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## Accepted Manuscript

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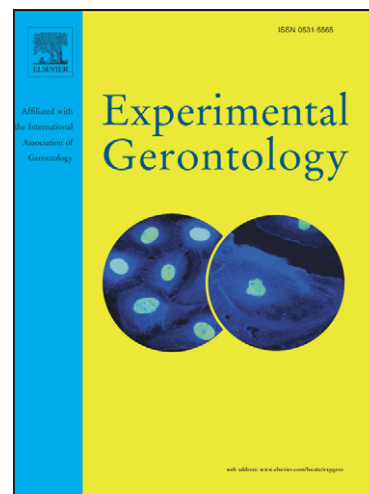
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1 Presence of low-grade inflammation in old rats does not worsen skeletal muscle  
2 loss under an endotoxemic and dietary stress

3

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16

17 Running title: Response to a stress in inflamed or not old rats

18

19 *Keywords:* Ageing; Inflammation; Lipopolysaccharide;  $\alpha_2$ -macroglobulin; Fibrinogen;  
20 Albumin.

21

22 Abbreviations used: CRP: C-reactive protein, BW: body weight, EDL: *extensor digitorum*

23 *longus*, IL-6: interleukin-6, LGI-Con: low-grade inflamed rats submitted to the control

24 treatment, LGI-Str: low-grade inflamed rats submitted to the stress, NI-Con: non-inflamed

- 25 rats submitted to the control treatment, NI-Str: non-inflamed rats submitted to the stress,  
26 TNF- $\alpha$ : tumor necrosis factor-alpha.

ACCEPTED MANUSCRIPT

27 **Abstract**

28           The study aimed to determine if age-associated low-grade inflammation aggravates the  
29 response to a stress, especially regarding to sarcopenia. Initial inflammatory status in 22  
30 month-old rats was based on plasma  $\alpha_2$ -macroglobulin and fibrinogen concentrations. The  
31 stress applied was a single intra-peritoneal injection of lipopolysaccharide followed by a 23-  
32 day period of malnutrition, *i.e.* a 4% casein diet distributed in quantity limited to 50% of  
33 spontaneous food intake. The response to the stress was analyzed in non-inflamed and low-  
34 grade inflamed rats and compared to non-inflamed and low-grade inflamed rats, which  
35 received the control treatment (*i.e.* no lipopolysaccharide injection and an 18% casein diet).  
36 The stress-induced body weight loss was higher in inflamed than non-inflamed rats, but the  
37 decrease in muscle weight was not worsened. Muscle protein turnover was not affected by the  
38 stress. Plasma  $\alpha_2$ -macroglobulin levels increased after the stress, whatever the initial  
39 inflammatory status. However, fibrinogen levels decreased more in inflamed than non-  
40 inflamed rats and albumin levels were not affected by the stress. Independently of the initial  
41 inflammatory status, the liver glutathione content was strongly depleted by the stress. These  
42 results extend and support our previous findings by demonstrating that age-associated low-  
43 grade inflammation does not aggravate sarcopenia in old rats.

## 44 1. Introduction

45 Frailty syndrome is an age-associated physiological state of vulnerability, resulting  
46 from impaired homeostatic reserves and a reduced capacity to withstand stress (Fried and  
47 Walston, 2003). Frailty consists of multi-system decline and is considered to be a  
48 consequence of changes in neuromuscular, endocrine and immune functions that occur as  
49 people progress in age (Fried and Walston, 2003; Vanitallie, 2003; Ferrucci et al., 2005;  
50 Paganelli et al., 2006). Frailty often results in several “vicious loops”: neuromuscular  
51 impairment induces increased rate of falls and fractures in old age; the act of falling causes  
52 itself hospitalization and immobilization, which aggravates sarcopenia; sarcopenia leads to  
53 deficit in continuous neuromuscular training which brings about neuromuscular impairment  
54 (Bales and Ritchie, 2002; Fried and Walston, 2003; Mühlberg and Sieber, 2004). The  
55 unintentional body weight loss, which is a frailty criterion (Fried et al., 2001), usually results  
56 from poor responses to successive various stresses (Vellas et al., 1992).

57 Frail subjects exhibit evidence of increased inflammation revealed by modified acute  
58 phase protein concentrations (Walston et al., 2002; Puts et al., 2005). Age-associated increase  
59 in the levels of inflammatory markers, such as interleukin-6 (IL-6) and CRP, are associated  
60 with disability and mortality (Ferrucci et al., 1999; Harris et al., 1999; Ferrucci et al., 2002;  
61 Cohen et al., 2003). Since age-associated inflammation appears to be an important component  
62 of frailty (De Martinis et al., 2006), it could worsen the aged subjects’ response to a stress. In  
63 the same way, acute phase proteins, such as  $\alpha_2$ -macroglobulin and fibrinogen have been  
64 recently identified as markers of frailty in old rats (Mayot et al., 2007).

65 CRP, IL-6 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) receptor-2 levels were negatively  
66 correlated with the rate of skeletal muscle protein synthesis (Toth et al., 2005). Similarly, in  
67 frail human skeletal muscle, TNF- $\alpha$  mRNA and protein levels decreased in concert with  
68 increases in strength and protein synthesis after 3 months of resistance exercise (Greiwe et al.,

69 2001). These results support the idea that age-associated low-grade inflammation affects  
70 skeletal muscle metabolism and could participate in sarcopenia development. However, they  
71 do not prove the causal relationship within the old population. Indeed, inflamed/frail subjects  
72 are often older than control groups (Walston et al., 2002; Barbieri et al., 2003). Thus, these  
73 observations may be independent of inflammatory status and more directly related to age.  
74 Indeed we observed that low-grade inflammation, lasting at least one month, does not  
75 enhance sarcopenia in old rats (Mayot et al., 2006). Since this investigation was performed in  
76 rats kept in conditions minimizing any kind of stress, it is not excluded that age-associated  
77 low-grade inflammation could impact the old rats' response to a stress.

