Resistance training regulates gene expression of molecules associated with intramyocellular lipids, glucose signaling and fiber size in old rats

Manoel Benício Teixeira Ribeiro1, Vinicius Guzzoni2, Jeffrey M. Hord3, Giselle Nunes Lopes4, Rita de Cássia Marqueti5, Rosângela Vieira de Andrade6, Heloisa Sobreiro Sellistre-de-Araujo4 & João Luiz Q. Durigan6

Sarcopenia is a complex multifactorial process, some of which involves fat infiltration1 and a reduction in skeletal muscle cross sectional area (CSA)2. Intramyocellular lipid (IMCL) accumulation is postulated to play a role in the progression of sarcopenia with aging3. Evidence indicates that IMCL accumulation blunts muscle glucose transport activity and glycogen synthesis4. Accordingly, age-induced changes in mitochondrial biogenesis may affect fatty acid oxidation and result in accumulation of lipids in skeletal muscle cells leading to an alteration in glucose uptake and glycogen synthesis5. However, the mechanisms those mediate IMCL and glucose homeostasis during age-related muscle loss have yet to be elucidated.

Various transcription factors and intracellular pathways have been implicated in the regulation of fat and glucose metabolism. For example, peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer binding proteins (C/EBPs), such as C/EBPα, are key early regulators of adipogenesis6. PPARγ also regulates lipogenesis in skeletal muscle7. Additionally, PGC-1α, a PPARγ binding protein plays a role in the transcriptional control of oxidative metabolism8 and fiber type switching9. PGC-1α also increases lipogenesis and lipid

1College of Physical Education, University of Brasilia, Distrito Federal, Brazil. 2Postdoctoral Fellowship, University of Brasilia, Distrito Federal, Brazil. 3Department of Molecular Physiology and Biophysics, Carver College of Medicine, University of Iowa, Iowa City, United States. 4Department of Physiological Sciences, Center of Biological and Health Science, Federal University of São Carlos, São Carlos, Sao Paulo, Brazil. 5Graduate program of Rehabilitation Sciences, University of Brasilia, Distrito Federal, Brazil. 6Graduate program of Genomics and Proteomics, Catholic University of Brasilia, Distrito Federal, Brazil. Manoel Benicio Teixeira Ribeiro and Vinicius Guzzoni contributed equally to this work. Correspondence and requests for materials should be addressed to V.G. (email: vinicius.guzzoni@gmail.com)
catabolism in skeletal muscle. Another factor is known as lipoprotein lipase (LPL), which is a key enzyme responsible for fatty acid and lipoprotein metabolism in muscle. The regulation of age-related alterations in glucose and fat metabolism have been documented in mice. However, the role of key regulatory components of glucose homeostasis, such as glycogen synthase type 1 (Gly-Syn-1), glucose-6-phosphate dehydrogenase (G6PDH), hexokinase type 2 (Hk-2) and glucose transporter 4 (GLUT-4) with aging is not fully understood. Considering the complexity of the crosstalk between adipogenic transcriptional factors, glycogen metabolism and atrophy/hypertrophy signaling pathways, a better understanding of molecular pathways that regulate the aging-induced phenotypes is needed.

Skeletal muscle atrophy is complex process that is due in part to proinflammatory cytokines, which includes TNF-α (tumor necrosis factor-α), TWEAK (tumor necrosis factor apoptosis inducing) and its receptor, Fn14 (growth factor-inducible 14 receptor fibroblasts). Further downstream, activation of forkhead box protein O1 (FOXO-1) promotes the expression of E3 ubiquitin ligases, Atrogin-1 and muscle ring finger protein-1 (MURF-1), which are key players in the ubiquitin-proteasome system. The loss of muscle mass during aging is also under influence of various growth factors, such as those in the TGF-β (transforming growth factor-β) family, mostly notably, myostatin. Along with elevated rates of protein degradation, sarcopenia has also been associated with a reduction in muscle protein synthesis. Anabolic signaling is primarily attributed to the activation of anabolic signaling axis which includes IGF-1 (Insulin-like growth factor 1), mTOR (mammalian target of rapamycin) and activation of p70S6K-1 (p70S6 kinase 1). Furthermore, altered sensitivity of satellite cells is implicated in sarcopenia.

