Lower recovery of muscle protein lost during starvation on old rats despite a stimulation of protein synthesis

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Lower recovery of muscle protein lost during starvation in old rats despite a stimulation of protein synthesis

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D. ATTAIX, AND P. PATUREAU MIRAND
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Mosoni, L., T. Malmezat, M. C. Valluy, M. L. Houlier, D. Attaix, and P. Patureau Mirand. Lower recovery of muscle protein lost during starvation in old rats despite a stimulation of protein synthesis. *Am. J. Physiol.* 277 (*Endocrinol. Metab.* 40): E608–E616, 1999.—Sarcopenia could result from the inability of an older individual to recover muscle lost during catabolic periods. To test this hypothesis, we compared the capacity of 5-day-refed 12- and 24-mo-old rats to recover muscle mass lost after 10 days without food. We measured gastrocnemius and liver protein synthesis with the flooding-dose method and also measured nitrogen balance, 3-methylhistidine excretion, and the gene expression of components of proteolytic pathways in muscle comparing fed, starved, and refed rats at each age. We show that 24-mo-old rats had an altered capacity to recover muscle proteins. Muscle protein synthesis, inhibited during starvation, returned to control values during refeeding in both age groups. The lower recovery in 24-mo-old rats was related to a lack of inhibition of muscle proteolysis during refeeding. The level of gene expression of components of the proteolytic pathways did not account for the variations in muscle proteolysis at both ages. In conclusion, this study highlights the role of muscle proteolysis in the lower recovery of muscle protein mass lost during catabolic periods.

sarcopenia; aging; proteolysis; flooding dose; 3-methylhistidine

IN HUMANS, alterations in muscle protein metabolism occur during the aging process, leading to a loss of muscle protein or sarcopenia. This sarcopenia is responsible for decreased mobility in elderly people and also reduces their ability to cope with nutritional, infectious, or traumatic stresses (32). Thus it is important to understand the mechanisms of sarcopenia to slow its development.

Sarcopenia results from an imbalance between muscle protein synthesis and degradation. This imbalance occurs over an extended period of time [years in humans and months in rodents where an age-related loss of muscle proteins has also been described at the level of hindlimb muscles (16)]. Thus very slight variations in muscle protein balance each day over a long period could easily account for the amount of protein lost. These small variations will probably be difficult to detect. In rats, no change with age in postabsorptive muscle protein synthesis rates has been detected (24). In humans, total, myofibrillar, and mitochondrial vas-

tus lateralis muscle protein synthesis rates were reported to be lower in old vs. young adults in the postabsorptive state (27, 29, 31). However, it is likely that the age-related loss of muscle proteins also results from a succession of more intense catabolic periods (infections, trauma, and anorexia). Sarcopenia could result from an altered capacity of older individuals to recover muscle proteins lost during catabolic periods. Indeed, refed, undernourished patients aged over 65 yr have been shown to have a lower fat-free mass recovery than those under 65 yr (14). This lower recovery is probably related to a lower stimulation of protein synthesis and/or a lower inhibition of proteolysis during refeeding; these changes are likely to be more easily detected than those involved in the gradual decrease in muscle protein mass (they are probably similar).

Thus, to test if the age-related loss of muscle protein could be related to a lower recovery of muscle mass lost during catabolic periods, we compared the capacity of 12- and 24-mo-old rats to recover from a 10-day starvation-induced loss of muscle proteins. Animals were refed for 5 days, and we analyzed how variations in muscle protein synthesis (measured in vivo with the flooding-dose method) and muscle proteolysis (derived from 3-methylhistidine urinary excretion and gene expression of several components of proteolytic pathways) could explain variations in muscle protein mass.

We have shown that there is a decrease in the ability of 24-mo-old rats to recover muscle mass lost after a catabolic event and that this decrease was related more to a dysregulation of muscle proteolysis than to a lack of stimulation of muscle protein synthesis during refeeding.

MATERIALS AND METHODS

Animals and experimental design. These experiments were performed in accordance with current legislation on animal experimentation in France. We used male Sprague-Dawley rats (obtained from Iffa Credo, Lyon, France) aged 12 or 24 mo at the end of the experimentation. They were maintained in individual metabolism cages at 22°C with a 12:12-h light-dark cycle (lights on from 9 PM to 9 AM) with free access to water; they were weighed daily and were offered a semiliquid casein-based diet containing 18.7 g protein/100 g dry matter, as described previously (23). The animals had free access to food between 9 AM and 5 PM. Food consumption was recorded daily by the measurement of the amounts of dry matter offered and remaining. After a 15-day acclimatization period (weight and food consumption were stable), starvation was initiated. Our aim was to induce a significant loss of muscle proteins to be able to analyze recovery. We first used 4-day starvation, but no significant loss occurred (23). We had to use 10-day starvation to obtain a significant but moderate

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(20%) loss of muscle proteins. Food deprivation was well tolerated by 12- and 24-mo-old rats, and there was no mortality related to food deprivation.

Six groups of animals were used: 1) in 12-mo-old rats, we used control fed rats (n = 6; fed), 10-day-starved rats (n = 6; starvation), and 10-day-starved 5-day-refed rats (n = 5; refed); and 2) in 24-mo-old rats, we used similar groups: fed (n = 6), starved (n = 5), and refed (n = 6).

