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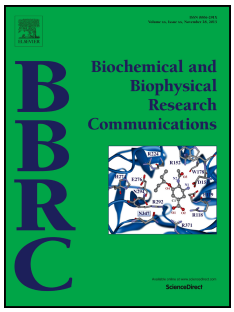

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Aging related ER stress is not responsible for anabolic resistance in mouse skeletal muscle

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Abstract

Anabolic resistance reflects the inability of skeletal muscle to maintain protein mass by appropriate stimulation of protein synthesis. We hypothesized that endoplasmic reticulum (ER) stress contributes to anabolic resistance in skeletal muscle with aging. Muscles were isolated from adult (8 mo) and old (26 mo) mice and weighed. ER stress markers in each muscle were quantified, and the anabolic response to leucine was assessed by measuring the phosphorylation state of S6K1 in soleus and EDL using an ex vivo muscle model. Aging reduced the muscle-to-body weight ratio in soleus, gastrocnemius, and plantaris, but not in EDL and tibialis anterior. Compared to adult mice, the expression of ER stress markers BiP and IRE1α was higher in EDL, and phospho-eIF2α was higher in soleus and EDL of old mice. S6K1 response to leucine was impaired in soleus, but not in EDL, suggesting that anabolic resistance contributes to soleus weight loss in old mice. Pre-incubation with ER stress inducer tunicamycin before leucine stimulation increased S6K1 phosphorylation beyond the level reached by leucine alone. Since tunicamycin did not impair leucine-induced S6K1 response, and based on the different ER stress marker regulation patterns, ER stress is probably not involved in anabolic resistance in skeletal muscle with aging.

Keywords: unfolded protein response; leucine; tunicamycin; S6K1; sarcopenia

Abbreviations

ATF4, activating transcription factor 4; BiP, binding immunoglobulin protein; CHOP, C/EBP homologous protein; EDL, extensor digitorum longus ; eIF2α, eukaryotic initiation factor 2 α; eEF2, eukaryotic elongation factor 2; ER, endoplasmic reticulum ; Leu, leucine ; mTORC1, mammalian target of rapamycin complex 1; IRE1α, inositol-requiring kinase 1α ; Rpl19, ribosomal protein L19; S6K1, ribosomal protein S6 kinase; TN, tunincamycin; UPR, unfolded protein response; sXBP1, spliced X-Box protein 1; lXBP1, unspliced X-Box protein 1

Introduction

Sarcopenia is the decline in skeletal muscle mass, strength and function with advancing age [1]. The impact of sarcopenia on older people is far reaching as it leads to frailty, loss of mobility, increased risk of cardiovascular and metabolic diseases, and mortality [2]. To date, effective strategies to escape from, or at least to attenuate sarcopenia remain limited. Resistance exercise and adequate protein-based nutrition are the current most effective means to limit the loss of muscle mass with aging [3]. However, the benefits of exercise and nutritional interventions to counteract sarcopenia may be partially diminished by a blunted responsiveness to these stimuli [4]. This phenomenon has been termed 'anabolic resistance' to reflect the inability of the muscle to maintain its protein mass by appropriate stimulation of protein synthesis, a major mechanism contributing to sarcopenia [5].

At the molecular level, the mammalian target of rapamycin complex 1 (mTORC1) is an essential site of integration for anabolic signals to stimulate muscle protein synthesis in human skeletal muscle via, amongst other downstream targets, ribosomal protein S6 kinase 1 (S6K1) [6]. It has been shown that basal total protein levels of mTOR and S6K1 are down-regulated in the elderly [7]. In addition essential amino acids-induced mTOR and S6K1 phosphorylation is reduced in muscle of old individuals [8]. The question remains why the mTOR pathway responds less to anabolic stimuli in skeletal muscle with aging. One possible molecular mechanism is an increase of the so-called endoplasmic reticulum (ER) stress, as ER stress induces anabolic resistance in a myogenic cell culture model through blockade of mTORC1 [9], it contributes to the impaired response of cyclo-oxygenase 2 to pulsating fluid flow, a known anabolic stimulus in bone, in cultured osteocytes of old compared with adult mice [10], and because ER stress markers are higher in skeletal muscle of old compared with adult mice [11].

