Sarcopenia by a Dual Mechanism: Improvement of Protein Balance and of Antioxidant Defenses

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The aim of our study was to elucidate the role of growth hormone (GH) replacement therapy in three of the main mechanisms involved in sarcopenia: alterations in mitochondrial biogenesis, increase in oxidative stress, and alterations in protein balance. We used young and old Wistar rats that received either placebo or low doses of GH to reach normal insulin-like growth-factor-1 values observed in the young group. We found an increase in lean body mass and plasma and hepatic insulin-like growth factor-1 levels in the old animals treated with GH. We also found a lowering of age-associated oxidative damage and an induction of antioxidant enzymes in the skeletal muscle of the treated animals. GH replacement therapy resulted in an increase in the skeletal muscle protein synthesis and mitochondrial biogenesis pathways. This was paralleled by a lowering of inhibitory factors in skeletal muscle regeneration and in protein degradation. GH replacement therapy prevents sarcopenia by acting as a double-edged sword, antioxidant and hypertrophic.

Key Words: Mitochondrial biogenesis—p70S6K—Myostatin—IGF-1.

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Sarcopenia is a syndrome characterized by progressive and generalized loss of skeletal muscle mass and strength with a risk of adverse outcomes such as physical disability, poor quality of life, and death (1). This loss of muscle occurs at a rate of 3%–8% per decade after the age of 30 with a higher rate of muscle loss at advanced age (2). Recent estimates show that one-quarter to one-half of men and women aged 65 and older are likely sarcopenic (3). Progressive sarcopenia is ultimately central to the development of frailty, an increased likelihood of falls, and impairment of the ability to perform activities of daily living (1). The logical endpoint of severe sarcopenia is loss of quality of life and ultimately institutionalization (4).

The importance of maintaining muscle mass and physical and metabolic functions in the elderly adults is well recognized. Less appreciated are the diverse roles of muscle throughout life and the importance of muscle in preventing some of the most common and increasingly prevalent clinical conditions, such as obesity and diabetes (4). Skeletal muscle atrophy is a common feature in several chronic diseases and conditions. It reduces treatment options and positive clinical outcomes as well as compromising quality of life and increasing morbidity and mortality (4). Individuals with limited reserves of muscle mass respond poorly to stress (4). In support of the importance of maintaining skeletal muscle mass, strength, and function, a recent study has demonstrated that all-cause, as well as cancer-based, mortality is lowest in men in the highest tertile of strength, an indicator of high muscle mass (5).

If there is a preexisting deficiency of muscle mass before trauma, the acute loss of muscle mass and function may push an individual over a threshold that makes recovery of normal function unlikely to ever occur. For this reason, more than 50% of women older than 65 years who break a hip in a fall never walk again (6).

Several hormones have been suggested to have an impact on muscle mass, strength, and function (7). Among them, growth hormone (GH) has been one of the most studied (7). Levels of GH are usually lower in the elderly subjects, and the amplitude and frequency of pulsatile GH release are significantly reduced (7). Thus, it has been hypothesized that GH would be useful in preventing the age-related loss of muscle mass (8).

In our study, we aimed to elucidate the role of GH replacement therapy in four of the main mechanisms involved in the onset and progression of sarcopenia: alteration in mitochondrial biogenesis, increase in oxidative stress, increase
in protein degradation, and lowering in the rate of protein synthesis (9,10).

In this study, we present the existing evidence behind the argument that restoration of GH profile is a good intervention to improve or preserve skeletal muscle mass in old animals.

**Materials and Methods**

**Animals and Treatment**

Ten young (aged 1 month) and 20 old (aged 22 months) male Wistar rats, maintained under controlled light and temperature conditions, were used in the study. We chose 22-month-old rats because previous studies have reported that sarcopenia is evident at this age in this species (11). The animals were fed a normal rat chow (A.04; Panlab, Barcelona, Spain) and had free access to tap water. Half of the old animals ($n = 10$) were treated daily with two subcutaneous doses of GH (2 mg/kg/day) diluted in saline; Omnitrope, Sandoz, Spain), one at 10.00 hours and another at 17.00 hours for 8 weeks. Control animals were injected with the same amount of vehicle (saline solution) as GH-treated rats. After 8 weeks of treatment, rats were sacrificed by cervical dislocation followed by decapitation, and troncular blood was collected and processed to measure plasma insulin-like growth factor-1 (IGF-1). Gastrocnemius muscle, liver, and heart were collected and immediately frozen in liquid nitrogen. The study was conducted following recommendations from the Institutional Animal Care and Use Committee, according to the Guidelines for Ethical Care of Experimental Animals of the European Union. The Committee of Ethics in Research from the University Complutense of Madrid granted ethical approval.