Measurements of in vivo protein synthesis rates. Protein synthesis rates were measured according to the flooding-dose method (10), which reduces uncertainty over the labeling of the tracer amino acid in the precursor pool for protein synthesis. The method was adapted for mature rats as described previously (23): each rat was injected subcutaneously with a flooding dose of valine (300 μmol/100 g body wt) to flood the precursor pools, with 50% L-[1-^{13}C]valine (Mass-Trace, Woburn, MA). The incorporation time was 40 min. There was a <10% difference between free valine enrichments in plasma, gastrocnemius, and liver (88–89%, 84–87%, and 78–85% of injected valine, respectively). We also demonstrated that free valine enrichment in plasma reached 89% of the plateau value within 5 min after injection and remained stable for more than 45 min (23). These measurements were made between 1:30 PM and 4:30 PM in fed or refed rats (postprandial state) and between 9 AM and 11 AM in starved rats.

A lethal intraperitoneal injection of pentobarbital sodium (Sanofi, Libourne, France) was given just before killing. As soon as anesthesia occurred, the rats were bled. The gastrocnemius, soleus, and tibialis anterior of both hind legs were quickly excised. The liver was cut into small pieces, dipped in cold saline to remove the blood, and wiped. The digestive tract was emptied, cleaned, and dried. All tissues were weighed, frozen in liquid nitrogen, and stored at −20°C or −80°C until analysis.

Free and protein-bound valine enrichment and RNA and protein content were measured in the liver and gastrocnemius as described previously (23).

Nitrogen balance, 3-methylhistidine, and creatinine excretion. Urine and feces were collected, weighed daily, and kept frozen at −20°C until analysis. Urine was collected in tubes containing 5 ml of 1 M HCl. For analysis, urine and feces samples from 1) the 5 days before starvation, 2) the first 4 days of starvation, 3) the last 6 days of starvation, and 4) the first 4 days of renutrition were pooled. Food and feces samples were freeze-dried and ground before analysis. The nitrogen content in urine, food, and feces was measured with the Kjeldhal method, and the nitrogen balance was calculated as the difference between ingested nitrogen and nitrogen excreted in urine and feces. The urine 3-methylhistidine content was measured by liquid chromatography with an automatic amino acid analyzer after deproteinization with 20% trichloroacetic acid and hydrolysis with 6 M HCl at 110°C for 17 h. The urine creatinine content was obtained after reaction with picric acid and measurement of absorbency at 492 nm (kit, Merck, Nogent sur Marne, France). 3-Methylhistidine excretion was then calculated per 100 g initial body weight or per milligrams of creatinine.

Northern blot analysis. To further elucidate the role of proteolysis in the adaptation to starvation and refeeding, we examined the expression of the mRNAs encoding proteinases and cofactors involved in lysosomal (cathepsin D), Ca^{2+}-dependent (m-calpain), and ubiquitin-proteasome-dependent (ubiquitin, 14-kDa ubiquitin carrier protein E2, and C2 and C8 proteasome subunits] proteolytic pathways. Total RNA was extracted from 0.25–0.35 g of tibialis anterior muscle with the Chomczynski and Sacchi (7) method. Twenty micro-

grams of total RNA were then electrophoresed in 1% agarose gels containing formaldehyde and electrophoretically transferred to a nylon membrane (GeneScreen, NEN, Boston, MA). RNA was covalently bound to the membrane by ultraviolet cross-linking. The membranes were hybridized with cDNA probes encoding human cathepsin D and m-calpain, chicken polyubiquitin, and rat 14-kDa ubiquitin-conjugating enzyme E2, and C2 and C8 proteasome subunits as described previously (28). Hybridizations were conducted overnight at 65°C with [^{32}P]cDNA fragments labeled by random priming. After being washed at the same temperature, the membranes were autoradiographed at −80°C with intensifying screens on Hyperfilm-MP (Amersham). Autoradiographic signals were quantified by digital image processing and analysis (National Institutes of Health Image 1.54) and were normalized with the corresponding 18S rRNA signals to correct for uneven loading.

Statistics. Results are given as means ± SE. Two-way ANOVA was performed to discriminate between the effect of age (12- or 24-mo-old rats) and the effect of nutritional state (fed, starved, and refed). The evolution of the nitrogen balance and 3-methylhistidine excretion during starvation and refeeding was analyzed in refed 12- and 24-mo-old animals by repeated-measures ANOVA. When ANOVA detected a significant effect, means were compared by the Student's t-test. The nonparametric Wilcoxon's test was used to compare means for mRNA level measurements. When not otherwise stated, the significance level was set at 0.05. These analyses were performed with the SAS computer program.

RESULTS

Food intake, body, and tissue weights. At the end of the acclimatization period and before any nutritional treatment, food intake was 20.4 ± 0.5, 18.1 ± 0.9, and 19.6 ± 0.7 g dry matter in the three groups of 12-mo-old rats (to be fed, to be starved, and to be refed, respectively) and 17.4 ± 1.0, 18.1 ± 0.7, and 18.7 ± 0.6 g dry matter in the three groups of 24-mo-old rats (to be fed, to be starved, and to be refed, respectively). There was a tendency for a lower food intake in 24-mo-old rats compared with 12-mo-old rats (P = 0.06). After food deprivation and during the first 2 days of refeeding, food intake was lower in refed rats than in control rats in both age groups and then became higher than in control rats (Table 1). The total amount of dry matter consumed during the first 4 days of refeeding was the same in all groups. Food intake recorded in fed and refed rats during the day of killing was lower than on the previous day because the feeding period was shorter (6 vs. 8 h). Food intake on this day was also higher in refed rats than in control fed rats. Food intake during refeeding was similar for 12- and 24-mo-old rats.