The ER is an organelle that plays a central role in folding newly synthesized proteins that enter its lumen. The rate of naive proteins entering and folding is stringently regulated. When protein synthesis exceeds the rate of folding, the unfolded protein response (UPR) is activated [12]. In turn, the UPR reduces protein synthesis and up-regulates the expression of genes such as X-box binding protein 1 (XBP1), C/EBP homologous protein (CHOP), and activating transcription factor 4 (ATF4), which encode proteins that ameliorate the ER protein-folding capacity and help to discard unfolded proteins via different proteolytic pathways [12].

Therefore, we hypothesized that ER stress could be involved in the reduced response to anabolic stimuli in skeletal muscle with aging.

Material and Methods

Animals

All experiments were approved by the local animal use and care committee of the VU University Amsterdam, and conform the Dutch Research Council's guide for care and use of laboratory animals. Muscles were extracted from male C57BL/6J adult (8 months, n = 8) and old mice (26 months, n = 8, Janvier Labs, Saint-Berthevin, France). The mice arrived at the age of 6 months and 24 months, and were kept for 2 months at the "Universitair Proefdier Centrum VU/Vumc" in individually ventilated cages, and fed a maintenance chow which was not enriched with proteins (RN-01-20K12, Carfil, Belgium). Fifteen minutes prior to surgery, mice received a subcutaneous injection of 0.06 ml 1% Temgesic (Reckitt Benckiser, UK) as an analgesic and were anesthetized with 4% isoflurane, 0.1 $l \cdot min^{-1}$ O_2 and 0.2 $l \cdot min^{-1}$ air. After nociceptive responses had ceased, the level of anesthesia was maintained with 1.5–2.5% isoflurane [13].

Experimental setup

Gastrocnemius, tibialis anterior, extensor digitorum longus (EDL), soleus, and plantaris muscles were extracted and weighed. Gastrocnemius and tibialis anterior muscles were used to assess ER stress and the UPR in adult and old mice both at the protein and mRNA level. To assess anabolic resistance and the effect of ER stress, an *ex vivo* muscle model of EDL and soleus was used. Soleus was split in the middle, whereas EDL was split at the interface between head IV and V [14] and halved longitudinally. The muscle bundles were mounted a their slack length in chambers by attaching the tendons at both ends to small rods, and kept metabolically active *ex vivo* by saturating the tyrode solution (containing: NaCl 7.5 g/l; KCl 0.35 g/l; $MgCl_2.6H_2O$ 0.214 g/l; $NaH_2PO_4.H_2O$ 0.058 g/l; $NaHCO_2$ 1.7 g/l; $CaCl_2.H_2O$ 0.2 g/l; glucose 2.2 g/l) with a carbogen gas mixture (95% O_2 and 5% CO_2) at 36°C. Medium (5 ml) was refreshed every 30 min. Two-third of the muscles were then incubated for 5 h in tyrode solution combined with minimum essential medium (MEM, Control (Ctrl)) while one third was treated with 10 µg/ml tunicamycin (TN, Sigma-Aldrich, Zwijndrecht, The Netherlands), a known inducer of ER stress [15]. At the end of the 5 h-incubation, 4 mM leucine (Leu; Sigma-Aldrich), known to induce an anabolic response in skeletal muscle by activating S6K1 [16], was added for the last 30 min to the TN-treated muscles and to half of the muscles kept under Ctrl conditions. The muscles were then instantly cryopreserved and stored at -80°C. Preliminary experiments were performed earlier and revealed that the optimal concentration of Leu was 4 mM and that of TN 10 µg/ml based on the phosphorylation state of S6K1 and the expression of ER stress markers.

