Antioxidant Supplementation Restores Defective Leucine Stimulation of Protein Synthesis in Skeletal Muscle from Old Rats[1,2]

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Abstract

Aging is characterized by a progressive loss of muscle mass that could be partly explained by a defect in the anabolic effect of food intake. We previously reported that this defect resulted from a decrease in the protein synthesis response to leucine in muscles from old rats. Because aging is associated with changes in oxidative status, we hypothesized that reactive oxygen species–induced oxidative damage may be involved in the impairment of the anabolic effect of leucine with age. The present study assessed the effect of antioxidant supplementation on leucine-regulated protein metabolism in muscles from adult and old rats. Four groups of 8- and 20-mo-old male rats were supplemented or not for 7 wk with an antioxidant mixture containing rutin, vitamin E, vitamin A, zinc, and selenium. At the end of supplementation, muscle protein metabolism was examined in vitro using epitrochlearis muscles incubated with increasing leucine concentrations. In old rats, the ability of leucine to stimulate muscle protein synthesis was significantly decreased compared with adults. This defect was reversed when old rats were supplemented with antioxidants. It was not related to increased oxidative damage to 70-kDa ribosomal protein S6 kinase that is involved in amino acid signaling. These effects could be mediated through a reduction in the inflammatory state, which decreased with antioxidant supplementation. Antioxidant supplementation could benefit muscle protein metabolism during aging, but further studies are needed to determine the mechanism involved and to establish if it could be a useful nutritional tool to slow down sarcopenia with longer supplementation.   J. Nutr. 138: 2205–2211, 2008.

Introduction

Aging is characterized by a gradual loss of muscle protein, which leads to sarcopenia (1,2). This atrophy is associated with a progressive loss of muscle strength that directly affects the mobility and health of elderly people. The origin of sarcopenia is multifactorial and may be a consequence of undernutrition, decreased activity, oxidative stress, inflammation, and endocrine changes.

Over the past several years, it has become evident that muscle protein loss during aging may be partly explained by a decreased ability of old muscle to respond appropriately to anabolic stimuli, such as food intake (3,4). This defect mainly results from a decreased response and/or sensitivity of protein synthesis and degradation to amino acids in old muscle (5–8). Among amino acids, leucine can stimulate muscle protein synthesis to the same extent as all amino acids by improving activation of translation initiation [(5); reviewed in (9)]. We previously showed a defect in the ability of leucine to stimulate protein synthesis in muscles from old rats, which was correlated with a defect in the ability of leucine to stimulate 70-kDa ribosomal protein S6 kinase (S6K)[5] activity (5). Recent studies have demonstrated that these age-related alterations may be overcome by dietary leucine supplementation. Indeed, postprandial stimulation of muscle protein synthesis and inhibition of protein degradation in old rats can be restored by leucine-supplemented meals (10–12). Similarly, increasing leucine intake has been shown to improve muscle protein synthesis in the elderly (13), which can be restored to that obtained in younger subjects (14). Overall, these data suggested that increasing leucine availability may represent a nutritional strategy for limiting muscle protein loss during aging.

Alternatively, oxidative damage induced by oxidative stress increases during aging (15,16) and can induce alterations in DNA, lipids, and proteins (17), resulting in a decrease of their biological function. Several in vitro studies demonstrated that various cell cultures exposed to oxidative stress reduced metabolic insulin

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action associated with impaired insulin signaling (18–23). Moreover, oxidative stress is often associated with inflammation (24) and is more frequent in the elderly (2,25). Because oxygen-derived free radicals are responsible for damage at the cellular and tissue levels, they could be involved in the impairment of the anabolic effect of leucine during aging and contribute to sarcopenia. To support our hypothesis, it has been suggested that oxidative protein damage was associated with impaired muscle strength in older women (26). However, the effect of oxidative stress on muscle protein metabolism is not well defined, except in the modulation of muscle proteolysis [see review (27)]. To our knowledge, its impact on muscle protein synthesis is still unknown.

