

Systemic low-grade inflammation does not decrease skeletal muscle mass and protein synthesis in old rats

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Abstract

Age-associated low-grade systemic inflammation may contribute to sarcopenia. We hypothesized that skeletal muscle mass and protein synthesis rate would be reduced in old rats exhibiting persistent low-grade inflammation compared to age-matched controls. Male 24-month-old Wistar rats exhibiting a low-grade systemic inflammation for at least one month (LGI group) were compared to non-enflamed rats (C group). Tissue protein synthesis rates were quantified using the L-[1-¹³C]-valine flooding dose method. Body weight, gastrocnemius muscle and spleen weights were not significantly different between groups, but liver and small intestine weights were 13 and 14% higher in LGI than in C. Fractional and absolute protein synthesis rates were not significantly different between groups for gastrocnemius, spleen and small intestine, but higher for liver in LGI than in C. Despite an increase in liver protein synthesis, low-grade inflammation did not reduce skeletal muscle mass, suggesting that age-associated low-grade systemic inflammation occurs independently of sarcopenia.

Keywords: Acute Phase Proteins, Aging, Liver, Protein Synthesis Rate, Skeletal Muscle

Introduction

Skeletal muscle mass is gradually lost during aging, a process referred to as sarcopenia¹. Low-grade inflammation, which also appears with aging, has been suggested to contribute to sarcopenia²⁻⁴.

Age-associated low-grade systemic inflammation is characterized by an increase in circulating inflammatory parameters in a lower range than in acute inflammation^{5,6}. It results from a dysregulation in *in vivo* cytokine production⁷. Accordingly, plasma concentrations of acute phase proteins (APP) such as C-reactive protein (CRP), fibrinogen and α_1 -acid glycoprotein increase, whereas concentration of the negative APP albumin consistently decreases during healthy aging in humans⁸⁻¹⁰.

In elderly populations, higher levels of inflammatory bio-

markers are associated with lower muscle mass and lower muscle strength¹¹⁻¹⁶. Older women with high interleukin-6 levels have a higher risk of developing a physical disability and experience a steeper decline in walking ability compared to those with lower levels¹⁷. These observations raise the question of a possible link between low grade-inflammation and sarcopenia in the elderly.

Age-associated inflammation may affect skeletal muscle protein metabolism based on the well-established negative effect of acute and chronic inflammation on skeletal muscle mass and protein metabolism¹⁸⁻²⁰. TNF- α , which is one of the main inducers of the acute phase response²¹, plays an important role in alterations of muscle protein metabolism in the model of sepsis²². TNF- α impairs skeletal muscle protein synthesis, at least in part, by decreasing mRNA translational efficiency resulting from impairment in translation initiation²³.

A recent study including young and old, female and male, volunteers shows that levels of circulating inflammatory biomarkers were negatively correlated with the rate of skeletal muscle protein synthesis²⁴. This study supports the idea that age-associated low-grade inflammation affects skeletal muscle metabolism in a way that would lead to sarcopenia. However, it does not prove the causal relationship within the old population since the study included young and old subjects. The correlation may be independent of inflammatory

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status within the old subjects and more directly related to age.

We hypothesized that, if age-associated low-grade systemic inflammation decreases skeletal muscle mass, then muscle mass and protein synthesis rate are lower in old rats exhibiting persistent inflammation compared to non-inflamed age-matched controls. Such stratification is realistic since a wide range of inflammation occurs among aged individuals^{17,25}. The inflammatory status of old rats can be graded on the basis of plasma fibrinogen. Indeed, in the rat, CRP has been shown to be a poor biomarker of inflammation compared to fibrinogen²⁶ and circulating TNF- α and interleukin-6 were not detectable (ultra sensitive tests not available) in old low-grade inflamed rats²⁵. Moreover, we have recently shown that fibrinogen is a predictor of persistent inflammatory status in old rats and demonstrated a relationship between fibrinogen and TNF- α receptor-I²⁵, which is now recognized as a better biomarker of inflammation than TNF- α itself²⁷. The aim of the present study was to quantify muscle mass and protein synthesis in old rats with or without persistent low-grade inflammation. Performing this investigation in old rats from the same cohort instead of in elderly humans allows us to discard confounding factors that are known to modulate skeletal muscle mass and protein synthesis: age, environment and behavioural habits, specifically diet, physical activity (type and intensity), smoking and/or medication^{28,29}.