78 Diseases, such as infections, and periods of malnutrition, which are frequent in elderly  
79 patients, are stressor events that may accelerate frailty (Fried and Walston, 2003). Sarcopenia  
80 is associated with a doubled risk of nosocomial infection (Cosqueric et al., 2006). Poor daily  
81 energy intake and protein intake adjusted for energy intake are related to frailty (Bartali et al.,  
82 2006). Four to 31% of free-living and up to 60% of geriatrics patients on admission in  
83 hospitals or nursing homes have shown evidence of malnutrition (Seiler, 2001). Most of these  
84 deficits affecting ill elderly subjects are related to proteins and micronutrients. An low protein  
85 diet (0.45 vs. 0.92 g/kg/d) reduced muscle mass and function in elderly women (Castaneda et  
86 al., 1995). Acute diseases and malnutrition decrease skeletal muscle mass, which provides  
87 amino acids for most vital functions (Heymsfield et al., 1982; Obled et al., 2002).

88 The aim of the present study was to evaluate whether age-associated low-grade  
89 inflammation impacts the old rats' response to a stress. The chosen stress combines a short-  
90 lasting inflammation (endotoxin injection) and protein-energy malnutrition. This double stress  
91 was used to mimic a situation in elderly subjects, who frequently face any kind of transient  
92 febrile episode followed by a period of protein-energy malnutrition. The investigation was  
93 primarily focused on body weight change, skeletal muscle weight and protein metabolism.

94 The acute phase protein response, weights of various organs and the glutathione liver status  
95 were considered as complementary targets.

96

## 97 **2. Material and methods**

### 98 *2.1. Animals*

99 Wistar male rats were produced and bred in our conventional, non specific-pathogen-  
100 free, animal facility (Unité Expérimentale de Nutrition Comparée, INRA, Theix, France).  
101 Before the experiment, rats were maintained in collective cages (3 to 4 per cage) under  
102 controlled conditions (temperature 21°C, relative humidity 55%, 12-h dark period starting at  
103 2000 h). They had free access to water and standard pellets (A04 from SAFE (Scientific  
104 Animal Food and Engineering), Villemoisson-sur-orge, France). The composition of the diet  
105 was 16% protein, 3% fat, 60% carbohydrates, 12% water, fibers, vitamins and minerals. The  
106 study was performed according to the current legislation on animal experiments in France.

107

### 108 *2.2. Experimental design*

#### 109 *2.2.1. Pre-experimental period*

110 At the age of 22 months, rats which were apparently ill and those exhibiting acute  
111 phase proteins levels that are predictive of old rat mortality (Mayot et al., 2007) [i.e.  $\alpha_2$ -  
112 macroglobulin and fibrinogen concentrations higher than 500 mg/l and 5.4 g/l, respectively]  
113 were excluded. Thirty-four apparently healthy rats were housed in individual cages and  
114 acclimatized to their new environment for 3 weeks. They received a diet containing 18%  
115 casein over 4 daily periods of 3 hours each, regularly spaced. The quantity of food distributed  
116 during the light and dark periods were adjusted to represent about 30 and 70% of the *ad*  
117 *libitum* daily intake, respectively. Four rats died during this period and two rats, which did not



118 eat, were excluded. Blood was withdrawn from a lateral tail vein 7 days before the end of the  
119 pre-experimental period.

### 120 2.2.2. Main experiment

121 Rats were distributed into 2 classes (non-inflamed (NI) and low-grade inflamed (LGI),  
122 n = 14, each) based on their inflammatory status that was analyzed by hierarchical clustering  
123 using Ward distance on  $\alpha_2$ -macroglobulin and fibrinogen variables (Mayot et al., 2007). At  
124 day 0, each class was sub-divided into two groups receiving either the control (Con) or the  
125 stress (Str) treatment. The control treatment, consisting in maintaining rats in the pre-  
126 experimental conditions, was applied to 6 non-inflamed rats (NI-Con group) and 5 low-grade  
127 inflamed rats (LGI-Con group). The stress consisted in a single intra-peritoneal injection of  
128 lipopolysaccharide from *Escherichia coli* 0127:B8 (0.4 mg/kg) followed by a 23-day period  
129 of protein-energy malnutrition, during which rats received a diet containing 4% casein in  
130 quantity individually limited to 50% of spontaneous food intake (distributed in four meals as  
131 for control treatment). It was applied to 8 non-inflamed rats (NI-Str group) and 9 low-grade  
132 inflamed rats (LGI-Str group). Rats were weighed at day 0 then three times per week. Blood  
133 was sampled at day 8 at day 21 for plasma analyses. At day 23, all surviving rats (n = 5 for  
134 NI-Con, LGI-Con and NI-Str groups and n = 7 for LGI-Str group) were anaesthetized with an  
135 intra-peritoneal injection of ketamine (150 mg/kg). *Epitrochlearis* muscle was quickly  
136 removed and incubated to measure *in vitro* protein metabolism, as already described  
137 (Combaret et al., 2003). Rats were then killed by aortic blood withdraw. Posterior leg skeletal  
138 muscles, liver, spleen, kidneys, epididymal adipose tissue, mesentery, small intestine, colon  
139 were dissected and weighted. Liver was frozen at  $-80\text{ }^{\circ}\text{C}$  until glutathione analysis.

### 140 2.3. Biochemical measurements

141 Plasma  $\alpha_2$ -macroglobulin, fibrinogen and albumin were measured as already described  
142 (Mayot et al., 2007). Total glutathione was quantified spectrophotometrically using a standard

143 enzymatic recycling procedure and 5,5'-dithio-bis-2-nitrobenzoic acid as oxidant (Malmezat  
144 et al., 1998).

