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Effect of aging on the rate of muscle protein turnover in rat

G. Frühbeck, B. Muguerza, I. Castilla-Cortázar and S. Santidrián

Departamento de Fisiología, Facultad de Medicina
Universidad de Navarra, 31080 Pamplona (Spain)

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The urinary excretion of N^t-methylhistidine (3-methylhistidine, 3-MH, an index related to myofibrillar protein breakdown), as well as the rate of L-[^{14}C]-tyrosine incorporation into gastrocnemius muscle (k_s, fractional rate of muscle protein synthesis, evaluated by the constant infusion method) have been measured to assess the effect of aging on the rate of skeletal muscle protein turnover. In addition, nucleic acids, muscle protein and serum corticosterone levels were determined. Weaning rats were fed a 10 % lactalbumin diet and killed in groups of seven when they were 35, 60, 120 and 300 days old. Apart from the rate of growth, no major differences were found between 35- and 60-day old animals. However, as compared to the youngest rats, 120-day old rats showed a significant reduction in the relative weight of the four muscles excised. Plasma corticosterone levels increased as the animals became older. Finally, in the 300-day old rats, the reduced rate of growth was accompanied by a significant reduction in the relative organ weight (with the exception of soleus), 3-MH and k_s. It is concluded that aging caused a reduction in the rates of both protein breakdown and synthesis. The reduced muscle breakdown may not be due to a relative reduced muscle mass in elder rats since urinary 3-MH remained low even when expressed per creatinine output.

Key words: Aging, Protein turnover, 3-Methylhistidine, Rat.

Investigations on the effect of aging on protein metabolism are quite extensive and show that there is a progressive loss of lean body mass throughout middle and old ages, with tendencies for the rate of loss of lean body mass to increase in later life (12). Aging is associated with a slow but continual change in body composition. These changes can be characterized by an increasing amount of body fat and a

Correspondence to S. Santidrián.

concomitant decline in lean body mass (10). Reviews on this subject have been frequently published (e.g., 11, 22, 34). In humans, these losses are largely accounted for by skeletal musculature which can shrink as much as 40 % between the ages of 20 and 80 years (8). This has been confirmed by the reduced urinary output of N^t-methylhistidine (3-methylhistidine, 3-MH) in elderly people (26), reduced number of contractile fibers in humans (29), and reduced fractional rates of muscle protein synthesis and degradation as measured by the administration of a flooded dose of radioactive labeled aminoacids (13).

The main specific goal of this work was first to report data on the effect of aging on myofibrillar protein breakdown in Wistar male rats as measured by the urinary output of 3-MH. And secondly, to evaluate the effect of aging on the rates of skeletal muscle protein synthesis as measured by the constant infusion method. The study was longitudinally conducted and showed that aging in rats brings about a noteworthy reduction in the rate of muscle protein turnover, degradation rates being slightly faster than synthesis reduction. This effect seems to be independent of the reduced muscle mass observed in the older animals since urinary excretion of 3-MH remains low even when expressed per unit of creatinine output.

Materials and Methods

Animals and experimental design.– Intact and immediately-post-weaning Wistar male rats were obtained from Panlab Breeding Center (Barcelona, Spain) and housed in individual, suspended, wire bottomed cages. The animal room was equipped with a 12-h light:dark cycle timer and maintained at 20-23 °C and 45-70 % relative humidity. Animals were fed *ad libitum a* purified adequate free-meat protein diet containing 10 % lactalbumin (Dyets Inc., Bethlehem, PA, USA) that followed the recommendations of the American Institute of Nutrition *Ad Hoc* Committee (33). Mineral and vitamin mixtures were purchased from Dyets Inc. and added to de diet, as well as the other ingredients, according to the aforementioned criteria. Diet was prepared as previously published (38). The animals were fed by giving them the same amount of diet at different daily intervals (four times every 24 h). This feeding procedure was applied to avoid differences in the daily repartition of meals occurring among freely fed rats. Water supply was offered *ad libitum.* Animals were adquired, cared for and handled in conformance with the Public Health Service's Guide for the Care and Use of Laboratory Animals and the Guiding Principles for Research Involving Animals and Humans approved by the Council of the American Physiological Society. Animals were randomly assigned into four age-groups of seven animals each so that at the killing time they were 35, 60, 120 and 300 day-old. Body weight changes were recorded daily (during the first month of the experiment) or weekly (from that time on). Within each age-group, five days prior to sacrifice, the rats were transferred to individual metabolic cages. Daily urine excretion was collected under 0.1 mL of 0.1 N HCl as preservative and pooled for subsequent determination of creatine, creatinine and 3-MH. At the end of each age-period, rats were killed by decapitation. A sample of blood was taken directly from the decapitated body in chilled heparinized tubes. Immediately, livers as well as total gastrocnemius, soleus, tibialis anterior and extensor digitorum longus muscles from rear hindlimbs were carefully excised and weighed.

