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Autophagy is induced by resistance exercise in young men but unfolded protein response is induced regardless of age

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Abstract

Aim

Autophagy and unfolded protein response (UPR) appear to be important for skeletal muscle homeostasis and may be altered by exercise. Our aim was to investigate the effects of resistance exercise and training on indicators of UPR and autophagy in healthy untrained young men (n = 12, 27 ± 4 years) and older men (n = 8, 61 ± 6 years) as well as in resistance-trained individuals (n = 15, 25 ± 5 years).

Methods

Indicators of autophagy and UPR were investigated from the muscle biopsies after a single resistance exercise bout and after 21 weeks of resistance training.

Results

Lipidated LC3II as an indicator of autophagosome content increased at 48 hours post resistance exercise ($P < 0.05$) and after a resistance-training period ($P < 0.01$) in untrained young men but not in older men. Several UPR_{ER} markers, typically induced by protein misfolding in endoplasmic reticulum, were increased at 48 hours post resistance exercise in untrained young and older men ($P < 0.05$) but were unaltered after the 21-week resistance-training period regardless of age. UPR was unchanged within the first few hours after the resistance exercise bout regardless of the training status. Changes in autophagy and UPR_{ER} indicators did not correlate with a resistance-training–induced increase in muscle strength and size.

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Conclusion

Autophagosome content is increased by resistance training in young previously untrained men, but this response may be blunted by aging. However, unfolded protein response is induced by an unaccustomed resistance exercise bout in a delayed manner regardless of age.

Keywords: Autophagy, resistance training, unfolded protein response

List of abbreviations: ACC, Acetyl-CoA carboxylase; ATF4, Activating transcription factor 4;

BCL-2, Apoptosis regulator; Cyt C, Cytochrome C; CRYAB; αB-crystallin, eIF2α, Eukaryotic initiation factor 2 subunit α; ER, Endoplasmic reticulum; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GRP75, Glucose-regulated protein 75; GRP78, Glucose regulated protein 78; HSP10, Heat shock protein 10; HSP27, Heat shock protein 27; IRE1α, Inositol-requiring enzyme 1α; JNK**,** Jun-amino-terminal-kinase; LC3, Microtubule-associated protein 1 light chain 3; MT, Mitochondria; PDI, Protein disulfide isomerase; PERK, Protein kinase R-like endoplasmic reticulum protein kinase**,** PGC- 1α, Proliferator-activated receptor gamma coactivator-1alpha; P62, Sequestome-1; RE, Resistance exercise; RT, Resistance training; ULK1, Uncoordinated 51-like kinase; UPR, Unfolded protein response; VL, Vastus lateralis

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Introduction

An acute bout of resistance exercise (RE) with sufficient load and volume induces skeletal muscle remodelling that leads within few months of training to increases in muscle size and strength.[1, 2] These adaptations are accompanied by numerous benefits for health and performance,[3, 4] yet many of the underlying cellular mechanisms leading to the specific adaptations are not yet fully understood.

Disruptions of cellular homeostasis (e.g. due to mechanical, genotoxic and/or heat stress) may induce damage to proteins and cellular organelles.[5, 6] To maintain homeostasis, the damaged structures are degraded in a stress response.[5] More specifically, disrupted cellular homeostasis may also induce misfolding of premature proteins in the endoplasmic reticulum (ER), an organelle responsible for protein folding, trafficking and calcium homeostasis.[7] ER stress activates an unfolded protein response (UPR), which tries to balance the homeostasis in ER. If ER stress is chronic and cannot be resolved by UPR, metabolic impairments or apoptosis may occur.[8] Rodent studies suggest that UPR plays a regulatory role in skeletal muscle metabolism and possibly also in exercise adaptations.[9, 10] Resistance exercise may also induce UPR in humans regardless of age,[11] but the effect of longer periods of resistance training is unknown.

UPR induces – among other biological processes – autophagy,[12] which is a major catabolic route in cells responsible for clearance of proteins and organelles.[13] In the process of macroautophagy (hereafter called autophagy), proteins or organelles are surrounded by double-membraned vesicles that deliver their cargo to lysosomes in which they are degraded.[13] High, and thus, pathological levels of autophagy are associated with muscle-

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wasting conditions,[6] whereas inhibition of autophagy in skeletal muscles can also lead to diminished muscle mass and dysfunction due to aggregation of damaged cellular organelles and proteins.[14]

Transgenic rodent studies suggest that autophagy is required to induce some exercise adaptations,[15] maintain functional mitochondria after repeated exercise bouts,[16] and regulate muscle glucose metabolism during endurance exercise.[17] Indicators of autophagy have been reported to be induced by a single endurance exercise bout and chronic endurance training in rodent skeletal muscle.[16–22] However, the effect has not been as consistent in humans.[23–25] The limited data from human resistance exercise (RE) studies, suggests that autophagy is unchanged or decreased within the first few hours[26–28] and even at 24 hours post-RE,[29, 30] but may increase at 48 hours post-RE in young and old adults.[30] Decreased markers of autophagy have been reported in aging rodents and sedentary older males compared with young males.[31] Resistance training may increase autophagy in aged rats,[32] but the long-term effect of resistance-training interventions on autophagy is currently unknown in humans.

Our main purpose was to investigate the acute and prolonged effects of resistance exercise on autophagy and UPR in healthy young adults as well as in healthy older individuals. On the basis of the previous literature, we hypothesized that UPR is activated after a resistance exercise bout in a delayed manner but that long-term resistance training has no effect on UPR at resting state. Autophagosome content markers are also hypothesized to be induced by resistance exercise and training.

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Results

Resistance training increases muscle size and strength (Experiments 1 and 2)

As a result of a 21-week supervised resistance-training period (RT), muscle strength and fibre size were significantly increased in 21 young males and 18 older males.[33] The increase in muscle strength and muscle fibre size was also significant with the smaller sample size used in this study for young men (27 ± 4 years, n = 12) (Experiment 1) and for older men (Experiment 2) (n = 8, 61 ± 6 years, $P < 0.05$) (data not shown).

Resistance exercise (RE) bout leads to acute decrease in muscle force (Experiments 1 and 2)

In the young men of Experiment 1, after an unaccustomed RE bout of 5 x 10 repetition maximum (RM) until failure on a leg press device, isometric bilateral leg extension force significantly decreased from pre-values 3513 ± 1434 N to 2203 ± 765 N ($P < 0.001$) immediately post-RE and to 2748 ± 1063 N ($P < 0.001$) at 1 hour. Muscle strength remained slightly decreased at 48 hours post-RE bout (3265 ± 1382 N, $P < 0.05$). In the older men of Experiment 2, isometric bilateral leg extension force was significantly decreased from the pre-values 2567 ± 451 N to 1808 ± 334 N ($P < 0.001$) immediately after the RE bout. After the RT period the force values were PRE = 2803 ± 349 N and post 48 h = 2132 ± 125 N, respectively ($P < 0.001$). Isometric muscle force was slightly more decreased in young men compared to older men immediately after the RE bout (young: 36 ± 7 % vs. older 30 ± 7 %, $P < 0.05$). There was no significant difference in the force decline between the RE bouts that were conducted before and after the RT period in older men.

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UPR increases 48 hours after the resistance exercise bout in young men (Experiment 1)

Of the UPR indicators, one bout of unaccustomed RE increased PERK, ATF4, GRP78, p-JNK, *Xbp1s*, *Xbp1t* (main effect: $P < 0.05$). More specifically, acutely 1 hour after the RE bout, the protein content of PERK and IRE1α signalling pathway markers were unchanged (Fig. 2a–g). At 48 hours, from the PERK arm of the UPR, PERK and ATF4 proteins increased ($P < 0.05$) (Fig. 2a,b). To support the finding that UPR is induced 48 hours after the RE bout, downstream of IRE1α pathway the mRNA level of spliced *Xbp1* (*Xbp1s*) as well as total *(Xbp1t)* were increased (Fig. 2e,f). However, not all of the UPR markers increased at this time point, because the IRE1α protein and the phosphorylation of eIF2α at ser51 were unchanged (Fig. 2c,d). The protein content of ER-located chaperone GRP78/BiP increased 48 hours after the RE bout (Fig. 2g), but the content of two other ER-located chaperones, PDI and calnexin, was unchanged (supplementary Fig. 1a,b). From the apoptotic arm of the UPR, the phosphorylation of JNK ($P < 0.01$) and the mRNA level of *Chop* increased already 1 hour after the RE bout. At 48 hours after the RE bout, JNK phosphorylation remained elevated while *Chop* mRNA decreased back to baseline (Fig. 2h, supplementary Fig. 2a).

The post 48-hour biopsy was from the same leg as the PRE-biopsy, although 3 cm above the biopsy scar. In the non-exercised subjects (No RE; $n = 4$), there was no consistent biopsy effect on PERK, *Xbp1s* or *Xbp1t* (Fig. 2a,e,f). However, the protein content of GRP78 seemed to be systematically increased in the No RE subjects 48 hours after the RE bout (Fig. 2g).

UPR is unaltered by 21 weeks of resistance training in young men

Unlike at 48 hours after the unaccustomed RE bout, the 21-week RT period did not alter relative content of UPR markers or ER chaperones at 4–5 days after the last RE session (Fig. 2, supplementary Fig. 1).

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Autophagy markers are increased 48 hours after an unaccustomed resistance exercise bout and remain elevated after 21 weeks of resistance training in young men

Resistance exercise had a consistent effect on the protein content of following indicators (LC3II, LC3I, Beclin-1, P62, BCL-2 and p-ULK1 at ser^{555} (main effect: $P < 0.05$). More specifically, 48 hours after the RE bout, LC3II, LC3I and P62 were increased ($P < 0.05$) and Beclin-1 protein tended *(*$P = 0.06$*)* to be elevated (Fig. 3a–d). Protein expression levels of LC3I, LC3II and P62 are probably not explained by their transcription/transcript levels, because the mRNA levels of *lc3b* and *p62* were unchanged (supplementary Fig. 3a,b). BCL-2 and the phosphorylation of ULK^{ser555} and ULK1^{ser757} as well as total ULK1 were unchanged at 48 hours post-RE (Fig. 3e-g). At 48 hours after the RE bout, the increase in autophagy markers may be in part due to repeated biopsy effect because P62 and LC3II tended to be increased in some No RE subjects as well (Fig. 3a,d). At 1 hour after the RE bout, LC3II was decreased ($P < 0.05$; Fig. 3a) whereas p-ULK1^{ser757} tended to be increased ($P = 0.12$; Fig. 3e) and p-ULK1^{ser555} was decreased ($P < 0.05$; Fig. 3f). Additionally, LC3I, Beclin-1, P62, total ULK1, BCL-2 proteins (Fig. 3b,c,d,e,g) and the mRNA level of *P62* and *lc3b* were unchanged (supplementary Fig. 3a,b).

After the 21-week RT period, LC3II as a marker of autophagosome content increased ($P < 0.01$), whereas BCL-2 decreased ($P < 0.05$; Fig. 3a,g). The RT period had no effect on the protein content of LC3I, P62, Beclin-1, p-ULK1^{ser757} and total ULK1 (Fig. 3b–e) or *lc3b* and *p62* mRNA levels (supplementary Fig. 3a,b), but the p-ULK1^{ser757} per total ULK (p-ULK1^{ser757}/ULK1) increased at 21 weeks, whereas p-ULK1^{ser555} was decreased ($P < 0.05$, Fig. 3e–f).

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Unaltered UPRmt and markers of oxidative stress in younger men

From the indicators of oxidative stress there was no effect of the RE bout or 21 weeks of RT on protein carbonyls or glutaredoxin (Fig. 4a, supplementary Fig. 3c). Heat shock protein 27 (HSP27) and αB-crystallin significantly decreased 1 hour after the RE bout (supplementary Fig. 3d,e). From the mitochondrial UPR (UPR_{MT}) markers, glucose-regulated protein 75 (GRP75) and heat-shock protein 10 (HSP10) were analysed. Even though the RE bout seemed to increase ER-stress–induced UPR, neither RE nor RT had any effect on GRP75 or HSP10 (Fig. 4c,d).