Resistance training (RT) has been consistently recommended to minimize the age-related muscle adaptations. In this regard, sarcopenia, along with fatty acid infiltration, is associated with physical inactivity. Moreover, it has emerged that exercise training confers beneficial effects on glucose homeostasis and modulates IMCL. However, the effects of RT on molecules related to glucose homeostasis and lipogenesis in skeletal muscle with advancing age are unknown. Furthermore, how RT affects intracellular signaling and transcription factors that control glucose homeostasis, lipogenesis and morphology of skeletal muscle in the aging is not understood.

Based on our assumptions, we hypothesized that RT could protect against the age-induced IMCL accumulation, glucose homeostasis and muscle atrophy via downregulation of adipogenic factors (CEBP-α, LPL, PPAR-γ and PGC-1α), and atrophy-associated molecules (TNF-α, TWEAK/Fn14 axis; FOXO-1, Atrogin1, MURF-1 and myostatin) concomitant to increases in hypertrophy-associated factors (IGF-1/mTOR/p70S6k-1/MyoD) and glucose homeostasis (Gly-Syn-1, G6PDH, GLUT-4). Thus, we sought to investigate the effects of RT on mRNA levels of atrophy/hypertrophy signaling, intracellular fatty infiltration and glucose metabolism in the soleus and gastrocnemius muscles of aged rats.

### Results

#### Body weight in old and trained rats.

The iBW and fBW are shown in Table 1. The iBW of old rats was higher than young groups at the beginning of experiment - first day of RT session. After 12 weeks of RT, fBW of YS and YT rats were 57.8% and 44.4% higher in comparison to their matched iBW. On the other hand, fBW of OS and OT groups decreased (6.8% and 15%, respectively) when compared to their matched iBW.

#### CSA and IMCL and glycogen content of gastrocnemius (GAS) and soleus (SOL) muscles in old and trained rats.

**Table 1.** Body weight (BW), percentage of gain of BW, gastrocnemius (GAS) and soleus (SOL) weights, glycogen content of GAS and SOL muscles from experimental groups. iBW = initial body weight; fBW: final body weight; Gain = percentage of increasing or decreasing of BW after 12 weeks; GAS weight = Glycogen content of gastrocnemius; SOL weight = Glycogen content of soleus. Groups: Young Sedentary (YS), Young Trained (YT), Old Sedentary (OS) and Old Trained (OT). Values are expressed as mean ± SEM. Two-way ANOVA, p < 0.05: a vs. YS; b vs. YT; c vs. OS. n = 6/group.
mRNA levels of adipogenic factors in response to aging and exercise in SOL muscle. CEBP-α, LPL, PPAR-γ and PGC-1α mRNA levels were elevated with aging (Fig. 3A–D). In response to exercise training, RT elevated mRNA levels of CEBP-α and PGC-1α in SOL muscle (Fig. 3A and D), whereas PPAR-γ and LPL were reduced in OT in relation to OS animals (Fig. 3B and C). In young rats, RT led to decreases in CEBP-α and PPAR-γ mRNA levels, even though PGC-1α was increased in YT rats in relation to their age-matched counterparts.

mRNA levels of glucose metabolism regulators of SOL muscle in response to aging and exercise. Aging did not affect GLUT-4, G6PDH, Hk-2 and Gly-synt-1 mRNA levels in SOL muscle (Fig. 3E–H). However, all those transcripts were elevated in trained rats when compared with their matched sedentary group (OT vs. OS and OT vs. YS).

mRNA levels of atrophy and hypertrophy-related factors of SOL muscle in response to aging and resistance training. RT was effective at mitigating the age-associated increase of TNF-α, TWEAK, Atrogin-1 and MURF-1 mRNA levels in SOL muscle (OT vs. OS) (Fig. 3I, J, M and N). Likewise, RT decreased FOXO-1 and myostatin in OT rats when compared with OS and YS animals (Fig. 3L and O). Moreover, TWEAK, Fn-14, FOXO-1, MURF-1, and myostatin mRNA levels were reduced after RT in young rats (YT vs. YS) (Fig. 3K, L, N and O). Interestingly, Fn-14 transcript was lower in OS and OT rats in relation to YS (Fig. 3K). On the other hand, IGF-1, mTOR and MyoD mRNA levels were not affected by aging (Fig. 3P, Q and S), even though
p70S6k-1 transcript was significantly decreased in OS rats when compared with YS (Fig. 3R). Conversely, each of those transcripts was substantially elevated in OT animals in comparison with OS rats (Fig. 3P–S).

