Effects of acute and chronic strength training on skeletal muscle autophagy in frail elderly men and women

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ABSTRACT

Aging is associated with alterations in skeletal muscle autophagy, potentially affecting both muscle mass and quality in a negative manner. Strength training with protein supplementation has been reported to improve both muscle mass and quality in frail elderly individuals, but whether improvements are accompanied by alterations in protein quality control is not known. To address this issue, we investigated protein degradation markers in skeletal muscle biopsies (*m. vastus lateralis*) from twenty-four frail elderly men and women (86 ± 7 yr) after acute and chronic (10 weeks) strength training with protein supplementation (ST+PRO) or protein supplementation alone (PRO). Acute increases in mRNA expression of genes related to the ubiquitin proteasome system (MuRF-1, MUSA1), autophagy (ATG7, LC3, p62), and mitochondrial fission (DRP1) were observed after the first, but not after the last training session in ST+PRO. Acute changes in gene expression were accompanied by changes in protein levels of both LC3-I and LC3-II. Hence, the acute training-induced activation of proteasomal degradation and autophagy seems to depend on training status, with activation in the untrained, but not trained state. The ten-week training intervention did not affect basal levels of autophagy mRNAs and proteins, and neither markers of the ubiquitin-proteasome system. This suggests that a relatively short period of strength training may not be sufficient to increase the basal rate of protein degradation in frail elderly.

Keywords: Resistance exercise, aging, frailty, anabolic resistance, protein breakdown

1. INTRODUCTION

Skeletal muscle of older individuals display increased levels of heat shock proteins [58], mitochondrial protein carbonylation [5], intramyocellular lipids [27] and lipofuscin aggregation [41], indicating deficits in protein quality control. It is possible that these changes in part reflect disturbances in skeletal muscle autophagy, and may altogether contribute to the age-related reduction in muscle mass and quality [2,12]. In contrast, strength training and protein supplementation has been shown to improve both muscle mass and quality, even in very old and frail individuals [19,11]. However, because muscle biopsies are rarely obtained in frail elderly, we do not know if training-induced improvements in muscle function are accompanied by improved protein quality control, for example through an up-regulation of autophagic activity. Elucidation of how exercise and protein intake affect autophagy and other mechanisms of protein quality control may aid in the development of individually tailored training programs, and perhaps also in the development of medicinal products aiming to attenuate or offset the functional decline. Hence, clinical trials should combine functional outcomes with investigation of adaptation at the cellular level.

Protein degradation in skeletal muscle is mainly controlled by the ubiquitin-proteasome pathway (UPP) and the autophagosome-lysosome system. UPP is largely responsible for the degradation of myofibrillar proteins [48], and depend on the activity of ubiquitin ligases such as muscle ring finger-1 (MuRF-1) [6], atrogin-1 (Fbox32/MAFbx) [26] and MUSA1 (Fbox30) [46]. These enzymes mark proteins with ubiquitin molecules, targeting them for degradation by the 26S proteasome. Studies have consistently reported increased gene expression of MuRF-1 the first hours after strength training in both young and healthy elderly [23,45,50]. To our knowledge, the UPP response to strength training has never been investigated in frail elderly individuals. Whether the acute response depends on training status is also unknown.

The autophagosome-lysosome system can degrade intracellular protein aggregates, but also macromolecules and organelles [52]. First, a membrane structure called a phagophore is formed; thereafter, the phagophore expands, a process requiring the conjugation of microtubule-associated protein 1 light chain (LC3-I) to PE (phosphatidylethanolamine) to form its lipidated form, LC3-II. Cargo receptors such as p62 recruit proteins and organelles to the growing autophagosome, which eventually will fuse with lysosomes to form an autolysosome, where the content is degraded [52]. Dysfunctional mitochondria normally undergo fission, and are thereafter removed by mitophagy, a selective form of autophagy [60].

Recent observations of a reduced LC3-II/LC3-I ratio the first hours after strength training suggest acute inhibition of autophagy [23,14]. In contrast, training-induced increases in mRNA levels for both LC3 and p62 indicate autophagy activation [40,14]. Moreover, chronic strength training has been shown to increase several autophagic markers, both in rodents [37] and humans [30,53]. Clearly, the autophagic response to strength training deserves further investigation, and acute responses should be interpreted in light of chronic effects, to better elucidate the role of autophagy in strength training adaptation.

The overall aim of this study was to investigate acute and chronic effects of strength training and protein supplementation on protein quality control in frail elderly individuals, with emphasis on autophagy. Since much uncertainty still exists with regards to the interpretation of several autophagic markers [38], the strength training group was compared to a non-training control group with only the protein supplement, a stimulus known to acutely reduce overall muscle protein breakdown [25]. Moreover, since catabolic signaling is closely linked to the anabolic pathways [47], investigation of certain anabolic proteins were included to assist in the overall interpretation. We hypothesized that 1) the acute upregulation of mRNA related to the ubiquitin-proteasome pathway and autophagy would be more pronounced after resistance exercise and protein supplementation than after protein supplementation alone, and 2) autophagy-related proteins would indicate increased autophagic activity at rest following 10 weeks of strength training with protein supplementation.