At the end of the acclimatization period and before any nutritional treatment, body weights were 698 ± 36, 662 ± 27, and 655 ± 28 g in the three groups of 12-mo-old rats (to be fed, to be starved, and to be refed, respectively) and 686 ± 28, 727 ± 40, and 672 ± 18 g in the three groups of 24-mo-old rats (to be fed, to be starved, and to be refed, respectively). There were no significant differences between groups. After 10 days without food, 12-mo-old rats lost 20% body weight and 24-mo-old rats lost 19%. Refeeding for 5 days produced

Table 1. *Evolution of food intake after starvation in refed rats: comparison with control fed rats*

	12-mo-old Rats		24-mo-old Rats	
	Fed ($n=6$)	Refed ($n=5$)	Fed ($n=6$)	Refed ($n=6$)
R1	20.4 ± 0.5	11.8 ± 0.7*	17.2 ± 1.0	13.4 ± 1.0*
R2	19.4 ± 1.1	17.2 ± 0.3	18.2 ± 1.0	16.9 ± 0.9*
R3	17.9 ± 0.9	20.1 ± 0.9	17.9 ± 0.9	20.0 ± 1.1
R4	19.7 ± 0.9	22.5 ± 0.4*	17.0 ± 1.1	21.8 ± 0.9*
Sum R1–4	74.4 ± 3.8	71.6 ± 1.5	70.2 ± 3.9	72.1 ± 3.8
R5	12.4 ± 1.1	19.9 ± 0.4*	13.6 ± 1.2	18.7 ± 1.3*

Values are means $\pm$ SE. Food intake was measured daily (expressed in g dry matter) during refeeding period from *day 1* (R1) to *day 4* (R4), and total amount consumed from *day 1* through *day 4* (Sum R1–4) was calculated. Sampling was performed on *day 5* (R5) in postprandial state after 6 h of feeding. Therefore, food intake was slightly lower on this day than during previous days when feeding period was 8 h. Data obtained in refed rats were compared with values obtained in control fed rats. *Significantly different from fed group within each age ($P \leq 0.05$). There was no significant effect of age.

a 10–15% increase in body weight (15.0 ± 1.4% in 12-mo-old rats; 10.1 ± 0.8% in 24-mo-old rats).

During starvation, 12-mo-old rats lost 25% gastrocnemius muscle weight (Fig. 1), and recovery was started but not completed after 5 days (+7%). In 24-mo-old rats, loss was similar (−21%) but there was no recovery within 5 days. As previously observed, muscle mass in the 24-mo-old rats was less than in the 12-mo-old rats. The effect of starvation was very marked in the liver and in the digestive tract (Fig. 1). After 10 days without food, liver weight decreased by 49% in 12-mo-old rats and by 34% in 24-mo-old rats. Whatever the age, recovery was complete. At time of death, liver wet weight was not different between 12- and 24-mo-old rats. The results were similar in the digestive tract with a 24% decrease in weight after starvation in 12-mo-old rats (19% in 24-mo-old rats), and there was complete recovery at both ages. The weight of the digestive tract was greater in 24-mo-old rats than in 12-mo-old rats.

Nitrogen balance. Because it was possible to measure nitrogen balance continuously in the same rat, we could take into account individual variability and follow the nitrogen balance in 12- and 24-mo-old refed rats, which were successively fed, starved, and refed. Nitrogen balance became negative during food deprivation, reflecting a loss of total body proteins (Fig. 2). Refeeding induced a restoration of positive nitrogen balance in both groups. However, nitrogen balance was significantly lower during refeeding in 24-mo-old rats compared with 12-mo-old rats. Nitrogen balance became significantly higher during refeeding than during the control period in 12-mo-old rats. We confirmed this significantly lower recovery by calculating the total amount of nitrogen regained during refeeding minus the amount lost during food deprivation in each group (12-mo-old refed rats $= -138 \pm 111$ mg N; 24-mo-old refed rats $= -1,182 \pm 175$ mg N). It should be underlined that recovery in the intestine and the liver (Fig. 1) was complete in 24-mo-old rats. Thus it is likely that it was muscle proteins that recovered less in 24-mo-old rats, which is confirmed by the gastrocnemius muscle results (Fig. 1).

Gastrocnemius protein metabolism. Protein metabolism in the gastrocnemius muscle is shown in Table 2. After 10 days without food, 12-mo-old rats lost 19% protein, which was partially recovered after 5 days of refeeding, reaching 89% of the control value. In 24-mo-old rats, there was a 21% decrease in mean protein mass after starvation and there was no recovery. Protein mass in 24-mo-old rats was less than in 12-mo-old rats in all groups. Adult rats lost 25% of total RNA during starvation, and there was no recovery (+3%). In 24-mo-old rats, variations in total RNA during starvation and refeeding were not significant. Capacity for protein synthesis, which is the ratio of total RNA to total protein, was not modified by nutritional treatments at both ages but was significantly greater in 24-mo-old rats than in 12-mo-old rats only in refed rats. Absolute protein synthesis rates were markedly lower during starvation in 12-mo-old rats (−57%) and to a