mRNA levels of adipogenic factors in response to aging and exercise in GAS muscle. RT alleviated the age-induced increases of CEBP-α, PPAR-γ and LPL transcripts in GAS muscle, although mRNA levels of PGC-1α increased in OT in relation to OS rats (Fig. 4A–D). PGC-1α transcript was also elevated in YT rats when compared with YS (Fig. 4D).

mRNA levels of glucose metabolism regulators of GAS muscle in response to aging and exercise. GLUT-4 transcript was found to be greater in OS than in YS rats in GAS muscle (Fig. 4E). Similar to SOL, GLUT-4, G6PDH, Hk-2 and Gly-synt-1 mRNA levels increased in GAS muscle of trained rats when compared with their matched sedentary group (OT vs. OS and OT vs. YS) (Fig. 4E–H).

mRNA levels of atrophy and hypertrophy-related factors of GAS muscle in response to aging and resistance training. TNF-α, Fn-14, FOXO-1 and Myostatin mRNA levels were significantly greater in GAS muscle of OS compared with YS rats (Figs 4I,K and 5E). No changes were observed in mRNA levels of MURF-1 and Atrogin-1 among the groups (Fig. 4M,N). Whereas RT further elevated the age-induced increases of TNF-α mRNA levels (Fig. 4I), Fn-14, FOXO-1 and Myostatin transcripts were reduced in GAS muscle of OT rats when compared with OS rats (Fig. 4K,L and O). While TWEAK mRNA levels were increased, Fn-14 transcript was significantly reduced in OT rats (vs. OS) (Fig. 4J,K). RT also reduced FN-14 transcript in young

Figure 2. Light microscope images of hematoxilin-eosin stained sections of GAS muscle at 10X magnification. * and ** means atrophied and hypertrophied fibers respectively (A). Quantification of cross sectional area (CSA) normalized by fBW (B). Light microscope images of intramyocellular lipids (IMCL) of GAS muscle at 20X magnification (C). Quantification of IMCL content (D). Groups: young sedentary (YS), young trained (YT), old sedentary (OS) and old trained (OT) rats. Values are expressed as means ± SEM. Two-way ANOVA, p < 0.05: *vs. YS; b vs. YT; c vs. OS. n = 6/group.
As expected, based on the evidence from atrophic markers, RT in aged rats resulted in marked increases of IGF-1, mTOR, p70S6k-1 and MyoD transcripts in GAS muscle (OT vs OS) (Fig. 4P–S). However, while aging evoked decreases in mRNA levels of IGF-1 (Fig. 4P), MyoD transcript was elevated in OS rats when compared with YS group (OS vs YS) (Fig. 4S). In young rats, RT was able to increase mTOR, p70S6k-1 and MyoD mRNA levels in relation to YS group (Fig. 4Q–S).

Discussion
To our knowledge, we have demonstrated for the first time that RT lowers IMCL content, which was associated with downregulation of PPAR-γ gene expression in skeletal muscle. In addition, our results indicate that PGC-1α seems to transcriptionally mediate intracellular signaling related to age-dependent changes in glucose homeostasis and fiber size in skeletal muscle. Accordingly, these responses were related to decreased expression of key catabolic genes and increased expression of key anabolic genes. Indeed, our findings contribute to a better understanding of sarcopenia and its relation with skeletal muscle metabolism during aging. Our findings demonstrate the effect of RT in aged muscle is an effective physiological intervention capable of counteracting lipid accumulation within the muscle, and thus, sarcopenia.

In fact, IMCL accumulation has been reported with advancing age3, which supports our findings. Although endurance exercise training led to an increase in IMCL content in the elderly23, the effects of RT are unknown. Here, we demonstrated that RT decreased the age-induced IMCL accumulation in SOL, but not in GAS, suggesting that RT plays a distinct role on IMCL content that likely depends on fiber composition of the muscle24. Concomitant to age-induced increases in IMCL content, elevated adipogenic markers were observed in GAS or SOL muscles of old rats, suggesting that aging affects the early stages of cell signaling of adipocyte differentiation and lipoprotein metabolism in skeletal muscle. PPAR-γ and C/EBP-α are key adipogenic transcription factors5 that regulate the expression of genes related to lipogenesis in skeletal muscle. PPAR-γ mRNA expression has
been positively correlated with triglyceride concentration in skeletal muscle. Indeed, we demonstrated increased mRNA levels of PPARγ with aging (OS vs. YS) in both muscles. Our findings suggest that age-induced IMCL accumulation could be associated with elevated PPARγ and C/EBP-α mRNAs expressions. However, mRNA levels of PPARγ has been shown to decrease with aging in skeletal muscle of old rats.