Materials and methods

Animals

Male Wistar rats were produced and bred in our conventional (not specific-pathogen-free) animal facility (Unité Expérimentale de Nutrition Comparée, INRA, Saint-Genès-Champanelle, France). They were maintained in collective cages (3 to 4 rats per cage) under controlled conditions (temperature 21 °C, relative humidity 55%, 12-h light/dark cycles) with free access to water and standard pelleted food (A04 from Scientific Animal Food and Engineering, Villemoisson-sur-Orge, France). The composition of the diet was 16% protein, 3% fat, 60% carbohydrates, 12% water, fibers, vitamins and minerals (diet contained 0.83% calcium and 0.59% phosphorus). The study was performed according to the current legislation on animal experimentation in France.

Experimental design

The experiment was performed with male Wistar rats at the age of 24 months, which corresponds to their mean life expectancy³⁰. Rats were maintained in their breeding conditions for a one-month follow-up period, during which skeletal muscle mass decreases³⁰⁻³³. The absence or the presence of low-grade inflammation were appreciated on the basis of plasma fibrinogen levels observed in adult and old rats²⁵. The absence of inflammation corresponds to plasma fibrinogen levels in the range of the mean value $\pm 2SD$ observed for 8-

month-old male Wistar rats i.e., mean=3.0, SD=0.3 g/L. Low-grade inflammation corresponds to fibrinogen levels above the highest reference value i.e., 3.6 g/L and below a pathological cut-off set at 5.4 g/L. Indeed, fibrinogen levels higher than 5.4 g/L are predictive of an increased rate of mortality in old rats. Inflammation status was presently based on plasma fibrinogen levels measured from blood withdrawn at the age of 24.0, 24.5 and 25.0 months. None of the rats exhibited obvious pathologies (tumor, infectious abscess) or a variation in fibrinogen level between two measurements higher than 33%. Control group (C) and low-grade inflamed group (LGI) consisted of 11 and 8 rats, respectively. At the age of 25 months, masses and protein synthesis rates were measured in skeletal muscle, in liver, which is the site of APP synthesis, and in small intestine and spleen, which also play a role in the inflammatory process due to their secondary lymphoid cells.

Measurement of *in vivo* protein synthesis

Rates of protein synthesis were measured in tissues using the flooding dose method³⁴. In the morning, a flooding dose (150 μ mol/100 g body weight, 0.37 ml/100 g body weight, 80 atom % excess) of L-[1-¹³C]-valine (99 atom % excess, Eurisotop, group CEA, Saclay, France) was injected into a lateral tail vein, at the beginning of the lighted period. General anesthesia was induced by intraperitoneal injection of pentobarbital (6 mg/100 g body weight, 0.1 ml/100 g body weight, Sanofi, Libourne, France) 5 minutes before killing. Rats were exsanguinated by sampling from abdominal aorta 20 minutes after valine injection. A 50 μ L aliquot of blood was collected for white blood cell counting. Plasma was separated by centrifugation and kept at -80 °C until measurements of free [¹³C]valine enrichment and APP levels. The liver, the whole intestine, from duodenum to colon, the spleen, and the gastrocnemius muscle were quickly removed. Intestine was flushed with ice-cold NaCl (9 g/L). All tissues were blotted dry, weighed, frozen in liquid nitrogen and stored at -80 °C until analysis. Soleus, extensor digitorum longus (EDL) and tibialis anterior muscles and thymus were removed and their masses measured.

Frozen tissues were powdered in liquid nitrogen in a ball mill (Danguomeau, Prolabo, Paris, France). Measurements of free and protein-bound [¹³C]valine enrichments were measured according to Mercier et al.³⁵ with minor modifications. A ~ 0.5 g aliquot of frozen tissue powder or ~ 0.2 mL of plasma was homogenized in 8 volumes of ice-cold 0.6 mol/L trichloroacetic acid. Enrichment of [¹³C]valine into protein was measured as its N-acetyl-N-propyl derivative by gas chromatography-combustion-isotope ratio mass spectrometry (Hewlett Packard 5890 series II gas chromatograph interfaced with a MAT 252 isotope ratio mass spectrometer (Thermo Electron, Bremen, Germany)). Nitrogen contents of gastrocnemius muscle, liver, small intestine and spleen were determined by the Kjeldahl method, and protein was calculated as N x 6.25.

Variables (means±SEM)	Age (month)	Group	
		Control	Low-grade inflamed
Fibrinogen (g/L)	24.0	3.0±0.2	4.3±0.3‡
	24.5	3.2±0.1	4.7±0.3‡
	25.0	3.1±0.1	4.5±0.3‡
α_2 -macroglobulin (mg/L)	25.0	19±5	83±18†
α_2 -acid glycoprotein (mg/L)	25.0	99±5	109±24
Albumin (g/L)	25.0	20±1	18±1*
White blood cells (10 ⁶ cells/ml)	25.0	2.8±0.3	3.9±0.4*

* $P \leq 0.05$, † $P \leq 0.002$, ‡ $P \leq 0.0003$ vs. control group, n=11 for control group and 8 for low-grade inflamed group.