#### 145 2.4. Statistical analysis

146 Values are given as means  $\pm$  SE. The significance of difference between non-inflamed  
147 and low-grade inflamed rats during the pre-experimental period was analyzed with Student's t  
148 test for unpaired data. The significance of the initial inflammatory status and the treatment  
149 effects was analyzed with 2-way ANOVA. For parameters measured before and at various  
150 times after stress induction ANOVA for repeated measurements was performed with the  
151 initial inflammatory status and the treatment as the between-rat factors and the time as the  
152 within-rat factor. The significance of differences among the four groups was further analyzed  
153 by Fisher's PLSD test, when appropriate. Linear regression was used to determine the  
154 relationship between acute phase protein variation and body weight change.  $P$  values  $\leq 0.05$   
155 were considered significant. Statistical analyses were performed using StatView for  
156 Windows, version 5 software (SAS Institute, Cary, NC), excepted the hierarchical clustering  
157 using Ward distance, which was performed with XLSTAT, version 7.5 software (Addinsoft,  
158 Paris, France).

159

### 160 3. Results

#### 161 3.1. Old rats' characteristics before the experimental period

162 According to the experimental design, low-grade inflamed rats exhibited 287 and 41%  
163 higher initial plasma concentrations of  $\alpha_2$ -macroglobulin ( $147 \pm 21$  vs.  $38 \pm 6$  mg/l,  $P =$   
164  $0.0002$ ) and fibrinogen ( $4.5 \pm 0.2$  vs.  $3.2 \pm 0.1$  g/l,  $P = 0.0002$ ), respectively (Table 1). As  
165 expected, low-grade inflamed rats exhibited 13% lower initial concentration of albumin than  
166 non-inflamed rats ( $12.1 \pm 0.6$  vs.  $13.9 \pm 0.6$  g/l,  $P = 0.055$ ); however this difference was only  
167 a trend. Maximal initial concentrations of  $\alpha_2$ -macroglobulin and fibrinogen were 307 mg/l

168 and 5.6 g/l, respectively, and minimal initial concentration of albumin was 8.3 g/l. The  
169 relative body weight loss occurring over the pre-experimental period (day -22 to day 0) was  
170 74% higher in low-grade inflamed than non-inflamed rats ( $-6.7 \pm 1.3$  vs.  $-3.9 \pm 1.2$  %,  $P =$   
171  $0.134$ ) without change in food intake; however the effect of the initial inflammatory status did  
172 not attain statistical significance. Based on 2-way ANOVA, groups did not differ with respect  
173 to mean body weights at days -22 and 0, and food intake before day 0 (Table 1). No  
174 interaction between treatment and initial inflammatory status was significant for any  
175 measured parameter (Table 1) indicating the absence of any bias related to the configuration  
176 of the groups.

### 177 *3.2. Stress-induced mortality and body weight change in low-grade inflamed and non-* 178 *inflamed old rats*

179 The stress (endotoxin injection followed by protein-energy malnutrition) applied to the  
180 old rats induced 37 and 22% of mortality in NI-Str and LGI-Str groups, respectively. Indeed,  
181 three rats from NI-Str group deceased at days 1, 2, and 6 and two rats from LGI-Str group  
182 deceased at days 1 and 22. No mortality was observed in non-stressed rats, but one rat from  
183 NI-Con group was euthanized at day 17 due to its huge body weight loss. As expected, mean  
184 relative body weight losses (%) over 23 days were very much higher in stress groups (NI-Str:  
185  $21.0 \pm 1.0$  and LGI-Str:  $24.0 \pm 1.3$ ) than in control groups (NI-Con:  $2.2 \pm 0.6$  and LGI-Con:  
186  $2.2 \pm 1.4$ ). Based on the ANOVA for repeated measurements, cumulative body weight loss  
187 was dependent on the treatment and the time but not on the initial inflammatory status of rats  
188 (Fig. 1). The significant interaction between the treatment, the initial inflammatory status and  
189 the time likely reflects the fact that relative body weight losses over time were higher in LGI-  
190 Str than NI-Str, whereas they were similar in NI-Con and LGI-Con groups. Comparison of  
191 results from LGI-Str and LGI-Con groups with the ANOVA for repeated measurements  
192 indicated that the relative body weight losses over time was higher in LGI-Str than in NI-Str

193 (there was a significant interaction between the initial inflammatory status and time). It is  
194 worthwhile to note that stress-induced body weight loss (NI-Str and LGI-Str groups) was  
195 correlated with initial concentration of either  $\alpha_2$ -macroglobulin ( $r^2 = 0.545$ ,  $P = 0.006$ ) or  
196 fibrinogen ( $r^2 = 0.331$ ,  $P = 0.050$ ) and inversely correlated with the initial albumin  
197 concentration ( $r^2 = 0.340$ ,  $P = 0.047$ ).

### 198 3.3. Stress effects on skeletal muscles in low-grade inflamed and non-inflamed old rats

199 Based on two-way ANOVA, the stress (endotoxin injection followed by protein-  
200 energy malnutrition) applied to the old rats decreased absolute and relative *gastrocnemius*  
201 muscle weights by 12% ( $2.02 \pm 0.07$  vs.  $2.31 \pm 0.06$  g) and 15% ( $323 \pm 11$  vs.  $379 \pm 17$   
202 mg/100g body weight at day 0 (BW)), respectively (Table 2). The same trend was observed  
203 for the absolute and relative weight of *tibialis anterior* (-9% ( $744 \pm 26$  vs.  $815 \pm 24$  g) and -  
204 11% ( $119 \pm 5$  vs.  $134 \pm 5$  mg/100 g BW)). The stress had no effect on *extensor digitorum*  
205 *longus* (EDL) and *soleus*. The initial inflammatory status did not affect the muscle weights,  
206 excepted for the relative weight of *soleus*, which was 18% higher in inflamed ( $30.1 \pm 0.8$   
207 mg/100 mg BW) than in control rats ( $25.5 \pm 1.9$  mg/100 mg BW). Neither protein synthesis,  
208 nor protein degradation was affected by the treatment or the initial inflammatory status (Fig.  
209 2).