Fig. 1. Evolution of tissue weight during starvation and refeeding. Shown are gastrocnemius and liver and digestive tract fresh wt in 12-mo-old fed (fed; $n = 6$), starved (starvation; $n = 6$), and refed (refed; $n = 5$) rats and 24-mo-old fed ($n = 6$), starved ($n = 5$), and refed ($n = 6$) rats. [a,b]Means within same age group with same letter are not significantly different; *values obtained in 24-mo-old rats are significantly different from corresponding values in 12-mo-old rats. Significant effects by ANOVA were age and nutritional state for gastrocnemius and digestive tract wt and nutritional state only for liver.
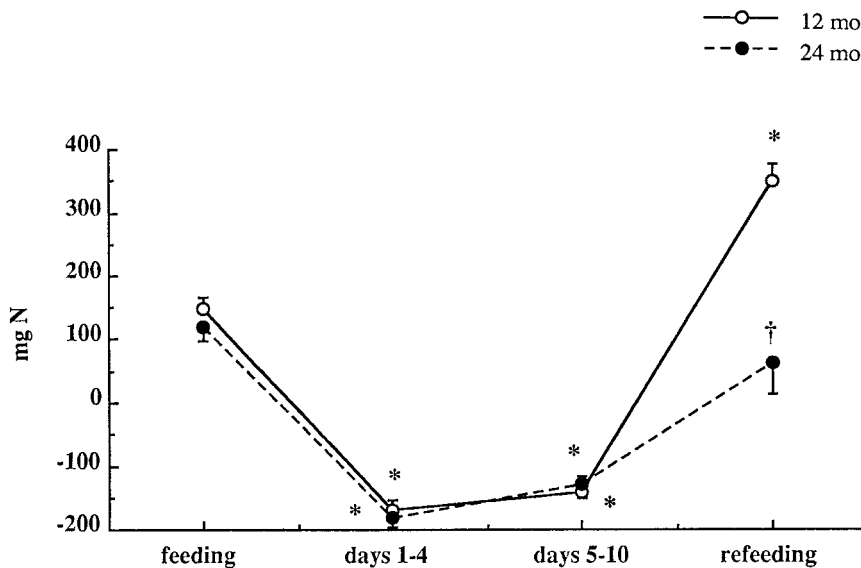
○—— 12 mo
●--- 24 mo
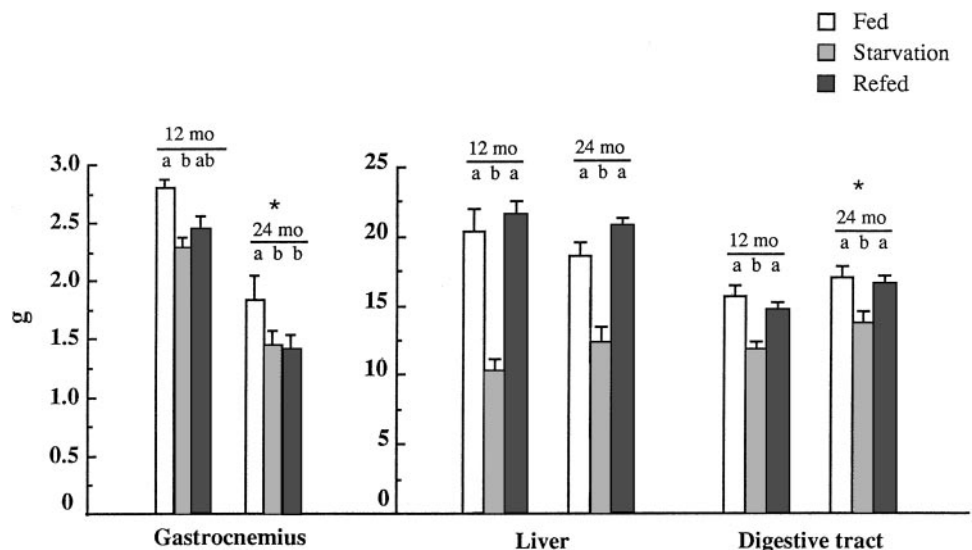
Fig. 2. Evolution of nitrogen balance during starvation and refeeding in 12- and 24-mo-old rats. Nitrogen balance was followed in 12- ($n = 5$) and 24-mo-old ($n = 6$) rats that were food-deprived for 10 days and then refed for 5 days. Mean nitrogen balance was measured during control period (feeding, average of 5 days of measurements), first 4 days of starvation (*days 1-4*), last 6 days of starvation (*days 5-10*), and first 4 days of refeeding (refeeding). Repeated-measures ANOVA showed that effects of age, nutritional treatment, and interaction of age and nutritional treatment were highly significant ($P \leq 0.001$). *Significantly different from control period for each age; †significant effect of age for a given nutritional period ($P \leq 0.05$).

lesser extent in 24-mo-old rats (−35%). Refeeding allowed a return to control values whatever the age. Absolute synthesis rates, when similar at both ages except during starvation when they were significantly higher in 24-mo-old rats. Ribosomal efficiency, which is the amount of protein synthesized per unit RNA and per day, was significantly decreased during starvation in 12-mo-old rats (−37%) and tended to decrease in 24-mo-old rats (−13%). It was restored during refeeding at both ages. Ribosomal efficiency was significantly higher in 24-mo-old rats than in 12-mo-old rats.

Liver protein metabolism. Protein metabolism in the liver is shown in Table 3. Total liver protein mass was markedly lower in starved animals than in control animals for both age groups (−40% in 12-mo-old rats; −46% in 24-mo-old rats. Recovery was good at both ages, but in 24-mo-old rats, liver protein mass reached slightly higher values in refed rats than in control rats (a significant interaction between age and nutritional treatments was detected by ANOVA). A significant effect of age on liver protein mass was also detected.

Starvation markedly affected liver total RNA (−40% in 12-mo-old rats; −52% in 24-mo-old rats). Refeeding induced a partial recovery at both ages. Total RNA was greater in 24-mo-old rats than in 12-mo-old rats. There was no effect of age and nutritional treatment on the capacity for protein synthesis. Liver protein absolute synthesis rates were markedly lower in starved animals than in control animals (−58% in 12-mo-old rats; −52% in 24-mo-old rats). Refeeding restored control values at both ages. Synthesis rates were greater in 24-mo-old rats than in 12-mo-old rats. In 12-mo-old rats, liver ribosomal efficiency was significantly lower in starved rats than in the other groups. In 24-mo-old rats, ribosomal efficiency was significantly higher in refed rats than in the other groups. ANOVA detected no effect of age but a significant interaction between age and nutritional treatment effect.