Protein expression

Frozen muscle tissue (5-7 mg) was homogenized with a Polytron mixer in ice-cold buffer [17]. Homogenates were then centrifuged at 10,000 g for 10 min at 4°C. The supernatant was collected and immediately stored at -80°C. Protein concentration was measured using the

Detergent Compatible protein assay kit (Bio-Rad, Nazareth, Belgium). Forty microgram of protein was separated by SDS-PAGE (10 or 12% gels) and transferred to polyvinylidene fluoride (PVDF) membranes. Then membranes were blocked with 5% non-fat milk for 1 h and incubated overnight at 4°C with the following antibodies (1:1000): total eukaryotic elongation factor 2 (eEF2), S6K1, binding immunoglobulin protein (BiP), inositol-requiring kinase 1 α (IRE1α), phospho and total eukaryotic initiation factor 2 α (eIF2α), CHOP, caspase 12 (Cell Signaling Technology, Leiden, The Netherlands). Horseradish peroxidase-conjugated anti-mouse (1:10000) or anti-rabbit (1:5000) secondary antibodies (Sigma-Aldrich, Bornem, Belgium) were used for chemiluminescent detection of proteins. Protein bands were quantified using GeneSnap and GeneTools (SynGene, Cambridge, UK). The values obtained for phosphorylated forms of proteins were normalized with their respective total forms and the remainder of the markers were normalized with eEF2.

RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted using Trizol (Invitrogen, Vilvoorde, Belgium) from approximately 20mg of muscle samples [18]. The quantity and purity of RNA were determined by NanoDrop® spectrophotometer (Isogen Life Science, Belgium). cDNA was synthesized from 1 μg of RNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Gent, Belgium) according to manufacturer's instructions. Real-time quantitative PCR reactions were performed using the ABI PRISM 7300 (Applied Biosystems). Mouse specific primers for ATF4, sXBP1 and lXBP1 were designed (Table 1). Samples were analyzed in triplicate and the values normalized for the housekeeping gene Rpl19. Melting curves were systematically analyzed to ensure the specificity of the amplification process. Relative quantities were calculated with the standard curve method.

Statistics

Student's t-test was used for testing the difference between old and adult values except for Leu/TN experiments for which a mixed model two-way ANOVA for repeated measures was used with age as a between subjects factor and treatment as within subjects factor. Results are presented as the means ± SEM. The statistical threshold was set to $p<0.05$.

Results

Aging promotes skeletal muscle atrophy

The ratio muscle weight to body weight was 13% lower in soleus ($p=0.023$), 15% in plantaris ($p=0.005$), and 14% in gastrocnemius ($p=0.0006$) of old compared to adult mice. This ratio tended to be lower in EDL ($p=0.071$) and in tibialis anterior ($p=0.105$) of old compared to adult mice (Table 2).

Aging regulates the expression of UPR and ER stress markers

Several well described markers of the UPR and ER stress were measured both at the mRNA level (Fig. 1a,b) and at the protein level (Fig. 1c-j) in different muscles of old and adult mice. In tibialis anterior, mRNA levels of ATF4 (-49%), sXBP1 (-37%) and sXBP1/lXBP1 (-26%) were reduced in old mice compared to adult mice ($p<0.05$, Fig. 1a). The opposite regulation was observed in gastrocnemius where ATF4 mRNA levels were about 2 times higher in old than in adult mice ($p<0.05$), and sXBP1 and lXBP1 levels about 50% more abundant ($p<0.05$; Fig. 1b). In tibialis anterior, the protein expression levels of BiP (-26%), cleaved caspase 12 (-52%), and cleaved caspase 12/total caspase 12 (-46%) were lower in old compared to adult mice ($p<0.05$; Fig. 1c,d). Only CHOP showed an increasing trend ($p=0.099$; Fig. 1c,d). In gastrocnemius, IRE1α and CHOP expression was about 50% higher while cleaved caspase 12 was about 50% less abundant in old compared to adult mice ($p<0.05$; Fig. 1e,f). The ratio peIF2α/total was about 2 fold higher in soleus (Fig. 1g,h) and EDL (Fig. 1i,j) of old compared to adult mice ($p<0.05$). BiP expression tended to be higher in soleus of old mice ($p=0.068$;

Fig. 1g,h). In EDL, BiP (1.5 fold) and IRE1α (2.6 fold) expression was higher in old compared to those in adult mice ($p<0.05$; Fig. 1i,j).