### 210 3.4. Stress effects on organs and adipose tissues in low-grade inflamed and non-inflamed old 211 rats

212 The stress (endotoxin injection followed by protein-energy malnutrition) applied to  
213 old rats decreased absolute (and relative) weights of spleen, liver, kidneys and small intestine  
214 by about 33 (34), 27 (28), 26 (28) and 10% (12%), respectively (Table 3). The significance  
215 was not reached for the stress-induced decreases in absolute and relative weights of colon,  
216 mesentery and epididymal adipose tissue respectively. Inflamed rats exhibited higher absolute  
217 (42%) and relative (48%) weights of kidneys and 11% higher relative weight of liver than

218 non-inflamed rats. Interaction between stress and initial inflammatory status was significant  
219 for absolute weight of kidneys and relative weights of kidneys and liver, indicating that stress-  
220 induced decreases in these organ weights were higher in inflamed than non-inflamed rats. The  
221 initial inflammatory status had no significant effect on absolute or relative weights of colon,  
222 mesentery and epididymal adipose tissue.

### 223 3.5. Stress effects on acute phase proteins and liver glutathione in low-grade inflamed and 224 non-inflamed old rats

225 The stress, the initial inflammatory status and the time had significant effects on both  
226  $\alpha_2$ -macroglobulin and fibrinogen plasma concentrations (Fig. 3), and the interaction between  
227 the stress and the time was significant for fibrinogen. Only initial inflammatory status had a  
228 significant effect on albumin plasma concentration. As expected,  $\alpha_2$ -macroglobulin,  
229 fibrinogen and albumin were stable over time in non-stressed rats (NI-Con and LGI-Con  
230 groups). In stressed rats (NI-Str and LGI-Str groups),  $\alpha_2$ -macroglobulin was higher at days 8  
231 and 21 than day -7. In NI-Str group, it was higher at day 8 than day 21. However, due to high  
232 variability, differences were not significant in LGI-Str group. The decrease in fibrinogen at  
233 days 8 and 21 was higher in LGI-Str than NI-Str group to reach similar values as in NI-Con  
234 group. Albumin was stable in stressed rats.

235 The stress, but not the initial inflammatory status, had a significant effect on  
236 glutathione status (Fig. 4). Glutathione concentration and content were 59% ( $3.04 \pm 0.6$  vs.  
237  $7.50 \pm 0.21$   $\mu\text{mol/g}$ ) and 70% ( $37 \pm 7$  vs.  $126 \pm 8$   $\mu\text{mol}$ ) lower in stressed than non-stressed  
238 rats, respectively.

239

## 240 4. Discussion

241 The present data indicate that age-associated low-grade inflammation renders old rats  
242 more vulnerable to stress-induced body weight loss, although without further decrease in

243 skeletal muscle weight. Stress-induced decreases in some abdominal organ weights and in  
244 fibrinogen plasma concentrations were enhanced by the initial low-grade inflammatory status,  
245 but responses of the other acute phase proteins to the stress and stress-induced decreases in  
246 liver glutathione concentration and content were independent of the initial inflammatory  
247 status of old rats.

248 In agreement with our previous characterization of a cohort of old rats regarding their  
249 inflammatory status (Mayot et al., 2007), rats belonging to the non-inflamed groups NI-Con  
250 and NI-Str were clearly non-inflamed and those belonging to the inflamed groups LGI-Con  
251 and LGI-Str exhibited low-grade inflammation at the beginning of the experiment. Indeed for  
252 the formers, their initial plasma  $\alpha_2$ -macroglobulin and fibrinogen levels did not differ from  
253 mean values previously observed for each corresponding first quartile in old rats or for adult  
254 rats (8 months). Conversely, the others groups exhibited  $\alpha_2$ -macroglobulin and fibrinogen  
255 levels similar to mean values previously observed in third quartiles in old rats. Of note, these  
256 concentrations reflect low-grade inflammation since they are much lower than those reported  
257 in acute (Breuillé et al., 1998; Breuillé et al., 1999) and chronic (Mercier et al., 2002)  
258 inflammation. In addition, initial plasma concentrations of acute phase proteins in inflamed  
259 old rats are consistent with those observed with age in human. Indeed, in old (66-76 years)  
260 healthy subjects fibrinogen level was ~ 47% greater and albumin level ~ 12% lower than in  
261 young (22-26 years) healthy subjects (El Yousfi et al., 2005). We have recently shown that  
262 increased fibrinogen and  $\alpha_2$ -macroglobulin levels to the range observed in present inflamed  
263 rats were associated with increased levels of soluble TNF- $\alpha$  receptor-1 (Mayot et al., 2007).  
264 An activation of the TNF- $\alpha$  signaling is likely to occur in inflamed rats and might be active on  
265 skeletal muscle protein metabolism, as recently suggested in human (Toth et al., 2005).

266 Interestingly, old rats submitted to the control treatment (NI-Con and LGI-Con  
267 groups) exhibited stable levels of plasma acute phase protein concentrations over time. Thus,

268 the inflammatory status defined at the beginning of the experiment was maintained all along  
269 the experiment in non-stressed rats. In parallel, final relative weights of liver, kidney and  
270 spleen were higher in inflamed than non-inflamed old rats submitted to control treatment.  
271 These organs play key role in the control of inflammation and immunity. Taken together, our  
272 results support the fact that the spontaneous low-grade inflammation observed in old rats is a  
273 chronic inflammation, which could affect the immune system and potentially weaken old rats.  
274 This is further supported by the observation that body weight loss observed after the stress  
275 was higher in inflamed than non-inflamed old rats.

276 In this context, the response of skeletal muscle to the stress regarding its mass and  
277 protein turnover was interesting to compare between initially inflamed and non-inflamed old  
278 rats. We recently showed that low-grade inflammation did not aggravate sarcopenia in control  
279 conditions (Mayot et al., 2006). In the present study, we confirmed our precedent results since  
280 skeletal muscle masses and protein turnover rates were not different between the two control  
281 groups NI-Con and LGI-Con. Moreover, present results are going one step further, showing  
282 that age-associated low-grade inflammation does not induce any negative effect in regards to  
283 skeletal muscle mass and protein metabolism in case of additional stress.

