Effect of experimental hyperthyroidism on skeletal-muscle proteolysis

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It is not clear whether the muscle wasting commonly observed in hyperthyroidism is due to alteration in the rate of protein synthesis or degradation. The effect of experimental hyperthyroidism on skeletal-muscle proteolysis in the rat was studied by measuring alanine and tyrosine release from isolated skeletal muscles *in vitro* and 3-methylhistidine excretion *in vivo*. Alanine release from the isolated epitrochlearis-muscle preparation was increased as soon as 24 h after a 25 µg dose of L-tri-iodothyronine *in vivo*. Conversely, alanine release from muscles of hypothyroid rats was decreased, but restored by L-tri-iodothyronine supplementation before death. Furthermore, 3-methylhistidine excretion was increased in hyperthyroid rats throughout an 18-day treatment period. The increased amino acid release from isolated muscles and the increased 3-methylhistidine excretion *in vivo* strongly suggests that hyperthyroidism increases skeletal-muscle proteolysis. Furthermore, the thyroid-hormone concentration may be an important factor in regulating muscle proteolysis.

Skeletal-muscle weakness and wasting are commonly observed in hyperthyroidism (Satoyoshi *et al.*, 1963). However, it is uncertain whether the wasting is due to changes in muscle protein synthesis or degradation. Several studies have suggested that hyperthyroidism increases protein degradation in skeletal muscle and that this may cause muscle wasting. Skeletal muscle isolated from rats given supraphysiological doses of thyroxine showed increased proteolysis as measured by amino acid release *in vitro* (Karl *et al.*, 1976). Similarly, muscle protein degradation was increased in skeletal muscle obtained from hypophysectomized rats given pharmacological doses of thyroid hormone (Goldberg *et al.*, 1977). Furthermore, the activity of lysosomal proteinases in skeletal muscle was increased by thyroid-hormone treatment (DeMartino & Goldberg, 1978). In contrast, other studies have reported no increased protein breakdown in skeletal muscle of hyperthyroid animals and have attributed the decreased muscle mass in hyperthyroidism to decreased protein synthesis (Flaim *et al.*, 1978). It is important to determine whether hyperthyroidism can in fact increase skeletal-muscle proteolysis, since changes in muscle mass induced by various other dietary and hormonal manipulations, such as insulin deficiency, hypercortisolism, protein

deficiency and total starvation, have been attributed to decreased protein synthesis rather than increased protein degradation (Millward *et al.*, 1976). Because the effect of hyperthyroidism on skeletal-muscle degradation is not clear, the present studies were directed at this question. We have measured the rate of amino acid release from isolated skeletal muscle obtained from hypothyroid, euthyroid and hyperthyroid rats. We have also examined 3-methylhistidine excretion in euthyroid and hyperthyroid rats. Our results strongly suggest that skeletal-muscle proteolysis is increased in hyperthyroidism and decreased in hypothyroidism.

Materials and methods

Materials

Alanine aminotransferase (EC 2.6.1.2), L-lactate dehydrogenase (EC 1.1.1.27), L-T₃, D-T₃, L-3-methylhistidine, L-1-methylhistidine, ε-N-methyl-L-lysine, bovine insulin and Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] were obtained from Sigma, St. Louis, MO, U.S.A. L-[U-¹⁴C]-Histidine (sp. radioactivity 363 Ci/mol) was purchased from ICN Chemical and Radioisotope Division, Irvine, CA, U.S.A. All other chemicals used were reagent grade. Purina laboratory chow and purified basal diet no. 5755 were purchased from Ralston Purina Co., St. Louis, MO, U.S.A. Normal and thyroidectomized male Sprague-

Abbreviations used: L-T₃ and D-T₃, 3,3',5-tri-iodo-L- or -D-thyronine.

Dawley rats weighing 125–175 g were obtained from Charles River Laboratories, Wilmington, MA, U.S.A. Unless otherwise indicated, the animals were fed on Purina laboratory chow *ad lib*. Hypothyroid rats were kept for 4 weeks before use and any animals not showing a marked decrease in growth rate were rejected.

Treatment of animals and muscle preparations used in alanine- and tyrosine-release experiments.