In this study, we examined the effect of antioxidant supplementation on muscle protein metabolism regulation by leucine in adult and old rats independently of increased leucine availability in vivo. Indeed, although antioxidant supplementation is receiving growing attention and has been shown to have beneficial effects in the aging process (cardiovascular disease, cancer, physical performance, and muscle strength) [reviews in (28–30)], the effect of antioxidant supplementation on muscle protein metabolism has not yet been studied.

Materials and Methods

Animals. These experiments were performed in accordance with current legislation on animal experimentation in France. Adult (8 mo) and old (20 mo) male Wistar rats produced and bred locally (Unité Expérimentale de Nutrition Comparée, INRA) were maintained in collective cages (2 rats per cage) under controlled environmental conditions (21°C, hygrometry 55%, 12-h dark period starting at 0700) and consumed water and food ad libitum. They were fed either a basal diet (Aox−) or the same diet supplemented with rutin (5 g/kg diet substituted with cellulose in the basal diet), vitamin E, vitamin A, zinc, and selenium (Aox+) for 7 wk (**Table 1**). The composition of the mineral and vitamin mixtures were based on the AIN-93M composition (31) with slight modifications to account for micronutrients associated with herring flour (Table 1). We chose rutin because it is thought to have antibacterial, antiviral, antioxidant, antiproliferative, antiinflammatory, and anticarcinogenic effects (32). In addition, rutin is a frequent component of human food, being one of the most abundant flavonols in vegetables and fruits, and is water soluble. Vitamin E is a fat-soluble protein used extensively in supplementation studies and is well

TABLE 1 Composition of the diets

Ingredients	Basal diet (Aox−)	Supplemented diet (Aox+)
	g/kg dry matter	
Herring flour	203	203
Wheat starch	450	450
Sucrose	127	127
Lactose	100	100
Cellulose	35	30
Peanut oil	20	20
Sunflower oil	20	20
Rutin	0	5
Mineral mixture[1]	35	35
Vitamin mixture[2]	10	10

[1] Composition of the mineral mixture was based on AIN-93M-MX composition (31) except for zinc carbonate (0.55 g/kg mineral mixture in Aox− and 1.65 g/kg mineral mixture in Aox+) and sodium selenate anhydrous (0 g/kg mineral mixture in Aox− and 0.14 g/kg mineral mixture in Aox+).

[2] Composition of the vitamin mixture was based on AIN-93-VX composition (31) except for folic acid (0.16 g/kg vitamin mixture in Aox− and Aox+ diets), vitamin E (all-*rac*-α-tocopheryl acetate, 500 UI/g) (2 vs. 60 g/kg vitamin mixture in Aox− and Aox+ diets, respectively), and vitamin A (all-*trans*-retinyl palmitate, 500,000 IU/g) (0.8 vs. 1.6 g/kg vitamin mixture in Aox− and Aox+ diets, respectively).

tolerated, preventing lipid peroxidation. Zinc, a cofactor of the cytosolic antioxidant enzyme superoxide dismutase, is thought to regulate its level and also to induce metallothioneins and prevent toxic reactions with transition metals (33). Selenium is also a cofactor of a key antioxidant enzyme, glutathione peroxidase. Finally, vitamin A is also considered a fat-soluble antioxidant enzyme. These antioxidants were already used separately, but to our knowledge were not used previously in combination to modulate several targets involved in the generation and/or protection against oxidative stress. The diets were prepared as pellets. Four groups of rats were studied: adult rats not supplemented (AAox −, $n = 16$), adult rats supplemented (AAox +, $n = 16$), old rats not supplemented (OAox −, $n = 16$), and old rats supplemented (OAox +, $n = 17$). Food intake and body weight were monitored once a week for 7 wk.

Before the experimental period (wk 0) and during the last week of the supplementation period (wk 7), rats were placed for 24 h in metabolism cages to collect urine. To avoid differences in food consumption, rats remained food deprived during these 24 h. In the evening, collected urine was stored at 4°C and pooled with urine collected during the night. An aliquot was taken and stored at −20°C until analysis.