In response to exercise training, we observed lower PPARγ transcripts in SOL and GAS muscles of OT rats (vs. OS), suggesting RT might play a critical role in reducing IMCL, which likely depends on fiber composition of the muscle, given that IMCL was not diminished in GAS of OT rats. Contrary to our findings, exercise training has been shown to increase PPARγ protein expression in EDL muscle of rats and mRNA levels in soleus and plantaris muscle of rats. While C/EBP-α transcripts increased both in SOL and GAS with advancing age, the effects of RT were distinct between the muscles in old rats. Thus, we hypothesize that C/EBPα would not be involved in the reduction of IMCL content induced by RT in old rats. Therefore, decreases of IMCL content induced by RT could be linked to lower mRNA levels of PPARγ, but not for C/EBPα transcripts.

Control of intramuscular triglyceride metabolism involves LPL activity. Interestingly, we observed increased LPL synthesis in both muscles, although LPL protein content and activity decreases in SOL muscle in old rats. Conversely, RT led to a marked reduction in LPL transcripts in old rats. However, LPL activity increases after exercise training. Other have shown that LPL mRNA levels were elevated after running training in rats. Thus, we postulate that LPL regulation in skeletal muscle seems to be regulated at either a post-transcriptional/pretranslational level (and not by LPL synthesis).

The PPARγ co-activator, PGC-1α has been shown to be a potent transcriptional factor that regulates lipid metabolism and glycogen content. A previous study found reduction in PGC-1α expression with aging. Importantly, overexpression of PGC-1α in the muscle protects against development of sarcopenia in old mice.
Figure 5. Integrative intracellular signaling of muscle atrophy/hypertrophy, IMCL accumulation and glycogen content in SOL and GAS muscles. IGF-1 has pleiotropic functions, some of which could be attributed to activation of satellite cells, as indicated by MyoD expression, and inhibition of ubiquitin ligases (Atrogin-1 and MURF-1) by FOXO1/3 mechanism. Myostatin seems to control MyoD levels in both muscles. Age-induced muscle atrophy and IMCL accumulation seem to be triggered by interplay between IGF-1 and PGC-1α as well as glucose uptake oxidation and storage through GLUT-4 and G6PDH. PGC-1α was inhibited by TWEAK/Fn-14 signaling in both muscles while TNF-α seems to modulate PGC-1α only in SOL muscle. LPL might be modulating IMCL content, although we demonstrated increases both in mRNA levels of LPL and IMCL in OS rats.
However, we found increased mRNA levels of PGC-1α with aging, even though it was not enough to prevent the reduction of CSA in GAS and SOL muscles in OS rats.

Indeed, PGC-1α has been shown to consistently increase in skeletal muscle after exercise training8, 37, whereas high intensity training elicited increased content of nuclear PGC-1α, while no changes in protein content was observed48. In this regard, PGC-1α in combination with exercise training improves glucose homeostasis in mice49. Rather than protein content, our findings demonstrate increased PGC-1α mRNA levels after RT, suggesting that mitochondrial biogenesis might be triggered by high intensity RT at the transcriptional level. Accordingly, while RT attenuated the age-associated reductions in CSA, elevated PGC-1α transcript was observed in SOL and GAS muscles of OT rats, suggesting that PGC-1α might be involved in protective mechanisms of RT in order to prevent muscle wasting52.

Aging did not affect glycogen content, Gly-Syn-1, G6PDH, HK-2 and GLUT-4 mRNA levels, either in GAS or SOL muscles. Similarly, glycogen content was not altered in aged SOL muscle50, while other observed a reduction in glycogen content in soleus of old rats41. In contrast, RT evoked greater glycogen content, which could be related with upregulation of GLUT-4, G6PDH, HK-2 and Gly-Syn-1 transcripts observed in this study. These data suggest that RT plays a large role in glycogen synthesis, glucose uptake, transport and metabolism, even at a transcriptional level.

Indeed, GLUT-4, G6PDH and HK-2 are determinants of glucose uptake within skeletal muscle during exercise52. Increased GLUT-4 has been consistently documented after exercise training42. In fact, endurance training induced greater glycogen concentration, which was associated with elevated protein abundance of GLUT-4, HK-2 and glycogen synthase in older people51. In this context, glucose-6-phosphate dehydrogenase (G6PDH) was recently associated with glucose uptake43, although this enzyme has been poorly studied in skeletal muscle50. Whereas aging did not affect G6PDH mRNA levels in this study, its activity and protein content was reduced in the gastrocnemius of aged rodents45.