Neither the RE nor the RT had effects on PGC1α1 protein, which is known to be associated with UPR[9] (Fig. 4b), or on the marker for AMPK activation, p-ACC, at measured time points (supplementary Fig. 4a). Similarly, no RT effect was observed on the marker for the mitochondria content cytochrome c (cyt c). However, 48 hours after the RE bout, cytochrome c protein was significantly decreased (supplementary Fig. 4b).

Unaltered markers of autophagy and oxidative stress, but increased markers of UPR in older men (Experiment 2)

As in the young men, some UPR markers were increased at 48 hours post-RE. This manifested as increased protein content of ATF4 ($P < 0.05$) and an increased trend ($P = 0.1$) in GRP78 after the RE bout conducted before RT (Fig. 5a,b). Also as in the young men, the RT period did not influence UPR, which was marked by unchanged levels of ATF4 and GRP78 proteins at 4–5 days after the last RE session (Fig. 5a,b). The protein content of GRP78 increased significantly 48 hours after the RE bout that was conducted after the 21-

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week RT period without changes in ATF4 (Fig. 5a,b). There were no RE or RT effects on PDI, IRE1α and PERK (Fig. 5c–e).

Unlike in the young men, neither the RE bout nor the RT period had an effect on LC3II, LC3I, P62, Beclin-1 or BCL-2 proteins (Fig. 6a–e). Similar to the young men, neither RE nor RT influenced protein carbonyl (Fig. 5f) or PDI protein content (Fig. 5c). PGC1-α1 protein content was unchanged after the 21-week training period and 48 hours after the unaccustomed RE bout, but not after the RE bout conducted after the RT period (supplementary Fig. 5a). Neither the RE bout nor the RT period influenced the UPR_{MT} marker HSP10 protein and the marker of mitochondria content cytochrome c (supplementary Fig. 5b,c).

Effects of RE bout in previously strength trained young individuals (Experiment 3)

To elucidate the acute effects of an RE bout on UPR and autophagy with a slightly longer acute time course and whether previous training background has any effects, we also analysed biopsies from 1.5 to 3 hours post-RE from a group of previously trained participants.[34] The RE bout had no effects on LC3II, LC3I protein or p-ULK1^{ser757}, but decreased the protein content of Beclin-1 at 150 minutes after the RE bout in these recreationally strength-trained individuals (Fig. 7d, supplementary Fig. 6b,c). Of the UPR_{ER} and UPR_{MT} markers, the bout of RE had any effects on IRE1α, PERK, PDI and p-eIF2α or HSP10 (Fig. 7a–c and supplementary Fig. 6a,d). Nevertheless, p-JNK54 and the ratio of p-JNK54/JNK54 were increased at both time points after the RE bout (Fig. 7e).

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Changes in autophagy and UPR markers are not associated with muscle hypertrophy, strength development and isometric force recovery

The main results of the three experiments are summarized in Fig 8. For Experiment 1, a computationally determined network was created between the main outcome variables. For this purpose, biologically similar variables were merged. The software Katiska/Himmeli running in a GNU Octave program environment (http://www.finndiane.fi/software/katiska/) was used for the analysis.[35] Interestingly, neither the change in UPR markers nor the autophagosome content at 48 hours post-RE and after the 21-week RT period were associated with improvement in leg press strength or muscle hypertrophy. These results were supported by the lack of correlation between these variables (supplementary Fig. 7). In addition, the autophagosome content and UPR markers at 48 hours did not correlate with the recovery of isometric muscle force at 48 hours post-RE.

Discussion

This study demonstrated, for the first time, that long-term hypertrophic resistance training increases the autophagosome content in previously untrained young men and has no effect on the content of UPR indicators relative to skeletal muscle total protein content at resting state or on ER-located chaperones in skeletal muscle. Additionally, two days after an unaccustomed bout of RE, indicators of UPR and autophagy were increased in previously untrained young and older men. However, the changes in autophagy and UPR indicators were not associated with oxidative stress or changes in muscle size and strength.

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Autophagy is a complex and dynamic process[36] that can be estimated from human biopsies by measuring lipidated LC3 protein (LC3II) as well as gene and protein expression of key proteins involved in various steps of autophagy.[37] In young men LC3II was increased 48 hours after the RE bout probably due to post-transcriptional regulation, suggesting increased autophagosome content. Moreover, increased LC3I and Beclin-1 suggest increased autophagic capacity and the induction of autophagy, respectively. However, the increased LC3II may also be due to decreased autophagosome degradation in the lysosomes and, thus, decreased autophagic flux.[36] To indirectly estimate autophagic flux, we also analysed the protein content of P62, which acts as an adaptor protein in the autophagosomes and is degraded when the autophagosomes release their cargo to the lysosomes.[38] P62 was increased at 48 hours post-RE indicating decreased clearance of the autophagosomes, or alternatively greater synthesis of P62 protein over its degradation. The present results are consistent with the previous research in which total protein of LC3b and P62 were increased 48 hours after an unaccustomed RE bout in young men.[30]

The effects of a long-term RT period on autophagy have never been investigated in human skeletal muscle.[39] In young men, RT increased LC3II, which indicates an increased number of autophagosomes and possibly increased autophagic flux, because the result was accompanied by unchanged LC3I and P62. However, the ratio of phosphorylated ULK1 at an inhibitory cite (ser757) to total ULK1 increased, whereas the phosphorylation of ULK1 at the activation cite at ser555 decreased. This may suggest downregulation of autophagosome precursor formation.[40] However, because after RT there was no change in Beclin-1, which is an autophagy induction marker, further studies are warranted to investigate the phenomenon further. The physiological increase in the autophagosome content may be considered as a positive adaptation[41] if we assume a greater recycling capacity of damaged organelles and

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proteins at rest or during recovery from a strenuous exercise bout. Previously in mice, 4–5 weeks of voluntary wheel running increased autophagic flux, as indicated by increased LC3II and decreased P62 content.[15] In the same study, in $Atg6^{+/-}$ (a critical protein in autophagy) mice, there was no increased autophagy flux, improved endurance performance, increased angiogenesis nor increased mitochondrial biogenesis as a result of 4–5 weeks of voluntary wheel running. This suggests that increased autophagy is needed to elicit endurance-training adaptations. In contrast to the present finding, pharmacological ways to increase muscle mass, such as blocking activins and myostatin, may not induce autophagy.[21]

The present study consisted of three separate experiments and only the relative changes induced by RE and RT within each experiment were analysed. Nevertheless, in contrast to the young men, in which 11/12 of the subjects showed an increase in LC3II content after the RT-period, in older healthy men 3 out of 8 subjects showed an increase in LC3II content and 5 out of 8 subjects showed a decrease, showing the clear difference in the response. In addition, several autophagy markers were induced 48 hours after an acute exercise bout in the young men but not in the older men. This suggests that aging may interfere with the RE/RT-induced increase in the regulation of autophagosome content. Previously, aging has been shown to decrease the markers of autophagy (i.e. ATG7 and LC3II/LC3I) in the skeletal muscle of sedentary humans.[31] They also reported that life-long participation in mainly endurance type of exercise prevented the decreases in autophagy, but the role of life-long exercise vs genomic effects remains speculative. Nevertheless, if autophagy or its response to exercise is interfered by aging, this may negatively affect cellular metabolism.[31, 42] Our data from the acute loading is contrary to the previous study by Ogborn *et al.*, which showed that autophagy was similarly induced within two days in both young and older men.[30] Furthermore, in older rodents RT enhanced markers of autophagy.[32] Therefore, more studies

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are needed to elucidate the effects of different types, intensities/loads, and volumes of RE and RT on autophagy, especially in humans at different ages.

There was a decrease in the content of lipidated LC3 (LC3II) 1 hour after the unaccustomed RE bout, a finding that supports earlier studies in humans.[23–25, 27–29] This may be explained by increased mTOR signalling that was previously observed in our subjects.[44] mTOR is known to inhibit autophagy by phosphorylating ULK1 at ser^{757},[45] and there was a non-significant trend for increase in p-ULK1^{ser757} content at 1 hour post-RE. In addition, p-ULK1^{s555}, which activates autophagosome precursor formation decreased at 1 h post RE. The decrease in LC3II did not occur in recreationally strength-trained individuals at 100–150 minutes post-RE, even though increased mTOR signalling was observed,[34] suggesting that the LC3 lipidation after an RE bout may vary by training status. However, the difference may also be due to the different RE protocols and the timing of the biopsies.

While the UPR markers in young individuals were unresponsive to the RE bout within the first 1–3 hours regardless of previous strength training background, many of the ER-stress–induced UPR indicators were increased 48 hours after the unaccustomed RE bout in young and older previously untrained men. This is consistent with a recent study reporting increased UPR markers at 24 and 48 hours after an unaccustomed RE bout in young and older men.[11] Thus, probably part of the muscle fibre remodelling following an unaccustomed RE bout may be mediated by UPR regardless of age. To support this, knockout mice lacking an upstream regulator of one UPR branch (ATF6α$^{-/-}$ mice) exhibit impaired ability to recover from exhaustive treadmill running and become exercise-intolerant after repeated treadmill running bouts.[9] On the other hand, UPR has been associated with acting as a molecular brake in

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rodents during rapid supraphysiological muscle hypertrophy.[10] The observed changes in UPR markers may be linked to the increased phosphorylation of JNK, which is suggested to occur during ER stress by IRE1α pathway. JNK phosphorylation has been suggested to promote apoptosis during ER stress,[46, 47] and has also been associated with mechanotransduction.[48]

Decreased muscle force and decreased protein content of cytochrome c were observed at 48 hours post-RE in young men, which previously has been related to mild muscle damage.[49] Unaccustomed exercise can cause disruption of the cytoskeleton structure of muscle fibres,[50] leading to the remodelling of muscle fibres,[51] a process in which integrins may have a role.[52, 53] Interestingly, the integrin-derived adaptations may involve activation of UPR,[54] but make firmer conclusions on this requires further research. Unlike UPR_{ER}, mitochondrial UPR (UPR_{MT} based on the protein contents of GRP75 and HSP10[55]) were unchanged. This suggests that an unaccustomed RE bout with predominant anaerobic energy production and a heavy load that induces mechanical strain on muscle tissue challenges the protein folding preferably in ER over mitochondria. To the authors' knowledge, this is the first publication examining the long-term effects of RT on UPR signalling and ER-resident chaperones. We did not observe changes in the UPR indicators or ER-resident chaperones in young and older previously untrained men after the 21-week RT period. This suggests that the content of these proteins is increased to the same extent as the overall muscle protein content when muscle size is increased after a long-term RT period. In previous studies, high-intensity endurance training has decreased the UPR markers in rodent skeletal muscle [56], whereas low-intensity endurance exercise did not [54, 55]. These results suggest that exercise training may suppress ER stress at resting state in some but not in all conditions.

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Both autophagy[15, 17] and UPR[9, 10] have been suggested to mediate part of the adaptation to exercise. However, the lack of significant correlations observed in the present study in young previously untrained young men suggests that the greater induction of UPR or autophagy markers did not translate into greater or minor muscle hypertrophy nor into strength in response to long-term resistance training. In addition, they were not associated with the recovery of maximal isometric force of the loaded muscles following the RE bout. Instead, when combined with the previous literature, [9, 14, 58] we suggest that these processes may be more likely related to the maintenance of skeletal muscle homeostasis than to increase or decrease muscle hypertrophy and/or strength. Because in our study protein carbonyls were unchanged, it is suggested that oxidative stress may not be the main contributor to the UPR induction[7] or autophagy[59] following RE.