Measurement of the fractional rate of muscle protein synthesis.– Rates of skeletal muscle protein synthesis were determined by the constant infusion method of [^{14}C]-tyrosine into one of the tail veins as described by GARLICK *et al.* (14, 15). The gastrocnemius muscle was chosen since it is reasonable large and easy to dissect out and, above all because it may be considered as a representative of the mixture of fibre types (I, slow oxidative, and II, fast glycolytic) present in the rodent (2, 23, 30). Protein and protein-free supernatant fractions were prepared from muscle tissue (7). The specific radioactivity of L-tyrosine in protein hydrolysates (radioactivity of the amino acid bound to protein, S_B) and in the protein free supernatant (radioactivity of the amino acid free in the intracellular pool, S_i) fractions were measured by the procedure of GARLICK *et al.*, (15). Details of the method and procedure have been repeatedly reported (16). In addition, muscle DNA, RNA and protein were determined according to the methods of BURTON (6), EISEMANN *et al.* (9) and BRADFORD *et al.* (5), respectively.

Urinary analysis.– After hydrolysis of the 3-MH N-acetyl derivative with 2 N HCl in boiling water for 2 h and subsequent desalting in a cation exchange column (Dowex AG50-X8), the urinary concentration of 3-MH was determined by HPLC (Model Waters 441) reverse phase with fluorescence detection as previously described (1). Urinary creatine and creatinine were evaluated by the method of HEINEGAR and TIDERSTRÖM (21).

Plasma analysis.– Plasma corticosterone was determined by radioimmunoassay procedure using a kit purchased from Interscience Institute (Los Angeles CA, USA). The hormone was extracted from the plasma with dichloromethane; then, the organic phase was separated from the aqueous one by centrifugation and evaporated to dryness in a vacuum oven at room temperature as previously described (38).

Statistical treatment of the data.– Data are expressed as mean±SEM. Statistical evaluations were carried out by conventional one-way analysis of the variance (ANOVA). Student's *t* test was applied for unpaired data in order to assess intragroup comparisons. Least significance differences between means were computed and accepted as significant at $p < 0.05$.

Results

Tables I and II show that no major differences were observed apart from the

Table I. *Effect of aging on body weight gain and liver and hindlimb muscles weights in male rats.* Values are means ± SEM from 7 rats in each group. G, gastrocnemius; S, soleus; T, tibialis anterior; EDL, extensor digitorum longus.

Age (days)	Body weight (g)	Weight gain (g/day)	Organs weight/100 g body weight				
			Liver (g)	G (g)	S (mg)	T (mg)	EDL (mg)
35	110±17^{a}	6.8±1.7^{a}	3.8±0.8^{a}	1.10±0.03^{a}	70±5^{a}	328±17^{a}	90±8^{a}
60	215±8^{b}	3.6±0.5^{b}	4.6±0.7^{b}	1.12±0.07^{a}	75±7^{a}	340±17^{a}	92±11^{a}
120	350±9^{c}	2.7±0.3^{c}	3.1±0.3^{a,c}	1.26±0.11^{b}	92±3^{b}	370±22^{b}	103±8^{b}
300	700±13^{d}	2.1±0.5^{d}	2.9±0.5^{c}	0.81±0.09^{c}	73±3^{a}	251±20^{c}	74±6^{a}

Values not sharing the same superscript are statistically significant ($p<0.05$, one-way ANOVA).