2. METHOD AND DESIGN

2.1 Participants and ethical approval

This investigation includes a subset of participants (n = 24) from a previously published study of 34 frail elderly men and women [1]. The study design, purpose and possible risks were explained to each subject before inclusion, and subjects gave their written consent to participate. The study was approved by the Regional Ethics Committee for Medical and Health Research of South-East Norway (2016/895/REK sør-øst C), and performed in accordance with the Declaration of Helsinki [57]. The study was registered at clinicaltrials.gov as NCT03326648.

2.2 Inclusion criteria

Subjects fulfilling three of the five Fried Frailty Criteria (FFC) were included [22]. In addition, subjects fulfilling two FFC criteria among "slowness", "weakness", "low activity level" were included. Furthermore, individuals with a score of ≤ 6/12 on the Short Physical

Performance Battery (SPPB) were included, regardless of categorization based on the FFC. The SPPB consists of timed standing balance, gait speed, and timed chair-rise assessment [28].

2.3 Study design

In the original study [1], nineteen subjects were allocated to ten weeks of strength training and protein supplementation (ST+PRO), whereas fifteen subjects constituted a non-training control group, only receiving protein supplementation (PRO). An acute experiment (described later) was conducted in twenty-four of these subjects, both before and after the ten-week intervention. Only results for these individuals are included in this article. A simplified flowchart is presented in **Figure 1**, and subject characteristics are presented in **Table 1.** For a full account of all subjects included in the original clinical trial, see the previous publication [1].

2.4 Training and supplementation

The strength training protocol performed in ST+PRO was a supervised progressive program, performed two times per week for 10 weeks. All training sessions were supervised by experienced training instructors (5^{th} year students in exercise physiology). The training program is presented in **Table 2**. In short, the program included leg press and knee extension, and participants performed 3 sets of 12 repetitions at submaximal loads the first week, and from week 2-10 subjects gradually progressed from 3 sets of 12 repetitions maximum (RM) to 4 sets of 6RM. Repetition maximum (RM) refers to the highest load that can be lifted for a defined number of repetitions. As an example, 6RM refers to a load that can be lifted maximally six times. Hence, from week 2-10 all training sets were performed until failure. This way of adjusting training loads represents an alternative to the most common way, using % of 1RM. Subjects were instructed to use approximately one second during the concentric phase, and two seconds on the eccentric phase. Each set was followed by 2-min rest periods. Strength training sessions were preceded by a 3-min lower extremity warm-up exercise, using a step platform.

Participants in both groups received one dietary supplement each day throughout the intervention period, containing 17 g of milk protein, 18 g of carbohydrate, and 1 g fat (149 kcal [627 kJ]). The supplement was consumed in the evening, except on training days, when the supplement was consumed within two hours after training. Participants were encouraged to continue their regular food routines in addition to the supplements.

2.5 Acute experiment

An acute experiment was conducted before and after the 10-week intervention period (**Figure 2**), to investigate the acute response to strength training followed by protein intake (ST+PRO) in the untrained and trained state. During the two weeks prior to the acute experiment, participants met twice in the lab to establish their 8RM in knee extension, and to perform familiarization to the standardized workout and the maximal voluntary contraction (MVC) test. At least three days of rest were given between the second familiarization session and the acute experiment. The PRO group also performed the same acute experiment before and after the ten-week intervention, except that the strength training session was replaced by a period of rest.

Subjects refrained from strenuous physical activity for three days before the acute experiment, and arrived at the laboratory by car or public transportation after an overnight fast. Participants received a standardized breakfast containing oatmeal, water, sugar and butter (20 kJ and 0.09 g protein per kg body mass). One hour after the breakfast, a percutaneous needle biopsy was obtained from the vastus lateralis muscle (explained in detail later). Thirty minutes later, both groups performed a 5-min warm-up on an exercise bike, followed by an assessment of maximal voluntary contraction (MVC) for the knee extensors. The MVC-test was followed by a leg strength training session in ST+PRO, and a rest period in PRO.

The exercise session in ST+PRO was designed to elicit a substantial stimulus for the vastus lateralis muscle, from which the muscle biopsies were obtained. The session began with 10 warm-up repetitions in knee extension at 65% of a load corresponding to 8 repetitions maximum (RM). Subsequently, subjects conducted 6 sets of 8RM in knee extension, with a new set starting every 3rd min. 8RM refers to a load that can be lifted maximally eight times. Hence, every training set was conducted until failure. For most subjects the load had to be reduced gradually from set 1 to set 6 to enable eight repetitions (due to fatigue). The exercise protocol had a duration of 20 min. Both groups received the protein supplement one hour after the baseline muscle biopsy. For ST+PRO, this coincided with the moment they had completed the training session. A second MVC-test was performed in both groups 10 minutes later. 150 minutes after consuming the supplement, a second muscle biopsy was obtained from vastus lateralis.