At the end of the experimental period, rats were killed by exsanguination through the abdominal aorta under anesthesia with sodium pentobarbital (6 mg/100 g) after overnight food deprivation. Epitrochlearis muscles were dissected intact for incubation. Posterior leg skeletal muscles [gastrocnemius, tibialis anterior, extensor digitorum longus (EDL), and soleus], heart, liver, spleen, and kidneys were quickly excised, weighed, and frozen in liquid nitrogen until further analysis.

Oxidative stress and inflammatory markers. To estimate the efficiency of antioxidant supplementation, TBARS concentrations were measured in the heart according to the method described in (34). Because aging is generally associated with chronic inflammation (35,36), we also measured plasma α2-macroglobulin, fibrinogen, and albumin, which are considered markers of low-grade inflammation in old rats (36).

S6K oxidation assays. Because an increase in oxidative stress during aging may alter protein functionality, we measured the oxidation state of S6K, which is a major kinase implicated in the leucine effect on protein metabolism. Change in carbonyl content of the S6K protein was used as a marker of oxidative damage. Tibialis anterior muscles from all groups were homogenized in ice-cold 50 mmol/L Tris-acetate, 50 mmol/L NaF, 2.5 mmol/L EDTA, 1 mmol/L EGTA, 5 mmol/L sodium pyrophosphate, 5 mmol/L β-glycerophosphate, 2 mmol/L dithiothreitol, 1 mmol/L benzamidine, 4 μg/L leupeptin, pH 7.2 (1.5 mL/g muscle) with polytron (Kinematica). Then, 10 μL/mL of 5% Triton X-100 was added, the homogenate was rotated at 4°C for 30 min, and centrifuged at 10,000 × g for 10 min at 4°C. An aliquot of supernatant was filtered at 0.2 μm and 3 mg protein was incubated overnight at 4°C with anti-S6K antibodies (1/250 Cell Signaling) and immunoprecipitated for 2 h with protein A agarose at 4°C. The mixture was centrifuged at 13,000 × g for 10 min at 4°C and the pellet was washed 4 times with PBS 1%, mixed with SDS 6%, dinitrophenyl hydrazine (DNPH), Tris/glycerol/bromophenol blue, and mercaptoethanol as described (37), and incubated 10 min at room temperature. DNPH associated with S6K protein was determined by Western blot analysis using anti-DNPH antibody (1/1000 Bethyl Laboratories), visualized using the enhanced chemiluminescence method, and quantified with NIH imageJ 1.37v. Total S6K was quantified after the membrane was dehybridized (incubation 30 min at 50°C in 100 mmol/L 2-mercaptoethanol, 2% SDS, Tris-HCl, pH 6.7, washing and blocking) and incubated with anti-S6K antibody as primary antibody (1/200 Santa Cruz). Values of the DNPH associated with S6K were normalized for total S6K content in samples.

To validate the method, muscle proteins were subjected to in vitro oxidation by incubating the whole supernatant (without immunoprecipitation) with horseradish peroxidase and H_2O_2. Western blots were then performed either with intact supernatant or after oxidation using anti-DNPH antibody as primary antibody and donkey anti-goat antibody as secondary antibody, as described above. Oxidation induced by peroxidase generated increased carbonyl contents (**Supplemental Fig. 1**).
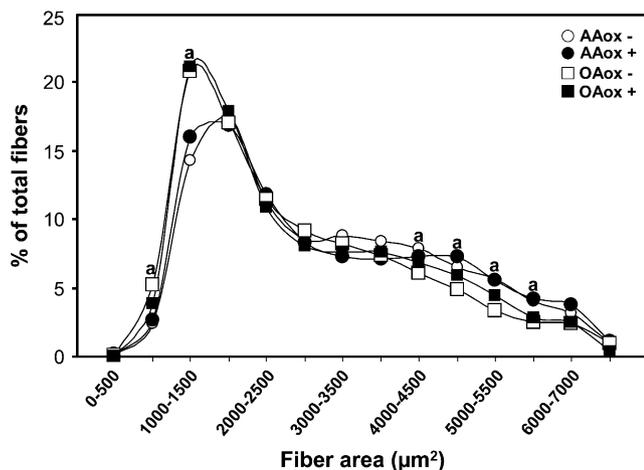
FIGURE 1 Muscle fiber area distribution in EDL muscles from adult (A) and old (O) rats supplemented (Aox+) or not (Aox−) with antioxidants for 7 wk. Values are means ± SEM, n = 10–12. The a indicates an effect of age, P < 0.05.