Evidence is scarce concerning the effects of physical training on G6PDH levels. However, it was shown that endurance training reduced G6PDH in adipose tissue of mice50 while our findings revealed that RT increased mRNA levels in G6PDH in both skeletal muscles tested. Given that glucose is transported into muscle cells, hexokinase plays an essential role in its conversion to glucose 6-phosphate and synthesis of glycogen, since glycogen synthase is activated by glucose 6-phosphate47. Considering hexokinase participates of glucose transport and uptake and hexokinase II is the most predominant isoform in rat skeletal muscle48, we evaluated HK-2 in GAS and SOL muscles. Whereas no changes were found in HK-2 transcript with advancing age in this study, reduction in HK-2 mRNA levels was observed49. Conversely, RT elevated HK-2 mRNA expression, either in GAS or SOL muscles, which is in partial agreement with other. For instance, increased HK-2 protein levels has been observed following running sessions in mice50.

Although our results demonstrated no changes in Gly-Syn-1 transcripts with aging, decreases in Gly-Syn activity and protein levels have been reported in skeletal muscle of old rats40, 41. It has been reported that chronic exercise training leads to an increase in both Gly-Syn activity and protein expression in rats51. Taken together, as similar effects of RT were observed in GLUT-4, G6PDH, HK-2 and Gly-Syn-1, we suggest that RT plays a crucial role in glucose homeostasis (uptake, transport and metabolism) at a transcriptional level along with upregulation of PGC-1α in aged skeletal muscle after RT. Furthermore, PGC-1α controls GLUT-4 gene expression, suggesting its importance on exercise training-mediated glucose uptake50. Considering PGC-1α transcripts were dramatically elevated in GAS muscle of YT rat, we suggest distinct effects of exercise training on PGC-1α signaling between skeletal muscle and aging.

It's noteworthy that PGC-1α is considered a fundamental intracellular target because of its importance in atrophy signaling37. This is supported, in part, by studies demonstrating that PGC-1α was inhibited by TWEAK/Fn14 signaling52 while PGC-1α activates the IGF-1-mTOR pathway53. In fact, our findings demonstrated increases in PGC-1α mRNA coincided with augmented IGF-1/mTOR/p70S6K transcripts, both in SOL and GAS muscles of OT rats. Increases in age-induced TNF-α, TWEAK, Atrogin-1 and MURF-1 mRNA levels were observed in SOL, while Fn-14, FOXO-1 were unchanged. These responses were accompanied by a reduction in fiber CSA of SOL muscle from OS rats. Inflammatory cytokines, such as TNF-α, initiate downstream signaling involved in muscle atrophy34. In this context, TWEAK-Fn14 system has emerged as a regulator of muscle atrophy, mitochondrial dysfunction and slow-to-fast fiber type switching13. In fact, our findings demonstrated increases in GAS muscle of YT rat, we suggest distinct effects of exercise training on PGC-1α signaling between skeletal muscle and aging.

In GAS muscle, elevated transcripts of TNF-α, Fas-14, FOXO-1 and myostatin were observed with aging, whereas no changes were observed in TWEAK, Atrogin-1 and MURF-1 mRNAs. The unchanged Atrogin-1 and MURF-1 transcripts observed in aged GAS muscle might not be contributing to the decreases in CSA and sarcopenia, as suggested by others56. Myostatin, a negative regulator of muscle growth, and shown to increase with aging57, 58, is also associated with upregulation of MURF-1 and Atrogin-152. Moreover, myostatin-induced upregulation of Atrogin-1 is mediated by FOXO-115. However, we did not demonstrate a similar fashion of crosstalk between signaling pathways. These discrepancies could be due to our findings solely rely on transcriptional mechanisms. Thus, aging might modulate atrophic signaling pathways, not only at a post-translational mechanism but also at transcriptional control.