2.6 Maximal voluntary contraction (MVC)

Unilateral maximal knee extension strength was assessed to obtain information about the extent of fatigue elicited by the exercise session. The test was performed in a custom-made knee-extension apparatus (Gym2000, Geithus, Norway). Participants were seated in a chair with a belt fixing the hips, with 90° in the hip and knee joints. Three attempts of 3-4 seconds per leg with 1 min rest between were given to reach MVC force. Force was measured with a force transducer (HMB U2 AC2, Darmstadt, Germany). To avoid potentially erroneous results due to biopsy-related pain, MVC data from the non-biopsied leg were used in the analysis.

2.7 Biopsy protocol

The biopsy procedure was conducted under local anesthesia (Xylocaine with adrenaline, 10 mg/ml lidocaine + 5 µg/ml adrenaline, AstraZeneca, London, UK), and approximately 200 mg (2–3 × 50–150 mg) of muscle tissue was obtained using a modified Bergström technique with suction. The two biopsies obtained during the acute experiment were obtained from the same incision, but in opposite directions. Tissue intended for immunoblot analyses was quickly rinsed in physiological saline, before fat, connective tissue and blood were removed and discarded. Subsequently, the sample was weighed and quickly frozen in isopentane cooled on dry ice (immunoblot analyses on cellular sub-fractions), or liquid nitrogen (immunoblot analyses of protein phosphorylation), before being transferred and stored at −80°C for later analyses. Samples intended for gene expression analyses were immersed into RNAlater® solution (Ambion, TX, USA), stored for 24-48 hours at 4 °C, and thereafter stored at −20 °C for later treatment and analysis.

2.8 Analyses

2.8.1 Pre-analytical analyses

Approximately 50 mg of muscle tissue was homogenized in 1 ml T-PER (Tissue Protein Extraction Reagent, Thermo Scientific, Rockford, IL, USA), 20 µl Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific), and 20 µl EDTA (Thermo Scientific). This homogenate was used in the analysis of anabolic proteins and ubiquitin. Another 50 mg of muscle sample was homogenized and fractionated into cytosolic, membrane, cytoskeletal, and nuclear fractions using a commercial fractionation kit (ProteoExtract Subcellular Proteome Extraction Kit, Cat#539790, Calbiochem, EMD Biosciences, Germany) according to the manufacturer's procedures. Analyses of autophagic proteins were performed using the cytosolic and membrane fractions. Protein concentration was measured in triplicates with a

commercial kit (BioRad DC protein microplate assay, Cat#0113, Cat#0114, Cat#0115, Bio-Rad Laboratories, Hercules, CA, USA), a filter photometer (Expert 96, ASYS Hitech, UK) and the software provided (Kim, ver. 5.45.0.1, Daniel Kittrich, Prague, Czech Republic).

2.8.2 Western blot

Equal aliquots of protein were loaded within a gel (5-30 µg) and separated by 4-20% gradient Mini-PROTEAN TGX Stain-Free Precast protein gels (4568093, Bio-Rad Laboratories). Electrophoresis was performed under denaturized conditions for 30-35 min at 200 volts in Tris/Glycine/SDS running buffer (161-0732, Bio-Rad Laboratories). After gel-electrophoresis, proteins were transferred onto a PVDF-membrane at 100 volts for 60 min (CriterionTM Blotter; Tris/Glycine buffer 161-0734, Bio-Rad Laboratories). Membranes were blocked at room temperature for 2 hours in a TBS solution with 5% fat-free skimmed milk and 0.1% Tween 20 (TBS, 170-6435, Bio-Rad Laboratories; Tween-20, 437082Q, VWR International, Radnor, PA, USA; Skim milk, 1.15363.0500, Merck, Darmstadt, Germany). Blocked membranes were incubated overnight at 4°C with primary antibodies (see below) followed by incubation with appropriate secondary antibodies for 1 h at room temperature (see below). Between stages, membranes were washed in 0.1% TBS-t. Protein stripping was conducted for phospho-proteins using Restore Western Blot Stripping Buffer (Pierce Biotechnology, Rockford, IL, USA). All samples were analyzed in duplicates, and bands were visualized using an HRP-detection system (Super Signal West Dura Extended Duration Substrate, 34076, Thermo Fisher Scientific). Chemiluminescence was measured using a ChemiDoc MP System (Bio-Rad Laboratories), and band intensities were measured with Image Lab (Bio-Rad Laboratories). All intensities were normalized to a control sample that was applied in each gel (in duplicates), to allow comparisons between gels. If difference between duplicates exceeded 20%, the analysis was performed over again.