Muscle protein synthesis and degradation response to leucine. Epitrochlearis muscles were used to determine muscle protein metabolism as previously described (5). Briefly, muscles were preincubated for 1 h in Krebs-Henseleit buffer (containing in mmol/L: 120 NaCl, 4.8 KCl, 25 NaHCO$_3$, 2.5 CaCl$_2$, 1.2 KH$_2$PO$_4$, and 1.2 MgSO$_4$; pH 7.4) supplemented with 5 mmol/L HEPES, 5 mmol/L glucose, and 0.1% bovine serum albumin (99% fatty acid free) in the absence or presence of leucine (100, 200, or 400 μmol/L) saturated with 95% O$_2$-5% CO$_2$ gas mixture. Muscles were then transferred into fresh medium of the same composition for 75 min. They were then incubated for an additional 75 min in fresh medium containing 0.5 mmol/L L-U[^{14}C] phenylalanine (5.55 kBq/L). At the end of incubation, muscles were weighed, blotted, and homogenized in 10% trichloroacetic acid (TCA). Homogenates were centrifuged at 10,000 × g for 10 min at 4°C and TCA-insoluble material (which represented the protein fraction) was washed 3 times with 10% TCA to eliminate free radioactivity. The resultant pellet was solubilized in 1 mol/L NaOH at 37°C for determination of radioactivity incorporated into muscle protein fraction. Protein-bound radioactivity was measured using liquid scintillation counting. Protein synthesis was calculated by dividing the protein-bound radioactivity by the specific activity of the phenylalanine into the incubation medium; it was expressed as nmol phenylalanine incorporated per 100 mg muscle per 75 min.

Muscle protein degradation was measured in samples without leucine (basal) or containing 200 μmol/L leucine (maximal effect) as described previously (38). Because tyrosine is neither synthesized nor degraded in muscle, the release of tyrosine into the incubation medium directly reflects net protein breakdown. Proteolysis was then estimated by the sum of net tyrosine release into the incubation medium and protein synthesis, after conversion of the rate of phenylalanine incorporation into proteins into tyrosine equivalents (39). Protein degradation was expressed in nmol tyrosine incorporated per mg muscle per 75 min.

Histochemical analyses of muscle. Entire EDL muscles were frozen in cooled isopentane (−160°C), respecting their physiological length, and stored at −80°C until use. Cross 10-μm-thick cryo-sections were performed using a cryostat (HM560 Microm) and stained using picro-Sirius red coloration (40), which reveals the collagen of perimysium and endomysium. Observations and image acquisitions were performed using a photonic microscope in bright field mode (Olympus BX 71) coupled to a high resolution digital camera (Olympus DP 71) and Cell F software. For each muscle section, 15–20 images, depending on the total area of the muscle, were acquired at a magnification of 200-fold. Image analysis was done using Visilog 5.4 software (Noesis) (**Supplemental Fig. 2**). For a given optical field, the areas of each cell and of connective tissue

were automatically calculated by counting the number of pixels. Fiber boundaries were manually corrected when necessary.

Biochemical measurements. Urinary creatinine concentration was measured after reaction with picric acid and measurement of absorbency at 500 nm (ABX, Horiba) on a COBAS MIRA system (Roche Diagnostics).

Statistical analysis. Values are means ± SEM. Two-way ANOVA (Statview) was performed to analyze the effects of age, antioxidant supplementation, and their interaction. When measurements could be repeated over time (body weight, food intake, creatinine excretion), 2-way repeated-measures ANOVA was performed. A 3-way analysis was performed to analyze the effects of age, diet, and leucine concentration and their interaction for muscle protein synthesis response to leucine measurements. For muscle fiber areas, distribution was determined using XLSTAT (Addinsoft, version 7.5.2). When ANOVA indicated a significant overall effect, differences among individual means were assessed using a Student's t test. P ≤ 0.05 was considered significant.