Aging reduces leucine-induced S6K1 phosphorylation in soleus

A trend toward a higher basal S6K1 phosphorylation was observed in soleus of old compared to adult mice (2-fold, $p=0.094$) (Fig. 2a,c). Compared to Ctrl, leucine alone increased S6K1 phosphorylation by about 3-fold in soleus of adult mice ($p<0.01$; Fig. 2a,c), while no increase was measured in old mice. Compared to Ctrl, addition of leucine in combination with the ER-stress inducer tunicamycin (TN+Leu) increased S6K1 phosphorylation in soleus of both adult (4-fold) and old (2-fold) mice (Fig. 2a,c, $p<0.001$). In EDL of old mice, no difference in S6K1 phosphorylation was observed at basal in the Ctrl condition (Fig. 2d,f). Leucine alone increased S6K1 phosphorylation by about 2 fold in EDL of both adult ($p<0.001$) and old ($p<0.05$) mice (Fig. 2d,f). After leucine treatment, S6K1 phosphorylation was higher in EDL of old compared to adult mice ($p<0.05$, Fig. 2d,f). Compared to Ctrl and Leu conditions, S6K1 phosphorylation was higher in TN+Leu in EDL of both adult and old mice ($p<0.001$; Fig. 2d,f). To further assess anabolic resistance, the response of S6K1 phosphorylation to leucine stimulation only was calculated in soleus (Fig. 2b) and in EDL (Fig. 2e). Compared to basal conditions, leucine increased S6K1 phosphorylation by 4-fold in soleus of adult mice, while the increase was less than 2-fold in old mice, which resulted in a lower response to leucine in old compared to adult mice ($p<0.05$; Fig. 2b). In EDL, no difference in the response to leucine was observed between old and adult mice (Fig. 2e). These results indicate that anabolic resistance is present in soleus but not in EDL of old mice.

Discussion

The purpose of the present study was to determine the role of ER stress in aging-induced anabolic resistance. We found that aging induces anabolic resistance in soleus but not in EDL

muscle of mice. Using an ex vivo muscle preparation, we could discard any impairment in intestinal absorption of leucine [19] or insulin production [20], suggesting that intramuscular mechanisms caused the reduced response to leucine. Having previously proposed that ER stress could contribute to anabolic resistance in myogenic cells in culture [9], we tested this hypothesis in isolated muscles. The present results, however, do not support that ER stress contributes to anabolic resistance with aging for two reasons. First, some ER stress and UPR markers were up-regulated in both soleus and EDL muscle, while anabolic resistance was only present in soleus. Second, according to this hypothesis, the activation of ER stress by tunicamycin should have decreased the response to leucine, while we observed the opposite response.

Interestingly, the muscle-to-body weight ratio of soleus was more affected by aging than that of EDL. Those results are in line with the role of anabolic resistance in the loss of muscle mass with age, as anabolic resistance was present in soleus but not in EDL. In the present study, the muscle-to-body weight ratio of soleus, plantaris, and gastrocnemius muscles decreased with age, but this ratio did not change in EDL and tibialis anterior muscles. Similar weight regulations have been found previously in mice [11, 13, 21-23]. Several hypotheses could be put forward, beginning with fiber type composition. One could argue that the difference observed between these muscles is due to a difference in type I and type II fiber type proportion. Aging is known to affect type II more than type I fibers [24]. However, in the present study, fiber typing does not likely explain the difference observed, as EDL and tibialis anterior contain more type II fibers than soleus and gastrocnemius muscles [25], while the weight-to-body ratio of those muscles was less affected with aging.

When assessing skeletal muscle mass and anabolic resistance, an important factor to be taken into consideration is physical activity, which usually considerably decreases with age, thereby exacerbating anabolic resistance [3]. However, the activation pattern has only been measured

in quadriceps and hamstrings during locomotion in mice [26, 27]. Based on results obtained in rats [28], muscles from the hindlimb are more recruited during locomotion than muscles from the forelimb. Although speculative, it is possible that hindlimb muscles suffer more than forelimb muscles from the decrease in locomotion with age, thereby favouring anabolic resistance as observed in soleus. However, additional investigation is needed to test whether decreased physical activity with age impacts specific muscles more than others.