In young men, the changes in the autophagy and UPR markers were more robust and more consistent in the RE group than they were in the individual responses in the individuals who did not do RE. However, in human muscle biopsy studies, not all of the acute exercise-induced changes in the gene expression are simply due to the exercise session, but may be induced by repeated biopsy obtainment or due to some unknown non-exercise stimulus.[60] Even though the 48-hour biopsy was obtained approximately 3 cm above the PRE-biopsy to avoid the residual effects of the repeated biopsy obtainment, some of the UPR markers as well as LC3II and P62 proteins were increased in a few non-exercisers as well. Due to this, we analysed normal variation in these proteins between two PRE-biopsies obtained from different legs in Experiment 3. Although there was a good correlation between the results from the two biopsies at the pre-RE situation, there were some differences suggesting that part of the changes in the control subjects are just normal variation. The increase in some of the UPR and autophagy markers in non-exercisers may also be induced by inflammation after

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repeated biopsies from the same leg.[61–63] Therefore, the present study recommends future studies to always include a non-exercise group or non-training limb for the acute experiments with multiple biopsies when studying unfolded protein response and/or autophagy.

In conclusion, the present study indicated that resistance training increases autophagosome content in the trained skeletal muscle, but this may be interfered with by aging. Additionally, a single bout of RE induced UPR in a delayed manner, but long-term RT had no effect on the UPR indicators or ER-located chaperone content at resting state regardless of age. Changes in autophagy and UPR indicators were not associated with the increase in muscle size, strength or isometric force recovery. When combined with the previous literature, our data suggest that, in response to resistance exercise, the induction of autophagy and UPR may be more important for maintaining skeletal muscle homeostasis than it is for regulating muscle growth or strength adaptations.

Materials and Methods

Ethical approval

The University of Jyväskylä Ethical committee approved Experiments 1 and 2, and the Regional Ethics Committee for Medical and Health Research of South-East Norway approved Experiment 3. All the subjects were carefully informed of the design, possible risks and discomfort related to the study and signed a written informed consent to participate. All the studies were conducted according to the Declaration of Helsinki.

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Overall study design

The present study consisted of three separate experiments. The first two experiments investigated the effects of the resistance exercise (RE) and resistance training (RT) -induced changes on UPR and autophagy in healthy previously untrained young men (Experiment 1) and older men (Experiment 2) (Fig. 1a,b). Experiment 3 examined the RE-induced acute changes on UPR and autophagy markers in healthy previously trained subjects with a slightly longer acute time-course than in Experiment 1 (Fig. 1c). In all of these experiments, previously collected muscle samples were analysed.

Subjects

Experiments 1 and 2. Healthy, normal weight or slightly overweight and untrained young men (26 ± 4 years, *n* = 16 BMI: 19.6–28.9) and older men (BMI: 23.4–28.8, *n* = 8, 61 ± 6 years) were selected from a larger group of subjects from previous studies[33, 64] based on muscle sample availability. In Experiment 1, the young men were originally randomized to either a whey protein group (26 ± 6 years, *n* = 6 in this study), a placebo group (27 ± 2 years, *n* = 6 in this study) or into non-exercise control group (26 ± 4 years, *n* = 4).[64] There were no significant effects of protein supplementation on the measured variables ($P > 0.05$). More specifically, the differences in the major variables were small and the *P* value was > 0.2 in more than 97% of the results, including the autophagy and UPR findings reported in the present study. Thus, for the sake of simplicity and to improve the statistical power for the RE/RT effects, the groups were pooled (27 ± 4 years, *n* = 12). The subjects have previously been described in more detail elsewhere.[33, 64] Subjects were excluded from the study if they had previous regular strength training experience or if they had cardiovascular or pulmonary diseases, malfunctions of the thyroid gland, diabetes, were overweight (body mass index >

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30), or were unable to perform the exercise training or tests due to some other disease. In addition, medication known to influence the endocrine system, heart rate, cardiovascular or neuromuscular performance were used as exclusion criteria.

Experiment 3. To elucidate the effects of acute resistance exercise with a slightly longer time course for the biopsy obtainment, with a slightly different exercise protocol than in Experiment 1, and if previous training status has an effect in the acute experiment, we also analysed muscle samples from another group of young, healthy and normal-weight recreationally strength-trained subjects (five females and ten males) (25 ± 5 years, n = 15).[32] Originally subjects were randomized to either a placebo (24 ± 2 years, *n* = 8, in this study) or antioxidant (26 ± 9 years, *n* = 7, in this study) group. There was no antioxidant effect on measured variables (P > 0.05). In order to simplify the study and to improve statistical power, groups were pooled.

Experiments 1 and 2

Experimental resistance exercise (RE)

In Experiments 1 and 2, a resistance exercise bout (RE) was 5 x 10 RM performed on a leg press device, as previously described.[64, 65] Maximal isometric bilateral leg extension force was measured before and after the last set with an electrodynamometer with a knee angle of 107 degrees. Additionally, in Experiment 1, isometric leg extension force was also measured 1 hour after the RE bout as well as 48 hours after the bout.

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Resistance training (RT)

The resistance training was conducted in the same manner as in Experiments 1 and 2. During the 21-week RT period, participants conducted two RE sessions per week as previously described.[33, 64] All the RE sessions were supervised by experienced trainers and there was at least two days between each session. The training programme focused especially on knee extensor muscles (i.e. vastus lateralis, VL) because the muscle cross-sectional area (CSA) was measured and biopsies were obtained from that muscle. In each RE session, bilateral leg press, knee extension and bilateral knee flexion were conducted. In addition, exercises for chest, shoulder, upper back, trunk extensor and flexor, upper arm, ankle extensor, hip abductor and adductor muscle groups were performed during the RT period. Of these exercises, leg press and knee extension activate the vastus lateralis (VL) muscle, which was the muscle mainly studied in the present study.

RE in Experiment 3

The participants were tested in an acute experiment at a time point where they had conducted 4–6 weeks of RT intervention. The acute exercise sessions included 4 x 10 RM of both leg press and knee-extension, with 1 minute of rest between sets and 3 minutes between exercises, as previously described.[34]

Muscle biopsies

Experiments 1 and 2. Biopsies were obtained from the VL muscle with a 5-mm Bergström biopsy needle, midway between the patella and greater trochanter as previously described.[64, 65] In both experiments the subjects fasted similarly for three hours before the morning

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biopsies (pre, 48h and pre-RE biopsy at post 21 wk) obtained and were not allowed to eat before the 1h post-RE biopsy. In Experiment 1, muscle biopsies were obtained 30 minutes before (PRE), 1 hour after and 48 hours after the first RE bout as well as 4–5 days after the last RE session of the 21-week RT period (Fig. 1a). In Experiment 2, biopsies were obtained before the RE bout and 48 hours after at both pre- and post-RT (Fig. 1b). The resting state post-RT biopsy was obtained 4–5 days after the last RE session in both experiments. In both experiments, the PRE and 48-hour biopsies were obtained from the same leg and the post 1-hour biopsy was from the other leg. To avoid the residual effects of repeated biopsies, the 48-hour biopsy was obtained approximately 3 cm above the PRE biopsy scar. The 21-week biopsy was taken from the same leg as the PRE and 48-hour biopsies.

Experiment 3. Muscle biopsies were obtained from the VL muscle twice before the acute exercise bout (two PRE samples from different legs) and twice after at 100 and 150 minutes (Fig. 1c), as previously described.[34] The repeated biopsies were always obtained approximately 3 cm proximal to the previous insertion.

In all the experiments muscle biopsies were flash frozen in liquid nitrogen after being rinsed of blood and any visible connective and adipose tissue. Muscle biopsies were stored at –80°C for future protein and mRNA analysis.

Protein extraction

In Experiments 1 and 3, muscle biopsies were hand-homogenized in ice-cold buffer [20 mM HEPES (pH 7.4), 1 mM EDTA, 5 mM EGTA, 10 mM MgCl2, 100 mM β-glycerophosphate, 1 mM Na3VO4, 2 mM DTT, 1% Triton X-100, 0.2% sodium deoxycholate, 30 μg ml^{-1}

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leupeptin, 30 μg ml^{-1} aprotinin, 60 μg ml^{-1} PMSF, and 1% phosphatase inhibitor cocktail (P 2850; Sigma, St. Louis, USA)] at a dilution of 15 μl mg^{-1} of wet weight muscle as previously described.[44] To remove cell debris, homogenates were rotated for 30 minutes at 4° C, centrifuged at 10,000 g for 10 minutes at 4°C and subsequently stored at –80°C for future analysis. Bicinchonic acid protein assay (Pierce Biotechnology, Rockford, IL) and BioRad DC protein microplate assays (Bio-Rad, Hercules, CA, USA) were used to determine total protein amount.

In the Experiment 2, muscle biopsies were hand-homogenized on ice with a buffer that is rather similar, but not identical as in Experiments 1 and 3: 50mM Hepes pH 7.4, 0.1% Triton X-100, 4 mM EGTA, 10 mM EDTA, 15 mM Na4P2O7·10H2O,100 mM β-glycerolphosphate, 25 mM NaF, 1 mMNa3VO4, 0.5 μg ml^{-1} leupeptin, 0.5 μg ml^{-1} pepstatin and 0.3 μg ml^{-1} aprotinin) in a 4 % (w/v) solution. Lowry-based method was used (Bio-Rad, Hercules, CA) to determine protein concentrations of the supernatants. Since no phosphatase inhibitor cocktails were used for the older men samples, only total protein levels were analysed from these samples.

RNA extraction and cDNA synthesis

In the Experiment 1, another part of the muscle biopsy was homogenized with FastPrep (Bio101 Systems, USA) for the RNA-analysis. Tubes and total RNA was extracted using Trizol-reagent (Invitrogen, Carlsbad, CA, USA) as previously described.[64] The yielded RNA extraction was un-degraded and DNA free based on an OD260/OD280 ratio (1.8–2.0) and electrophoresis, respectively. RNA (3 μg) was reverse transcribed to cDNA by High Capacity cDNA Archive Kit (Archive Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

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Western immunoblot analyses

For all the three experiments muscle homogenates were mixed with Laemmli sample buffer and were heated at 95°C for 10 minutes to denaturize proteins. Approximately 30 µg of total protein was separated by SDS-PAGE and was transferred to a PVDF membrane. The membrane was blocked with 5 % milk (TBS including tween 0.1 %) for 2 hours in room temperature and incubated overnight with primary antibodies in 4°C. Membrane was washed with TBS-T and incubated with secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h in RT. After secondary antibody incubation, membranes were washed with TBS-T and proteins were visualized with ECL (SuperSignal west femto maximum sensitivity substrate, Pierce Biotechnology, Rockford, IL, USA). The chemiluminescent signal was quantified by ChemiDoc XRS device with Quantity One software (version 4.6.3. Bio-Rad laboratories, Hercules, CA, USA). The average of Ponceau-S staining and GAPDH (Abcam, Cambridge, UK) was used to normalise the protein loading and blotting efficiency. There were two PRE-samples in the Experiment 3 and the CV (%) and intraclass correlations are shown in the Table 1.

Protein carbonyls. As a marker of oxidative stress, protein carbonyl content was measured with Oxyblot Protein Oxidation Detection kit (Merck Millipore, S1750) according to manufacturer's instructions with 10 µg of total protein used for the derivatization reaction. The blots were visualised and quantified as described above. The membrane was stained with Ponceau-S to normalise protein loading and blotting efficiency.