284 The stress applied to old rats, consisting in a single endotoxin injection followed by a  
285 23-day period of protein-energy malnutrition, induced severe metabolic disturbances  
286 leading to increased mortality rate, loss of body weight and decreased abdominal organ  
287 weights. The effect of the stress on acute phase protein response was protein-dependent.  
288 Indeed, plasma levels of  $\alpha_2$ -macroglobulin increased, whereas fibrinogen levels decreased  
289 and albumin levels did not change in stressed old rats. The increase in  $\alpha_2$ -macroglobulin is  
290 consistent with the fact that sepsis and protein-deficient diet are known to stimulate its  
291 synthesis in young rats (Breuillé et al., 1998; Lyoumi et al., 1998; Breuillé et al., 1999). If  
292 the stress would have been only an inflammatory stress, an increase in fibrinogen would

293 have been also expected. The fact that its plasma concentration decreased under the stress  
294 suggests that either its utilization increased or its synthesis was limited by the malnutrition  
295 in these old rats. The decrease in fibrinogen concentration is in agreement with data  
296 obtained in piglets that were fed with a protein-deficient diet (Jahoor et al., 1999). Taken  
297 together, these observations suggest that the transient inflammatory stress (*i.e.* endotoxin  
298 injection) and the following protein-energy malnutrition stress used in the present study in  
299 old rats may have had opposite effects on some metabolic aspects. Albumin levels were not  
300 affected by the stress applied to old rats. By contrast, sepsis and protein-deficient diet are  
301 known to decrease it in young rats (Lyoumi et al., 1998; Ruot et al., 2002). Thus, as we  
302 already suggested (Mayot et al., 2007), albumin may not be a good nutritional marker in old  
303 rats. Glutathione which is known to be very sensitive to protein intake (Hum et al., 1992)  
304 and to decrease during infection (Breuillé et al., 1994) was highly depleted in the stressed  
305 old rats. The overall effect of the present stress applied in old rats on plasma levels of acute  
306 phase proteins and liver glutathione status was largely consistent with known effects of  
307 protein-deficient nutrition in young animals, albumin excepted.

308 Unfortunately, the effect of low-grade inflammation on stress-induced mortality in old  
309 rats could not be statistically analyzed because of too low number of animals. Low-grade  
310 inflammation increased the susceptibility of old rats to body weight loss. Indeed, low-grade  
311 inflamed rats lost almost 30 g more body weight under the stress than non-inflamed rats. The  
312 higher stress-induced body weight loss in inflamed vs. non-inflamed rats could not be  
313 explained by differences in adipose tissue or skeletal muscle weights. Part of the higher body  
314 weight loss can be explained by higher decreases in liver, spleen kidneys and colon weights,  
315 (Table 3). However, the results obtained from longitudinal observations (*i.e.* body weights  
316 measured in the same rats before and during the treatment) are difficult to explain by the  
317 transversal observations concerning mean skeletal muscles and organs weights from different



318 groups of rats. Indeed, transversal observations are generally associated with higher  
319 variability than measurements performed in longitudinal observations. Finally, tissues and  
320 organs that have not been dissected such as the skin, whose weight contributes to ~ 20% of  
321 body weight (Obled and Arnal, 1992), might have been more affected in inflamed than non-  
322 inflamed old rats.

323         Regarding the acute phase reaction, the stress applied in old rats modified differently  
324 the fibrinogen kinetic in inflamed as compared to non-inflamed rats. The fibrinogen  
325 concentration was decreased by the stress in both groups, but to a greater extend in inflamed  
326 than non-inflamed rats. The stress-induced increase in  $\alpha_2$ -macroglobulin might also have been  
327 higher but variability was too high to observe a significant effect in LGI-Str group. Finally,  
328 the inflammatory status of the old rats did not impact the stress response regarding the  
329 glutathione status and the skeletal muscle protein turnover. Taken together, the initial  
330 inflammatory status of old rats did not have a strong impact on the metabolic response to the  
331 present stress and for sure did not enhance the stress-induced skeletal muscle loss.

332         The failure of low-grade inflammation to worsen the stress-induced skeletal muscle  
333 loss extends our previous observation made in non-stressed rats showing that low-grade  
334 inflammation does not aggravate sarcopenia (Mayot et al., 2006). The present stress applied in  
335 old rats had more effects on splanchnic organs than on skeletal muscles. This is consistent  
336 with decreases observed in splanchnic organ weights but not in skeletal muscle weights after a  
337 50% dietary restriction in old rats (Chambon-Savanovitch et al., 1999). Similarly, higher  
338 decreases in liver (49%) than in *gastrocnemius* (25%) weights were observed after 10 days of  
339 starvation in old rats (Mosoni et al., 1999). Since muscle wasting and weakness are late  
340 symptoms of malnutrition (Seiler, 2001), a longer malnutrition period might be required to  
341 see differential reduction of skeletal muscle weight according to the initial inflammatory  
342 status. In young rats, when starvation is initiated, there is a period of “saving” before skeletal

343 muscle loss becomes significant (Chérel et al., 1991). However, there is a large decrease in  
344 skeletal muscle protein synthesis observed in fasting young rats, even during the early phase  
345 during which muscle weight is preserved. A decrease in skeletal muscle protein synthesis is  
346 also observed in piglets fed a protein-deficient diet (Jahoor et al., 1999). In contrast, skeletal  
347 muscle protein turnover was not affected in our stressed old rats. Taken together, these  
348 observations suggest that the present double stress (endotoxin injection combined with  
349 protein-energy malnutrition) might have induced some early metabolic effects that might have  
350 been counteracted by the 23-day period of malnutrition. This assumption seems to be  
351 emphasized by the observation of the decreased fibrinogen concentration under the stress, as  
352 opposed to the increase usually observed after an inflammatory stress. Thus, to definitively  
353 conclude that low-grade inflammation does not worsen sarcopenia in aged, it would be  
354 probably necessary to test the impact of another type of stress, for example a stress inducing a  
355 more marked skeletal muscle loss than the present stress or a stress for which no opposite  
356 metabolic effects are observed.