We have previously shown that young animals do not show any effect when submitted to our GH treatment because they have high endogenous GH levels and also do not show alterations that could get ameliorated (12–14). This is why this experimental group has not been included in the study.

**Body Composition Study**

All rats were weighted weekly to determine changes in body weight during the study. After the rats were sacrificed, total body fat was determined by the specific gravity index (SGI), which shows the proportion between lean mass and body fat (15). This can be calculated comparing the animal’s carcass weight (animal without head, hair, and viscera) in the air ($W_{a}$) and in the water ($W_{w}$), using the following formula: $\text{SGI} = W_{a} / (W_{a} - W_{w})$ (assuming the specific gravity of water at $21^\circ\text{C}$ to be 1) (15).

**IGF-1 Levels**

Plasma and hepatic IGF-1 levels were measured as previously described (16) by an specific radioimmunoassay, using reagents kindly provided by the National Hormone and Pituitary Program from the National Institute of Diabetes and Digestive and Kidney Diseases and a secondary antibody obtained in our laboratory.

**Determination of Oxidative Damage in Gastrocnemius Muscle**

Oxidative modification of total proteins in gastrocnemius muscles was assessed by immunoblot detection of protein carbonyl groups using the “OxyBlot” protein oxidation kit (Millipore, MA) as previously described (17).

Oxidative DNA damage was measured by 8-hydroxy-2'-deoxyguanosine (8-OHdG). A commercially available enzyme-linked immunosassay (Highly Sensitive 8-OHdG Check; Japan Institute for the Control of Aging, Japan) was used to measure oxidized DNA in isolated muscle DNA samples. DNA was extracted from the muscle via the High Pure PCR Template Preparation Kit (Roche, GmbH, Germany) according to the manufacturer’s protocol. DNA was used if it had a minimum 260:280 ratio of 1.8. The assay was performed following the manufacturer’s directions. Briefly, 50 µL of DNA were incubated with the primary antibody, washed, and then incubated in secondary antibody. The chromogen (3,3′,5,5′-tetramethylbenzidine) was added to each well and incubated at room temperature in the dark for 15 minutes. The reaction was terminated, and the samples were read at an absorbance of 450 nm. Samples were normalized to the DNA concentration measured via a plate spectrophotometer for nucleic acids (ND-2000; NanoDrop, Wilmington, DE). All analyses were done in triplicate.

**Determination of Citrate Synthase and Glucose-6-Phosphate Dehydrogenase Activities in Gastrocnemius Muscle**

Citrate synthase assay was performed in the gastrocnemius muscle following the method of Srere (18). Results were obtained in nmol × mg of protein$^{-1} \times$ minute$^{-1}$. Values were normalized to those observed in the samples obtained from the young group, which were assigned a value of 100%.

Glucose-6-phosphate dehydrogenase (G6PDH) activity was determined following the method of Waller and coworkers (19). Results have been expressed in nmol × mg of protein$^{-1} \times$ minute$^{-1}$.

Protein concentrations were determined by Bradford’s method (20) by using bovine serum albumin as standard.