3-Methylhistidine excretion. 3-Methylhistidine excretion is an index of skeletal muscle proteolysis, although it also derives from the degradation of smooth muscles from the splanchnic area. It can be expressed as a

Table 2. *Effect of starvation and refeeding and age on gastrocnemius muscle protein metabolism*

Parameter	Age	Fed	Starved	Refed	Sign Effects
Total protein, mg	12 mo	522 ± 15^a	424 ± 12^b	462 ± 20^{ab}	age, nutr
	24 mo	$345 \pm 44^{a*}$	$273 \pm 23^{b*}$	$266 \pm 21^{b*}$	
Total RNA, mg	12 mo	2.99 ± 0.14^a	2.23 ± 0.25^b	2.29 ± 0.10^b	age, nutr
	24 mo	$1.98 \pm 0.18^{a*}$	$1.57 \pm 0.15^{a*}$	$1.74 \pm 0.06^{a*}$	
Cs, mg RNA/g prot	12 mo	5.72 ± 0.13^a	5.20 ± 0.35^a	4.97 ± 0.14^a	age
	24 mo	5.90 ± 0.40^a	5.78 ± 0.41^a	$6.72 \pm 0.50^{a*}$	
ASR, mg prot/day	12 mo	25.6 ± 1.7^a	11.0 ± 1.8^b	23.8 ± 1.1^a	nutr
	24 mo	26.2 ± 2.9^a	$16.9 \pm 1.1^{b*}$	23.3 ± 1.0^a	
K_{RNA}, mg prot·mg RNA^{-1}·day^{-1}	12 mo	8.6 ± 0.5^a	5.4 ± 0.8^b	10.4 ± 0.4^a	age, nutr
	24 mo	$13.3 \pm 0.7^{a*}$	$11.6 \pm 1.1^{a*}$	$13.4 \pm 0.4^{a*}$	

Values are means ± SE. Presented are gastrocnemius total protein, total RNA, capacity for protein synthesis (Cs), absolute (ASR) synthesis rates, and ribosomal efficiency (K_{RNA}, amount of protein synthesized per day per mg RNA), in 12-mo-old fed (fed; $n = 6$), starved (starved; $n = 6$), refed (refed; $n = 5$) rats and 24-mo-old fed ($n = 6$), starved ($n = 5$), and refed ($n = 6$) rats. Data were analyzed by two-way ANOVA to discriminate between effects of age and nutritional state (nutr): significant effects are noted in table. [a,b] Means within same age group with same letter are not significantly different; *values obtained in 24-mo-old rats are significantly different from corresponding values in 12-mo-old rats.

Table 3. *Effect of starvation and refeeding and age on liver protein metabolism*

| Parameter	Age	Fed	Starved	Refed	Sign Effects
Total protein, g	12 mo	3.09 ± 0.24^a	1.86 ± 0.18^b	2.60 ± 0.11^a	age, nutr
	24 mo	3.51 ± 0.13^a	1.90 ± 0.10^b	$3.60 \pm 0.16^{a*}$	age × nutr
Total RNA, mg	12 mo	119 ± 11^a	71 ± 11^b	100 ± 6^{ab}	age, nutr
	24 mo	$157 \pm 12^{a*}$	76 ± 9^b	115 ± 13^c	
Cs, mg RNA/g prot	12 mo	38.6 ± 1.8^a	37.4 ± 1.9^a	38.3 ± 1.0^a	
	24 mo	44.5 ± 2.1^a	39.8 ± 3.1^{ab}	32.2 ± 4.0^b	
ASR, g prot/day	12 mo	1.71 ± 0.15^a	0.72 ± 0.05^b	1.58 ± 0.12^a	age, nutr
	24 mo	1.98 ± 0.14^a	0.95 ± 0.12^b	$2.13 \pm 0.11^{a*}$	
K_{RNA}, mg prot·mg RNA^{-1}·day^{-1}	12 mo	14.4 ± 0.7^{ab}	11.9 ± 0.6^b	15.9 ± 0.8^a	nutr
	24 mo	12.7 ± 0.6^a	12.5 ± 0.6^a	$19.5 \pm 2.0^{b*}$	age × nutr

Values are means ± SE. See Table 2 for explanation of abbreviations. [a,b] Means within same age group with same letter are not significantly different; *values obtained in 24-mo-old rats are significantly different from corresponding values in 12-mo-old rats.

fractional rate, per milligrams of creatinine, an index of muscle mass (Fig. 3, *top*), or as an absolute rate, per body weight. Here, we used initial body weight (Fig. 3, *bottom*) to take into account variations in body weight during starvation. In the same way as for nitrogen balance, we measured 3-methylhistidine and creatinine excretion in refed animals. 3-Methylhistidine excretion tended to decrease during starvation, in particular during the first few days. This decrease was significant only for 12-mo-old rats when 3-methylhistidine was expressed per 100 g initial body weight. During refeeding, 3-methylhistidine excretion was either unchanged or decreased compared with control values in 12-mo-old rats. In 24-mo-old rats, regardless of the method of expression, 3-methylhistidine excretion was always significantly higher during refeeding than during the control period (the interaction between age and nutritional state effects was significant). Thus the effect of refeeding on 3-methylhistidine excretion was significantly different in 12- and 24-mo-old rats.

3-Methylhistidine excretion adjusted to creatinine excretion was significantly higher in 24-mo-old rats compared with 12-mo-old rats. This was not the case when 3-methylhistidine excretion was adjusted to initial body weight. This is related to the lower creatinine excretion in 24-mo-old rats, reflecting a loss of muscle mass.

Expression of components of proteolytic pathways. There was no marked effect of nutritional treatments on mRNA levels for various critical components of proteolytic pathways (Fig. 4). These levels tended to increase during starvation; the increases were significant for ubiquitin, 14-kDa E2, m-calpain in 12-mo-old rats, and for cathepsin D in 24-mo-old rats. Values obtained in refed rats were similar to values obtained in control rats in all cases. Regarding the effect of age (Fig. 5), there was often a tendency for an increase with age, but this increase was significant only for the C2 subunit of the 20 S proteasome.