Table 1. Inflammatory status of control and low-grade inflamed groups.

Calculations

The fractional synthesis rates in tissues (FSR, defined as the percentage of tissue protein synthesized each day, i.e., %/d) were then calculated as follows: $FSR = 100 \times (Sb - Sb_0) / (Sa' \times t)$, where t is the time interval between the end of the bolus injection and the killing of the rats (incorporation period expressed in day), Sb_0 is the mean basal enrichment of protein-bound [¹³C]valine from four additional rats fed with the same diet and without receiving tracer injection (atom% excess), Sb is the enrichment of protein-bound [¹³C]valine at the end of the incorporation period, and Sa' is the enrichment of free [¹³C]valine calculated at a time halfway between injection and killing, to take into account the decline in Sa during measurement³⁵. The mean Sa' enrichment was the $Sa(t_{1/2})$ value calculated from the linear regression obtained in tissue between the time 0 and time t . The absolute synthesis rate (ASR) was calculated from the product of FSR and protein content of the tissue and expressed in milligrams per day.

Plasma acute-phase protein and white blood cells

Plasma fibrinogen level was measured by turbidimetry³⁶ on a Cobas Mira analyzer (ABX Diagnostics, Montpellier, France). Concentration is expressed as g human equivalent/L since human fibrinogen was used as a reference (Ingen, Rungis, France). Variability of the method was 7%, intra-group and inter-group variability 10 and 14 %, respectively. Plasma levels of α_2 -macroglobulin, albumin and α_1 -acid glycoprotein were measured by single radial immunodiffusion¹⁹. White blood cell count was determined on scil Vet abc (scil animal care company, Holtzheim, France).

Statistical analysis

Values are given as means±SEM, n=8-11. The significance of differences was analyzed using the Student's t-test

for unpaired data. P values ≤ 0.05 were considered significant. All statistical analyses were performed using StatView for Windows, version 5 software (SAS Institute, Cary, NC).

Results

Old rats' inflammatory status

As anticipated, fibrinogen level was 44-48% higher in LGI than C groups all over the one-month follow-up, and remained stable within each group. At the end of the follow-up, the number of white blood cell counts and α_2 -macroglobulin concentration were 38 and 326% greater, respectively, while plasma albumin concentration was 12% lower in LGI than C groups (Table 1). Plasma α_1 -acid glycoprotein concentration was not different between the groups.

Body and tissue weights

Body weights of C and LGI groups of rats were similar and did not change during the follow-up period (Table 2). Gastrocnemius, tibialis anterior, EDL and soleus muscles masses did not differ between the two groups (Table 2). Liver, small intestine and spleen weights were 13, 14 and 13% higher in LGI than C groups, respectively (Table 2). However the difference in spleen weight did not attain statistical significance. Thymus weights were not different between the groups. Relative weights of liver and small intestine were also higher in LGI than in C groups, 10 and 11%, respectively (data not shown). Gastrocnemius, liver, small intestine, and spleen total protein contents were not significantly different between the groups (Table 3).

Tissue protein synthesis

Fractional protein synthesis rates in gastrocnemius muscle, spleen and small intestine were similar in both groups (Table 3). By contrast, liver fractional protein synthesis was

Variables (means±SEM)	Group	
	Control	Low-grade inflamed
Body weight at 24 months (g)	666±11	670±18
Body weight at 25 months (g)	658±13	674±18
Gastrocnemius weight (g)	2.83±0.07	2.86±0.10
Tibialis anterior weight (mg)	985±20	996±29
EDL weight (mg)	236±5	237±6
Soleus weight (mg)	186±7	181±13
Liver weight (g)	19.7±0.4	22.3±1.2*
Small intestine weight (g)	10.4±0.3	11.8±0.4†
Spleen weight (g)	1.33±0.11	1.52±0.14
Thymus weight (mg)	418±32	422±35

* $P\leq 0.05$, † $P\leq 0.01$ vs. control group, n=11 for control group and 8 for low-grade inflamed group, EDL=extensor digitorum longus.

Table 2. Body and tissue weights of control and low-grade inflamed group.