**Immunoblot Analysis**

Aliquots of muscle lysate (50–120 µg of proteins) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The whole gastrocnemius was used to ensure homogeneity. Proteins were then transferred to
nitrocellulose membranes, which were incubated overnight at 4°C with appropriate primary antibodies: anti-myf5 (1:200; Santa Cruz Biotechnology Inc., Santa Cruz, CA); anti-p70S6K (1:1,000; Cell Signaling); anti-phosphorylated p70S6K (1:1,000; Cell Signaling); anti-myostatin (1:1,000; Abcam, UK); anti-catalase (1:5,000; Sigma Aldrich, MO); anti-G6PDH (1:1,000; Abcam); anti-Gpx (1:2,000; Abcam); anti-cytochrome c (1:1,000; Santa Cruz Biotechnology); anti-PGC-1α (1:1,000; Cayman); anti-AKT (1:1,000; Cell Signaling); anti-phosphorylated AKT (1:1,000; Cell Signaling); anti-p38 (1:1,000; Cell Signaling); anti-phosphorylated p38 (1:1,000; Cell Signaling); anti-MuRF-1 (1:200; Santa Cruz Biotechnology Inc.); anti-MAFbx (1:500; Abcam); anti-Nrf1 (1:200; Santa Cruz Biotechnology Inc.); and anti-p21 (1:200; Santa Cruz Biotechnology Inc.). Thereafter, membranes were incubated with a secondary antibody for 1 hour at room temperature. Specific proteins were visualized by using the enhanced chemiluminescence procedure as specified by the manufacturer (Amersham Biosciences, Piscataway, NJ). Autoradiographic signals were assessed by using a scanning densitometer (Bio-Rad, Hercules, CA). Data were represented as arbitrary units of immunostaining. To check for differences in loading and transfer efficiency across membranes, an antibody directed against α-actin (1:1,000; Sigma Aldrich) was used to hybridize with all the membranes previously incubated with the respective antibodies. For the western blotting quantifications, we first normalized all the proteins measured to α-actin. Samples from each group were run on the same gel.

Statistical Analysis
Statistical analyses were performed using the SigmaStat 3.1 Program (Jandel Corp., San Rafael, CA). Results are expressed as mean ± SD. Normality of distribution was checked with the Kolmogorov test, and homogeneity of variance was tested by Levene’s statistics. We used one-way analysis of variance to compare group differences. If overall analysis of variance revealed significant differences, post hoc (pairwise) comparisons were performed using Tukey’s test. Differences were considered significant if $p < .05$.

RESULTS
Effect of Aging and GH Replacement Therapy on Body Composition and IGF-1 Plasma and Hepatic Levels in Rats
Table 1 shows the effect of aging on body composition of rats. In the 2-month study period, young animals increased their weight by 20 g (6.9% of their initial weight), whereas old animals lost weight by approximately 60 g (~9.8% of their initial weight). However, when old animals were treated with GH, they showed an increase in weight of approximately 9 g (1.5% of their initial weight), that is, very significantly different from the loss of weight that occurred in untreated old rats. This loss in weight was mainly due to changes in lean mass because the SGI fell from 5 in young animals to 3 in old ones, which mean that adiposity is augmented and lean body mass reduced. Old animals treated with GH had an intermediate SGI, that is, 4. We measured the gastrocnemius atrophy by weighting the muscle, and we found a significant (30%) decrease in the relative muscle weight in the old animals, that was significantly prevented in the old treated ones. It is well known that GH increases the weight of the heart. We show that the relative weight of the heart of old animals fell in the study period, and that treatment with GH resulted in a significant increase in the relative heart weight (see Table 1).

Table 1. Effect of Aging and GH Replacement Therapy on Body Composition and IGF-1 Plasma and Hepatic Levels in Rats

<table>
<thead>
<tr>
<th></th>
<th>Young (n = 10)</th>
<th>Old (n = 10)</th>
<th>Old + GH (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total final body weight (g)</td>
<td>311.2 ± 14.1</td>
<td>569.5 ± 75.0 *</td>
<td>616.7 ± 26.6 *</td>
</tr>
<tr>
<td>Weight change during the 2-month study period (g)</td>
<td>+19.9 ± 4.4</td>
<td>−61.9 ± 36.2 †</td>
<td>+9.3 ± 5.1</td>
</tr>
<tr>
<td>Weight change during the 2-month study period (% of initial weight)</td>
<td>+6.9 ± 4.8</td>
<td>−9.8 ± 5.7 †</td>
<td>+1.5 ± 0.1</td>
</tr>
<tr>
<td>Relative gastrocnemius muscle weight (g/100 g body weight)</td>
<td>0.52 ± 0.03</td>
<td>0.36 ± 0.02 *</td>
<td>0.41 ± 0.01 †</td>
</tr>
<tr>
<td>SGI</td>
<td>5.0 ± 0.1</td>
<td>3.0 ± 0.6 *</td>
<td>4.0 ± 0.3 †</td>
</tr>
<tr>
<td>Relative cardiac weight (g/100 g of body weight)</td>
<td>0.26 ± 0.02</td>
<td>0.21 ± 0.00 †</td>
<td>0.25 ± 0.01 †</td>
</tr>
<tr>
<td>Plasma IGF-1 levels (ng/mL)</td>
<td>1103 ± 61</td>
<td>590 ± 70 ‡</td>
<td>1180 ± 90 ‡</td>
</tr>
<tr>
<td>Hepatic IGF-1 levels (ng/mL)</td>
<td>237 ± 8</td>
<td>192 ± 19 †</td>
<td>324 ± 51 †</td>
</tr>
</tbody>
</table>