Although ER stress probably did not influence the response to leucine, some markers were differently expressed in old compared to adult mice. With age, there is a general trend to down-regulate the folding capacity and the management of misfolded protein in the ER due to a decrease in the expression of chaperones [29]. Consequently, the apoptotic pathways are activated, leading to cell death. In several tissues, a decrease in BiP expression, which is essential for sensing misfolded proteins, and an increase in CHOP expression as well as activation of caspase 12, two key proteins in the ER-activated protein degradation, are often observed alongside [29]. Only very few data exist in skeletal muscle where BiP, CHOP, and protein disulphide isomerase have been reported to increase in gastrocnemius of 24 months-old mice compared to 6 months-old mice [11]. Our results only partially confirm those findings as CHOP increased while BiP was unchanged in gastrocnemius of old mice. Due to the scarcity of available results in skeletal muscles with age, it is difficult to make further comparison with previous reports. However, based on the present results, it seems that each muscle has its own regulation pattern to ER stress with some markers increasing while others decreasing. Another key point is the age of the animal as, at old age, a difference of a few weeks can have severe physiological consequences [30]. This could explain why BiP was high at 24 months in a previous report [11], while we did not observe any difference at 26 months. One can speculate that more misfolded proteins accumulate with age. To counteract this accumulation, a higher expression of chaperones is favoured to a certain point of

disruption when misfolded proteins further increase while the pool of chaperones becomes exhausted, leading to ER homeostasis disruption and further activation of ER stress [29]. However, more data is needed to confirm or reject this hypothesis. Further investigation should also take the degree of physical activity into consideration as physical activity regulates ER stress and the downstream UPR [31-33] while it decreases with age.

Tunicamycin was used to activate ER stress and to test whether leucine-induced S6K1 activation was impaired in the presence of ER stress in our model, as previously observed in myogenic C2C12 cells [9]. Unexpectedly, the opposite regulation was observed, i.e. an increase in leucine-induced S6K1 phosphorylation when the muscles were pre-treated with tunicamycin. These results suggest that tunicamycin increased S6K1 sensitivity to leucine, or that under the present conditions, pre-incubation with tunicamycin increased S6K1 phosphorylation before being further increased by leucine. To test the latter possibility, we measured S6K1 phosphorylation in the samples collected during the preliminary experiment, in which we determined the dose of tunicamycin needed to activate ER stress in our model. S6K1 phosphorylation remained unchanged in soleus and decreased in EDL when tunicamycin was added (data not shown), thereby refuting our second hypothesis. Although the molecular mechanisms remain to be determined, tunicamycin seems to hypersensitize S6K1 to leucine in isolated skeletal muscles, at least in EDL. Similarly, a higher activation of S6K1 phosphorylation by insulin has been reported when L6 myotubes were pre-treated with tunicamycin for 3h [34]. A major difference between the previous studies in L6 (3h) [34] and C2C12 myotubes (17h) [9], and the present study (5h) is the duration of the pre-treatment with tunicamycin before stimulating the cells with leucine or insulin. Unfortunately, the experimental set-up used here does not allow a longer incubation with tunicamycin. It is therefore impossible to test whether longer pre-incubation with tunicamycin (up to 17h)

would have given similar results to what we observed previously in cultured cells, i.e. an impairment of S6K1 response to leucine.

In conclusion, the muscle-to-body weight ratio was decreased in the hindlimb muscles more than in the forelimb muscles of old mice. Anabolic resistance could contribute to the decreased muscle weight of soleus as S6K1 response to leucine was impaired in this muscle. However, contrary to our hypothesis, ER stress is probably not involved in the reduced response to leucine in skeletal muscle of old compared to adult mice.

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Legends

Figure 1. ER stress markers in the skeletal muscle of old versus adult mice

mRNA level of ER stress markers in tibialis anterior (a) and gastrocnemius (b) of adult and old mice. Protein expression of ER stress markers in tibialis anterior (c) and gastrocnemius (e) of adult and old mice with their respective representative western blot images (d and f). Protein expression of ER stress markers in soleus (g) and EDL (i) of adult and old mice with their respective representative western blot images (h en j). Values are expressed as mean ± SEM. *p<0.05, **p<0.01. Cl. Casp 12, cleaved caspase 12; Ucl. Casp 12, uncleaved caspase 12 ; A, adult ; O, old ; EDL, extensor digitorum longus.