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Primary antibodies. Antibodies were purchased from several companies. The antibodies from Cell Signaling Technology (Danvers, MA, USA) were as follows: inositol-requiring enzyme 1α (IRE1α, #3294, 1:1000), protein disulfide isomerase (PDI, #3501, 1:3000), protein kinase R-like endoplasmic reticulum protein kinase (PERK, #5683, 1:1000), eukaryotic initiation factor 2 subunit α (eIF2α, #5324, 1:1000) and its phosphorylated form at ser51 (p-eIF2α, #3398, 1:1000), glucose regulated protein 78 (GRP78, #3177, 1:1000), calnexin (#2679, 1:1000), serine/threonine protein kinase (ULK1, #8054, 1:1000) and its phosphorylated form at serine 757 (p-ULK1, #14202, 1:1000) and at ser555 (p-ULK1, #5869, 1:1000), Sequestome-1/P62 (P62, #5114, 1:2000) Beclin-1 (#3738, 1:1000) apoptosis regulator BCL-2 (BCL-2, #3498, 1:1000) activating transcription factor 4 (ATF4, #11815, 1:1000), jun-amino-terminal-kinase (JNK, #9252, 1:1000) and its phosphorylation at Thr183/Tyr185 (p-JNK, #4668, 1:1000), crystallin-αB (CRYAB, #45844; 1:3000) and phosphorylated acetyl-CoA carboxylase at ser79 (p-ACC, #8578, 1:2000). Antibody against GAPDH (ab9485, 1:10 000) was from Abcam (Cambridge, UK). Antibodies that recognize the inducible forms of heat shock protein 27 (HSP25, SPA-801, 1:1000) and glucose-regulated protein 75 (GRP75, SPS-825, 1:1000) were from Enzo Life Sciences Inc (Farmingdale, NY, USA). Antibody against HSP10 (SAB4501465, 1:1000) was from Sigma-Aldrich (St. Louis, MO, USA). For measuring protein levels of the non-truncated full-length splice variants of PGC-1α, the antibody (1:5000, Calbiochem, Merck KGaA, Darmstadt, Germany) against C-terminus of protein (amino acids 777–797) was used. LC3I and LC3II protein levels were measured by using an antibody (L7543, 1:1000) from Sigma-Aldrich (St. Louis, MO, USA). Cytochrome C was measured with an antibody (SC-8385, 1:1000) from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Dallas, USA). Antibodies against glutaredoxin HSP27 and GRP75 were kindly provided by Dr. Mustafa Atalay.

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RT-qPCR

RT-qPCR with standard procedures using iQ SYBR Supermix (Bio-Rad laboratories) and CFX96 real-time PCR Detection system (Bio-Rad laboratories) were used to measure mRNA expression level in triplicates. Total X-box binding protein (*Xbp1t*) and spliced variant (*Xbp1s*) were analysed using SYBR green primers: *Xbp1s*: forward: tgctgagtccgcagcaggtg, reverese: gctggcaggctctggggaag, product length: 169 bp and *Xbp1t*: forward: aggagaaggcgctgaggaggaaact, reverse: accacttgctgttccagctcactca, product length: 100 bp. The protocol of *Xbp1s* and *Xbp1t* was initiated at 95°C for 3 min, which was followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 62°C for 15 s and extension at 72°C for 30 s. *Xbp1s* and *Xbp1t* end-products were verified by DNA electrophoresis. qPCR for C/EBP homologous protein (*Chop/Ddit3*, assay ID (qHsaCED0056908), *Lc3b* (assay ID qHsaCED0038576) and sequestome 1 (*P62/SQSTM1*, assay ID qHsaCED0045925) were conducted by using pre-designed and validated Bio-Rad PrimePCRtm SYBR Green Assays using the recommended protocol by the manufacturer. GAPDH was used to normalise the mRNA expression levels because it was unaffected by RE and RT and was better than 18SRNA as previously described.[66] Delta delta Ct (ddCt) method was used to measure gene expression levels at the exponential amplification phase.

Statistical analysis

For all three experiments, the main effect of RE/RT was investigated with a General Linear Model with repeated measures (IBM SPSS statistics version 24). For the post-hoc tests, the RE- and RT-induced fold changes from the PRE condition were evaluated by a Holm-Bonferroni adjusted *t* test or a related samples Wilcoxon rank-test (when not normally distributed). In the Experiment 1, three comparisons were included in the Bonferroni

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adjustments (PRE vs. 1 hour, PRE vs. 48 hours and PRE vs. 21 weeks). In the Experiment 2, the comparisons were PRE vs. 48 hours, PRE vs. post 21 weeks (PRE RT) and PRE vs. 48 hours (POST RT). In the Experiment 3, the comparisons were PRE (an average of 2 PRE samples) vs. 100 and PRE (an average of 2 PRE samples) vs. 150.

Due to the small sample size in the No RE group in Experiment 1 (n = 2–4), statistical tests were not performed for it and instead the individual values are shown. Due to the nature of separate experiments with minor differences between the experiments, direct comparisons were not conducted between the experiments. Pearson's product moment coefficient was used to analyse associations between the variables. In the figures, open bars depict the mean of that time point whereas circles and triangles depict individual values. The statistical significance was set at $P < 0.05$.

Competing interests

No conflicts of interest, financial or otherwise, are declared by the authors.

Author contributions

The Experiments 1 and 2 were designed and carried out by JJH, JPA, AAM and KH, while the present study was designed by JH, JJH and JPA. The Experiment 3 was originally designed and carried out by GP and TR. JH analysed the data in the present study with the supervision of JJH. The first version of the manuscript was drafted by JH, which was then commented by other authors and revised by JH, JJH and JPA. All the authors read and approved the final manuscript.

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References

1. Ahtiainen JP, Walker S, Peltonen H, Holviala J, Sillanpää E, Karavirta L, et al. Heterogeneity in resistance training-induced muscle strength and mass responses in men and women of different ages. *Age (Omaha)* 2016;**38**:10.

2. Ralston GW, Kilgore L, Wyatt FB, Baker JS. The Effect of Weekly Set Volume on Strength Gain: A Meta-Analysis. *Sport Med*. 2017.

3. Westcott WL. Resistance training is medicine: effects of strength training on health. *Curr Sports Med Rep*. 2012;**11**:209–16.

4. Wolfe RR. The underappreciated role of muscle in health and disease. *Am J Clin Nutr*. 2006;**84**:475–82.

5. Schneider K, Bertolotti A. Surviving protein quality control catastrophes - from cells to organisms. *J Cell Sci.* 2015;**128**:3861–9.

6. Sandri M. Protein breakdown in muscle wasting: role of autophagy-lysosome and ubiquitin-proteasome. *Int J Biochem Cell Biol*. 2013;**45**:2121–9.

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7. Cao SS, Kaufman RJ. Endoplasmic reticulum stress and oxidative stress in cell fate decision and human disease. *Antioxid Redox Signal*. 2014;**21**:396–413.

8. Hetz C, Chevet E, Oakes SA. Proteostasis control by the unfolded protein response. *Nat Cell Biol.* 2015;**17**:829–38.

9. Wu J, Ruas JL, Estall JL, Rasbach KA, Choi JH, Ye L, et al. The unfolded protein response mediates adaptation to exercise in skeletal muscle through a PGC-1alpha/ATF6alpha complex. *Cell Metab.* 2011;**13**:160–9.

10. Hamilton DL, Philp A, MacKenzie MG, Patton A, Towler MC, Gallagher IJ, et al. Molecular brakes regulating mTORC1 activation in skeletal muscle following synergist ablation. *Am J Physiol Endocrinol Metab.* 2014;**307**:E365-73.

11. Ogborn DI, McKay BR, Crane JD, Parise G, Tarnopolsky MA. The unfolded protein response is triggered following a single, unaccustomed resistance-exercise bout. *Am J Physiol Regul Integr Comp Physiol.* 2014;**307**:R664-9.

12. Yorimitsu T, Nair U, Yang Z, Klionsky DJ. Endoplasmic reticulum stress triggers autophagy. *J Biol Chem.* 2006;**281**:30299–304.

13. Sandri M. Autophagy in skeletal muscle. *FEBS Lett.* 2010;**584**:1411–6.

14. Masiero E, Agatea L, Mammucari C, Blaauw B, Loro E, Komatsu M, et al. Autophagy Is Required to Maintain Muscle Mass. *Cell Metab.* 2009;**10**:507–15.

15. Lira VA, Okutsu M, Zhang M, Greene NP, Laker RC, Breen DS, et al. Autophagy is required for exercise training-induced skeletal muscle adaptation and improvement of physical performance. *FASEB J Off Publ Fed Am Soc Exp Biol.* 2013;**27**:4184–93.

16. Lo Verso F, Carnio S, Vainshtein A, Sandri M. Autophagy is not required to sustain exercise and PRKAA1/AMPK activity but is important to prevent mitochondrial damage during physical activity. *Autophagy.* 2014;**10**:1883–94.

17. He C, Bassik MC, Moresi V, Sun K, Wei Y, Zou Z, et al. Exercise-induced BCL2-regulated autophagy is required for muscle glucose homeostasis. *Nature.* 2012;**481**:511–5.

18. Grumati P, Coletto L, Schiavinato A, Castagnaro S, Bertaggia E, Sandri M, et al. Physical exercise stimulates autophagy in normal skeletal muscles but is detrimental for collagen VI-deficient muscles. *Autophagy.* 2011;**7**:1415–23.

19. Pagano AF, Py G, Bernardi H, Candau RB, Sanchez AMJ. Autophagy and protein turnover signaling in slow-twitch muscle during exercise. *Med Sci Sports Exerc.* 2014;**46**:1314–25.

20. Vainshtein A, Tryon LD, Pauly M, Hood DA. Role of PGC-1alpha during acute exercise-induced autophagy and mitophagy in skeletal muscle. *Am J Physiol Cell Physiol.* 2015;**308**:C710-9.

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21. Hulmi JJ, Oliveira BM, Silvennoinen M, Hoogaars WMH, Pasternack A, Kainulainen H, et al. Exercise restores decreased physical activity levels and increases markers of autophagy and oxidative capacity in myostatin/activin-blocked mdx mice. *Am J Physiol Endocrinol Metab.* 2013;**305**:E171-82.

22. Salminen A, Vihko V. Autophagic response to strenuous exercise in mouse skeletal muscle fibers. *Virchows Arch B Cell Pathol Incl Mol Pathol.* 1984;**45**:97–106.

23. Fritzen AM, Madsen AB, Kleinert M, Treebak JT, Lundsgaard A-M, Jensen TE, et al. Regulation of autophagy in human skeletal muscle: effects of exercise, exercise training and insulin stimulation. *J Physiol.* 2016;**594**:745–61.

24. Moller AB, Vendelbo MH, Christensen B, Clasen BF, Bak AM, Jorgensen JOL, et al. Physical exercise increases autophagic signaling through ULK1 in human skeletal muscle. *J Appl Physiol.* 2015;**118**:971–9.

25. Schwalm C, Jamart C, Benoit N, Naslain D, Premont C, Prevet J, et al. Activation of autophagy in human skeletal muscle is dependent on exercise intensity and AMPK activation. *FASEB J Off Publ Fed Am Soc Exp Biol.* 2015;**29**:3515–26.

26. Smiles WJ, Areta JL, Coffey VG, Phillips SM, Moore DR, Stellingwerff T, et al. Modulation of autophagy signaling with resistance exercise and protein ingestion following short-term energy deficit. *Am J Physiol Regul Integr Comp Physiol.* 2015;**309**:R603-12.

27. Fry CS, Drummond MJ, Glynn EL, Dickinson JM, Gundermann DM, Timmerman KL, et al. Skeletal muscle autophagy and protein breakdown following resistance exercise are similar in younger and older adults. *J Gerontol A Biol Sci Med Sci.* 2013;**68**:599–607.

28. Glynn EL, Fry CS, Drummond MJ, Dreyer HC, Dhanani S, Volpi E, et al. Muscle protein breakdown has a minor role in the protein anabolic response to essential amino acid and carbohydrate intake following resistance exercise. *Am J Physiol Regul Integr Comp Physiol.* 2010;**299**:R533-40.