In these experiments, rats were given intraperitoneal injections of L-T₃ or D-T₃ dissolved in 5 mM-NaOH/0.9% NaCl as indicated in the Tables. The thyroid-hormone preparations had pH approx. 10.5 and were injected in a volume of 0.10 ml/100 g body wt. Control animals were given an equivalent volume of diluent. After the animals were killed by decapitation, both epitrochlear muscles were removed and incubated *in vitro* as previously described (Garber *et al.*, 1976a). The muscles were quickly rinsed in normal saline (0.9% NaCl), blotted, weighed, and placed in 10 ml incubation flasks containing 1 ml of modified Krebs–Henseleit (1932) buffer, pH 7.4. This buffer contained half the usual Ca²⁺ concentration, and also 5 mM-glucose, 100 munits of insulin/ml and 5 mM-Hepes. In tyrosine-release experiments, the buffer also contained 0.1 mM-cycloheximide. The flasks were gassed for 1 min with O₂/CO₂ (19:1), stoppered, and incubated for 3 h at 37°C in a shaking water bath. At the end of the incubation, the muscles were removed and the medium was heated to 100°C for 2 min. These incubation conditions were chosen to duplicate standard conditions previously used in the study of alanine release from epitrochlear muscles. In previous studies, insulin and glucose additions to the reaction mixture *in vitro* did not alter alanine release (Garber *et al.*, 1976a), although induction of diabetes *in vivo* greatly increased alanine release from this preparation (Karl *et al.*, 1976).

Measurement of alanine release from muscle preparations

Alanine released into the incubation medium was determined by a modification of a previous method (Karl *et al.*, 1972). A coupled enzymic reaction was used in which alanine reacts with α -oxoglutarate in the presence of alanine aminotransferase to form pyruvate plus glutamate. Pyruvate is then converted into lactate by lactate dehydrogenase, with stoichiometric oxidation of NADH. A 0.25 ml portion of incubation medium was added to 0.75 ml of reaction mixture containing 140 mM-sodium phosphate buffer, pH 7.5, 1.3 mM- α -oxoglutarate, 0.2 mM-NADH and 4 units of lactate dehydrogenase. This mixture was pre-incubated for 90 min at room temperature to convert any preformed pyruvate into lactate. After

measurement of the A₃₄₀, 2 units of alanine aminotransferase was added, followed by a second 90 min incubation and measurement of A₃₄₀. The alanine content was calculated from the decrease in A₃₄₀ that appeared after the alanine aminotransferase addition. Standard alanine solutions assayed with each experiment yielded A₃₄₀ decreases that were $94.7 \pm 0.9\%$ (mean $\pm$ S.E.M., $n = 12$) of theoretical values.

Measurement of tyrosine release from muscle preparations

A sample of the muscle incubation medium was deproteinized by addition of sufficient ice-cold 50% (v/v) trichloroacetic acid to produce a final concentration of 10%. The mixture was placed on ice for 15 min and the clear supernatant taken for tyrosine assay by a fluorimetric procedure (Waalkes & Udenfriend, 1957).

Treatment of animals used in 3-methylhistidine studies

To test the effect of hyperthyroidism on 3-methylhistidine excretion, two groups each of eight rats weighing 125–175 g were placed in metabolism cages and fed *ad lib*. on Purina basal diet no. 5755, which does not contain 3-methylhistidine. After a 4-day adaptation period, one group of animals was given daily intraperitoneal injections of L-T₃ (25 μ g/100 g body wt.) dissolved in 5 mM-NaOH/0.9% NaCl. Control animals were given an equivalent volume of diluent. During the 18-day treatment period, 24 h urine samples were collected in plastic cups containing 3 ml of conc. HCl and frozen until the time of assay.

Measurement of 3-methylhistidine excretion

The 3-methylhistidine content of the rat urine was determined as follows. The urine samples were hydrolysed in 6 M-HCl for 20 h at 100°C under nitrogen to convert acetylated 3-methylhistidine into the free form. The hydrolysate was decolorized by addition of 300 mg of powdered activated charcoal, followed by Millipore filtration. HCl was removed from the hydrolysate by repeated evaporation to dryness under vacuum. The hydrolysate was desalted by ion-exchange chromatography (Asatoor, 1969), and 3-methylhistidine was isolated by a modification of the pyridine elution technique described by Nishizawa *et al.* (1977). The desalted urine was evaporated to dryness, dissolved in 5.0 ml of 0.2 M-pyridine and applied to a column (1.0 cm $\times$ 30 cm) of Dowex 50 (X 8; 200–400 mesh) equilibrated with 0.2 M-pyridine. The column was eluted with 130 ml of 0.2 M-pyridine to remove acidic and neutral amino acids, and then eluted with 1.0 M-pyridine at a flow rate of 0.25 ml/min, and 5.0 ml fractions were collected. Under these condi-

tions, 3-methylhistidine emerges as a sharp, distinct peak near the 1.0M-pyridine front, followed by a peak containing histidine. The amino acid peaks were identified and quantified by the ninhydrin reaction described by Rosen (1957).