Age-stimulated increases in mRNA of TNF-α, TWEAK, Atrogin-1 and MURF-1 were blunted in SOL muscle after RT. On the other hand, RT reduced the age-induced increases of Fn-14, FO XO-1 in GAS muscle. Myostatin mRNA expression was significantly decreased after RT, in agreement with the finding from previous report56. Indeed, RT attenuated the age-associated elevations of TNF-α in vastus lateralis from elderly humans50.
Furthermore, recent evidence has shown that TWEAK-Fn14 transcripts are responsive to RT\(^6\). Similarly, others reported decreases in MURF-1 protein content after endurance training\(^5\). To our knowledge, this is the first evidence that RT downregulated the major intracellular regulators of age-induced atrophy of SOL and GAS muscles. Therefore, we propose that RT modulates transcription in aged muscles in order to minimize the age-associated atrophic signaling.

RT has been shown to alleviate the age-induced reduction in CSA in GAS in SOL muscles, which is likely due in part to pro-growth signaling events that result in increased rates of protein synthesis\(^43\). In fact, IGF-1, mTOR, p70S6k-1 and MyoD were markedly upregulated after RT, either in both GAS and SOL muscles of old rats. These findings are in support of the hypothesis that RT modulates the hypertrophic signaling, even at transcriptional mechanisms. p70S6k-1 transcript was elevated in OT rats, suggesting RT plays crucial role on downstream hypertrophy signaling, which are supported by other\(^44\). Our findings corroborate with other, once MyoD transcripts were elevated in vastus lateralis after RT in old women\(^34\). IGF-1 has pleiotropic functions, some of which could be attributed to activation of satellite cells, as indicated by MyoD expression\(^44\) and inhibition of ubiquitin ligases (Atrogin-1 and MURF-1)\(^65\). With advancing age, diminished activation of IGF-AKT-mTOR axis occurs, resulting in FOXO1/3-mediated activation of Atrogin-1 and leading to protein degradation\(^35\). Furthermore, IGF-1 favors free fatty acid uptake and oxidation in skeletal muscle\(^45\). Thus, these responses might be dependent on, at least in part, a complex interplay of PGC-1\(^\alpha\), GLUT-4 and IGF-1. Accordingly, our findings suggest that RT plays a vital role at a transcriptional level on intramuscular metabolic metabolism and signaling related to myofiber size during aging, as proposed by the intracellular signaling. Considering that intracellular crosstalk between lipogenesis, glucose homeostasis and muscle development might be occurring in response to aging and RT, we have proposed an outline of the pathways, including events occurring at the transcriptional level (Fig. 5).

Finally, it is important to point out some limitations of the present study. Although we evaluated regulators of intramuscular lipogenesis and glucose homeostasis, along with muscle atrophy and hypertrophy pathways, we did not measure protein content and subcellular localization. In addition, we evaluated RPLP0 expression, which was found to vary according in the muscles and groups (data not shown). Thus, RPLP0 might not be a good housekeeping gene for GAS and SOL muscles in a model of aging and RT in mice.

Conclusions
In conclusion, RT attenuated the age-associated accumulation of IMCL concomitant to a downregulation of PPAR\(^\gamma\) gene expression and enhanced expression of glucose homeostasis regulators (GLUT-4, G6PDH, HK-2 and Gly-Syn-1). These responses were also linked to decreasing catabolic (TNF-\(\alpha\), TWEAK/Fn14 axis; FOXO-1, Atrogin-1 and MURF1; myostatin) and increasing anabolic (IGF-1-mTOR-p70S6k-1 axis; MyoD) signaling effectors. Our results point out the importance of RT on modulation of gene expression of intracellular regulators related to age-related morphological and metabolic adaptations of skeletal muscle.

Methods
Twenty-eight male Wistar rats with 3 (\(n=14\); 298.74 ± 32 g) and 20 months old (\(n=14\); 517.8 ± 76 g) of age were housed in plastic cages under controlled environmental conditions (12-hour light/dark cycle) with free access to water and standard chow (Sociol, Sao Paulo, Brazil). Rats were randomly distributed into four experimental groups with 6 animals per group in the following order: young sedentary (YS), young trained (YT), old sedentary (OS) and old trained (OT). The experimental procedures received approval from the Animal Experimentation Ethics Committee of the Federal University of Sao Carlos, SP, Brazil (number 056/2010), and the study was conducted in accordance with the National Guide for the Care and Use of Laboratory Animals.

Resistance Training (RT) protocol. The description of RT protocol was recently reported by our laboratory\(^46\).