29. Dickinson JM, Reidy PT, Gundermann DM, Borack MS, Walker DK, D'Lugos AC, et al. The impact of postexercise essential amino acid ingestion on the ubiquitin proteasome and autophagosomal-lysosomal systems in skeletal muscle of older men. *J Appl Physiol.* 2017;**122**:620–30.

30. Ogborn DI, McKay BR, Crane JD, Safdar A, Akhtar M, Parise G, et al. Effects of age and unaccustomed resistance exercise on mitochondrial transcript and protein abundance in skeletal muscle of men. *Am J Physiol Regul Integr Comp Physiol.* 2015;**308**:R734-41.

31. Carnio S, LoVerso F, Baraibar MA, Longa E, Khan MM, Maffei M, et al. Autophagy impairment in muscle induces neuromuscular junction degeneration and precocious aging. *Cell Rep.* 2014;**8**:1509–21.

This article is protected by copyright. All rights reserved.

Accepted Article

32. Luo L, Lu A-M, Wang Y, Hong A, Chen Y, Hu J, et al. Chronic resistance training activates autophagy and reduces apoptosis of muscle cells by modulating IGF-1 and its receptors, Akt/mTOR and Akt/FOXO3a signaling in aged rats. *Exp Gerontol.* 2013;**48**:427–36.

33. Mero AA, Hulmi JJ, Salmijarvi H, Katajavuori M, Haverinen M, Holviala J, et al. Resistance training induced increase in muscle fiber size in young and older men. *Eur J Appl Physiol.* 2013;**113**:641–50.

34. Paulsen G, Hamarsland H, Cumming KT, Johansen RE, Hulmi JJ, Borsheim E, et al. Vitamin C and E supplementation alters protein signalling after a strength training session, but not muscle growth during 10 weeks of training. *J Physiol.* 2014;**592**:5391–408.

35. Makinen V-P, Forsblom C, Thorn LM, Waden J, Kaski K, Ala-Korpela M, et al. Network of vascular diseases, death and biochemical characteristics in a set of 4,197 patients with type 1 diabetes (the FinnDiane Study). *Cardiovasc Diabetol.* 2009;**8**:54.

36. Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy.* 2016;**12**:1–222.

37. Loos B, du Toit A, Hofmeyr J-HS. Defining and measuring autophagosome flux—concept and reality. *Autophagy.* 2014;**10**:2087–96.

38. Bjorkoy G, Lamark T, Brech A, Outzen H, Perander M, Overvatn A, et al. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *J Cell Biol.* 2005;**171**:603–14.

39. Martin-Rincon M, Morales-Alamo D, Calbet JAL. Exercise-mediated modulation of autophagy in skeletal muscle. *Scand J Med Sci Sports.* 2017.

40. Zachari M, Ganley IG. The mammalian ULK1 complex and autophagy initiation. *Essays Biochem.* 2017;**61**:585–96.

41. Levine B, Klionsky DJ. Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell.* 2004;**6**:463–77.

42. Salminen A, Kaarniranta K. Regulation of the aging process by autophagy. *Trends Mol Med.* 2009;**15**:217–24.

43. Wohlgemuth SE, Seo AY, Marzetti E, Lees HA, Leeuwenburgh C. Skeletal muscle autophagy and apoptosis during aging: effects of calorie restriction and life-long exercise. *Exp Gerontol.* 2010;**45**:138–48.

44. Hulmi JJ, Tannerstedt J, Selanne H, Kainulainen H, Kovanen V, Mero AA. Resistance exercise with whey protein ingestion affects mTOR signaling pathway and myostatin in men. *J Appl Physiol.* 2009;**106**:1720–9.

This article is protected by copyright. All rights reserved.

45. Kim J, Kundu M, Viollet B, Guan K-L. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol.* 2011;**13**:132–41.

46. Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP, et al. Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science.* 2000;**287**:664–6.

47. Szegezdi E, Logue SE, Gorman AM, Samali A. Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO Rep.* 2006;**7**:880–5.

48. Frey JW, Farley EE, O'Neil TK, Burkholder TJ, Hornberger TA. Evidence that mechanosensors with distinct biomechanical properties allow for specificity in mechanotransduction. *Biophys J*. 2009;**97**:347–56.

49. Zager RA, Johnson ACM, Hanson SY. Proximal tubular cytochrome c efflux: determinant, and potential marker, of mitochondrial injury. *Kidney Int.* 2004;**65**:2123–34.

50. Lehti TM, Kalliokoski R, Komulainen J. Repeated bout effect on the cytoskeletal proteins titin, desmin, and dystrophin in rat skeletal muscle. *J Muscle Res Cell Motil*. 2007;**28**:39–47.

51. Malm C, Yu J-G. Exercise-induced muscle damage and inflammation: re-evaluation by proteomics. *Histochem Cell Biol*. 2012;**138**:89–99.

52. Lueders TN, Zou K, Huntsman HD, Meador B, Mahmassani Z, Abel M, et al. The alpha7beta1-integrin accelerates fiber hypertrophy and myogenesis following a single bout of eccentric exercise. *Am J Physiol Cell Physiol.* 2011;**301**:C938-46.

53. Zou K, Meador BM, Johnson B, Huntsman HD, Mahmassani Z, Valero MC, et al. The alpha(7)beta(1)-integrin increases muscle hypertrophy following multiple bouts of eccentric exercise. *J Appl Physiol.* 2011;**111**:1134–41.

54. Mahmassani ZS, Son K, Pincu Y, Munroe M, Drnevich J, Chen J, et al. alpha7beta1 Integrin regulation of gene transcription in skeletal muscle following an acute bout of eccentric exercise. *Am J Physiol Cell Physiol.* 2017;**312**:C638–50.

55. D'Amico D, Sorrentino V, Auwerx J. Cytosolic Proteostasis Networks of the Mitochondrial Stress Response. *Trends Biochem Sci.* 2017.

56. Kim K, Kim Y-H, Lee S-H, Jeon M-J, Park S-Y, Doh K-O. Effect of exercise intensity on unfolded protein response in skeletal muscle of rat. *Korean J Physiol Pharmacol Off J Korean Physiol Soc Korean Soc Pharmacol*. 2014;**18**:211–6.

57. Hulmi JJ, Hentilä J, DeRuisseau KC, Oliveira BM, Papaioannou KG, Autio R, et al. Effects of muscular dystrophy, exercise and blocking activin receptor IIB ligands on the unfolded protein response and oxidative stress. *Free Radic Biol Med*. 2016;**99**:308–22.

58. Masiero E, Sandri M. Autophagy inhibition induces atrophy and myopathy in adult skeletal muscles. *Autophagy*. 2010;**6**:307–9.

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Accepted Article

59. Lee J, Giordano S, Zhang J. Autophagy, mitochondria and oxidative stress: cross-talk and redox signalling. *Biochem J*. 2012;**441**:523–40.

60. Vissing K, Andersen JL, Schjerling P. Are exercise-induced genes induced by exercise? *FASEB J*. 2005;**19**:94–6.

61. Malm C, Nyberg P, Engstrom M, Sjodin B, Lenkei R, Ekblom B, et al. Immunological changes in human skeletal muscle and blood after eccentric exercise and multiple biopsies. *J Physiol.* 2000;**529 Pt 1**:243–62.

62. Van Thienen R, D'Hulst G, Deldicque L, Hespel P. Biochemical artifacts in experiments involving repeated biopsies in the same muscle. *Physiol Rep.* 2014;**2**:e00286.

63. Zhang K. Integration of ER stress, oxidative stress and the inflammatory response in health and disease. *Int J Clin Exp Med.* 2010;**3**:33–40.

64. Hulmi JJ, Kovanen V, Selanne H, Kraemer WJ, Hakkinen K, Mero AA. Acute and long-term effects of resistance exercise with or without protein ingestion on muscle hypertrophy and gene expression. *Amino Acids.* 2009;**37**:297–308.

65. Ahtiainen JP, Hulmi JJ, Lehti M, Kraemer WJ, Nyman K, Selänne H, et al. Effects of resistance training on expression of IGF-I splice variants in younger and older men. *Eur J Sport Sci*. 2016;**16**:1055–63.

66. Hulmi JJ, Ahtiainen JP, Kaasalainen T, Pollanen E, Hakkinen K, Alen M, et al. Postexercise myostatin and activin IIb mRNA levels: effects of strength training. *Med Sci Sports Exerc.* 2007;**39**:289–97.

Figure legends

Figure 1. Study design of the three experiments. A) In young previously untrained (Experiment 1) and B) older previously untrained men (Experiment 2) resistance exercise (RE) was 5 x 10 repetitions in leg press until failure. Resistance training (RT) was 21 weeks of supervised heavy whole body resistance training and was conducted similarly in young and older men. C) In previously recreationally strength trained subjects (Experiment 3) RE was 4 x 10 RM of both leg press and knee-extension, with 1 minute of rest between sets and 3 minutes between exercises. Needles in the figure depict the time points when biopsies were

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obtained from the vastus lateralis (VL) muscle. The biopsy after the RT period (Experiment 1 and 2) was obtained 4–5 days after the last RE session to represent resting state and to avoid the acute effects of the last RE session of RT period.

Figure 2. UPR indicators before, 1 hour, and 48 hours after an unaccustomed resistance exercise bout (PRE RT) as well as after 21-wk RT period (RT) and in non-exercised controls (No RE) in young men (Experiment 1). A) PERK protein, B) the ratio of p-eIF2α and eIF2α, C) ATF4 protein, D) IRE1α protein, E) spliced Xbp1 mRNA, F) total Xbp1mRNA, G) GRP78 protein and H) the ratio of p-JNK and JNK (46 kDa and 54 kDa averaged). Open bars depict means. Circles and triangles depict individual values. The symbol * depicts the statistical significance $P < 0.05$ from PRE. n = 10–12 in RT and n = 3–4 in No RE subjects except in Xbp1s and Xbp1t n = 13–15 in RT and n = 2–3 in No RE subjects and in p-JNK/JNK n = 7–9 in RT and n = 2–4 in No RE.

Figure 3. Autophagy indicators before, 1 hour, and 48 hours after an unaccustomed resistance exercise bout (PRE RT) as well as after 21-wk RT period (RT) and in non-exercised controls (No RE) in young men (Experiment 1). A) LC3II protein, B) LC3I protein, C) Beclin-1 protein, D) P62 protein, E) the ratio of p-ULK1^{ser757} and total ULK1, F) p-ULK1^{ser555} and G) BCL-2 protein. Open bars depict means. Circles and triangles depict individual values. The symbols * and ** depict the statistical significance $P < 0.05$, $P < 0.01$; respectively. n = 10–12 in RT and n = 3–4 in No RE for all except p-ULK1^{ser555} n = 7–10 in RT and 2–4 in No RE.

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Figure 4. Indicators of oxidative stress, aerobic metabolism and mitochondrial UPR before, 1 hour, and 48 hours after an unaccustomed resistance exercise bout (PRE RT) as well as after 21-wk RT period (RT) and in non-exercised controls (No RE) in young men (Experiment 1). A) Protein carbonyl content, B) PGC1α1 protein content, C) GRP75 protein content, D) HSP10 protein content. Open bars depict means. Circles and triangles depict individual values. In protein carbonyls n = 8 in RT and n = 2–3 in No RE. In PGC1α1, GRP75 and HSP10 n = 10–12 in RT and n = 3–4 in No RE.

Figure 5. UPR indicators before and 48 hours after an unaccustomed resistance exercise bout (PRE RT) and before and 48 h after RE bout after 21-wk RT period (POST RT) in older men (Experiment 2). A) GRP78 protein, B) ATF4 protein, C) PDI protein, D) IRE1α protein, E) PERK protein, F) Protein carbonyls. Open bars depict means. Circles and triangles depict individual values. The symbol * depicts the statistical significance $P < 0.05$ from PRE state. In all of the variables n = 6–8.