This initial peak appeared to contain only 3-methylhistidine. Paper chromatography of this peak, with a mixture of 30 g of *m*-cresol, 15 g of phenol and 7.5 ml of 0.1 M-sodium borate buffer, pH 8.3, run on borate-buffered paper (Asatoor, 1969), revealed a single ninhydrin spot which migrated with authentic 3-methylhistidine and was distinct from other basic amino acids expected in urine, including histidine, ϵ -N-methyl-lysine, lysine, ornithine and arginine. Furthermore, 1-methylhistidine emerged from the column as a separate peak behind 3-methylhistidine.

To correct for losses of 3-methylhistidine occurring during the hydrolysis, decolorization and desalting steps, 50 000 d.p.m. of L-[U- 14 C]histidine was added to each urine specimen before hydrolysis and the radioactivity recovered in the histidine peak was measured by liquid-scintillation counting. The measured 3-methylhistidine content was corrected for histidine recovery, which averaged $61 \pm 0.7\%$ (mean $\pm$ S.E.M.). In a recovery experiment performed to demonstrate the adequacy of this procedure, four identical rat urine specimens were prepared and 540 μ g of 3-methylhistidine was added to each of two specimens. On analysis, the 3-methylhistidine content of the unsupplemented specimens was 438 μ g and that of the supplemented specimens was 1012 μ g for a recovery of 106%.

Results

Effect of thyroid state on alanine release from isolated epitrochlaris muscles

The isolated rat epitrochlaris muscle has been

used as a model *in vitro* to study skeletal-muscle proteolysis (Garber *et al.*, 1976a,b; Karl *et al.*, 1976). This very thin skeletal muscle maintains physiological concentrations of phosphocreatine, ATP, ADP, lactate and pyruvate *in vitro* for periods up to 6 h (Garber *et al.*, 1976a). This preparation releases amino acids *in vitro* in the same relative proportions and amounts as does perfused human skeletal muscle *in vivo*, with alanine and glutamine predominating (Garber *et al.*, 1976a). The release of alanine and glutamine appeared to reflect their formation within the muscle from the transformation of other amino acids made available by proteolysis (Garber *et al.*, 1976a,b). Inhibition of protein synthesis by adding cycloheximide *in vitro* did not influence the release of these amino acids (Karl *et al.*, 1976). Although pyruvate produced by glycolysis is a potential substrate for alanine formation, pyruvate produced in this manner is unlikely to be rate-limiting for alanine formation in this preparation (Snell, 1980).

In the present study, alanine release into the incubation medium has been used to indicate muscle protein degradation. As shown in Table 1, L-T₃ (25 μ g/100 g) given daily for 6 days increased alanine release from 15.6 to 25.0 nmol/min per g of muscle. Similarly, the same dose of L-T₃ given for 3 days increased alanine release from 14.9 to 20.6 nmol/min per g, and a single dose of L-T₃ given 24 h before death increased alanine release from 11.2 to 15.6 nmol/min per g. Conversely, alanine release from muscles of hypothyroid rats was decreased from control values of 14.6 to 5.93 nmol/min per g of muscle. Supplementation of the hypothyroid rats with a daily dose of 5 μ g of L-T₃/100 g for 7 days returned alanine release to control values. To determine if this apparent catabolic effect of L-T₃ is a non-specific, perhaps toxic, effect of the

Table 1. *Effect of thyroid-hormone status in vivo on alanine release from isolated epitrochlaris muscles in vitro*
The animals were given the indicated dose of L-T₃ dissolved in 5 mM-NaOH/0.9% NaCl by intraperitoneal injection for the indicated interval before death. Saline-injected controls were given an equivalent volume of diluent. For each experimental condition, two to four control and experimental animals were treated simultaneously and their muscles incubated at the same time. The epitrochlaris muscles were removed, incubated, and alanine release into the incubation medium was measured as described in the Materials and methods section.

Experimental condition	No. of animals per group	Alanine released (nmol/min per g)	
		Control	Experimental
1. 25 μ g of L-T ₃ /100 g daily for 6 days, versus saline-injected controls	8	15.6 $\pm$ 0.8	25.0 $\pm$ 1.4**
2. 25 μ g of L-T ₃ /100 g daily for 3 days, versus saline-injected controls	8	14.9 $\pm$ 0.7	20.6 $\pm$ 1.3*
3. 25 μ g of L-T ₃ 24 h before death, versus saline-injected controls	22	11.2 $\pm$ 0.6	15.6 $\pm$ 1.0*
4. Hypothyroid rats, versus age-matched controls	15	14.6 $\pm$ 0.8	5.93 $\pm$ 0.6**
5. Hypothyroid rats given 5 μ g of L-T ₃ /100 g daily for 7 days, versus saline-injected hypothyroid rats	8	6.86 $\pm$ 1.4	13.9 $\pm$ 1.3*

* $P < 0.01$, ** $P < 0.001$ by two-tailed Student's *t* test.