357 In conclusion, the present study clearly indicates that low-grade inflammation  
358 increases the body weight loss induced by the endotoxin injection followed by malnutrition, a  
359 stress that mimics a transient febrile episode followed by a period of protein-energy  
360 malnutrition, which frequently occurs in elderly. Stress-induced decreases in organ weights  
361 and fibrinogen plasma concentration were enhanced by the initial low-grade inflammatory  
362 status. The response of skeletal muscle (weight and protein turnover) was not worsened by the  
363 low-grade inflammation. This extends and supports our previous findings that age-associated  
364 low-grade inflammation does not aggravate sarcopenia.

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368

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ACCEPTED MANUSCRIPT



494 **Figure legends**

495

496 **FIGURE 1** *Time-course of cumulative relative body weight changes in non-inflamed and*  
497 *low-grade inflamed old rats submitted to the control or the stress treatment.*

498 Means  $\pm$  SE (n = 5 or 7). There are significant effects of the stress and the time on body  
499 weight changes and significant interactions between the stress and the time, between the  
500 initial inflammatory status and the time, and between the stress, the initial inflammatory status  
501 and the time (ANOVA for repeated measurements with the stress and the initial inflammatory  
502 status as the between-rat factors and the time as the within-rat factor). NI-Con: non-inflamed  
503 rats submitted to the control treatment, NI-Str: non-inflamed rat submitted to the stress  
504 treatment, LGI-Con: inflamed rats submitted to the control treatment, LGI-Str: inflamed rats  
505 submitted to the stress treatment.

506

507 **FIGURE 2** *Skeletal muscle protein turnover in non-inflamed and low-grade inflamed old rats*  
508 *submitted to the control or the stress treatment.*

509 Means  $\pm$  SE (n = 5 or 7). Two-way (initial inflammatory status and treatment) ANOVA:  
510 *epitrochlearis* protein synthesis and protein degradation: none significant effect,  $P < 0.05$ , NI-  
511 Con: non-inflamed rats submitted to the control treatment, NI-Str: non-inflamed rat submitted  
512 to the stress treatment, LGI-Con: inflamed rats submitted to the control treatment, LGI-Str:  
513 inflamed rats submitted to the stress treatment.

514

515 **FIGURE 3** *Time-course of plasma  $\alpha_2$ -macroglobulin, fibrinogen and albumin concentrations*  
516 *in non-inflamed and low-grade inflamed old rats receiving the control or the stress treatment.*

517 Bars represent means and SE for 5 or 7 rats in each group at each time.  $\alpha_2$ -macroglobulin:  
518 significant effects of the stress, the initial inflammatory status and the time, (interaction

519 between the stress and the time  $P = 0.056$ ); fibrinogen: significant effects of the stress, the  
520 initial inflammatory status and the time, significant interaction between the stress and the time  
521 (interaction between the stress, the initial inflammatory status and the time  $P = 0.072$ );  
522 albumin: significant effect of the initial inflammatory status (ANOVA for repeated  
523 measurements with the stress and the initial inflammatory status as the between-rat factors  
524 and the time as the within-rat factor). Within a group, bars not sharing a common letter are  
525 different (ANOVA for repeated measurement, followed by Fisher's PLSD test). NI-Con: non-  
526 inflamed rats submitted to the control treatment, NI-Str: non-inflamed rat submitted to the  
527 stress treatment, LGI-Con: inflamed rats submitted to the control treatment, LGI-Str: inflamed  
528 rats submitted to the stress treatment.

529

530 **FIGURE 4** *Liver glutathione status in non-inflamed and low-grade inflamed old rats*  
531 *submitted to the control or the stress treatment.*

532 Means  $\pm$  SE (n = 5 or 7). Two-way (initial inflammatory status and treatment) ANOVA:  
533 significant effect of the treatment for both concentration and content,  $P < 0.05$ , NI-Con: non-  
534 inflamed rats submitted to the control treatment, NI-Str: non-inflamed rat submitted to the  
535 stress treatment, LGI-Con: inflamed rats submitted to the control treatment, LGI-Str: inflamed  
536 rats submitted to the stress treatment.

Table 1

Old rats' characteristics before the experimental period

Initial inflammatory status	Non-inflamed		<u>Low-grade</u> inflamed		Two-way ANOVA
	Treatment	Stress	Control	Stress	
	<u>NI-Con</u>	<u>NI-Str</u>	<u>LGI-Con</u>	<u>LGI-Str</u>	
$\alpha_2$ -macroglobulin at D-7(mg/l)	36 ± 4	40 ± 12	167 ± 37	133 ± 26	I
Fibrinogen at D-7 (g/l)	3.24 ± 0.18	3.12 ± 0.22	4.42 ± 0.39	4.51 ± 0.28	I
Albumin at D-7 (g/l)	14.6 ± 0.9	13.3 ± 0.7	12.2 ± 0.8	12.1 ± 1.0	I ( $P = 0.066$ )
Body weight at D-22 (g)	693 ± 68	627 ± 24	613 ± 33	704 ± 45	ns
Body weight at D0 (g)	663 ± 59	603 ± 22	576 ± 17	648 ± 35	ns
D-22 to D0 body weight change (%)	- 4.0 ± 1.9	- 3.8 ± 1.8	- 5.5 ± 2.9	- 7.6 ± 1.0	ns
Food intake before D0 (g/d)	22.0 ± 1.4	21.1 ± 1.2	21.3 ± 0.9	22.1 ± 0.7	ns

Means ± SE (n = 5 or 7). Two-way (initial inflammatory status and treatment) ANOVA: ns: none significant effect, I: significant effect of the initial inflammatory status,  $P \leq 0.05$ ,  $P$  value is in parenthesis when a trend is observed. NI-Con: non-inflamed rats submitted to the control

treatment, NI-Str: non-inflamed rat submitted to the stress treatment, LGI-Con: low-grade inflamed rats submitted to the control treatment, LGI-Str: low-grade inflamed rats submitted to the stress treatment.