Figure 6. Autophagy indicators before and 48 hours after an unaccustomed resistance exercise bout (PRE RT) and before and 48 h after RE bout after 21-wk RT period (POST RT) in older men (Experiment 2). A) LC3II protein content, B) LC3I protein content, C) Beclin-1 protein content, D) P62 protein content, E) BCL-2 protein content. Open bars depict means. Circles and triangles depict individual values. In all of the variables n = 6–8.

Figure 7. UPR and autophagy indicators before, 100 and 150 minutes after resistance exercise bout in recreationally resistance trained young individuals (Experiment 3). A) PERK protein, B) the ratio of p-eIF2α and eIF2α protein, C) IRE1α protein, D) LC3II and I, E)

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Ratio of p-JNK54 and total JNK54 protein. PRE value is average of two biopsies before the RE bout. Open bars depict means. Circles and triangles depict individual values. The symbol * depicts the statistical significance $P < 0.05$ from PRE state. n = 14–15.

Figure 8. Summary figure of the resistance exercise (RE) and resistance-training (RT) induced changes in unfolded protein response (UPR) and autophagy markers in young previously untrained (Experiment 1) and previously recreationally strength trained (Experiment 3) and older previously untrained individuals (experiment 2). The symbol of autophagy (autophagosome) is depicting autophagosome content, whereas the ellipse entitled: "UPR" depicts ER stress induced overall unfolded protein response. Autophagy indicators were either decreased or unchanged acutely after the RE bout. In addition, UPR indicators were unaltered acutely after the RE bout. Several autophagy markers increased at 48 hours post RE in young men indicating activation of autophagy and an increase in autophagosome content (LC3II). LC3II content increased after the RT period in untrained young men indicating increased autophagosome content and possibly autophagic flux (unchanged LC3I and P62). The ratio of phosphorylated ULK1 at an inhibitory cite (ser757) to total ULK1 increased, whereas the phosphorylation of ULK1 at the activation cite at ser555 decreased. This would suggest inhibition of autophagy initiation but the suggestion is not supported by unchanged content of Beclin-1. Autophagy markers were unaltered by RE and RT in older individuals suggesting that aging may blunt the resistance training induced increase in autophagy. Several UPR markers, typically induced by protein misfolding in endoplasmic reticulum, were increased at 48 hours after RE bout in untrained individuals but were unaltered after the 21-week RT period regardless of age. The arrows in the boxes depict changes in UPR and autophagy markers; ↓=relative decrease ↑=relative increase ↔ no change. Markers that were significantly changed are highlighted as bolded.

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Supplementary tables and figures

Table 1. Coefficient of variation (CV %) and intraclass correlation values of the results between muscle biopsies from different legs in the Experiment 3.

Supplementary Figure 1. ER-resident chaperones before, 1 hour, and 48 hours after an unaccustomed resistance exercise bout (PRE RT) as well as after 21-wk RT period (RT) and in non-exercised controls (No RE) in young men (Experiment 1). A) Calnexin protein content and B) PDI protein content. Open bars depict means. Circles and triangles depict individual values. n = 11–12 in RT and n = 3–4 in No RE.

Supplementary Figure 2. Indicators of UPR related apoptosis before, 1 hour, and 48 hours after an unaccustomed resistance exercise bout (PRE RT) as well as after 21-wk RT period (RT) and in non-exercised controls (No RE) in young men (Experiment 1). A) CHOP mRNA, B) p-JNK46 protein, C) JNK46 protein, D) p-JNK54 E) JNK54 protein. The symbol * depicts the statistical significance from PRE state, $P < 0.05$. Open bars depict means. Circles and triangles depict individual values. In p-JNK/JNK n = 7–9 in RT and n = 2–4 in No RE. In CHOP n = 8–11 in RT. There were No RE cDNA samples left for Chop analysis.

Supplementary Figure 3. mRNA level of A) P62 and B) LC3, C) protein content of glutaredoxin, D) HSP27 and αB-crystallin before, 1 hour, and 48 hours after an unaccustomed resistance exercise bout (PRE RT) as well as after 21-wk RT period (RT) and in non-exercised controls (No RE) in young men (Experiment 1). The symbols * and ***

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depict the statistical significance from PRE state, $P < 0.05$, $P < 0.001$, respectively. Open bars depict means. Circles and triangles depict individual values. In LC3 and P62 $n = 9–11$ and in glutaredoxin, αB-crystallin and HSP27 $n = 7–12$ in RT and $n = 2–4$ in No RE. There were not no No RE cDNA samples left for Lc3b and P62 analysis.

Supplementary Figure 4. Protein content of A) p-ACC and B) Cyt C, b before, 1 hour, and 48 hours after an unaccustomed resistance exercise bout (PRE RT) as well as after 21-wk RT period (RT) and in non-exercised controls (No RE) in young men (Experiment 1). The symbol * depicts the statistical significance from PRE state, $P < 0.05$. Open bars depict means. Circles and triangles depict individual values. $n = 10–12$ in RT and $n = 3–4$ in No RE.

Supplementary Figure 5. Protein content of A) PGC1α1, B) Cytochrome C and C) HSP10 before and 48 hours after an unaccustomed resistance exercise bout (PRE-RT) and before and 48 hours after RE bout after 21-wk RT period (POST-RT) in older men (Experiment 2). The symbol * depicts the statistical significance from PRE state, $P < 0.05$. Open bars depict means. Circles and triangles depict individual values. In all of the variables $n = 6–8$.

Supplementary Figure 6. A) PDI protein, B) p-ULK1^{ser757}, C) Beclin-1 and D HSP10 protein content before, 100 and 150 minutes after resistance exercise bout in recreationally resistance trained individuals (Experiment 3). The symbol * depicts the statistical significance ($P < 0.05$) from PRE state. $n = 13–15$.

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Supplementary Figure 7. Correlations in Experiment 1 between: A) the relative change in UPR indicators (at post 48 h RE) and relative change in muscle strength as a result of 21-week resistance training period B) the relative change in UPR indicators (at post 48 h RE) and relative change in vastus lateralis (VL) muscle size as a result of 21 week resistance training period C) the relative change in LC3II content (at post 48 h RE) and relative change in muscle strength as a result of 21-week resistance training period D) the relative change in LC3II content (at post 48 h RE) and relative change in vastus lateralis (VL) muscle size as a results of 21 week resistance training period E) the relative change in LC3II content (at post 21 wk RT) and relative change in muscle strength as a result of 21 week resistance training period F) the relative change in LC3II content (at post 21 wk RT) and relative change in vastus lateralis (VL) muscle size as a results of 21 week resistance training period G) relative change from baseline in 1 RM leg press at 48 h post RE and the relative change in UPR indicators (at post 48 h RE) H) relative change from baseline in 1 RM leg press at 48 h post RE and the relative change in LC3II content (at post 48 h RE). In all variables, PRE = 1.

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Table. 1

Protein	CV (%)	Intraclass correlation (average measures)
PERK	24	R = 0.91, *P* < 0.001
IRE1α	29	R = 0.81, *P* < 0.01
PDI	18	R = 0.65, *P* < 0.05
p-eIF2α	23	R = 0.55, *P* = 0.08
eIF2α	10	R = 0.72, *P* < 0.05
HSP10	27	R=0.805, *P*<0.01
LC3I	17	R = 0.78, *P* < 0.01
LC3II	20	R = 0.84, *P* < 0.01
p-ULK1ser757	23	R = 0.81, *P* < 0.01
p-JNK54	22	R = 0.85, *P* < 0.01
p-JNK46	38	R=0.11, *P* = 0.42
JNK54	13	R = 0.68, *P* < 0.05
JNK46	22	R = 0.57, *P* = 0.07
Beclin-1	15	R = 0.85, *P* < 0.01

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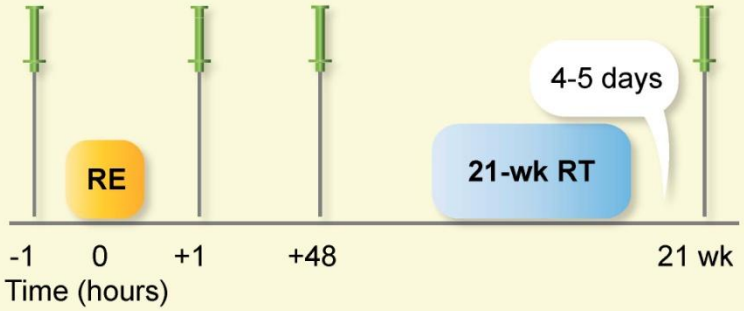
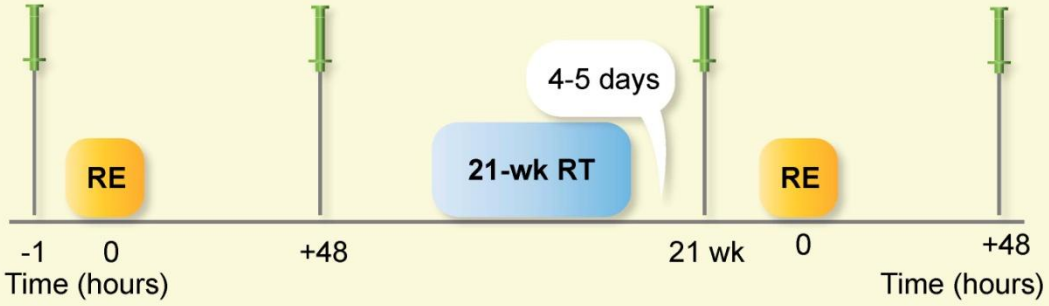
Young men (Experiment 1)



Older men (Experiment 2)



(Experiment 3)