Figure 2. S6K1 response to leucine in the skeletal muscle of old versus adult mice

Phosphorylation of S6K1 in response to Leu or TN+Leu in soleus (a) and EDL (d) of adult and old mice. The response to leucine alone is also reported as 'fold increase' compared to the Ctrl conditions of the respective age in soleus (b) and EDL (e). Representative western blot images are shown in (c) for soleus and in (f) for EDL. Values are expressed as mean ± SEM. *p<0.05 versus adult; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 versus Ctrl same age; $^{\$\$\$}$p<0.001 versus Leu same age. TN, tunicamycin; Leu, leucine ; EDL, extensor digitorum longus.

Table 1: Primers sequences

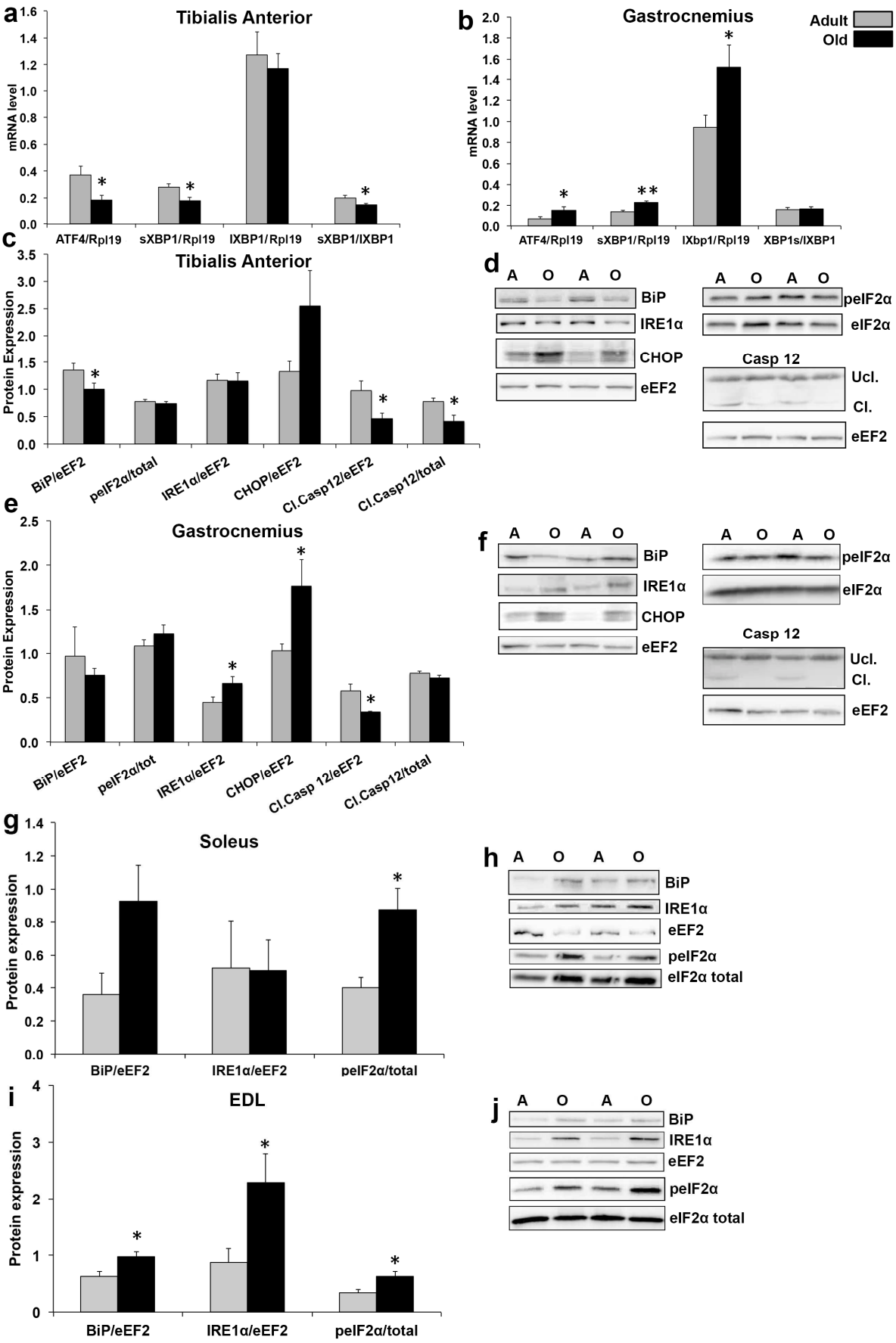
Gene	Forward	Reverse
ATF4	GAGCTTCCTGAACAGCGAAGTG	TGGCCACCTCCAGATAGTCATC
sXBP1	TGAGAACCAGGAGTTAAGAACACGC	CCTGCACCTGCTGCGGAC
lXBP1	TGAGAACCAGGAGTTAAGAACACGC	CACATAGTCTGAGTGCTGCGG
Rpl19	GAAGGTCAAAGGGAATGTGTTCA	CCTTGTCTGCCTTCAGCTTGT