Results

Rat characteristics. Before the experimental period, body weight was higher in old (663 ± 11 g) than in adult rats (607 ± 4 g; P < 0.0001). Total body weight increased in all groups during the experimental period (P < 0.0001), but it increased more in adult than in old rats (81 ± 9 and 99 ± 12 g in AAox− and AAox+, respectively, vs. 17 ± 14 and 12 ± 18 g in OAox− and OAox+, respectively; P < 0.0001). Body weights did not differ among groups at the end of the experimental period. Antioxidant supplementation did not affect body weight in either adult or old rats (**Table 2**).

Over the experimental period, mean food intake was slightly higher in adult than in old rats but was not modified by antioxidant supplementation (29.0 ± 0.5 and 29.8 ± 0.6 g dry matter ingested per day in AAox− and AAox+, respectively, vs. 26.2 ± 1.1 and 26.1 ± 1.1 in OAox− and OAox+, respectively; P < 0.05 for age effect).

Skeletal muscle weights were lower during aging (P < 0.0001) but were not affected by antioxidant supplementation (Table 2). Urinary creatinine excretion, a nondestructive index of total muscle mass, was also measured before and at the end of the experimental period (**Table 3**). Before supplementation, creatinine excretion was lower in old rats than in adult rats (P < 0.002). It increased during the experimental period in all groups (P < 0.0001) (Table 3). There was no direct effect of antioxidant supplementation on creatinine excretion; however, there was a trend for a greater increase in creatinine excretion from wk 0 to wk 7 in old rats supplemented with antioxidants (P = 0.0975 for interaction age × time).

Based on 2-way ANOVA, aging did not induce changes in heart and liver weights, whereas it increased spleen and kidney weights (Table 2). Antioxidant supplementation did not affect liver and kidney weights but significantly reduced heart and spleen weight (Table 2).

Muscle fiber area and distribution. Muscle fiber area distribution differed in old compared with adult rats (**Fig. 1**). Muscles from old rats contained a higher proportion of small area fibers (<2000 μm^2) than those from adult rats (43.1 ± 1.9 and 42.9 ± 1.7% vs. 34.2 ± 2.1 and 35.5 ± 3.3% in OAox− and OAox+ vs. AAox− and AAox+, respectively; P < 0.002). Conversely, a significant decrease in the percentage of larger area fibers (between 4000 μm^2 and 6000 μm^2) was observed in old compared with adult rats (16.8 ± 2.2 and 20.0 ±

TABLE 2 Body, skeletal muscle, and organ weights in adult and old rats supplemented (Aox+) or not (Aox−) with antioxidants[1]

	Adult		Old		
	Aox−	Aox+	Aox−	Aox+	2-Way ANOVA[2]
Body weight, g	692 ± 11	704 ± 12	675 ± 24	681 ± 23	
Gastrocnemius, g	5.96 ± 0.15	6.11 ± 0.16	4.95 ± 0.08	5.20 ± 0.11	A
Tibialis anterior, g	1.98 ± 0.05	2.04 ± 0.06	1.72 ± 0.06	1.81 ± 0.05	A
EDL, mg	510 ± 14	508 ± 12	439 ± 11	458 ± 10	A
Soleus, mg	415 ± 11	433 ± 13	360 ± 15	371 ± 18	A
Heart, g	1.69 ± 0.02	1.60 ± 0.03	1.78 ± 0.09	1.59 ± 0.07	D
Liver, g	16.9 ± 0.5	16.9 ± 0.4	16.0 ± 0.8	16.1 ± 0.6	
Spleen, g	1.18 ± 0.05	1.10 ± 0.03	1.59 ± 0.08	1.42 ± 0.05	A, D
Kidneys, g	3.42 ± 0.10	3.17 ± 0.07	4.94 ± 0.70	4.05 ± 0.39	A

[1] Values are means ± SEM, n = 10–12.
[2] A: Significant effect of age, $P < 0.005$; D: significant effect of diet, $P < 0.05$.