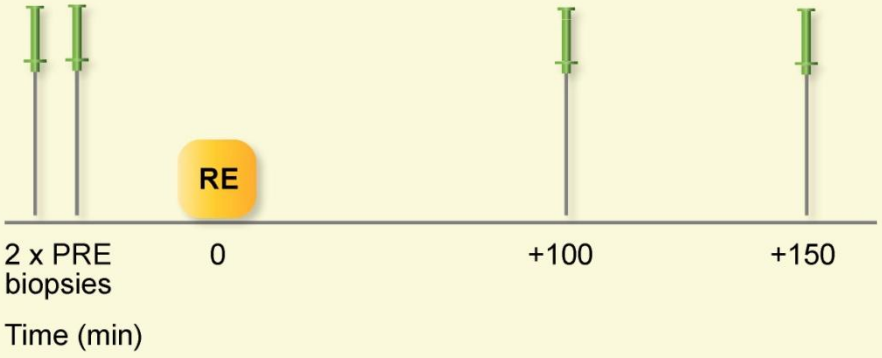


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Figure 2

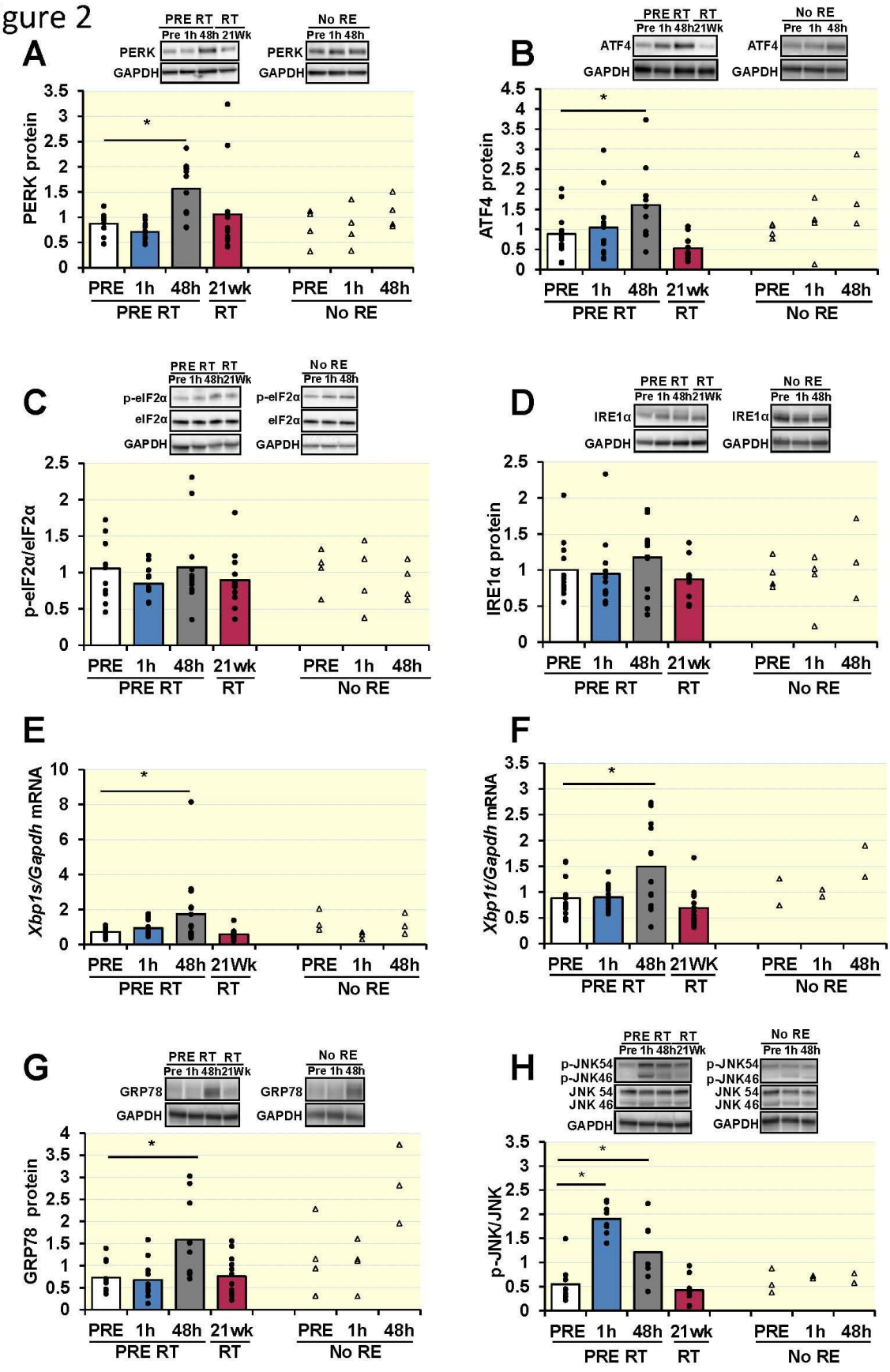

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Figure 3

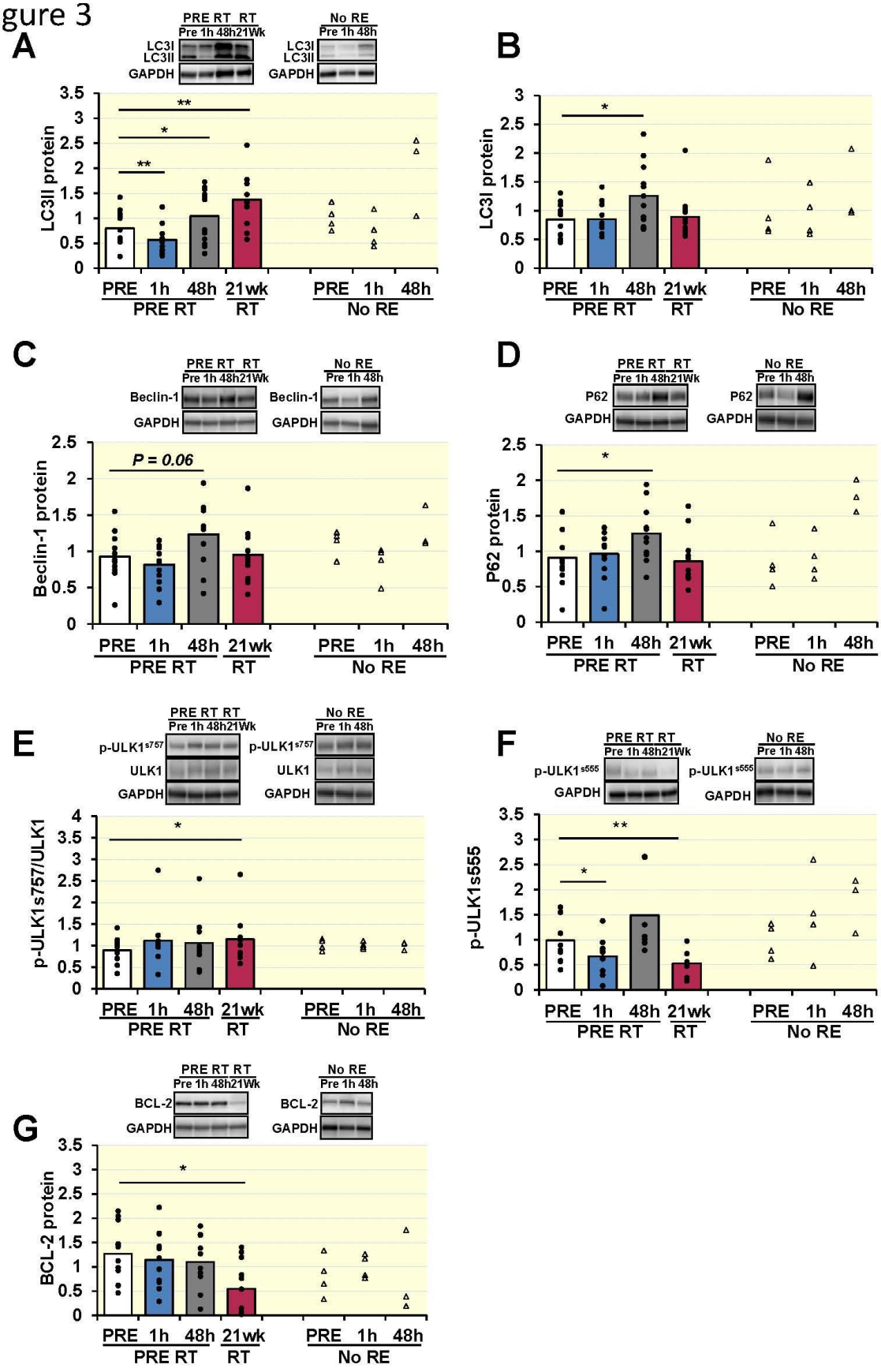

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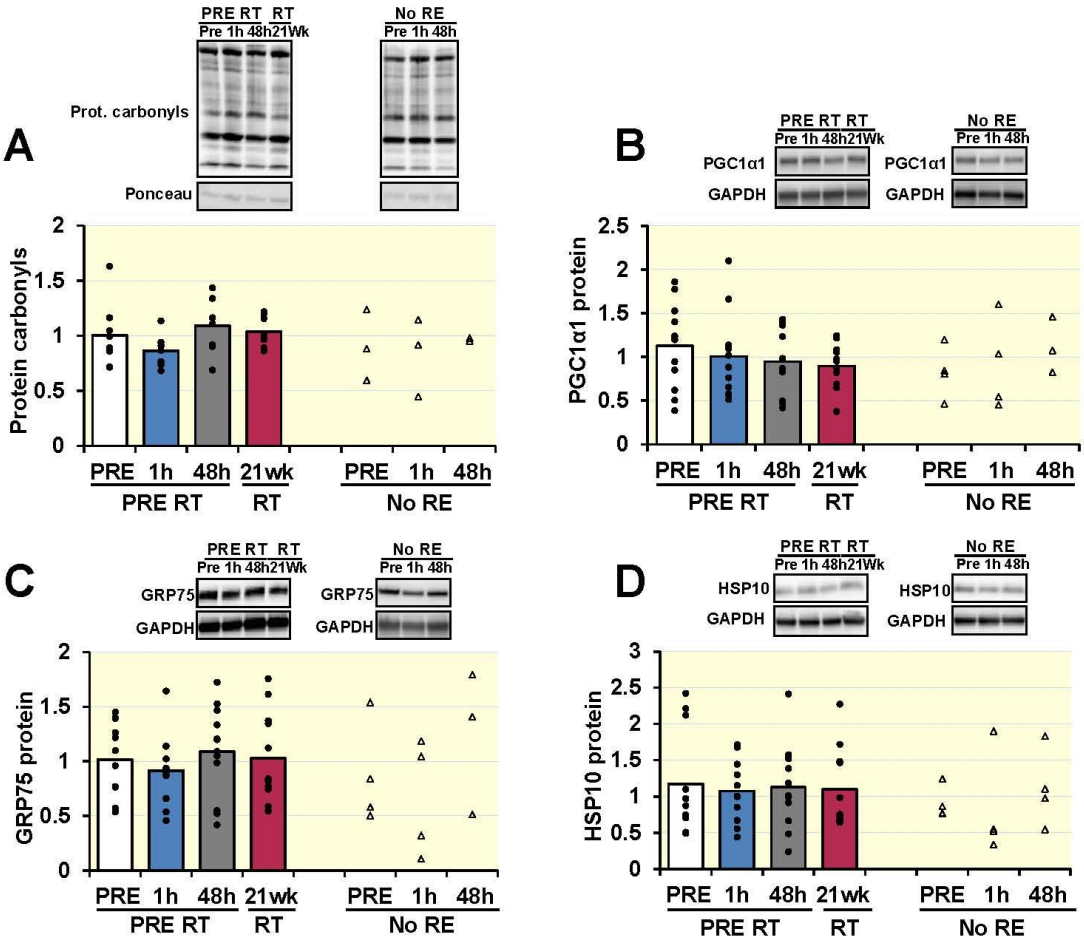
Figure 4



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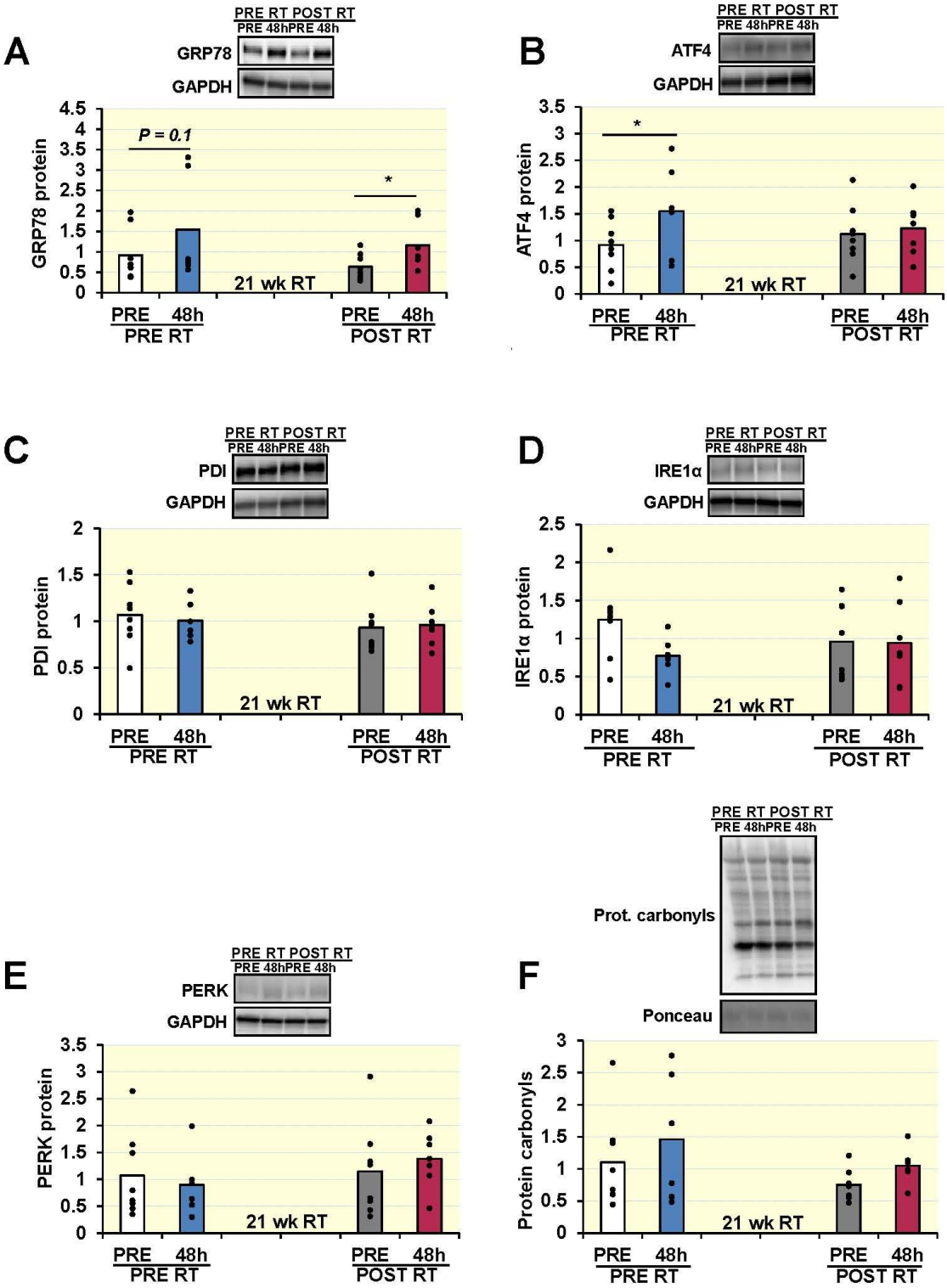