Tissue	Variables (means±SEM)	Group	
		Control	Low-grade inflamed
Gastrocnemius	Protein (mg)	636±16	648±26
	FSR (%/d)	4.39±0.12	4.45±0.17
	ASR (mg/d)	27.9±0.7	28.7±1.2
Liver	Protein (g)	3.34±0.07	3.59±0.17
	FSR (%/d)	55.7±0.9	63.3±3.0*
	ASR (g/d)	1.86±0.06	2.27±0.14†
Small intestine	Protein (g)	1.51±0.05	1.75±0.14
	FSR (%/d)	70.3±1.8	70.9±3.3
	ASR (g/d)	1.07±0.05	1.25±0.13
Spleen	Protein (mg)	273±25	310±31
	FSR (%/d)	26.4±1.6	26.7±1.7
	ASR (mg/d)	68.1±4.3	80.0±5.3

* $P\leq 0.02$, † $P\leq 0.01$ vs. control group, n=11 for control group and 8 for low-grade inflamed group, FSR=fractional protein synthesis rate, ASR=absolute protein synthesis rate.

Table 3. Gastrocnemius, liver, small intestine, and spleen protein synthesis rates in control and low-grade inflamed groups.

higher in LGI than C groups. Gastrocnemius muscle and small intestine ASR did not differ between the groups, whereas liver and spleen ASR were greater (22%, $P<0.01$; 17%, $P<0.1$; respectively) in LGI than C groups.

Discussion

The present data indicate that age-associated low-grade systemic inflammatory state lasting for at least one month did not alter gastrocnemius muscle mass and protein synthesis rates in old rats. The inflammation grade was based on the

level of fibrinogen, which is a powerful biomarker of inflammation in old rats, compared to CRP or circulating cytokines^{25,26}. Rats belonging to the C group were not inflamed all over the one-month follow-up period since, as anticipated, their mean fibrinogen levels were never different from mean values previously observed for both adult rats and the first quartile of a cohort of old rats²⁵. Rats belonging to the LGI group definitively exhibited a persistent age-associated low-grade systemic inflammation and not an acute (disease-related) inflammation state. Firstly, their mean fibrinogen level was stable over the one-month follow-up period.

Secondly, it was different from the adult reference but in the range of the third quartile of a cohort of old rats²⁵. The LGI group exhibited higher plasma fibrinogen and α_2 -macroglobulin levels, and lower plasma albumin levels than the C group. The fact that α_1 -acid glycoprotein did not differ between LGI and C groups is consistent with the absence of any increase in this APP in old rats as compared to adult rats³⁷. Plasma fibrinogen, α_2 -macroglobulin and albumin levels have been shown to be very variable in old rats and can reach extreme values. However, values observed in the present study in the LGI group were different from values that have been shown to be predictors of increased mortality rate²⁵. The magnitude of the differences in APP between LGI and C groups was much less than reported in acute inflammation^{19,38} and also less than in chronic inflammation³⁵. For example, the plasma α_2 -macroglobulin level in low-grade inflamed old rats was less than half of its level in chronic inflamed rats³⁵. APP variations in the present study are consistent with those due to age-associated inflammation reported in humans. Indeed, plasma fibrinogen concentration were $\sim 47\%$ greater and plasma albumin concentration $\sim 12\%$ lower in old (66-76 years) than in young (22-26 years) healthy subjects³⁹. We have recently shown that increased fibrinogen and α_2 -macroglobulin levels to the same range as in LGI group²⁵ is associated with increased levels of soluble TNF- α receptor-1. Thirdly, weight differences observed for the liver, where APP are synthesized, and for the spleen and small intestine, which contain secondary lymphoid cells, are consistent with the presence of an inflammation in LGI rats¹⁹. By contrast to acute and chronic inflammations, the present inflammation did not induce body weight loss during the one-month follow-up. Finally, the increase in white blood cells in LGI suggests a low-grade inflammation, since its magnitude was lower than those observed in experimental models of chronic inflammation in rats^{10,35}. The etiology of age-associated inflammation occurring in the LGI group and the precise age at which it started are unknown, but it is not a critical point for our study (see below). As in humans^{6,7}, this inflammation could result from aging *per se* or from environmental factors, namely the breeding conditions of rats.

Low-grade inflamed rats were compared to age-matched rats issued from the same cohort and breed in the same conditions. This freed us from potential confounders that are present in many elderly human studies and complicate the statistical analysis. It is important to compare age-matched rats to avoid assessing the effect of age *per se*, instead of the effect of systemic inflammation. Indeed, age-associated low-grade inflammation is more important in older subjects^{12,40} and it is frequent to compare groups with different ages. Because muscle composition and metabolism are known to be modified during aging, it is imperative to compare groups of age-matched individuals to assess the effect of low-grade inflammation on muscle. Moreover, body weight, which was stable during the one-month follow-up, did not differ between LGI and C groups. Thus, the two present groups of rats differed only by their systemic inflammatory status.