1.7% vs. 24.1 ± 1.7 and 24.2 ± 2.2% in OAox− and OAox+ vs. AAox− and AAox+, respectively; $P < 0.01$). There was no significant effect of antioxidant supplementation.

Inflammation and oxidative stress markers. Confirming the existence of an age-associated, low-grade inflammation, plasma fibrinogen and α2-macroglobulin concentrations were higher in old rats than in adults and plasma albumin was lower ($P < 0.05$) (**Table 4**). Antioxidant supplementation did not change plasma fibrinogen and albumin concentrations, whereas the plasma α2-macroglobulin concentration was reduced in antioxidant-supplemented rats ($P < 0.05$).

As expected, heart TBARS were dramatically decreased in antioxidant-supplemented rats ($P < 0.0001$), whereas age had no effect (676 ± 58 and 64 ± 10 nmol/g tissue in AAox− and AAox+, respectively, vs. 674 ± 61 and 114 ± 23 in OAox− and OAox+, respectively).

Effect of leucine on muscle protein synthesis. In the absence of leucine in the incubation medium, protein synthesis was similar in muscles of adult and old rats supplemented or not with antioxidants. Increasing leucine concentration in the incubation medium significantly increased the rate of muscle protein synthesis in epitrochlearis muscles from all groups (**Fig. 2**) and the maximal effect was recorded at 200 μmol/L. However, the ability of leucine to stimulate protein synthesis was reduced in muscles of old rats compared with adults not supplemented [1.59 ± 0.10 vs. 1.91 ± 0.08 ($P < 0.05$) and 1.63 ± 0.07 vs. 1.90 ± 0.07 ($P < 0.05$) nmol phenylalanine/(100 mg muscle · 75 min) in OAox− vs. AAox− for 200 and 400 μmol/L leucine, respectively].

Antioxidant supplementation did not change basal and leucine-stimulated protein synthesis in muscles from adult rats. In contrast, antioxidant supplementation improved the response of protein synthesis to leucine in muscles from old rats [1.85 ± 0.06 vs. 1.59 ± 0.10 ($P < 0.05$) and 1.85 ± 0.08 vs. 1.63 ± 0.07 ($P < 0.05$) nmol phenylalanine/(100 mg muscle · 75 min) for 200 and 400 μmol/L leucine in OAox+ and OAox−, respectively] (Fig. 2). Actually, the dose-response curve of leucine-stimulated protein synthesis in muscles from old rats supplemented with antioxidant did not differ from those obtained in muscles from adult rats.

Effect of leucine on muscle protein degradation. Basal protein degradation did not differ between adult and old rats or between Aox− and Aox+ (**Table 5**). The addition of leucine to the incubation medium (200 μmol/L) decreased proteolysis in all groups. However, the effect of leucine tended to be lower in old than in adult rats not supplemented with antioxidant (Aox− groups). Indeed, the difference between values obtained in the presence of leucine and basal values, which represented the ability of leucine to inhibit muscle protein degradation, was lower in old than in adult rats not supplemented. Antioxidant supplementation improved the leucine-induced inhibition of protein degradation in the old rats (Table 5).

Oxidation state of S6K. The carbonyl content, a marker of oxidative damages, detected by DNPH associated to S6K (expressed as a ratio to total S6K) was similar in adult and old rats and did not change by Aox supplementation (data not shown).

TABLE 3 Urinary creatinine excretion in adult and old rats supplemented (Aox+) or not (Aox−) with antioxidants before (wk 0) and at the end (wk 7) of the experimental period[1]

	Adult		Old	
Wk	Aox−	Aox+	Aox−	Aox+
	mmol·kg^{-1}·24 h^{-1}			
0	0.262 ± 0.008	0.270 ± 0.008	0.234 ± 0.009	0.214 ± 0.014
7	0.291 ± 0.013	0.284 ± 0.013	0.260 ± 0.012	0.279 ± 0.018

[1] Values are means ± SEM, n = 10–16. Significant effects were observed for age, $P < 0.002$, and time, $P < 0.0001$ with 2-way repeated measures analysis.