Table II. *Plasma corticosterone (Cort., mmol/100 ml) and urinary of creatine, creatinine and 3-methylhistidine (3-MH).*
Within each age-group, 24-h urine excretion was collected from individual rats during five successive days before sacrifice. Collection were pooled in each group and then analyzed. Means (±SEM) correspond to these five days determinations.

Age days	Cortico-sterone	Creatine (mg/100 g b.wt.)	Creatinine (mg/100 g b.wt.)	3-MH (mmole/100 g b.wt.)	3-MH (mmole/mg creatinine)	3-MH (mmole/100 g muscle)
35	23±6[a]	0.17±0.01[a]	1.50±0.04[a]	0.91±0.01[a]	0.61±0.015[a]	58.1±2.5[a]
60	26±8[a]	0.22±0.01[b]	1.47±0.06[a]	0.90±0.02[a]	0.61±0.015[a]	56.2±3.1[a]
120	33±5[a]	0.18±0.01[a]	2.30±0.18[b]	0.82±0.01[b]	0.37±0.02[b]	20.1±2.3[b]
300	47±8[b]	0.11±0.01[a]	2.62±0.19[c]	0.67±0.02[c]	0.26±0.01[c]	21.5±3.1[b]

Values not sharing the same superscript are statistically significant ($p<0.05$, one-way ANOVA).

growth rate, between 35- and 60-day old rats. Only creatine output was significantly higher in the 60-day old animals as compared to the youngest rats. Total urinary output of creatinine was significantly reduced especially in the two oldest groups of rats which reflects losses in the skeletal muscle compartment of lean body mass

Nevertheless, when creatinine output is expressed per 100 g of body weight it became significantly increased in these two groups as compared to the younger animals. However, 120-day old rats, while displaying a significant reduction in both growth rate and relative liver weight, had a significant increase in the relative weight of the four muscles excised, as well as a reduction in the urinary output of creatine. Nevertheless, in this group urinary 3-MH (expressed per 100 g body weight, creatinine output and the sum of the four hindlimb muscles weight) were significantly lower than those of younger animals. 300-d old rats, which grew only at the rate of about 2 g/day showed, as compared to younger animals, a significant reduction both in the relative weight of liver, gastrocnemius, tibialis and extensor digitorum longus (but not in soleus weight) and 3-MH output, whatever way expressed. However, creatinine output was significantly increased in the oldest rats as compared to any of the younger animals. The same table shows that plasma corticosterone levels significantly increased as the animals grew older. Table III shows that total RNA concentration (mostly ribosomal RNA), expressed as the ratio RNA/DNA, remains relatively unchanged throughout the experimental period with a tendency to increase in the oldest animals. The ratio protein/DNA progressively increased along the different age-groups, this being increased almost

Table III. *Gastrocnemius RNA/DNA ratio and protein/DNA and ks, fractional rate of muscle protein synthesis (expressed as % of newly synthesized protein/24h).*
Entries are means (±SEM) from seven rats in each group. Values not sharing the same superscript are statistically significant ($p<0.05$, one-way ANOVA).

Age (days)	RNA/DNA	Protein/DNA	k_s
35	2.5±0.2[a]	120±15[a]	14.3±2.0[a]
60	2.4±0.1[a]	260±19[b]	15.6±1.9[a]
120	2.3±0.2[a]	440±30[c]	10.2±1.3[b]
300	2.5±0.5[a]	590±75[d]	8.9±0.9[b]

fivefold in the 300-day old rats as compared to 30-day old animals. Finally, the fractional rate of muscle protein synthesis, k_s (i.e., amount of newly synthesized protein per day), was significantly lower in the older animals.

Discussion

The rational and validation of measuring 3-MH as an index of myofibrillar protein breakdown have been extensively investigated (41). 3-MH arises from the carcass, 22 % from skin and 2 % from small intestine. 3-MH is not reutilized for protein synthesis and both in rodents and humans it is quantitatively excreted in the urine after its release (partly, as its acetyl derivative) from actin and myosin during protein breakdown. Therefore, it is possible to reasonably estimate *in vivo* degradation rates of the myosin-actin pool of skeletal musculature from the urinary excretion of 3-MH by taking into account the contribution of skin and intestine (27, 41, 42). Validation of the constant infusion method as a means of evaluating fractional rates of protein synthesis is well established (43).