Table 2. Comparison of L-T₃ and D-T₃ treatment in vivo on alanine release from isolated epitrochlearis muscles in vitro

The animals were given the indicated dose of L-T₃ or D-T₃ dissolved in 5mM-NaOH/0.9% NaCl by intraperitoneal injection, and saline-injected controls were given an equivalent volume of diluent. Two animals in each group were treated simultaneously, and their muscles were incubated at the same time. The epitrochlearis muscles were removed, incubated, and alanine release into the incubation medium was measured as described in the Materials and methods section. Values are means $\pm$ S.E.M. for eight animals.

Experimental condition	Alanine released (nmol/min per g)
1. Saline-injected controls	14.1 $\pm$ 1.5
2. 25 μ g of L-T ₃ /100g daily for 3 days	20.6 $\pm$ 1.0*
3. 25 μ g of D-T ₃ /100g daily for 3 days	14.0 $\pm$ 1.0

* $P < 0.01$ by two-tailed Student's *t* test for difference from controls.

injected material, the effects of L-T₃ and D-T₃ treatment on alanine release were compared (Table 2). Groups of six animals, including two saline-injected controls, two animals given daily doses of 25 μ g of L-T₃/100g for 3 days, and two animals given identical doses of D-T₃, were studied simultaneously. In the L-T₃-treated animals, alanine release was increased from 14.1 to 20.6 nmol/min per g of muscle, whereas no change occurred in the D-T₃-treated animals.

Effect of hyperthyroidism on tyrosine release from isolated epitrochlearis muscles

To confirm the supposition that increased alanine release from hyperthyroid muscles represents increased protein degradation, tyrosine release from control and hyperthyroid muscles was measured in the presence of 0.1 mM-cycloheximide. Hyperthyroid animals were given 25 μ g of L-T₃/100g daily for 3 days, and tyrosine release from isolated epitrochlearis muscles was measured as described in the Materials and methods section. Tyrosine release from muscles of eight control animals was 2.80 $\pm$ 0.14 nmol/min per g, whereas that from muscles of eight hyperthyroid animals was increased to 3.39 $\pm$ 0.21 (mean $\pm$ S.E.M.; $P < 0.05$ by Student's *t* test).

Effect of hyperthyroidism on 3-methylhistidine excretion in rats

The excretion of 3-methylhistidine is commonly used as an index of skeletal-muscle proteolysis because it is found primarily in contractile protein of skeletal muscle, it is methylated after incorporation into the peptide chain, and it cannot be reutilized

Fig. 1. Effect of hyperthyroidism on (a) body weight and (b) 3-methylhistidine excretion in rats

□, The experimental animals were given a daily dose of 25 μ g of L-T₃/100g by intraperitoneal injection for 18 days. ■, Control animals were given an equivalent volume of diluent. For each data point except day 18, values for 3-methylhistidine excretion were determined by using pooled urine samples obtained from the eight control and eight L-T₃-treated animals respectively. On day 18, 3-methylhistidine excretion was determined separately for each control and experimental animal. The vertical bars indicate means $\pm$ S.E.M.

after muscle proteolysis (Young & Munro, 1978). As shown in Fig. 1(a), the body weight of both control and L-T₃-treated animals increased during the 18-day treatment period, with control animals gaining at a faster rate. To demonstrate that the L-T₃-treated animals were indeed hyperthyroid, the heart/body-weight ratio in this group was 6.54 $\pm$ 0.02 mg/g (mean $\pm$ S.E.M.), compared with 3.43 $\pm$ 0.08 in the control animals. As shown in Fig. 1(b), urinary 3-methylhistidine excretion in the L-T₃-treated group was consistently increased by 24–33% over control values from day 2 until the end of the treatment period. Excretion of 3-methylhistidine rose progressively in both treatment groups as the animals grew and their muscle mass increased. For all data points except day 18, the values presented were obtained from pooled urine samples prepared from the eight control and eight L-T₃-treated animals respectively. On day 18, 3-methylhistidine excretion determined separately for each animal was 494 $\pm$ 19.8 μ g/24 h (mean $\pm$ S.E.M.) in the control group and 613 $\pm$ 19.1 μ g/24 h in the L-T₃-treated group.