TABLE 4 Plasma inflammatory reactive proteins in adult and old rats supplemented (Aox+) or not (Aox−) with antioxidants[1]

	Adult		Old		2-Way ANOVA[2]
	Aox−	Aox+	Aox−	Aox+	
Fibrinogen, g/L	3.65 ± 0.28	3.36 ± 0.14	3.89 ± 0.24	4.20 ± 0.32	A
α_2-Macroglobulin, mg/L	94 ± 32	34 ± 5	169 ± 39	108 ± 24	A, D
Albumin, g/L	21.4 ± 1.0	23.3 ± 0.9	18.1 ± 3.4	17.3 ± 1.4	A

[1] Values are means ± SEM, n = 10–12.
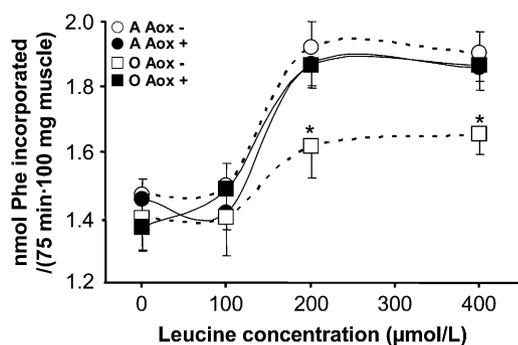[2] A: Significant effect of age, $P < 0.05$; D: significant effect of diet, $P < 0.05$.

FIGURE 2 Dose-response curves for leucine stimulated muscle protein synthesis in adult (A) and old (O) rats supplemented (Aox+) or not (Aox−) with antioxidants for 7 wk. Rate of protein synthesis was measured as in vitro incorporation of ^{14}C phenylalanine into epitrochlearis muscles in the presence of increasing leucine concentrations. Values are means ± SEM, $n = 10$–12. Significant effects were age ($P < 0.05$), leucine concentration ($P < 0.0001$), and age × diet ($P < 0.05$). *OAox− differs from other groups at that leucine concentration, $P < 0.05$.

Discussion

Muscle protein loss during aging may be partly explained by a decreased ability of old muscle to respond appropriately to food intake (10,11,41). This defect mainly results from a decreased response and/or sensitivity of protein synthesis and degradation to leucine in old muscle associated with a defect in S6K expression or activation (5,8). Because oxidative stress increased during aging (15,16), we hypothesized that it could be implicated in the age-associated impairment of the anabolic effect of leucine. In this study, we assessed the effect of a 7-wk antioxidant supplementation with rutin, vitamin E, vitamin A, zinc, and selenium on muscle protein metabolism response to leucine during aging in rats. In agreement with previous data, we observed a decreased ability of leucine to stimulate muscle protein synthesis in old rats (5,8). Previously, we demonstrated that a leucine concentration 2-fold of the postprandial level allowed protein synthesis to be stimulated normally in old muscles (5). In the present study, we showed that the defect in leucine-stimulated protein synthesis in muscles of old rats was reversed when they were supplemented with antioxidants, independently of an increase in leucine availability. Antioxidant supplementation also tended to improve the leucine-induced inhibition of protein degradation, especially in muscles from old rats. These data suggest that antioxidant supplementation, through synergistic effect with amino acids (leucine), can improve

muscle protein anabolism in old rats by restoring the leucine effect during the postprandial period.