The results obtained in this study showed that the excretion of this amino acid derivative, expressed per unit of body weight was markedly reduced as the animals became older. This could indicate possible age-dependent differences in the fractional rate of muscle protein breakdown, or simply, differences in the size of muscle mass, this being especially relevant in 300-day old animals. The urinary excretion of creatinine was evaluated both to provide an index of muscle mass and to express the output of 3-MH, trying thus to minimize variabilities from daily excretion. When the 3-MH output was expressed per unit of creatinine excreted, still the same qualitative differences persisted. Furthermore, in the almost one-year old rats, the marked reduction in the relative weight of liver and three of the four muscles excised (gastrocnemius and tibialis anterior, representing predominatly mixed fibers and extensor digitorum longus, containing almost exclusively white fibers), suggests that the observed reduction in rate of myofibrillar protein breakdown may be accompanied by a parallel reduction in the rates of liver and muscle protein synthesis.

It is known that the main difficulty, in the evaluation of the rate of muscle protein synthesis in tracer constant infusion experiments is the uncertainty on the specific radioactivity of the precursor (31). Former investigators showed that the direct estimation based on lysine data allows for better estimates of body protein turnover as compared to other methods (32). In our study the fractional rate of grastrocnemius protein synthesis decreased from 14.3 % /day to 8.9 %/day between 110 and 700 g of body weight, somewhat less than the observations reported by other investigators who assessed protein synthesis by different procedures (17, 24, 32). Such differences may be attributed to the fact that a single muscle is under study here and not the whole skeletal musculature while the experiments conducted by some of the cited investigators began with rats weighing 60 g. Besides, it must be pointed out that data obtained on protein turnover may be markedly depending on the strain of rats used in the metabolic studies (32).

On the other hand, whole body myofibrillar protein breakdown as qualitatively assessed by 3-MH urinary output, and expressed per unit of body mass decreased at a lesser rate (0.91 vs 0.67, approximately, 26 %) than synthesis (14.3 vs 8.9, about 38 %). Again, it must be borne in mind that a single-mixed-fiber type muscle is assumed to be representative in the evaluation of whole body mus-

cular protein synthesis. Taking these conditions into account, the present data seem to indicate that the aging process produces a decrease in the rate of protein deposition. Analogous observations have been reported by others (35, 43) In at least one of these reports (32) the efficiency of protein deposition has been demonstrated to decrease as the animals grew larger. The reduced muscle protein turnover displayed by old rats may be in correlation with the observation of NARICI *et al.* (28) who showed that aged human adductor pollicis is weaker, slower and tetanized at lower fusion frequencies than younger muscles, but paradoxically is more resistant to static fatigue, which suggests that changes in muscle composition, turnover and function could be the consequence of alterations of the endocrine system. Skeletal muscle metabolism is known to be under hormonal control as well as that glucocorticoids play an outstanding role in muscle protein metabolism. Elevated doses of these hormones seem to produce an increase in skeletal muscle protein degradation during the first days of administration; however, maintained high glucorticoid levels have been repeatedly reported to induce a marked decreased in muscle protein synthesis, which might be in agreement with the results found in this study (30, 38, 43). On the other hand, androgens and growth hormone (GH) secretion decrease with age (36), whereas insulin increases. Administration of testosterone to elderly men has been demonstrated to produce an increase in strength as well as a greater proportion of type II fibers in guinea pigs (39), whereas GH administration increase body mass (37). A progressively reduced action of these hormones may enhance muscle disuse in the elderly (20). The age-related reduction in skeletal muscle mass has been ascribed as well to other different factors, some of which cannot be modified while others are susceptible to change. Apart from the hormonal impact, it has been proposed that as a result of age-related changes in the nervous system, there may be a long-term denervation process resulting in deficient neural input to the muscle. Other hypotheses include changes in skeletal muscle, such as decreased capacity to regenerate new muscle fibers and reduced oxygen diffusion to muscular mitochondria due to a loss of intracellular water (10).