Fig. 3. Evolution of 3-methylhistidine excretion during starvation and refeeding in 12- and 24-mo-old rats. Shown is 3-methylhistidine excretion adjusted to creatinine excretion (*top*) or to initial body wt (IBW, *bottom*) during same time periods of nitrogen balance (see Fig. 2). Repeated-measures ANOVA indicates that for 3-methylhistidine excretion adjusted to creatinine excretion, effects of age ($P = 0.06$), nutritional state ($P = 0.07$), and interaction between age and nutritional treatment ($P = 0.04$) were significant; for 3-methylhistidine excretion adjusted to initial body wt, only effects of nutritional state ($P = 0.001$) and interaction between age and nutritional treatment ($P = 0.05$) were significant. *Significantly different from control period for each age ($P \leq 0.05$); $ significantly different from control period for each age ($P \leq 0.08$); †significant effect of age for a given nutritional period ($P \leq 0.05$).
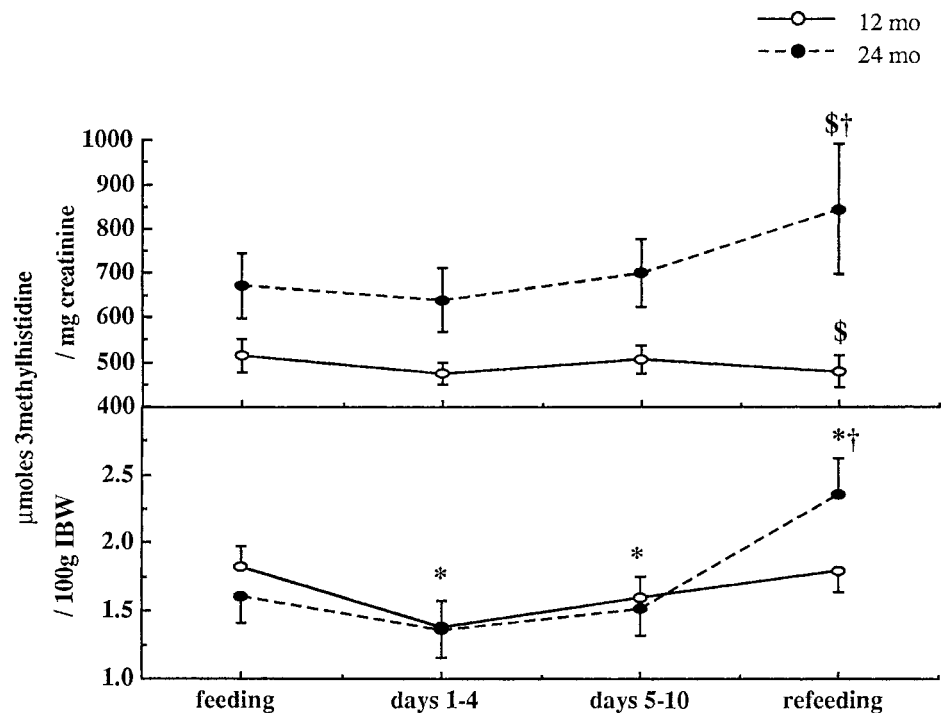
DISCUSSION

The loss of muscle protein observed during senescence could be related to an inability of older individuals to recover muscle mass lost during catabolic periods. To test this hypothesis, we analyzed how 12- and 24-mo-old rats could recover from a food deprivation-induced loss of muscle mass and examined whether variations in muscle protein synthesis and degradation
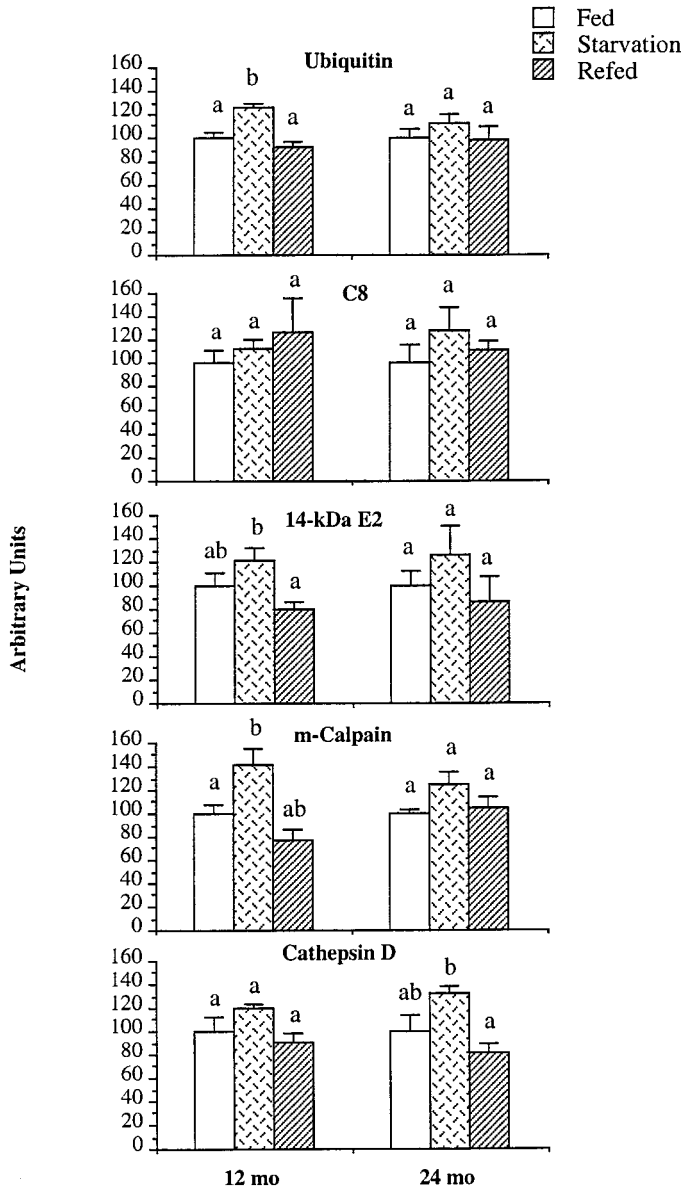