Primary and secondary antibodies were diluted in a 1% fat-free skimmed milk and 0.1% TBS-t solution. Antibodies against p70S6K (2708), phospho-p70S6K Thr389 (9234), eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) (9644), phospho-4EBP1 Thr37/46 (2855), eukaryotic elongation factor 2 (eEF2) (2332), phospho-eEF2 Thr56 (2331), LC3-I/II (2775) and ubiquitin (3933) were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibody against p62 (ab56416) was obtained from Abcam (Cambridge, UK). Secondary antibodies were purchased from Cell Signaling Technology (goat anti-rabbit IgG, 7074) and Thermo Scientific (goat anti-mouse, 31430). Representative western blots are presented in **Figure 3** and **Figure 4**.

2.8.3 RNA extraction and RT-PCR

Total RNA was extracted from biopsies dedicated to gene expression analysis by mean of Trizol reagent (Invitrogen, Carlsbad, CA, USA). Briefly, each muscle sample was rinsed from RNAlater® solution in cold sterile PBS and immediately homogenized with Trizol by means of a metal bead and Tissue Lyser apparatus (Qiagen, Venlo, Netherlands). Subsequent RNA extraction was carried out following Trizol manifacturer manual. Total RNA was resuspended in RNAse/DNAse free water (GIBCO) and quantified using RNA BR assay test for Qubit fluorometer (Invitrogen). 400 ng of total RNA were retrotranscribed using SuperScript IV kit (Invitrogen) following the manufacturer's guide. cDNA was finally resuspended in 50 µl with RNAse/DNAse free water and 1µl of diluted cDNA was used as a template for RT-qPCR analysis. qPCR analysis and Ct detection were carried out with QuantStudio5 machine (Applied Biosystems, CA, USA) using PoweUP SYBR green technology (Applied Biosystems). mRNA expression level of the following genes was detected: actin, MuRF-1, Atrogin1, MUSA1, ATG7, LC3, p62, BNIP3, BNIP3L, OPA1 and DRP1. Expression level is presented as fold change normalized to actin expression. Primer sequences are listed in **Table 3**.

2.9 Statistics

Statistical analyses were performed using GraphPad Prism6 software (GraphPad Software, Inc., La Jolla, CA). The limit for statistical significance was set at $P = 0.05$. Gene expression data were log-transformed prior to statistical analyses. All other non-normally distributed data (D'Agostino and Pearson omnibus normality test) were also log-transformed prior to statistical analyses. Between-group differences at baseline were investigated with unpaired Student's t-tests. A two-way ANOVA with Bonferroni's multiple comparison test was used to evaluate the effect of time and group (absolute values). In addition, paired t-tests were performed to investigate changes from baseline within the groups. Associations between variables were investigated using Pearson's correlation analysis. All data are presented as mean ± standard deviation.

3. RESULTS

Due to several drop-outs in ST+PRO, the subjects initially allocated to PRO were offered to perform 10 weeks of training after the initial intervention, and thereafter perform an acute experiment in the trained state. Two subjects did so, and these two subjects are therefore included in both groups for the acute experiment after the intervention period. In addition, for

some biopsies we were not able to obtain enough muscle tissue for all analyses. Hence, the number of subjects included for each variable differs and is represented by the number of lines in the presented graphs.

3.1 Subjects

The two groups were similar in terms of age, lean mass and functional capacity, but subjects in PRO had a higher body fat percentage and tended to have a higher body mass than the subjects in ST+PRO (**Table 1**).

3.2 Muscle hypertrophy and muscle strength

Changes in muscle size and strength following the 10-week training intervention have been described in detail previously [1]. In short, ST+PRO increased quadriceps femoris muscle cross-sectional area by 7%, knee extension MVC by 7% and knee extension 1RM by 17%. No changes were observed in PRO.

3.3 Training volume and fatigue during acute experiment

In ST+PRO, the training load (8RM) utilized during the acute experiment after the ten-week intervention was higher (37.2 ± 9.7 kg) than before the ten-week intervention period (32.6 ± 9.5 kg; $P = 0.002$). Thus, total weight lifted was also higher after the intervention (1733 ± 452 kg vs. 1486 ± 448 kg; $P = 0.002$). A reduced force-generating capacity measured as MVC force was evident in the training group 10 minutes after the training session both at the first (9 ± 10% reduction) and last acute experiment (11 ± 8% reduction).

3.4 Acute changes before the intervention period

Gene expression (**Table 4**). No group x time interactions were observed for any of the genes investigated. However, in ST+PRO, increased expression from baseline was observed for LC3 ($P = 0.002$), p62 ($P = 0.005$), MuRF-1 ($P = 0.037$), ATG7 ($P = 0.05$), MUSA1 ($P = 0.031$), and DRP1 ($P = 0.017$). No significant changes were observed in PRO.