Notes: The values are shown as mean ± SD. Differences were checked for statistical significance by a one-way analysis of variance. GH = growth hormone; IGF-1 = insulin-like growth factor-1; SGI = specific gravity index.

$p < .05$, $*p < .01$ young vs old.

$p < .05$, †$p < .01$ old vs old animals treated with GH.

$p < .01$ young vs old animals treated with GH.
**GH Replacement Therapy Prevents Age-Associated Oxidative Damage to Skeletal Muscle**

Figure 1 reports the effect of aging on protein and DNA oxidation and its prevention by GH replacement therapy. Figure 1A shows results of the effect of aging on skeletal muscle protein oxidation. Aging resulted in an increase in protein oxidation that was relatively small (~10%) but statistically significant. This was prevented by treatment with GH, thus old animals treated with GH had values of protein oxidation that were not distinguishable from those of young animals. In Figure 1B, we report results on DNA oxidation. Old animals had a significantly increased DNA oxidation (as determined by the levels of 8-OHdG) when compared with young controls. Treatment with GH completely prevented this increase.

Thus, protein, as well as DNA oxidation, is elevated in muscles of old animals, but this is prevented by treatment of old animals with GH.

**Effect of Aging and GH Replacement Therapy on Antioxidant Enzyme Levels in Skeletal Muscle of Rats**

To seek an explanation for the effect of GH replacement therapy in protecting against oxidative damage, we measured the levels of three important antioxidant enzymes: catalase, glutathione peroxidase, and G6PDH, the latter being an antioxidant because it generates NADPH required for normal functioning of the glutathione redox cycle and activates catalase (21). Treatment with GH increased the levels of antioxidant enzymes (see Figure 2). The effect of aging itself on the levels of these enzymes was marked and was significant from the statistical viewpoint in the case of G6PDH. We also determined the G6PDH enzymatic activity, and we confirmed our western blotting results. Young animals had a skeletal muscle G6PDH activity of $0.49 \pm 0.09$ nmol $\times$ mg of protein$^{-1} \times$ minute$^{-1}$. We found a significant ($p < .01$) decrease in its activity in the old animals ($0.28 \pm 0.04$ nmol $\times$ mg of protein$^{-1} \times$ minute$^{-1}$) that was recovered in the old group treated with GH ($0.44 \pm 0.08$ nmol $\times$ mg of protein$^{-1} \times$ minute$^{-1}$) ($p < .01$). However, we did not find a significant effect of aging on catalase and glutathione peroxidase protein levels. In any case, there was a clear upregulation of these enzymes when we treated old animals with GH. These effects explain the prevention of age-associated damage to muscle proteins and DNA by GH and indeed suggest a so far unknown antioxidant effect of GH.