Fig. 4. Evolution of mRNA levels for various proteolytic genes in tibialis anterior muscle during starvation and refeeding in 12- and 24-mo-old rats. mRNA levels were measured for ubiquitin, C8 subunit of 20 S proteasome, 14-kDa E2, m-calpain, and cathepsin D in 12-mo-old fed (fed), starved (starvation), and refed (refed) rats and 24-mo-old fed, starved, and refed rats. Densitometric signals are expressed as a % of 18 S rRNA signal: values for fed rats at each age were given value 100, and values obtained in other groups were expressed as a % of control. [a,b] Means within a same age group affected with same letter are not significantly different with nonparametric Wilcoxon's test ($P \leq 0.05$). Data for 12- and 24-mo-old rats presented in this figure were obtained from different membranes and therefore are not comparable.
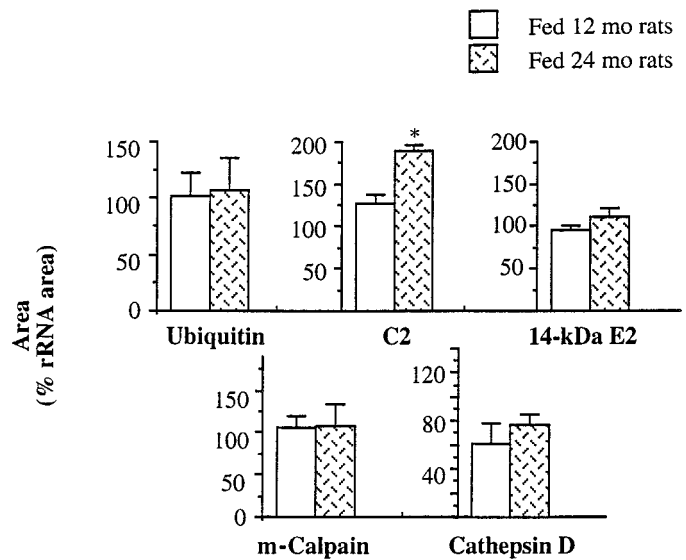

Fig. 5. Effects of age on mRNA levels for various proteolytic genes in tibialis anterior muscle in 12- and 24-mo-old fed rats. mRNA levels were measured for ubiquitin, C2 subunit of 20 S proteasome, 14-kDa E2, m-calpain, and cathepsin D in 12- and 24-mo-old control fed rats. Values are means ± SE for 2–4 rats of densitometric signals expressed as a % of 18 S rRNA signal. *Age effect was analyzed with nonparametric Wilcoxon's test ($P \leq 0.05$).

could explain variations in muscle mass in both age groups.

Our data showed that the capacity to recover lost muscle mass is impaired in old rats: *1*) 5 days of refeeding 10-day-starved 24-mo-old rats did not induce any increase in gastrocnemius muscle fresh weight and total protein. In 12-mo-old rats, gastrocnemius muscle weight and total protein increased during refeeding to a value not significantly different from control fed rats. *2*) The amount of body protein recovered during refeeding, as reflected by nitrogen balance, was significantly and markedly higher in 12-mo-old rats than in 24-mo-old rats. However, recovery was the same at both ages in liver and digestive tract. Muscle mass represents the predominant proportion of body protein. Thus it seems very likely that the lower recovery of body proteins detected by nitrogen balance represents a lower recovery of body muscle protein.

To our knowledge, no studies have compared the recovery capacities of 12- and 24-mo-old rats after prolonged periods of food deprivation. However, using dietary protein depletion to induce a decrease in muscle mass, Carter and Lynch (5) showed that hindlimb muscle mass was still unchanged compared with depleted animals after 3 wk of protein refeeding in 24-mo-old rats, whereas it was significantly higher in 3-mo-old rats. An age-related impaired capacity to recover muscle mass lost during catabolic periods has also been described in humans (14).

This lower capacity of muscle mass recovery after fasting in 24-mo-old rats could be related to alterations either in muscle protein synthesis or in muscle protein degradation pathways. In our study, muscle protein synthesis was stimulated to a similar extent in 12- and 24-mo-old refed rats by 5 days of refeeding. This

stimulation of muscle protein synthesis did not occur in the first hours of refeeding (23), and it is still possible that this stimulation occurred more slowly in 24-mo-old rats. However, based on protein measurements at *day 5*, it seems that the lower recovery of muscle proteins in 24-mo-old rats was not related to a lack of stimulation of muscle protein synthesis.

Because muscle protein mass is controlled by the balance between protein synthesis and degradation, if the lower recovery is not related to age-related differences in muscle protein synthesis, it must be related to protein degradation. Indeed, the muscle proteolysis response to refeeding, as reflected by 3-methylhistidine excretion, was significantly different in 12- and 24-mo-old rats.