Autophagic proteins. No group x time interactions were observed for any of the proteins measured, but the cytosolic level of LC3-I increased by 31% from baseline in ST+PRO ($P = 0.005$) (**Figure 5A**). Moreover, LC3-II in the membrane fractions was reduced to 33% of baseline values, both in ST+PRO ($P < 0.001$) and PRO ($P = 0.008$) (**Figure 5C**). Hence, also the LC3-II/LC3-I ratio was reduced from baseline in both groups ($P < 0.05$). No changes were observed for cytosolic p62 (**Figure 5E**).

Ubiquitination. The level of ubiquitinated proteins was reduced from baseline by 11% in ST+PRO ($P < 0.01$) (**Figure 6A**), but no group x time interaction was observed. The level of free (unbound) ubiquitin did not change in ST+PRO or PRO (**Figure 6C**).

Anabolic proteins. No group x time interactions were observed for p70S6K, 4E-BP1 or eEF2. However, phosphorylation ratio of p70S6K increased from baseline in ST+PRO ($P < 0.001$), and tended to increase from baseline in PRO ($P = 0.08$) (**Figure 7A**). Moreover, a 49% increase in the phosphorylation ratio of 4E-BP1 was observed in ST+PRO, but the change did not reach statistical significance ($P = 0.054$) (**Figure 7C**). Phosphorylation of eEF2 did not change in ST+PRO or PRO (**Figure 7E**). In ST+PRO, a correlation was observed between the change in p70S6K phosphorylation and the change in LC3-I ($r = 0.56$, $P = 0.037$). In addition, a tendency to an inverse correlation was observed between the change in p70S6K phosphorylation and change in LC3-II ($r = -0.48$, $P = 0.08$).

3.5 Acute changes after the intervention period

Gene expression. No group x time interactions were observed for any of the genes investigated (**Table 4**). However, Atrogin-1 expression decreased from baseline both in ST+PRO ($P < 0.001$) and PRO ($P = 0.019$).

Autophagic proteins. No group x time interactions were observed for cytosolic LC3-I (**Figure 5B**) or LC3-II (**Figure 5D**). However, LC3-II in the membrane fraction was reduced to 39 and 31% of baseline values, in ST+PRO ($P = 0.032$) and PRO ($P = 0.002$), respectively. Thus, the LC3-II/LC3-I ratio was also reduced in both groups ($P < 0.01$). No changes were observed for cytosolic p62 (**Figure 5F**).

Ubiquitination. No group x time interactions were observed for the level of ubiquitinated proteins (**Figure 6B**) or the level of free/unbound ubiquitin (**Figure 6D**).

Anabolic proteins. No group x time interactions were observed for p70S6K, 4E-BP1 or eEF2. Phosphorylation of p70S6K increased from baseline in ST+PRO ($P = 0.023$), and tended to increase from baseline in PRO ($P = 0.10$) (**Figure 7B)**. The phosphorylation ratio of 4E-BP1 increased by 38% from baseline in ST+PRO ($P = 0.011$), whereas a 31% non-significant increase was observed in PRO ($P = 0.07$) (**Figure 7D)**. Phosphorylation of eEF2 did not change in ST+PRO or PRO (**Figure 7F**).

3.6 Intervention effects on gene and protein expression at rest

Basal gene expression did not change in ST+PRO or PRO following the ten-week intervention (results not shown). No changes were observed for the anabolic proteins or autophagic proteins investigated (results not shown). A group x time interaction was observed for ubiquitination ($P = 0.03$), due to a small non-significant decrease in ST+PRO (-4%), and a non-significant increase in PRO (44%). Training-induced gains in muscle mass and strength did not correlate with changes in any of the genes or proteins investigated. Results from all biopsy analyses are summarized in **Table 5**.

4. DISCUSSION

The present study investigated acute and chronic effects of strength training with protein supplementation (ST+PRO), compared with protein supplementation alone (PRO), on markers of protein degradation in skeletal muscle of frail elderly individuals. The main finding was that a 20 min session of heavy load strength training performed in the untrained state increased expression of genes related to autophagy, mitochondrial fission and the ubiquitin-proteasome pathway. Additionally, protein levels of LC3-I were increased. In contrast, gene expression and levels of LC3-I did not change following the same training session performed in the trained state, indicating that the activation of these systems greatly depend on training status. Ten weeks of heavy-load strength training had minimal effects on basal gene and protein expression in the two groups.

4.1 Autophagy

In response to the first training session, increased gene expression of ATG7, LC3 and p62 was observed in ST+PRO. Ogborn and colleagues (2015) observed increased gene expression of LC3 and p62, but not ATG7, 3 hours after a strength training session in untrained young and old individuals [40]. The increase in gene expression was accompanied by increased protein levels of both LC3-I and p62 48 hours later [40]. In contrast, Fry and colleagues [23] did not observe changes in either LC3 gene expression or LC3-I protein expression 3, 6 and 24 hours after strength training in physically active young and elderly subjects. In the present investigation, the increases in gene expression were accompanied by increased cytosolic LC3-I 2.5 hours after the training session, potentially reflecting increased *de novo* synthesis. Indeed, the reduction in LC3-II at this time point could indicate increased autophagic flux, with enhanced lysosomal degradation of autophagosomes. In such a situation, increased synthesis of LC3 would be an expected response in order to replenish the cytosolic pool of

LC3-I. An increase in lysosomal degradation is also supported by the observation of unchanged p62 protein levels, despite an increase in p62 gene expression.