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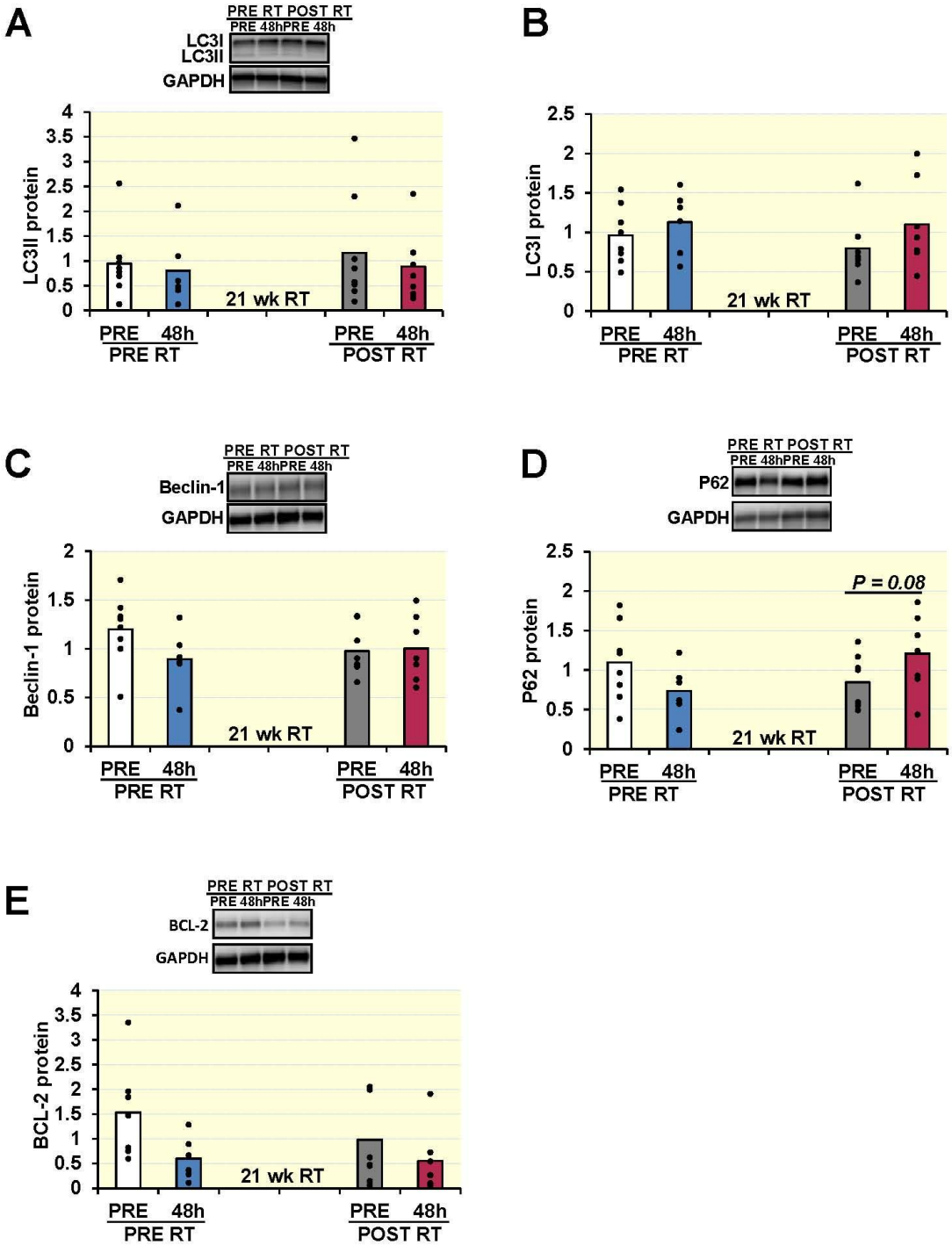
Figure 6



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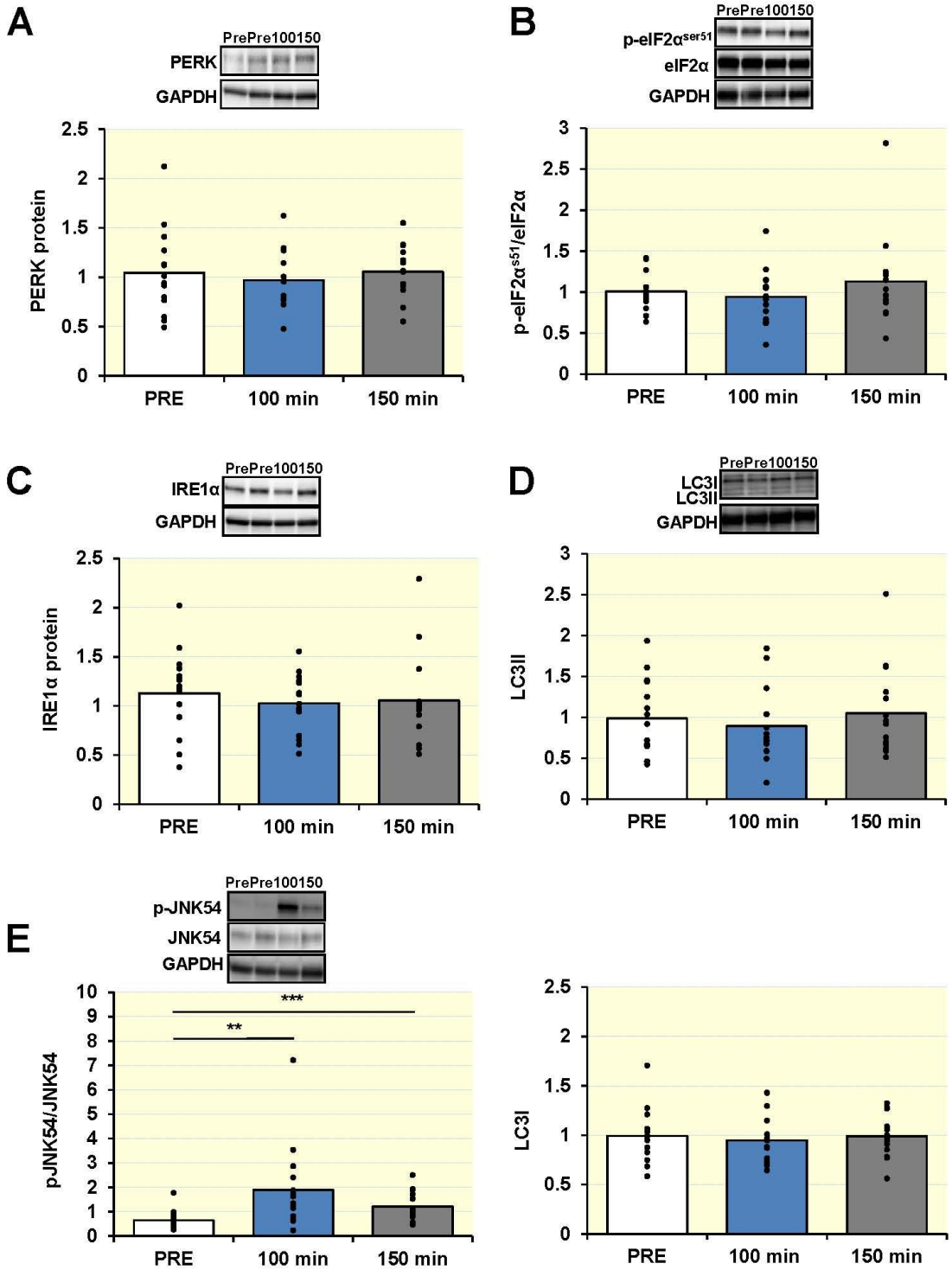

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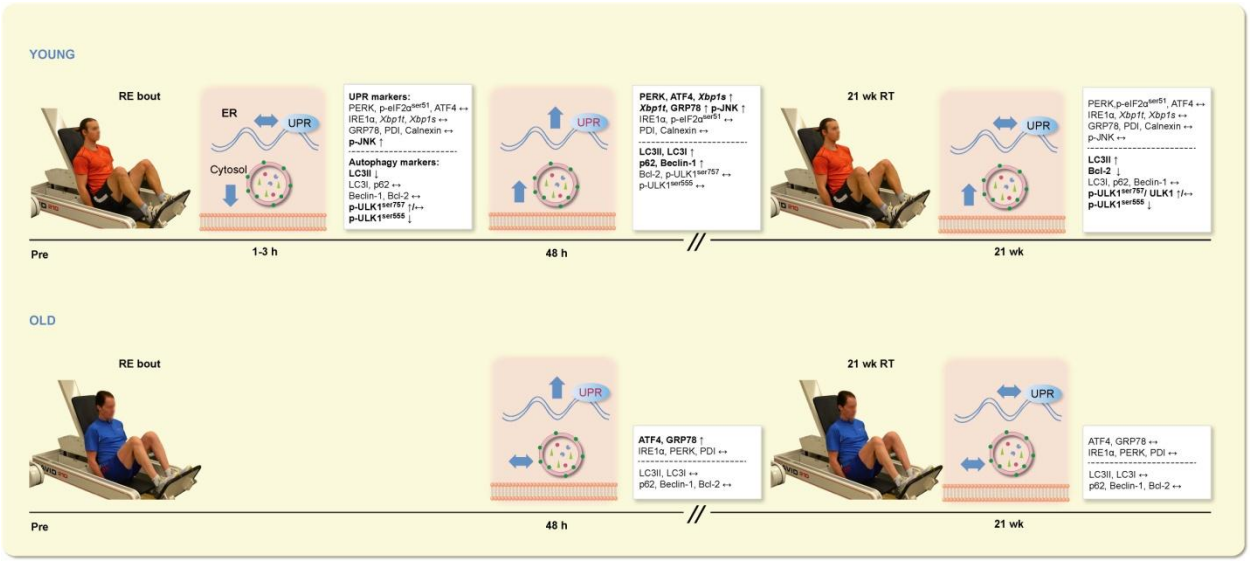

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