Oxidative damage generated by increased oxidative stress during aging may alter protein functionality. It has been reported in various in vitro studies that oxidative stress impaired insulin action (glucose transport, glycogen synthesis), which was associated with impaired insulin signaling (18–23). For example, Tirosh et al. (22) demonstrated that oxidative stress also impairs insulin activation of S6K in 3T3-L1 adipocytes, a kinase part of the mammalian target of rapamycin signaling pathway leading to protein synthesis modulation. Like insulin, leucine stimulates protein synthesis by activating specific kinases implicated in pathways involved in the regulation of translation initiation, such as S6K. The lower ability of leucine to stimulate protein synthesis in aged muscles was associated with a defect in S6K activation (5,8). However, the molecular mechanisms responsible for this alteration remain unknown. Because aging increased oxidative damage of proteins, we hypothesized that oxidative modifications of S6K protein could contribute to the alteration of its activity in old subjects. In fact, the absence of change with age and diet in the carbonyl content of muscle S6K protein suggested that the decrease in leucine effect on muscle protein synthesis in old rats was not associated with changes in oxidative damage of S6K protein. Furthermore, the beneficial effect of antioxidant supplementation was not due to an improvement in S6K. Alternatively, it has been shown that Zn is able to directly stimulate phosphorylation of S6K or mammalian target of rapamycin activity in adipocytes (42) or in vivo in mice (43). Zinc was included in the antioxidant cocktail at a low dose in the present experiment and we cannot exclude that it may partly explain the increase in leucine-stimulation of muscle protein synthesis in old rats. To answer this question, it would be necessary to investigate a zinc supplementation alone.

Aging is also characterized by the development of a low-grade inflammation with an increase of cytokines and acute phase protein levels (44,45), which has been suspected to be one of the components implicated in sarcopenic development (25). Recently, Cuthbertson et al. (8) have shown that the inflammation-associated protein nuclear factor-κB was increased in aged human muscle and the authors concluded that a chronic inflammation state occurred during aging. In a previous study, we demonstrated that the development of low-grade inflammation in old rats does not modify skeletal muscle mass (46) but impairs muscle protein synthesis stimulation by food intake (47). In this study, plasma fibrinogen and α2-macroglobulin concentrations were also significantly increased and albumin significantly decreased in old rats compared with adults, an evidence of the development of low-grade inflammation (36). Interestingly, antioxidant supplementation reduced the plasma α2-macro-

TABLE 5 Protein degradation in epithrochlearis muscles from adult and old rats supplemented (Aox+) or not (Aox−) with antioxidants[1,2]

	Adult		Old		
	Aox−	Aox+	Aox−	Aox+	2-Way ANOVA[3]
	nmol tyr/(75 min · mg muscle)				
Basal	0.183 ± 0.007	0.188 ± 0.010	0.177 ± 0.011	0.181 ± 0.012	
Leu 200 μmol/L	0.142 ± 0.006	0.139 ± 0.004	0.159 ± 0.008	0.139 ± 0.006	$P = 0.06$ for D
Leu − basal	−0.041 ± 0.006	−0.051 ± 0.009	−0.018 ± 0.007	−0.042 ± 0.009	A, D

[1] Values are means ± SEM ($n = 9$–12).
[2] Rate of protein degradation was measured in epitrochlearis muscles incubated in vitro without (basal) or with 200 μmol/L leucine (Leu 200 μmol/L).
[3] A: Significant effect of age, $P < 0.005$; D: significant effect of diet, $P < 0.05$.

globulin concentration and spleen and kidney weights of old rats, suggesting a decrease in inflammatory status. Whether improvement of inflammatory status by antioxidant supplementation can partly explain the improved leucine effect on muscle protein metabolism in old rats remains to be established.

As expected, muscle mass and fiber areas were significantly lower in old compared with adult rats, confirming the age-related sarcopenia. Unfortunately, when looking at individual muscles or creatinine excretion (an index of total muscle mass), the improvement of protein synthesis response to leucine after the 7-wk antioxidant supplementation did not reach significance in preventing the age-related decrease in muscle mass. It may be postulated that a longer period of supplementation would be necessary to have a significant effect on muscle mass.

In conclusion, our results suggest that antioxidant supplementation is able to improve the ability of leucine to stimulate protein synthesis in muscles of old rats independently of an increase in leucine availability and without modification of S6K oxidative status. We propose that this effect may be mediated through a reduction of the inflammation state and/or oxidative stress, but the mechanisms involved remain to be determined. Antioxidant supplementation was probably not long enough to observe a clear effect on muscle mass. Further experiments are needed to determine the best conditions for this supplementation to prevent the age-associated decrease in muscle mass and function without negative side effects. Indeed, not only efficacy of antioxidants but also their safety must be assessed [reviewed in (29,48)].

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