It should also be acknowledged that part of our results points toward suppressed autophagy 2.5 hours after strength training and protein supplementation. For example, a positive correlation was observed between the change in p70S6K-phosphorylation and the change in cytosolic LC3-I. Hence, it may be that enhanced mTORC1 activity suppressed lipidation of LC3-I to LC3-II, and that this was the reason for the increased LC3-I levels in ST+PRO. Still, if this was the case one would perhaps expect increased p62 protein levels, considering the increase in p62 gene expression at this time point. Instead, a non-significant 6% reduction was observed in p62 protein levels.

In summary, the gene expression data strongly suggest a training-induced activation of autophagy. However, whether or not autophagy was upregulated 2.5 hours after strength training and protein supplementation (when the post exercise biopsy was obtained) is uncertain. It could be that autophagic activity was enhanced *during* the training session, but that the autophagic flux was suppressed 2.5 hours later, due to a potent stimulation of the mTORC1 pathway. It is also possible that the increases in gene expression of ATG7, LC3 and p62 may have caused a later increase in autophagic proteins, as observed previously (Ogborn et al., 2015). It is important to acknowledge that static measurements of autophagy gene and protein levels does not give a clear picture of the autophagy flux. In mice and cell cultures, autophagy inhibitors are utilized to assess the autophagy flux, but this is not possible in human subjects. Hence, we must emphasize that the results from the present investigation should be interpreted with caution.

The ten-week training intervention did not change basal gene expression of the autophagy-related genes, and neither protein levels of LC3-I, LC3-II or p62. In a previous study, increased protein levels of LC3-II were observed in young, but not middle aged/older men (61 ± 6 yr.) after 21 weeks of strength training [30]. Collectively, these findings may indicate a suppressed potential of strength training to upregulate basal autophagy in older individuals. Further research should establish if perhaps a greater training frequency (>2 sessions per week) is needed to increase basal levels of autophagic markers in this population.

4.2 Mitophagy

BNIP3L mRNA tended to increase following the first training session in ST+PRO (P = 0.058), accompanied by a significant increase in DRP1 mRNA. BNIP3L mediates mitophagy

[61], whereas DRP1 regulates mitochondrial fission [21]. Evidence suggests that mitophagy is attenuated with aging [42,35], and it has been shown that autophagy inhibition leads to increased oxidative stress and mitochondrial dysfunction in mice [12,24]. Hence, it has been hypothesized that defects in mitophagy contribute to the development of sarcopenia [3]. Interestingly, alterations in mitochondrial dynamics may be involved in the age-related reduction of mitochondrial function, based on observations of increased abundance of small, fragmented mitochondria [33], as well as very large mitochondria [36] in muscles from old rodents. Indeed, both the mRNA and protein levels of important fusion and fission proteins have been reported to be lower with aging in both rodents [32] and humans [34,54]. Moreover, lower DRP1 expression was observed in frail, compared with non-frail elderly women [17], indicating that mitochondrial dynamics may be more affected in individuals with low functional capacity. In contrast, the results in the present study suggest that unaccustomed resistance exercise might activate mitophagy in frail elderly, and that increased mitochondrial fission might be involved. No changes were observed in response to the last training session, but whether this was due to increased basal levels of mitophagy proteins is not known. Hence, the effect of strength training on mitophagy and mitochondrial dynamics warrants further investigation.

4.3 Ubiquitin-proteasome pathway

No group x time interactions were observed for the UPP genes investigated, and the changes from baseline within the groups should therefore be interpreted with caution. Nevertheless, the 2.9-fold increase in MuRF1 expression 2.5 hours after the first training session is in line with previous reports [50,23,45,14]. Moreover, increased expression from baseline was observed for the ubiquitin ligase MUSA1. The increase in expression of these two genes were accompanied by a reduction in the level of ubiquitinated proteins, perhaps reflecting an increased degradation of ubiquitinated proteins by the 26S proteasome. It should neither be dismissed that increased autophagy may have contributed to the reduction in ubiquitinated proteins. Collectively, these results point toward a training-induced increase in proteasomal degradation following unaccustomed strength training in frail elderly subjects.

Expression of atrogin-1 did not change in any of the groups during the acute experiment conducted before the ten-week intervention. Increased expression of atrogin-1 has been observed in some [45,50], but not all studies [23,14]. The less consistent results for atrogin-1 compared to MuRF-1 might be due to the role of atrogin-1 in regulating the half-life of MyoD and eIF3f [7], factors involved in the MPS response. Hence, it is logical that increases in

atrogin-1 are observed more consistently in situations of increased MPB and attenuated MPS, such as the first days of immobilization/disuse [13,51,55] and muscle denervation [8].

