$ 370	1870 $\pm$ 113
Gastrocnemius protein/body-wt. ratio (mg/100g)		90.5 $\pm$ 2.3	94.9 $\pm$ 3.0	88.8 $\pm$ 3.6	86.7 $\pm$ 3.4
Heart/body-wt. ratio (mg/g)		3.07 $\pm$ 0.06	3.57 $\pm$ 0.08	5.61 $\pm$ 0.09**	5.70 $\pm$ 0.13**
Kidney/body-wt. ratio (mg/g)		6.68 $\pm$ 0.12	7.30 $\pm$ 0.15	9.87 $\pm$ 0.10**	12.6 $\pm$ 0.35**

Table 2. N^5 -Methylhistidine excretion by euthyroid and hyperthyroid rats fed on basal and protein-free diets
Groups of euthyroid and hyperthyroid rats were divided into subgroups which were fed on basal or protein-free diets *ad lib*. Values presented below represent pooled urine samples from 12 rats in each subgroup.

Thyroid status and diet	Time on protein-free diet (days) ...	Excretion (μ g/100g body wt. per 24h)			
		3	7	10	14
Euthyroid, basal		160	156	150	145
Euthyroid, protein-free		172	136	131	90
Hyperthyroid, basal		202	186	220	205
Hyperthyroid, protein-free		248	220	193	176

Table 3. Protein turnover in the gastrocnemius muscles of euthyroid and hyperthyroid rats fed on basal and protein-free diets

Groups of euthyroid and hyperthyroid rats were divided into subgroups, which were fed on basal or protein-free diets *ad lib*. The fractional rate of protein synthesis in the gastrocnemius muscle was determined by constant [14 C]tyrosine infusion. The fractional growth rate of muscle protein was estimated from fractional changes in body weight during the 3 days before death. Fractional degradation rates were calculated by subtracting growth rates from synthesis rates. Values presented in the Table are means $\pm$ S.E.M. for eight rats in each subgroup. * $P < 0.01$ compared with euthyroid basal group, and ** $P < 0.01$ and *** $P < 0.02$ compared with hyperthyroid basal group, by Student's t test.

Thyroid status and diet	Fractional rate (%/24h) of muscle protein		
	Synthesis	Growth	Degradation
Euthyroid, basal	6.8 $\pm$ 0.19	1.9 $\pm$ 0.12	4.9 $\pm$ 0.16
Euthyroid, protein-free	3.0 $\pm$ 0.16*	-0.9 $\pm$ 0.14*	3.9 $\pm$ 0.21
Hyperthyroid, basal	10.2 $\pm$ 0.64	1.8 $\pm$ 0.22	8.4 $\pm$ 0.48
Hyperthyroid, protein-free	5.9 $\pm$ 0.30**	-1.0 $\pm$ 0.10**	6.9 $\pm$ 0.29***

creased the fractional rate of muscle protein synthesis by 42% and the fractional rate of muscle protein degradation by 18%. Therefore both the [14 C]tyrosine-infusion and the N^5 -methylhistidine-excretion studies indicated that hyperthyroid animals decrease muscle protein turnover in response to protein depletion.

Effect of hyperthyroidism on urinary nitrogen excretion by rats fed on the protein-free diet

Table 4 shows urinary nitrogen excretion by euthyroid and hyperthyroid rats during 2 weeks on the protein-free diet. On day 3 of protein depletion,

the total urinary nitrogen excretion by hyperthyroid rats was increased 2-fold. During the remainder of the protein-depletion period, total nitrogen excretion by control rats did not change, whereas excretion by hyperthyroid rats fell progressively toward control values. On day 14, total nitrogen excretion by hyperthyroid animals was increased by only 24%. Urea excretion by hyperthyroid rats was increased by 65% on day 3 of the protein-free diet, but was not increased on days 7-14. In contrast, urinary ammonia excretion by hyperthyroid animals was increased 3-8-fold during the entire protein-depletion period. Excretion

Table 4. Urinary nitrogen excretion by euthyroid and hyperthyroid rats fed on the protein-free diet

Excretion of urinary nitrogen compounds by groups of euthyroid (C) and hyperthyroid (T₃) rats was measured on days 3, 7, 10 and 14 of feeding of the protein-free diet *ad lib*. Values for excretion of total N, urea N, ammonia N, creatinine N and creatinine N are means $\pm$ s.e.m. for 12 rats in each group. Values for α -amino N excretion are means $\pm$ s.e.m. for six rats in each group. **P* < 0.05 and ***P* < 0.01 compared with corresponding euthyroid group.

Parameter	Excretion (mg/100g body wt. per 24h)			
	Time on protein-free diet (days)		...	
	3	7	10	14
	C	T ₃	C	T ₃
Total N	17.6 $\pm$ 2.4	36.4 $\pm$ 2.2**	16.7 $\pm$ 1.8	23.4 $\pm$ 0.6**
Urea N	8.1 $\pm$ 1.1	13.4 $\pm$ 1.6*	7.7 $\pm$ 0.7	6.2 $\pm$ 0.3
Ammonia N	0.7 $\pm$ 0.05	2.4 $\pm$ 0.2**	0.3 $\pm$ 0.07	2.6 $\pm$ 0.2**
Urea plus ammonia N	8.8 $\pm$ 1.2	15.8 $\pm$ 1.6**	8.0 $\pm$ 0.4	8.8 $\pm$ 0.4
Creatinine N	0.4 $\pm$ 0.04	8.6 $\pm$ 0.6**	1.4 $\pm$ 0.5	3.6 $\pm$ 0.4**
Creatinine plus creatinine N	3.4 $\pm$ 0.09	5.6 $\pm$ 0.2**	3.4 $\pm$ 0.3	3.9 $\pm$ 0.2
α -Amino N	3.8 $\pm$ 0.1	14.2 $\pm$ 0.6**	4.8 $\pm$ 0.4	7.5 $\pm$ 0.6**
	0.4 $\pm$ 0.05	0.9 $\pm$ 0.05**	0.4 $\pm$ 0.01	0.6 $\pm$ 0.04**
				0.4 $\pm$ 0.03