**Effect of Aging and GH Replacement Therapy on Mitochondriogenesis in Rat Muscle**

We have previously observed that mitochondriogenesis (which is heavily dependent on the activity of PGC-1α) is seriously affected by oxidative stress (10). The observation that GH prevents age-associated oxidative stress in muscle prompted us to test whether PGC-1α was affected in old animals and whether treatment with GH could reverse this effect. Figure 3 shows that PGC-1α levels were significantly lower in old animals than in young ones. This decrement (see Figure 3A) was completely prevented by...
replacement therapy with GH. Similar results were found with the protein levels of NRF-1. PGC-1α coactivates NRF-1, and as expected, we found a significant decrease in the NRF-1 levels in the old animals that was prevented by treatment with GH (see Figure 3B). Because PGC-1α is the master regulator of mitochondriogenesis, we tested whether GH replacement therapy in old animals resulted in changes in mitochondrial mass, and for this, we used two markers: cytochrome c protein levels and citrate synthase activity. Figure 3C and D shows that levels of either cytochrome c or citrate synthase activity were significantly lower in old animals than in young ones, and that this decrease was fully prevented when old animals were treated with GH. So, we can conclude that mitochondriogenesis in old animals, which depends on PGC-1α, is seriously depressed in old animals (as already well established), but that this is prevented by treatment with replacing doses of GH.
Effect of Aging and GH Replacement Therapy on Skeletal Muscle Protein Synthesis

It is well established that skeletal muscle mass is lower in old animals than in young ones, and that GH may have an effect in preventing this (12–14). However, so far, it is not clear whether the effect of GH on muscle mass is due to suppression in protein degradation, increase in amino acid uptake, and/or stimulation of protein synthesis. The insulin family ligands can bind to the IGF-1 receptor, which then phosphorylates IRS-1 (22). This protein acts as a docking protein for activation of phosphoinositide-3 kinase (PI-3K) (22). PI-3K activation leads to phospholipid generation in the plasma membrane, which recruit and activate AKT Kinase. We found a significant decrease in the phosphorylation of AKT in the old muscles that was completely recovered when the old animals were treated with GH (see Figure 4A). AKT Kinase activation leads to activation of mammalian target of rapamycin (mTOR) and of the mitogen-activated serine/threonine kinase p70 ribosomal protein S6 kinase (p70S6K). Recent work in humans
BRIOCHE ET AL.

Figure 4. Growth hormone (GH) replacement therapy activates protein synthesis in the old skeletal muscle. Thirty animals were divided into three experimental groups: young (Y) \((n = 10)\), old (O) \((n = 10)\), and old animals treated with GH (OGH) \((n = 10)\). Western blotting analysis to detect Akt activation (A), p70S6K activation (B), and myf-5 (C) in rat gastrocnemius muscle were performed. Representative blots are shown. The content of α-actin, a housekeeping protein marker in skeletal muscle, was determined in all the experimental groups. For the densitometric analysis of the results, values are shown as mean \((± SD)\). Values were normalized to those observed in the samples obtained from the young group, which was assigned a value of 100\%. In panels A and B, the data are represented as a percentage of immunostaining values obtained for the phosphorylated form of the kinase relative to the total form. \(*p < .05\), \(**p < .01\), NS = nonsignificant.

has identified the mTOR complex I (mTORC1) as being required to stimulate muscle protein synthesis in humans (23). p70S6K, a downstream target of mTORC1, plays a critical role in cell growth and survival (24–27). We have found that the phosphorylation of p70S6K decreases with aging and is restored to young values with GH replacement therapy (see Figure 4B).

Myf-5 is a primary myogenic regulatory factor. It facilitates repair or regeneration and growth of mature myofibers. Figure 4C reports results on myf-5 protein levels in young and old animals and the effect of GH replacement therapy. Although aging did not affect the basal muscle levels of myf-5, we found a significant increase in this myogenic factor in the GH-treated group.

**Effect of Aging and GH Replacement Therapy on the Expression of Inhibitory Growth Factors in Skeletal Muscle**

Myostatin is one of the major inhibitory factors in skeletal muscle regeneration. It downregulates myf-5 and myoD.
We found a significant increase in myostatin in the old skeletal muscle that was prevented with GH replacement therapy (Figure 5A). Myostatin maintains satellite cell quiescence and represses cell renewal through the induction of p21 (28), which is a cell cycle inhibitor (29). p21 expression is significantly (40%) increased in old animals when compared with young ones, this is prevented by treatment with GH (see Figure 5B). Recently, we have shown that p38 signaling promotes skeletal muscle atrophy through the expression of E3 ubiquitin ligases (30). Figure 5C shows an increase in the phosphorylation of p38 mitogen-activated protein kinase (MAPK) in the muscle of old animals. GH treatment significantly prevented the p38 phosphorylation. To finally identify the mechanism by which GH prevents the loss of muscle mass during aging, we determined the expression, in gastrocnemius muscle, of the muscle atrophy F-box (MAFbx) and muscle RING finger-1 (MuRF-1). MAFbx and MuRF-1 are two well-known muscle-specific E3 ubiquitin ligases involved in proteolysis. We did not find a significant effect of aging on MAFbx protein levels (see Figure 5E). However, aging was associated with a significant increase in MuRF-1 that was prevented by treatment with GH.