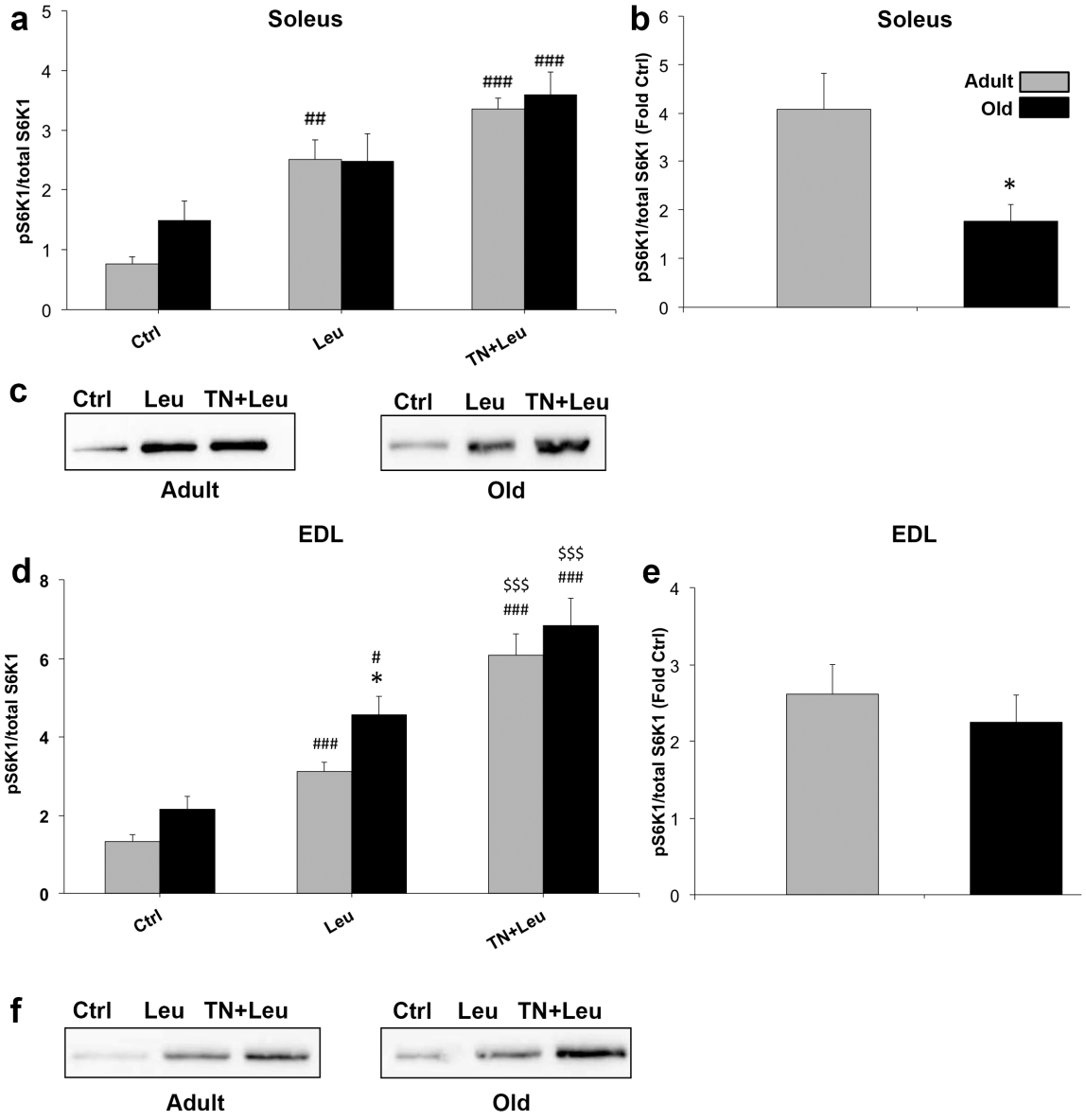
ATF4, activating transcription factor 4; sXBP1, spliced X-Box protein 1; lXBP1, unspliced X-Box protein 1; Rpl19, ribosomal protein L19