No changes were observed for MuRF1, MUSA1 or ubiquitin during the acute experiment conducted after the ten-week intervention, presumably indicating a lower activation of the ubiquitin-proteasome system at this stage of training. This corresponds well with the findings by Stefanetti and colleagues [49], who observed limited UPP-activation following a bout of strength training after a 10-week strength training program in young men. Also in line with our findings, Phillips and colleagues (1999) observed greater increases in fractional breakdown rate following a strength training session in untrained, compared with trained subjects (same relative training load) [44].

The ten-week intervention had minimal effects on basal gene and protein expression of the UPP components investigated. Because we did not measure protein levels of the ubiquitin ligases, we cannot exclude the possibility that increased mRNA expression following the first training sessions led to increased protein levels of these ligases, perhaps resulting in increased UPP activity at rest. For example, Phillips and colleagues [43] observed increased fractional breakdown rate (FBR) at rest following eight weeks of resistance training in young men. We did observe a group x time interaction for ubiquitination in the present study, but this interaction was primarily due to an increase in PRO. Changes in ubiquitination is nevertheless difficult to interpret, since the amount of ubiquitinated proteins at any specific time point will depend both on the activity of ubiquitin ligases and the rate of degradation by the 26S proteasome.

4.4 Signaling related to muscle protein synthesis

The phosphorylation ratio of p70S6K increased from baseline in ST+PRO both after the first and last training session (acute experiment 1 and 2). In addition, the phosphorylation ratio of 4E-BP1 increased by 34 and 37% before and after the intervention, respectively, although only the latter reached statistical significance. Both p70S6K and 4E-BP1 are under mTORC1 control [59], and promote translation initiation when phosphorylated and hyper-phosphorylated, respectively. In addition, p70S6K promote translation elongation, through phosphorylation of eEF2 kinase, resulting in increased activity of eEF2 [56]. The change in phosphorylation of p70S6K and 4E-BP1 has been observed to correlate with the change in fractional synthetic rate after a strength training session [10,9]. Thus, our results suggest that a session of heavy load strength training followed by protein intake upregulates muscle protein

synthesis in frail elderly individuals, and that this response occurs regardless of training status. In the group only ingesting a protein supplement, phosphorylation of p70S6K tended to increase both before and after the ten-week intervention. 4E-BP1 phosphorylation tended to increase during the second acute experiment. These results suggest that ingestion of 17 grams of milk protein also increase muscle protein synthesis in frail elderly, albeit perhaps to a lesser extent than when protein intake was preceded by exercise. Hence, our results correspond well with previous observations in young and non-frail elderly individuals [20]. The signaling data for p70S6K and 4E-BP1 correspond well also with the fact that quadriceps femoris CSA increased by 7% in ST+PRO, with no changes in PRO, as previously reported [1].

Phosphorylation of eEF2 did not change in either group. Results for eEF2 after strength training and protein intake have been ambiguous, with some observations of increased activation [15,16,18], and some with no change [31,29,4,39]. These discrepancies do not appear to be related to the population investigated, timing of biopsies or antibodies utilized. In summary, although no group x time interactions were observed for either p70S6K, 4E-BP1 or eEF2, it seems that skeletal muscle of frail elderly – as for younger individuals - is "sensitized" by a session of heavy-load strength training, increasing the anabolic response to protein ingestion.

4.5 Limitations

Even though this study was initially designed as a randomized controlled trial, we were forced to abandon this design in order to increase statistical power. Furthermore, owing to several dropouts and some analytical challenges, the results for some variables still represent a quite low number of subjects, increasing the risk of statistical type II errors. The low number of subjects also precluded the possibility of separate analyses for men and women. Furthermore, an important difference between the present study and a majority of the previous studies in this field is that participants received a standardized breakfast 1 h prior to the baseline biopsy (during the acute experiment). We therefore cannot exclude the possibility that some of the between-subject variation is at least partly due to differences in digestion, uptake, transport, or cellular signaling responses to the nutrients consumed. This may have introduced more variation to our data, negatively affecting statistical power. The lack of information about the participants' diet, as well as compliance to the supplement also represents a limitation of the study. Another limitation is that the PRO group had a higher body fat percentage and tended to have a higher body mass than the subjects in ST+PRO. This may have had an impact on the basis for group comparisons. We also acknowledge that between-subject variation in fiber

type composition may have introduced variation into our data, considering that this was not accounted for in our analyses.