Collectively, the data reported in Figures 4 and 5 show that the combined effect of the upregulation of myostatin, p21, p38, and MuRF-1 and the downregulation of AKT-p70S6K and myf-5 may be involved in the lowering in protein levels in the skeletal muscle of old rats. GH replacement therapy seems to be a good strategy to restore skeletal muscle regeneration and to combat the process leading to sarcopenia.

**DISCUSSION**

**Effect of Aging and GH Replacement Therapy on Body Composition of Rats**

There are three general approaches to hormone therapy. Hormones can be given to replace a deficiency, to raise their concentration above the normal value, and finally agents can be given to block hormone action by either reducing the rate of secretion or by blocking their action (4). Despite the large number of studies aiming at assessing the effects of GH supplementation on muscle mass, the controversial findings reported in the literature maintain the debate as to whether or not to use GH to treat sarcopenia (31). The contrasting findings reported may be explained by methodological differences such as dosing. High doses of GH cause high incidence of adverse effects (32,33). Thus, we have used relatively low doses of GH in our study. We need to take into consideration that the GH used was of human origin, so that the response was not the same as for rat GH. On the other hand, small animals need a much higher dosage than humans, as was demonstrated by Mordenti and coworkers (34). Plasma IGF-1 values were lower in old than in young animals, but IGF-1 levels in old animals treated with GH were not statistically different from young controls. We also determined the hepatic IGF-1 levels and found similar results, an age-associated decrease in the liver IGF-1 levels that was prevented by the treatment with GH (see Table 1).

In our hands, GH replacement therapy is useful in preventing the age-related muscle mass loss.

In the 2-month study period, we found that young animals increased their weight, whereas old animals lost weight (see Table 1). However, when old animals were treated with GH, they showed an increase in weight of approximately 9 g, that is, significantly different from the loss of weight that occurred in untreated old rats. This loss was mainly due to changes in lean mass because the SGI fell from 5 in young animals to 3 in old ones. SGI is an index that relates lean body mass and fat mass; the higher it is, the less fat the animal has. Our data also show that GH administration significantly increases SGI in old male rats, which means that GH, through its anabolic, antiapoptotic, and lipolytic properties, is able to increase muscle mass and reduce body fat (12,13). We also determined the gastrocnemius muscle atrophy by weighting the muscles, and we found a 30% decrease in the relative muscle weight in the old animals that was significantly prevented in the old treated ones. Finally, we also found that the relative weight of the heart of old animals fell along the study period, and that treatment with GH resulted in an increase in heart weight (see Table 1).

**The Antioxidant Effect of GH Replacement Therapy**

The free radical theory of aging has provided a theoretical background to devise experiments to understand aging (35). It is now well established that upregulating the endogenous antioxidant defenses is a useful mechanism for cells to prevent damage associated with excessive free radical production (36,37). The effects of GH on sarcopenia have been studied extensively (32,38), but so far, they have been completely dissociated with prevention of free radical damage. A major finding reported in this article is that GH supplementation can act as an antioxidant because it upregulates the expression of important intracellular antioxidant enzymes, such as catalase, glutathione peroxidase, and G6PDH (see Figure 2). The result of this upregulation is that, as reported in Figure 1, old animals treated with relatively small doses of GH suffer less oxidative stress than untreated old animals, both in terms of protein oxidation (measured as carbonylation) and DNA oxidation (measured by the levels of 8-OHdG). Our results show that supplementation with GH activates endogenous antioxidant enzymes, prevents oxidative damage to critical cellular structures, and thus behaves as an antioxidant. This may contribute to explain the protection against sarcopenia conferred by supplementation with GH as discussed in the following paragraphs. The mechanism by which GH activated the expression of antioxidant enzymes is beyond the scope of this article and is being studied in this laboratory.
Figure 5. Growth hormone (GH) replacement therapy attenuates the age-associated increase in protein degradation in skeletal muscle. Thirty animals were divided into three experimental groups: young (Y) (n = 10), old (O) (n = 10), and old animals treated with GH (OGH) (n = 10). Western blotting analysis to detect myostatin (A), p21 (B), P38 (C), MuRF-1 (D), and MAFbx (E) in rat gastrocnemius muscle were performed. Representative blots are shown. The content of α-actin, a housekeeping protein marker in skeletal muscle, was determined in all the experimental groups. For the densitometric analysis of the results, values are shown as mean (±SD). Values were normalized to those observed in the samples obtained from the young group, which was assigned a value of 100%. (C) The data are represented as a percentage of immunostaining values obtained for the phosphorylated form of the kinase relative to the total form. *p < .05, **p < .01, ***p < .001. Mafbx = muscle atrophy F-box; Murf-1 = muscle RING finger-1; NS = nonsignificant.
Protein Synthesis, Mitochondriogenesis, and the Prevention of Sarcopenia by GH