Table 2: Muscle weight normalized to body weight in adult and old mice

	Adult	Old
Soleus	0.00046± 0.00002	0.00040 ± 0.00001*
EDL	0.00044 ± 0.00001	0.00040 ± 0.00001
Plantaris	0.00082 ± 0.00003	0.00070 ± 0.00001**
Tibialis anterior	0.00201 ± 0.00003	0.00191 ± 0.00004
Gastrocnemius	0.00535 ± 0.00007	0.00461 ± 0.00011***

EDL, extensor digitorum longus. Results are expressed as the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 vs Adult.


a
Tibialis Anterior
mRNA level
ATF4/Rpl19
sXBP1/Rpl19
IXBP1/Rpl19
sXBP1/IXBP1
b
Gastrocnemius
Adult
Old
mRNA level
ATF4/Rpl19
sXBP1/Rpl19
IXbp1/Rpl19
XBP1s/IXBP1
c
Tibialis Anterior
Protein Expression
BiP/eEF2
peIF2α/total
IRE1α/eEF2
CHOP/eEF2
Cl.Casp12/eEF2
Cl.Casp12/total
d
A O A O
BiP
IRE1α
CHOP
eEF2
peIF2α
eIF2α
Casp 12
Ucl.
Cl.
eEF2
e
Gastrocnemius
Protein Expression
BiP/eEF2
peIF2α/tot
IRE1α/eEF2
CHOP/eEF2
Cl.Casp 12/eEF2
Cl.Casp12/total
f
A O A O
BiP
IRE1α
CHOP
eEF2
peIF2α
eIF2α
Casp 12
Ucl.
Cl.
eEF2
g
Soleus
Protein expression
BiP/eEF2
IRE1α/eEF2
peIF2α/total
h
A O A O
BiP
IRE1α
eEF2
peIF2α
eIF2α total
i
EDL
Protein expression
BiP/eEF2
IRE1α/eEF2
peIF2α/total
j
A O A O
BiP
IRE1α
eEF2
peIF2α
eIF2α total


a
Soleus
pS6K1/total S6K1
##
###
###
Ctrl
Leu
TN+Leu
b
Soleus
pS6K1/total S6K1 (Fold Ctrl)
Adult
Old
*
c
Ctrl Leu TN+Leu
Adult
Ctrl Leu TN+Leu
Old
EDL
d
pS6K1/total S6K1
$$$
###
$$$
###
#
*
###
Ctrl
Leu
TN+Leu
e
EDL
pS6K1/total S6K1 (Fold Ctrl)
f
Ctrl Leu TN+Leu
Adult
Ctrl Leu TN+Leu
Old

Highlights

- Muscle-to-body weight ratio was decreased in the hindlimb muscles of old mice.

- The regulation of ER stress markers with aging is not similar in all muscles.

- Anabolic resistance was present in soleus, not in EDL.

- Tunicamycin-induced ER stress does not impair the response of S6K1 to leucine.

- ER stress probably does not contribute to anabolic resistance in skeletal muscle of old mice.