of urea plus ammonia by hyperthyroid rats was increased by 80% on day 3, but was not increased during the remainder of the protein-depletion period. On day 3, excretion of creatine plus creatinine by hyperthyroid rats was increased 3.7-fold, and creatine excretion 20-fold. Excretion of creatine plus creatinine by hyperthyroid animals remained increased, at more modest values, throughout the protein depletion period. On day 14, excretion of creatine plus creatinine by hyperthyroid rats was increased by 49%, and creatine excretion 2-fold. α -Amino nitrogen excretion by hyperthyroid animals was increased 2-fold on day 3 and remained elevated by 50% during the remainder of the protein-depletion period. Although their response was delayed and less complete than in the controls, hyperthyroid animals greatly decreased total nitrogen excretion in response to protein depletion.

Discussion

We have used two independent methods to investigate the effect of hyperthyroidism on muscle protein turnover in rats fed on basal and protein-free diets. *N*³-methylhistidine is a component of the contractile proteins of skeletal muscle that cannot be reutilized after proteolysis and is excreted in the urine (Young & Munro, 1978). Since skeletal muscle is the predominant reservoir of this amino acid, its excretion has been used as an index of myofibrillar proteolysis (Young & Munro, 1978). As previously reported (Haverberg *et al.*, 1975), protein depletion decreased *N*³-methylhistidine excretion per unit body weight in euthyroid rats, suggesting that a decrease in muscle protein breakdown had occurred. Likewise, protein depletion decreased *N*³-methylhistidine excretion by hyperthyroid animals, although the decrease was delayed and smaller than that observed in euthyroid animals. However, *N*³-methylhistidine excretion may not accurately reflect muscle protein degradation (Millward *et al.*, 1980; Nishizawa *et al.*, 1977). Although skeletal muscle is the predominant reservoir of this amino acid, other sources, such as skin and intestine, turn over much more rapidly. Measurement of *N*³-methylhistidine turnover and pool sizes in skeletal muscle, skin, and intestine of adult rats suggested that less than half of the excreted *N*³-methylhistidine arose from skeletal muscle (Millward *et al.*, 1980). For this reason, protein turnover in the gastrocnemius muscle was measured by a [¹⁴C]tyrosine-infusion technique (Garlick *et al.*, 1973). Again, rates of protein synthesis and degradation were decreased in muscles of both euthyroid and hyperthyroid rats fed on the protein-free diet compared with corresponding animals fed on the complete diet. These studies in-

dicate that hyperthyroidism increases the rate of protein turnover in skeletal muscles, yet it does not prevent a decrease in muscle protein turnover in response to protein depletion.

A previous study (Burini *et al.*, 1981), examined the effect of a protein-free diet on *N*^ε-methylhistidine excretion by hypothyroid rats. Although *N*^ε-methylhistidine excretion per unit body weight was decreased in hypothyroid animals, a further decrease occurred with protein depletion. That study and the present one suggest that the baseline extent of muscle protein turnover is strongly influenced by thyroid status. However, the decrease in muscle protein turnover caused by protein depletion occurs independent of thyroid status, since protein depletion decreased muscle proteolysis in both hypothyroid and hyperthyroid animals.

The capacity of hyperthyroid animals to decrease obligatory nitrogen excretion was determined by measuring urinary nitrogen output of euthyroid and hyperthyroid rats placed on the protein-free diet. On day 3 of protein depletion, the total nitrogen excretion per unit body weight by hyperthyroid animals was increased 2-fold compared with controls. In contrast, the total nitrogen excretion by hyperthyroid animals on day 14 of protein depletion was increased by only 24%. Therefore hyperthyroid animals greatly decreased their initially high rates of nitrogen excretion during the 2-week period of protein depletion. This finding suggests that hyperthyroid animals decreased catabolism of total body protein as well as muscle protein during the period of protein depletion. An elevated excretion of creatinine plus creatine and α -amino nitrogen was a continuing source of increased nitrogen loss in protein-depleted hyperthyroid animals. On the other hand, the excretion of urea plus ammonia by these animals was not different from control values during the second week of the protein-free diet. Therefore increased amino acid catabolism to supply energy needs cannot explain the excess nitrogen loss by hyperthyroid animals during this interval.

Starvation decreased the rate of oxygen consumption in euthyroid and hypothyroid rats (Wimpfheimer *et al.*, 1979). Furthermore, the capacity of starved animals to increase oxygen consumption in response to T_3 was decreased. In protein-depleted rats, the calorogenic response to T_3 did not appear to be diminished (Tulp *et al.*, 1979). However, the current findings indicate that protein depletion decreases the capacity of rats to increase muscle protein turnover in response to T_3 .

This work was supported by Veterans Administration Research funds, project no. 1308-002, and by the

National Institutes of Health, grant no. 1-R01-AM20718-05. We acknowledge the able technical assistance of Mary E. Lynch.

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