The maintenance of skeletal muscle mass is regulated by a balance between protein synthesis and protein degradation (39). Muscle protein synthesis decreases with age (40). The involvement of p70S6K in skeletal muscle hypertrophy has been documented in various animal models (41). When activated via AKT Kinase, mTOR influences translation initiation by phosphorylation of p70S6K, which, in turn, phosphorylates the S6 ribosomal protein and allows the upregulation of a subclass of mRNAs encoding the translational apparatus (42). As shown in Figure 4, we found a significant decrease in the phosphorylation of AKT in the skeletal muscle of the old animals that was completely recovered when they were treated with GH. Similarly, phosphorylation of p70S6K was lower in old skeletal muscles than in young ones, and this was not caused by changes in total p70S6K protein levels. Old animals treated with GH showed similar phospho-p70S6K values than young animals (see Figure 4). Our results contradict previous studies showing that an intraperitoneal injection of IGF-1 increases phosphorylation of p70S6K in the young but not in the old skeletal muscle (43).

The attenuation in the capacity for muscle hypertrophy in old individuals has also been related to an age-related impairment in myogenic potential (44,45). Thus, we aimed to compare the myogenic response of gastrocnemius muscle in young and old rats treated with GH. Myf-5 is a well-known marker of myoblast/satellite cell differentiation and facilitates repair or regeneration and growth of mature myofibers (46). It has been shown that GH treatment upregulates not only liver IGF-1 but also skeletal muscle IGF-1 gene expression (47) that is involved in the activation of satellite cells (48). Figure 4 shows that although aging did not cause a decrease in the myf-5 skeletal muscle protein levels, GH replacement therapy significantly increased the levels of this myogenic factor.

We then focused our interest in myostatin, a negative muscle regulatory factor (48). This belongs to the transforming growth factor-β family, but its expression is restricted to muscle tissue (49). Absence or blockade of myostatin induces massive skeletal muscle hypertrophy that was initially attributed to the proliferation of the population of muscle fiber-associated satellite cells (50). However, it has been recently shown that myostatin regulates protein balance within the muscle fibers themselves. Several research groups have shown that hypertrophy, in the absence of myostatin, involves little or no input from satellite cells (51,52). Hypertrophic fibers contain no more myonuclei or satellite cells, and myostatin has no significant effect on satellite cell proliferation in vitro (51). As previously reported, we found an increase in myostatin and p21 in old muscles (53). GH replacement therapy significantly reduced them (see Figure 5). Thus, the effect of GH on these two factors may contribute to the prevention of muscle wasting. Phosphorylation of p38 MAPK has been reported after addition of myostatin in skeletal muscle fibroblasts (54). p38 is a stress-activated protein kinase that responds to a variety of stimuli, including oxidative stress and tumor necrosis factor-α (30), and has been identified as a likely mediator of catabolic signaling in skeletal muscle (55,56). Thus, we determined the phosphorylation of p38 in the old animals that was prevented by hormone replacement therapy with low doses of GH.