In terms of cellular growth, the rat muscle growth for this colony was characteristic, especially during the first 120 days of age. In this sense, and in agreement with previously reported data (25), the results of this study show an increase in cell size (hypertrophy), or more correctly, in DNA/unit size (muscle fibers are polynucleated and, therefore, the parameter protein/DNA correlates to the amount of cytoplasm controlled by or related to a single nucleus). These measurements would have included various non-contractile cell types such as connective tissue, adipocytes, etc., but it has long been assumed that these tissues account for no more than a small percentage of the total (44). Finally, it must be pointed out that muscle mass increased in rats from 30 to 120 days of age and that a marked reduction in muscle mass (with the exception of soleus) was observed only in the almost one-year old animals. This finding correlates with the classical observations of GORDON *et al.* (19) and SHORT (40) who indicated that both sarcoplasmic and myofibrillar protein concentration of rat skeletal muscle continues to increase until 140 days of age, accelerating at 60-65 days and then slowing down at 110-115 days of age. The fact that the soleus weight was the same in both young and relatively old rats may be explained by the following considerations. It is well known that skeletal musculature is not homogeneous

in terms of metabolic activity (18, 23). As mentioned above, muscles are usually a mixture of red-oxidative-type I fibers (slow-contractile and predominantly related to the positional and equilibrium movements of the medial-proximal motor system) and white-glycolytic-type IIa and IIb fibers (predominatly related to fast-well coordinated movements of the lateral-distal motor system). Red muscles, such as the soleus, have higher synthesis rates than white muscles (3, 42). However, both type of muscles have basically the same protein concentration, with the difference that type-I fiber muscles contain a higher proportion of myofibrillar and stromal proteins than type II, making the former more resistant to changes in experimental treatments. In fact the overall synthesis rate of myofibrillar proteins has been reported in rat skeletal muscle to be slower than that of sarcoplasmic proteins (4, 10). In this sense, it may be useful to consider that human *in vivo* studies, in which computerized tomography was used, revealed that aging is associated with a reduced cross-sectional area, as well as with a decrease in muscle density, associated, in turn, with increased intramuscular fat. These studies, in addition showed a decrease in both type I (red) and type II (white) fibers, but with the existence of a disproportionate earlier loss in type II fibers with age (10). All this correlates well with the soleus weight found in this study.

It is concluded that aging brings about a noteworthy reduction in the rate of muscle protein turnover in male rats, degradation rates being slightly faster than synthesis reduction. It seems that this effect is somehow independent of the reduced muscle mass observed in the older animals, since urinary output of 3-MH remains low even when expressed per unit of creatinine excreted.

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G. FRÜBECK, B. MUGUERZA, I. CASTILLA-CORTÁZAR y S. SANTIDRIÁN. *Efecto del envejecimiento sobre el turnover de proteínas musculares en rata.* J. Physiol. Biochem. (Rev. esp. Fisiol.), 52 (4), 207-214, 1996.

Se mide el efecto del envejecimiento sobre el turnover proteico muscular en rata, determinando la excreción urinaria de N^t-metilhistidina (3-metilhistidina, 3-MH), como indicador de la velocidad de degradación proteica miofibrilar, y la velocidad de síntesis de proteínas (k_S) mediante la técnica de infusión constante de L-[^{14}C]-tirosina. Se determina también la concentración de ácidos nucleicos en músculo y de corticosterona en plasma. Las ratas recién destetadas se alimentan con una dieta equilibrada (lactalbúmina, 10 %); posteriormente se sacrifican en grupos de 7 a los 35, 60, 120 y 300 días de edad. Aparte del crecimiento, no se encuentran diferencias significativas entre los grupos de 35 y 60 días de edad. Sin embargo, comparando con los animales más jóvenes, las ratas de 120 días de edad muestran una reducción significativa en el peso relativo de cuatro músculos diseccionados. Los niveles plasmáticos de corticosterona aumentan a medida que los animales crecen. Finalmente, en los animales de 300 días de edad, la reducción de la velocidad de crecimiento se acompaña de una reducción significativa del peso de los músculos diseccionados, (excepto en sóleo), 3-MH y k_S. Se concluye que en el proceso de envejecimiento se produce una reducción de las velocidades de síntesis y de degradación proteica que no parece estar relacionada con la disminución de la masa proteica muscular, ya que la 3-MH urinaria permanecía baja incluso cuando se expresa con referencia a creatinina urinaria.

Palabras clave: Envejecimiento, Turnover proteico, 3-Metilhistidina, Rata.

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