Discussion

The finding of increased alanine release from epitrochlearis muscles of L-T₃-treated rats confirms the report of Karl *et al.* (1976), who noted increased alanine release from these muscles of rats given

50 μ g of L-thyroxine daily for 4 days. Our findings also support those of Goldberg *et al.* (1977), who noted increased tyrosine release from the soleus and extensor digitorum longus muscles obtained from hyperthyroid rats, and those of DeMartino & Goldberg (1978), who found increased lysosomal proteinase activity in skeletal muscle of hyperthyroid rats. The present results and the work cited above strongly suggest that skeletal-muscle proteolysis is increased in hyperthyroidism. Furthermore, the present studies show increased alanine release as early as 24 h after a single dose of L-T₃. This suggests that the effect is not dependent on chronic adaptation to the hyperthyroid state and that thyroid hormone may act directly on muscle, perhaps at the nuclear level. The fact that alanine release from muscles of hypothyroid rats is decreased, whereas that from hyperthyroid rats is increased, suggests that thyroid-hormone concentrations may play a role in regulating skeletal-muscle proteolysis. Goldberg *et al.* (1977) also noted decreased tyrosine release from muscles of hypophysectomized rats, which was increased to normal by physiological doses of thyroid hormone and to supranormal values by pharmacological doses. Likewise, DeMartino & Goldberg (1978) found low activities of lysosomal proteinases in muscles of hypophysectomized rats, which were increased to normal values by anabolic doses of thyroid hormone and to supranormal values by catabolic doses. The failure of D-T₃ to influence alanine release suggests that this effect is restricted to biologically active thyroid-hormone analogues and is not a non-specific action unrelated to biological activity.

The finding of increased 3-methylhistidine excretion in hyperthyroid rats provides additional evidence that skeletal-muscle proteolysis is increased in hyperthyroidism. Similarly, 3-methylhistidine excretion was increased in human subjects with spontaneous hyperthyroid myopathy and decreased in those with spontaneous hypothyroid myopathy (McKeran *et al.*, 1979). Furthermore, L-T₃ administration increased 3-methylhistidine excretion in fasting obese human subjects (Burman *et al.*, 1979). Therefore 3-methylhistidine excretion appears to be increased in experimental and clinical hyperthyroidism. Since approx. 17% of the 3-methylhistidine excreted by the rat may come from protein turnover in the gastrointestinal tract and skin (Nishizawa *et al.*, 1977), it is possible that the increased 3-methylhistidine excretion in hyperthyroid rats results from greatly accelerated turnover of these tissues rather than increased muscle proteolysis. This possibility seems unlikely, in view of the present and previous studies showing increased amino acid release from skeletal muscle in hyperthyroidism.

The results of the present study differ from those

of Flaim *et al.* (1978), who reported that pharmacological doses of L-thyroxine given for 5 days did not increase protein degradation as measured by phenylalanine release from the rat hemicorpus in the presence of 0.1 mM-cycloheximide. Furthermore, these investigators found decreased protein synthesis in skeletal muscle and attributed the decreased muscle mass in hyperthyroidism to decreased protein synthesis rather than enhanced protein breakdown. Although decreased protein synthesis may play a role in decreasing skeletal-muscle mass in hyperthyroidism, the finding of increased amino acid release *in vitro* from skeletal muscle of hyperthyroid animals and increased 3-methylhistidine excretion by hyperthyroid animals *in vivo* strongly suggests that increased protein degradation is also important.

Although alanine release from isolated muscle preparations appears to reflect muscle proteolysis (Garber *et al.*, 1976a,b; Snell, 1980), pyruvate produced by glycolysis is also a potential substrate for alanine formation. Furthermore, alanine release was measured in the absence of protein-synthesis inhibitors, so that increased alanine release from muscles of hyperthyroid animals could conceivably result from either decreased protein synthesis or increased protein degradation. For these reasons, tyrosine release from epitrochlearis muscles of control and hyperthyroid animals was measured in the presence of 0.1 mM-cycloheximide and found to be elevated in the latter muscles. Since tyrosine is not synthesized or degraded within muscle (Goldberg *et al.*, 1980), the increased release of this amino acid in the presence of blocked protein synthesis strongly supports our conclusion that proteolysis is increased in skeletal muscle of hyperthyroid animals.

It should be pointed out that short-term treatment of normal animals with supraphysiological doses of thyroid hormone in the present study may not be directly comparable with the studies of Flaim *et al.* (1978) and Goldberg *et al.* (1977), who used thyroidectomized and hypophysectomized animals respectively. Differences in nutritional status, insulin concentrations and other factors in the various animal models may influence the response to thyroid-hormone injection.

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