Table 2

Skeletal muscle weights in non-inflamed and low-grade inflamed old rats submitted to the control or the stress treatment.

Initial inflammatory status	Non-inflamed		Inflamed		Two-way ANOVA
Treatment	Control	Stress	Control	Stress	
Group	<u>NI-Con</u>	<u>NI-Str</u>	<u>LGI-Con</u>	<u>LGI-Str</u>	
<i>Gastrocnemius</i> (g)	2.31 ± 0.06	2.00 ± 0.09	2.29 ± 0.10	2.03 ± 0.11	T
mg/100g BW	360 ± 32	333 ± 19	399 ± 13	316 ± 15	T
<i>Tibialis anterior</i> (mg)	844 ± 40	719 ± 35	786 ± 23	762 ± 38	T ( <i>P</i> =0.056)
mg/100g BW	130 ± 10	120 ± 8	137 ± 5	119 ± 6	T ( <i>P</i> =0.069)
EDL (mg)	189 ± 18	179 ± 8	196 ± 9	190 ± 12	ns
mg/100g BW	29.3 ± 3.7	29.7 ± 1.1	34.2 ± 2.0	29.3 ± 1.1	ns
<i>Soleus</i> (mg)	156 ± 17	163 ± 17	172 ± 6	188 ± 6	ns
mg/100g BW	23.9 ± 2.6	27.1 ± 2.8	29.9 ± 0.9	30.3 ± 1.3	I

Means  $\pm$  SE (n = 5 or 7). Two-way (initial inflammatory status and treatment) ANOVA: ns: none significant effect, I: significant effect of the initial inflammatory status, T: significant effect of the treatment,  $P < 0.05$ . P value is in parenthesis when a trend is observed. BW: body weight at day 0, NI-Con: non-inflamed rats submitted to the control treatment, NI-Str: non-inflamed rat submitted to the stress treatment, LGI-Con: low-grade inflamed rats submitted to the control treatment, LGI-Str: low-grade inflamed rats submitted to the stress treatment.

Table 3

Organ and adipose tissue weights in non-inflamed and low-grade inflamed old rats submitted to the control or the stress treatment.

Initial inflammatory status	Non-inflamed		Inflamed		Two-way ANOVA
Treatment	Control	Stress	Control	Stress	
Group	<u>NI-Con</u>	<u>NI-Str</u>	<u>LGI-Con</u>	<u>LGI-Str</u>	
Liver (g)	15.9 ± 1.3	11.8 ± 0.5	17.7 ± 1.3	12.5 ± 0.4	T
g/100g BW	2.41 ± 0.08 <sup>a</sup>	1.96 ± 0.02 <sup>b</sup>	3.06 ± 0.17 <sup>c</sup>	1.96 ± 0.09 <sup>b</sup>	T, I, TxI
Kidneys (g)	3.55 ± 0.17 <sup>ab</sup>	2.89 ± 0.14 <sup>a</sup>	5.66 ± 0.40 <sup>c</sup>	3.82 ± 0.25 <sup>b</sup>	T, I, TxI
mg/100g BW	548 ± 040 <sup>a</sup>	480 ± 19 <sup>a</sup>	986 ± 71 <sup>b</sup>	601 ± 50 <sup>a</sup>	T, I, TxI
Spleen (g)	1.43 ± 0.11	1.00 ± 0.02	1.62 ± 0.15	1.04 ± 0.08	T
mg/100g BW	220 ± 18	166 ± 4	280 ± 22	163 ± 14	T, I ( <i>P</i> =0.085), TxI ( <i>P</i> =0.063)
Small Intestine (g)	10.4 ± 0.5	8.5 ± 0.5	10.2 ± 0.3	9.7 ± 0.4	T
g/100g BW	1.60 ± 0.10	1.42 ± 0.03	1.78 ± 0.06	1.53 ± 0.11	T

Colon (g)	1.80 ± 0.10	1.78 ± 0.06	1.99 ± 0.22	1.67 ± 0.10	ns
mg/100g BW	279 ± 26	297 ± 16	344 ± 31	267 ± 28	TxI ( $P=0.098$ )
Mesentery (g)	7.36 ± 2.70	4.28 ± 0.87	6.78 ± 1.34	5.05 ± 1.28	ns
g/100g BW	1.07 ± 0.34	0.71 ± 0.14	1.18 ± 0.23	0.74 ± 0.17	T ( $P=0.091$ )
Epididymal adipose tissue (g)	12.1 ± 2.8	9.5 ± 2.1	9.1 ± 0.5	9.1 ± 1.3	ns
g/100g BW	1.77 ± 0.33	1.55 ± 0.32	1.58 ± 0.08	1.36 ± 0.17	ns

Means ± SE (n = 5 or 7). Two-way (initial inflammatory status and treatment) ANOVA: ns: none significant effect, I: significant effect of the initial inflammatory status, T: significant effect of the treatment, TxI: significant interaction between the two factors,  $P < 0.05$ .  $P$  value is in parenthesis when a trend is observed. <sup>a,b,c</sup>: values, within a row, not sharing a common letter are significantly different (Fisher's test). BW: body weight at day 0, NI-Con: non-inflamed rats submitted to the control treatment, NI-Str: non-inflamed rat submitted to the stress treatment, LGI-Con: low-grade inflamed rats submitted to the control treatment, LGI-Str: low-grade inflamed rats submitted to the stress treatment.