As anticipated, the rats with age-associated low-grade inflammation were associated with the higher liver fractional and absolute protein synthesis rates. This occurred without difference in total liver protein content suggesting that an increase in the synthesis of exported protein, namely APP, occurred. The magnitude of these effects was comparable to those observed in experimental models of chronic inflammation in rats^{10,35}. Fractional protein synthesis rates in the spleen and small intestine were not different between groups, while they are reported to increase in acute and chronic inflammation^{19,35}. Thus, the regulation of protein synthesis in these tissues appears to be less sensitive to systemic inflammation than in the liver.

We did not observe any effect of age-associated low-grade systemic inflammation on muscle mass or fractional protein synthesis rate. Consequently the absolute protein synthesis rate was similar in the muscles of the two groups. This is consistent with the fact that body weights were stable during the follow-up period. Taking into account size and variability of groups, we could significantly have detected differences of 10-15% for muscle mass and protein synthesis in the present cross-sectional study. Much lower differences could be detectable in longitudinal studies, but there is no accurate non-invasive measurement of either muscle mass or muscle protein synthesis rate. A possible reason for the non-association between low-grade inflammation and muscle mass and protein synthesis rate may be a too short duration of the persistent low-grade inflammatory state. However, it is likely that inflamed rats had exhibited inflammation for a longer period of time than one month, since we observed that inflammation is persistent in old rats between 22 and 24.5 months of age²⁵. We have actually observed that low-grade inflammation lasting up to 3 months does not decrease muscle mass too (unpublished data). Based on mean life expectancies in men⁴¹ and male rats, a period of one month in 24-month-old rats would correspond to 3.2 years in 76.7-year-old men. A 3-year longitudinal study in over 65-year-old humans showed a significant decrease in appendicular skeletal muscle mass⁴². A similar result was observed in another longitudinal study including 60-90 years old humans lasting 4.7 ± 2.3 years⁴³. In adult rats, significant effects on muscle mass (-11%) and protein synthesis (-23%) have been detected after 27 days of dextran sulfate sodium treatment, an experimental model of chronic inflammation³⁵. In acute inflammation, significant effects were observed after only two days¹⁹. Altogether our present results clearly support the fact that low-grade systemic inflammation, lasting at least one month, does not decrease muscle mass in old rats. This is in accordance with the fact that administration of low and intermediate doses of interleukin-6 did not induce skeletal muscle mass atrophy, although a high dose induced atrophy⁴⁴. Furthermore, a study with analyses stratified by plasma interleukin-6 tertiles, revealed that subjects with intermediate levels (2nd tertile) had similar total muscle strength than subjects in the lowest tertile, although those with highest levels (3rd tertile) had the lowest strength¹². Since neither

muscle mass nor muscle protein synthesis were different between C and LGI groups, muscle proteolysis was likely not affected by low-grade inflammation in old rats, despite the well-established effect of inflammatory diseases on proteolysis⁴⁵. Our results suggest that skeletal muscle protein synthesis and proteolysis are not sensitive to age-associated low-grade systemic inflammation in rats.

The present study has been performed in the basal state only, i.e., old rats were maintained in their environmental conditions and not submitted to any kind of treatment. It is not excluded that low-grade inflammation in old rats can have an effect on muscle in either anabolic, catabolic or recovery periods. Indeed, no marked change with age could be detected in muscle protein metabolism, either synthesis or proteolysis, in the post-absorptive state in rats^{46,47}. By contrast, in anabolic and catabolic situations, a number of dysregulations in muscle protein turnover became clearly apparent^{31,48,49}. Altered responses to nutrients and to catabolic treatments may contribute to explain sarcopenia⁵⁰. Muscle regenerative response to injury is also dramatically impaired in aged individuals⁵¹⁻⁵³. Age-associated low-grade inflammation may play a role by impairing the anabolic effects of nutrients, worsening the response to catabolic states and also impairing the ability to recover from catabolic stress due to a defective regeneration process. This deserves further investigation.

In conclusion, in old rats exhibiting low-grade inflammation and stable body weight for at least one month, synthesis of liver proteins, likely mainly APP, was increased, but skeletal muscle mass was not decreased, compared to age-matched rats. Our results suggest that age-associated systemic low-grade inflammation is not deleterious to muscle.

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