3-Methylhistidine excretion represents the myofibrillar component of muscle protein degradation and may also reflect turnover of 3-methylhistidine in the splanchnic region; the proportion of urine 3-methylhistidine originating from skeletal muscle was estimated to be from 50% (22) to 85% (4). Thus it is only an index of muscle proteolysis. It can be expressed either per milligrams of creatinine, an index of muscle mass, or per body weight. Expressed in either way, it was significantly higher during the refeeding period than during the control period in 24-mo-old rats, thus preventing muscle mass recovery. On the contrary, in 12-mo-old rats it was decreased (when expressed per mg creatinine) or unchanged (when expressed per 100 g initial body wt). Thus the effect on 3-methylhistidine excretion of refeeding was significantly different in 12- and 24-mo-old rats.

The tendency of a lower 3-methylhistidine excretion per milligrams of creatinine in 12-mo-old rats could have been related to overeating during refeeding. However, overeating was similar in refed 12-mo-old rats and refed 24-mo-old rats. To our knowledge, no studies have compared the rate of 3-methylhistidine excretion in 12- and 24-mo-old rats during starvation refeeding. However, a decrease in 3-methylhistidine excretion during refeeding after fasting has been described in 320-g rats (26) and in adult women (15). Other studies with younger animals, less resistant to food deprivation, showed that refeeding allowed a return to control values after increased excretion during starvation (12, 25). Thus the results that we obtained are consistent with results obtained in other studies.

It must be noted that 3-methylhistidine excretion per milligrams of creatinine, which is equivalent to a fractional degradation rate, was higher in 24-mo-old rats than in 12-mo-old rats whatever the group. Similarly, fractional synthesis rates (data not shown) were higher in 24-mo-old rats than in 12-mo-old rats. We propose that these higher fractional rates are mainly due to the age-related loss of muscle mass (lower creatinine excretion and lower muscle protein mass). Similar results for fractional synthesis rates have been obtained previously (24). As discussed in this paper, it is not clear whether this represents a true increase in muscle protein turnover or if this is a bias due to the calculation. Both 3-methylhistidine excretion per ini-

tial body weight and gastrocnemius absolute synthesis rates were unchanged with age.

These results are not consistent with data obtained in humans where total, myofibrillar, and mitochondrial vastus lateralis muscle protein synthesis rates were reported to be lower in old vs. young adults in the postabsorptive state (27, 29, 31). This discrepancy could be related to a species difference. However, human measurements were performed with a small sample of the same muscle and need to be confirmed in other muscles or with different techniques. Indeed, most human studies have not been able to detect a decrease with age in whole body protein synthesis rates per kilogram fat-free mass (2).

The effect of starvation on muscle protein metabolism was similar in 12- and 24-mo-old rats. Mobilization of muscle proteins was similar; muscle protein synthesis was inhibited, and 3-methylhistidine excretion tended to be reduced at both ages. Such a sparing of muscle during starvation through an inhibition of both protein synthesis and degradation has already been described (9, 11, 13, 18, 19, 23). Our results are characteristic of the effect of long-term fasting in moderately fat mature animals (fat content is 15% body wt in 12-mo-old rats, 20% body wt in 24-mo-old rats; unpublished data). There was no effect of starvation on the oxidative soleus protein mass (data not shown). This is consistent with results obtained previously (1, 23). Glycolytic or mixed muscles, which are predominant in the body, are more sensitive to starvation than oxidative muscle.

The increase in 3-methylhistidine excretion during refeeding in 24-mo-old rats was not related to a higher expression of mRNA encoding the components of proteolytic pathways (ubiquitin, C8, 14-kDa E2, m-calpain, and cathepsin D); it was unchanged or slightly lower in the refed group compared with the control group. Similarly, there was a small but significant increase for ubiquitin, 14-kDa E2, and m-calpain mRNA during starvation in 12-mo-old rats, whereas 3-methylhistidine excretion was unchanged or decreased in these animals. This could be related to the fact that 3-methylhistidine excretion reflected the mean of several days, whereas mRNA expression was measured on the 10th day of starvation and on the 5th day of refeeding. On the contrary, in young growing rats and 2-kg rabbits, there was a good correlation between the observed increase in protein degradation (measured in vitro or with 3-methylhistidine excretion) during starvation and variations of mRNA expression for proteasome subunits, ubiquitin, and 14-kDa E2 (21, 30) or for μ-calpain, m-calpain, cathepsin D, and proteasome C2 subunit (17). It is possible that the level of regulation of proteolysis may be different in older rats that are less sensitive to starvation. Indeed, Belkhou et al. (3) showed that muscle cathepsin B, H, and L activities fell during phase II of starvation during which protein degradation is inhibited in 375-g rats.

Liver protein mass and protein synthesis were markedly decreased during starvation and returned to fed values after refeeding at both ages. Overeating in refed

rats probably helped the recovery. Similar results were obtained previously with 4-day starvation (23). With longer starvation (10 days), an additional 8% loss of protein and an additional 26% loss of RNA occurred. In studies with similar methodologies, a decrease in liver protein synthesis during starvation has also been obtained (6, 20). However, De Blaauw et al. (8) with the arteriovenous dilution model obtained an increase in liver protein synthesis of 112-h starved 200- to 300-g rats. The reasons for this discrepancy between incorporation studies and the arteriovenous dilution model are still unclear.

Recovery of liver protein was favored relative to muscle protein. In 24-mo-old rats, recovery of liver and muscle proteins seemed better and worse, respectively, than in 12-mo-old rats. Whether the greater recovery of liver proteins leads to the lesser recovery of muscle proteins in 24-mo-old rats by decreasing amino acid availability for muscle (or vice versa) remains an open question.

In conclusion, the capacity of muscle to recover proteins lost during starvation seems to be impaired in 24-mo-old rats. Although we cannot exclude the possibility of a slower stimulation of protein synthesis during refeeding, after 5 days, the lower recovery seems to be mainly due to a different regulation of muscle protein degradation, which is stimulated during refeeding. Further studies are necessary to understand the mechanisms responsible for this dysregulation of muscle protein degradation.

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