FIGURE 1

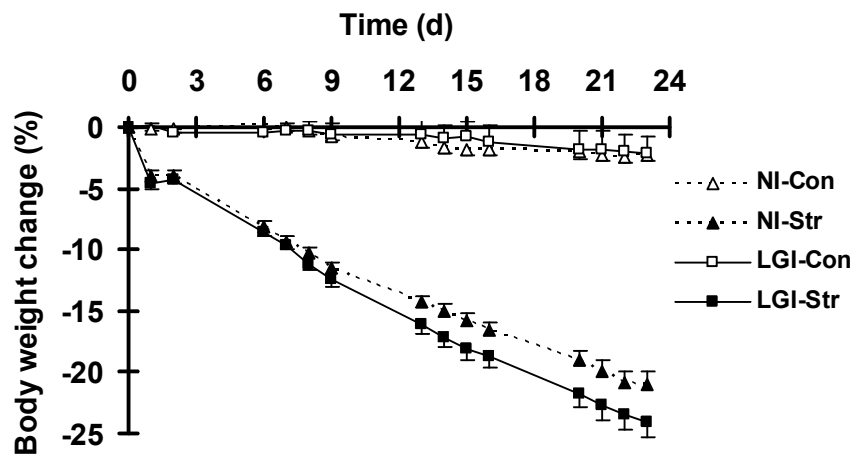


FIGURE 2

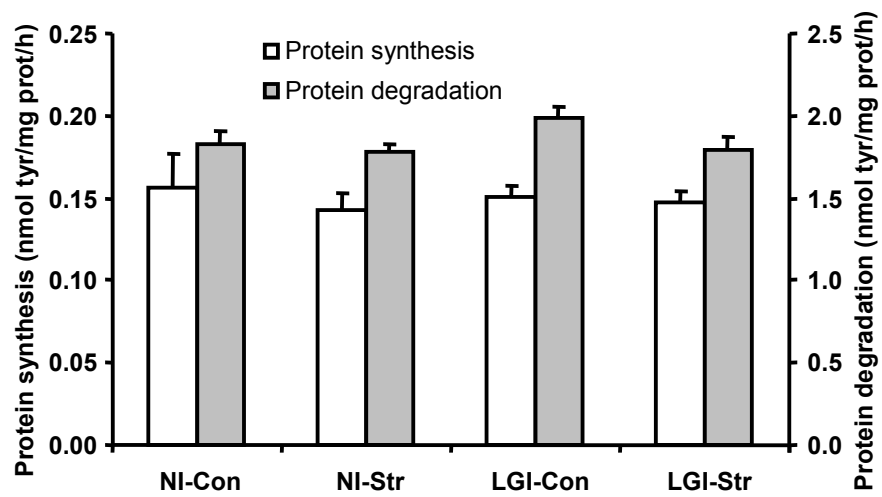


FIGURE 3

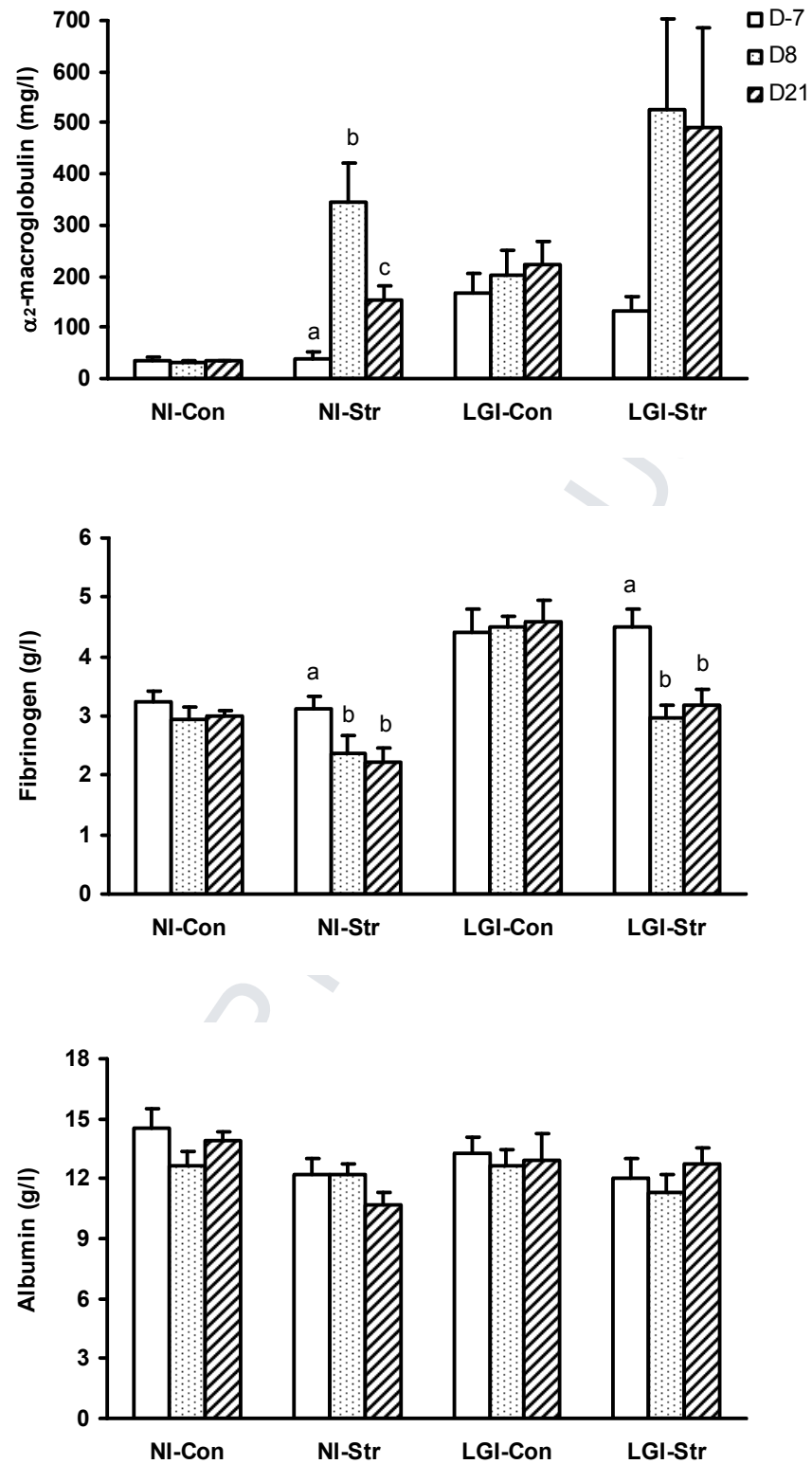


FIGURE 4