Body Weight and Muscle Sample Collection. Initial (iBW) and final (fBW) body weights were recorded in grams (g) before the first session of RT protocol and 48 hours after the last training session, respectively. Forty-eight hours after the last training session, animals were anesthetized using i.p injection with a solution of xylazine (12 mg/Kg of BW) and euthanized using ketamine (95 mg/Kg of body weight). Soleus (SOL) and Gastrocnemius (GAS) muscles were carefully removed and weighed. The muscles were then divided into two parts at the middle of the belly: the proximal (origin) and the distal (insertion) attachments were used for the histological analysis and mRNA analysis, respectively. For histological evaluation, the muscle fragment was immediately frozen in isopentane, pre-cooled in liquid nitrogen and stored at −80 °C (Forma Scientific, Marietta, Ohio). For mRNA analysis, the muscle fragment was frozen in liquid nitrogen and stored at −80°C.

Histological Analysis. Histological cross-sections (10\(\mu\)m) of each GAS and SOL muscles were obtained in a cryostat (Micron HE 505, Jena, Germany). IMCL content was determined using Oil Red staining and quantified as previously described\(^48\). Ten-\(\mu\)m slices were stained with Toluidine Blue/1% Borax (TB) in order to measure fiber cross-sectional area (CSA). Images of 5 different regions were obtained using a light microscope (AxioLab, Carl Zeiss, Jena, Germany) equipped with a digital camera (Sony DSC 775, Tokyo, Japan). The CSA of 100 randomly fibers were chosen from each picture and measured using the Axiovision 3.0.6 SP4 software (Carl Zeiss, Jena, Germany) totaling 600 muscle fibers per animal. Finally, CSA of muscle fibers were normalized by fBW of matched animal (CSA/body weight).

Glycogen content. SOL and GAS muscle samples were processed with hot 30% KOH and glycogen was precipitated by ethanol to determine muscle glycogen as previously described\(^49\).
efficient, with a slope value of −3.32 and r-value (n=6/group). GAPDH were used as reference genes for normalization. The gene expression assays were 100% ± SEM. Shapiro-Wilk and Levene's tests were used according to the manufacturer's instructions.

RNA Isolation - PCR. Frozen fragment of each muscle was homogenized (Omini Tip Plastic Homogenizer Probes® Kennesaw, GA, USA) and total RNA was isolated using Trizol reagent (Life Technologies). In order to obtain clean RNA with no contamination, samples were treated with DNase followed by removal treatment (Life Technologies). The amount of RNA was quantified by Qubit® (Life Technologies) using 1 µl of each sample. The integrity and quality of the total RNA obtained was tested in a Bioanalyser (Agilent Technologies Inc. USA). The RIN (RNA Integrity Number) value ranged from 8.0 to 10.0, and the ratio ranged from 1.8 to 2.0. This indicated that intact RNA, free of genomic DNA, was successfully isolated. Approximately 1 µg of total RNA from each sample was used to synthesize cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions.

Real Time-PCR. The differential expression of genes was validated by qPCR using Sybr Green PCR Master Mix (Life Technology®). Amplifications were performed by qPCR using 10–80 ng cDNA/µl added to a reaction containing 10 mM SYBR Green PCR master mix and 100–300 nM primers (sense and antisense) in a final volume of 30 µl solution in triplicate. The cycling conditions were in accordance to the standards of each primer according to the annealing temperature. The Cq value (Cycle Quantification) of each sample was calculated using the StepOne software (Applied Biosystems). The average Cq values of triplicates were used for the calculation of the Fold Change (Arbitrary Unit). Six animals from each experimental group were used for Real Time-PCR analysis (n = 6/group). GAPDH were used as reference genes for normalization. The gene expression assays were 100% efficient, with a slope value of −3.32 and r-value > 0.99.

Table 2. List of oligonucleotides primers. CEBP-α: CCAAT/enhancer binding proteins alpha; LPL: Lipoprotein lipase; PPAR-γ: Peroxisome proliferator activated receptor gamma; PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1-α; GLUT-4: Glucose transporter type 4; Hk-2: Hexokinase-2; TNF-α: Tumor necrosis factor alpha; FoxO1: Forkhead box protein O1; Atrogin-1: F-box protein 32; MuRF1: Muscle ring finger protein-1; IGF-1: Insulin-like growth factor 1; mTOR: Mamalian target of rapamycin; p70S6K: p70S6 kinase 1; Myostatin: Growth differentiation factor 8; MyoD: myogenic growth factor 1 RPLP0: Ribosomal protein lateral stalk subunit P0; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