To identify the final mechanism by which GH prevents the loss of muscle mass during aging, we determined the expression of two well-known muscle-specific E3 ubiquitin ligases involved in several in vivo models of skeletal muscle atrophy, MAFbx and MuRF-1 (58). Although controversial (59), the levels of both MuRF-1 and MAFbx mRNA are markedly upregulated in aged muscles (60). We found a significant increase in muscle MuRF-1 protein levels in the old animals that was prevented by GH treatment. However, we did not find any change on muscle MAFbx protein levels. Thus, MuRF-1 seems to be involved in the age-associated sarcopenia.

Aging causes a decrease in mitochondrial content and activity (61,62). PGC-1α is a master regulator of mitochondrial biogenesis (63,64) and itself is a molecule that responds swiftly to the changes in oxidative stress (36,64,65). Since, as described in the previous paragraph, we have seen that aging in muscle results in an increase in oxidative stress markers and that this is prevented by relatively low doses of GH, we tested whether aging resulted in a decrease in PGC-1α expression in muscle and this was indeed the case, as shown in Figure 3. Treatment with GH completely prevents the decrease in PGC-1α associated with aging. PGC-1α coactivates NRF-1, and we found that the levels of NRF-1 were significantly lower in the skeletal muscle. This was completely prevented when animals were treated with GH. We previously reported that PGC-1α does not respond to the normal activation by exercise when animals are old (10). This lack of responsiveness may be due to a blocking of the activating mechanisms by GH because when it is administered to animals, PGC-1α is activated and mitochondrial biogenesis resumes, as shown in Figure 3. Probably GH activates PGC-1α, acting in three ways. Stimulating the IGF→AKT→mTOR→p70S6K pathway, inhibiting the myostatin→p38→MuRF-1, and acting as an antioxidant (as described earlier). Recently, Vescovo coworkers, working in cardiac muscle, reported that GH activates PGC-1α via IGF-1 and calcineurin (66). Our previous work showing that PGC-1α could also be activated by MAPKs together with the antioxidant effects discussed in the previous paragraph may explain the unique capability of GH supplementation to maintain normal muscle levels in the old animal. Our previous work (10) showed that exercise, cold exposure, or even thyroid hormone treatment could not activate PGC-1α in the old animals. A marker of mitochondrial
mass, cytochrome c protein levels, were also lower in the muscle from old animals. We also found that the citrate synthase activity was 50% lower in the muscles of our old animals than in the muscles of young ones. It has been reported that mitochondrial isolation procedures induce preferential losses of matrix soluble enzymes, such as citrate synthase, in aged muscle mitochondria (67). In a recent study, Picard and coworkers examined the differences in mitochondrial function between permeabilized muscle fibers and isolated mitochondria from the same sample (68). The authors found that mitochondrial function was decreased in both isolated mitochondria and permeabilized fibers, with an exaggerated age-effect in isolated mitochondria (68). We cannot rule out the idea that the dramatic decrement found in the citrate synthase activity in our study might have been due to sample preparation. However, we consider that collectively the results reported in Figure 3 support the idea that GH replacement therapy prevents the age-associated decline in mitochondrial content in the old skeletal muscle.

In this respect, the activation by GH that we report here seems to be unique in maintaining normal muscle mass in the old animal and thus preventing sarcopenia.

In this study, we report results supporting the argument that restoration of GH profile is a good intervention to preserve skeletal muscle mass in the elderly subjects. A schematic interpretation of our results is shown in Figure 6. We would like to reiterate that the supplementation of GH that we have performed in the animals is a rather low one in that the aim is to return the levels to the normal physiological ones. We do not claim here that supplementation with high doses of GH should be recommended. However, small doses of GH supplementation may be a very useful way to prevent age-associated sarcopenia. If these results could be extrapolated to humans, one could suggest that the

Figure 6. GH replacement therapy and its effects in sarcopenia. GH = growth hormone; IGF-1 = insulin-like growth factor-1; mTOR = mammalian target of rapamycin; Murf-1 = muscle RING finger-1; ROS = reactive oxygen species.

(Table 1)
loosing of muscle mass observed in persons, even if they have performed exercise in their youth, could be prevented by hormone replacement therapy with low doses of GH. This interesting possibility remains to be studied in the clinical setting.

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**Conflict of Interest**

The authors declare that no conflict of interest exists.

**References**


**GROWTH HORMONE AS ANTIOXIDANT**

1197