| Primer | Forward | Reverse | NCBI (Reference Sequence) | Amplicon Size, bp |
|--------|---------|---------|--------------------------|------------------|
| CEBP-α | TCGTGTGATAAGAAGACGCAG | GGGGCCTGTCTTGGCCTTAT | NM_001287577.1 | 93 |
| LPL    | CTTCCTGATTTACACGGGAGGTT | ATGGCATTTCACAAACACCGT | NM_012598.2 | 229 |
| PPAR-γ | AAGGGGCGTCTGACCTTCTG | ATAAAGGGGGGGCAGGCTG | XM_006540115.1 | 630 |
| PGC-1α | TCTAGGAGCAATAAAGCCGAAG | TGGTGGTGGTGGTGGAGGAG | XM_006503779.1 | 117 |
| Gly-SynT | CCGGCTTCTGCTTGGT | CCGATCCAGAAGTAAATGCC | NM_030678.3 | 71 |
| GAPDH | GTTGGGAGGGCGGAACTTAA | GCCATCACCCCTGGTACACT | NM_017006.2 | 108 |
| GLUT-4 | CAAGAACAGCAGCACTCTGA | TGGACACACTGCTACTTACGG | XM_00656283.1 | 190 |
| Hk-2   | ACCAAGTCGAAAGGTTGACCA | TCTGTGGCGAGGGGAGAA | NM_013820.3 | 432 |
| TNF-α | GCCACACGGCCCTCTCTGGT | GTCTGGGGCCATGAGACTAT | NM_012675.3 | 101 |
| TWEAK | GTAGCAGACGGCAAGTTGGG | GCCAACACCGTTGCCACAG | NM_011614.3 | 130 |
| Fn14   | AAGTGGATGCTGGCTTCTTT | GGAAGCTAGAACAGCCGCAAC | NM_181086.3 | 154 |
| FoxO1  | TCAAGGATAAGGGGCCGACAG | GGTTCCTCATCTGCTGACGAAT | NM_019739.3 | 103 |
| Atrogin-1 | CCATCACAGAAGGTATCTATGT | GGCTCCCCAAATGCAGTA | NM_13352.1 | 75 |
| MuRF1  | TGTCTGAGGCTGTTCGCC | ATGGCCGGCTCATGACTT | NM_080903.1 | 59 |
| IGF-1  | GCCCTTCTGCTTGGTGGGAGA | AGATCACAGCCTGGGAGGCC | NM_184052.3 | 125 |
| mTOR   | CACCACAGGCTGGACCTTCA | GCCTGGTGGGGCTCATGATGGT | NM_019906.1 | 156 |
| p70S6K-1 | CTACAGAGGGCTGAGCCGGAAGA | AATGGTGGGCTGACTTGGTCTCAC | NM_031985.1 | 114 |
| Myostatin | CTACACCCGAAACAAATCATCCA | AGCAACATTGGGCTCCTTCA | NM_019151.1 | 78 |
| MyoD   | ACTACAGGCGCGGCGTACGAGC | AGCATGATGGGCGGCTGCT | NM_176079.1 | 122 |
| RPLP0  | AGGGTCTCTGCTTGGCTGTGG | AGCTGACAGGACGACAGTGG | NM_022402.2 | 135 |
| GAPDH | TGGACACCAACACTGGT | GGAATGGGAGGAGATGATTC | NM_017008.4 | 177 |

Data standardization. We measured GAPDH and RPLP0 as internal controls. However, we observed a high variability of RPLP0 mRNA than GAPDH. Therefore, the GAPDH gene was chosen as the internal control, assuming that the GAPDH mRNA was expressed constitutively.

Statistical Analysis. Results were expressed as means ± SEM. Shapiro-Wilk and Levene’s tests were used to investigate whether the data were normally distributed. As all included variables were normally distributed, a
two-way ANOVA (training x age) followed by a Tukey HSD post-hoc test were performed to compare treatments. Differences were considered significant when \( p < 0.05 \). Statistical analysis was performed using the Statistica 7.0 software package (StatSoft Inc., Tulsa, OK, USA).

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**Author Contributions**

Ribeiro M.B.T. performed the experiments as well as analyzed data. Guzzoni V. analyzed data and wrote the manuscript. Hord J.M. analyzed data and wrote the manuscript - contributed with the English language. Lopes G.N. performed the experiments. Marqueti R.d.C., Andrade R.d.V., Selistre-de-Araujo H.S., Durigan J.L.Q. designed the research, analyzed data and contributed with writing of the manuscript.

**Additional Information**

**Competing Interests:** The authors declare that they have no competing interests.

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