4.6 Conclusions

This study shed light on acute and chronic effects of strength training with protein supplementation on protein quality control in frail elderly individuals, a population in which muscle biopsies are rarely obtained. Gene expression data indicated activation of both the ubiquitin proteasome system and autophagy after strength training in the untrained state, but such a response was absent after ten weeks of regular exercise. Furthermore, strength training performed twice weekly for ten weeks did not affect basal gene expression or basal levels of the autophagic and anabolic proteins investigated. Further research should investigate if a greater training frequency, or perhaps a stronger metabolic stimulus (15-20 RM), is required to elicit changes in basal autophagy in frail elderly individuals.

FUNDING

The study was funded by the Norwegian School of Sport Sciences.

ACKNOWLEDGEMENTS

The authors gratefully appreciate the contributions of the participants. The authors also thank Kristoffer Toldnes Cumming, Hege Nymo Østgaard and Ingrid Ugelstad for their assistance during collection of muscle biopsies.

CONFLICT OF INTEREST

SNA, DT, SG, MN, AA, MS, HBB and TR declare that they have no conflict of interest regarding the publication of this article.

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TABLES AND TABLE LEGENDS

Table 1. Subject characteristics.

Characteristic	ST+PRO	PRO	*P*-value
N (men/women)	10/8	6/4	-
Age (years)	87 ± 7	84 ± 6	0.18
Body mass (kg)	64.4 ± 13.3	74.7 ± 13.4	0.06
Lean body mass (kg)	43.0 ± 8.0	45.7 ± 9.8	0.44
Lean leg mass (kg)	14.2 ± 3.0	15.6 ± 4.4	0.32
Body fat (%)	30.4 ± 7.1	37.0 ± 6.0	0.02
SPPB score	5.7 ± 2.8	6.5 ± 2.8	0.46

N, Number; SPPB, Short Physical Performance Battery. Data shown as mean ± SD. *P*-values show between-group differences.

Table 2. The ten-week strength training program performed in ST+PRO. RM, Repetition Maximum.

Week	Exercise	Session 1			Session 2		
		Sets	Reps	Load	Sets	Reps	Load
1	Leg press	3	12	<RM	3	12	<RM
	Knee extension	3	12	<RM	3	12	<RM
2-4	Leg press	3	12	RM	3	10	RM
	Knee extension	3	12	RM	3	10	RM
5-7	Leg press	3	10	RM	3	8	RM
	Knee extension	4	10	RM	4	8	RM
8-10	Leg press	3	8	RM	4	6	RM
	Knee extension	4	8	RM	4	6	RM

Table 3. Primer sequences employed for reverse transcription-polymerase chain reaction.

Gene	Forward	Reverse
Actb	GGGAAATCGTGCGTGACA	GGACTCCATGCCCAGGA
Atrogin-1	GCAGCTGAACAACATTCAGATCAC	CAGCCTCTGCATGATGTTCAGT
MuRF1	CTTCCCTTCTGTGGACTCTTCCT	CCTGAGAGGCATTGACTTTGG
MUSA1	GGGGAAAAGGAAGTATCCAGA	GCCATGCTTAGGATGTCAGC
OPA1	GGCTCCTGACACAAAGGAAA	TCCTTCCATGAGGGTCCATT
DRP1	GGCGCTAATTCCTGTCATAA	CAGGCTTTCTAGCACTGAGC
BNIP3	GGGATGCAGGAGGAGAGC	ACTCCGTCCAGACTCATGC
BNIP3L	CATGAATCAGGACAGAGTAGTTCC	CCTTCTCTCCTTCTACAACTTCTTC
ATG7	AGGAGATTCAACCAGAGACGG	TGGTGAGGCACAAGCCCAA
P62	GCTTCCAGGCGCACTACC	CATCCTTCACGTAGGACATGG
LC3	TCGAGAGCAGCATCCAAC	GCTGTGTCCGTTCACCA

Table 4. mRNA analyses of regulators of proteasomal degradation, autophagy/mitophagy, and mitochondrial fusion and fission after strength training with protein supplementation (ST+PRO), or protein supplementation alone (PRO), before (Acute experiment 1) and after (Acute experiment 2) the ten-week intervention.

	Acute experiment 1 (fold change from pre biopsy)		Acute experiment 2 (fold change from pre biopsy)	
mRNA	ST+PRO	PRO	ST+PRO	PRO
Positive regulators of autophagy / mitophagy				
ATG7	1.41 ± 0.69*	1.07 ± 0.33	1.03 ± 0.37	1.84 ± 1.72
LC3	2.90 ± 3.77*	1.30 ± 0.54	1.05 ± 0.35	2.41 ± 2.81
p62	1.89 ± 1.90*	1.27 ± 0.28	1.14 ± 0.25	1.49 ± 1.78
BNIP3	2.35 ± 4.83	1.25 ± 1.06	1.31 ± 0.61	1.30 ± 1.64
BNIP3L	3.71 ± 9.04	1.05 ± 0.27	1.24 ± 0.70	1.87 ± 2.38
Regulators of mitochondrial fusion and fission				
OPA1	1.68 ± 1.63	1.07 ± 0.22	0.88 ± 0.24	1.41 ± 1.07
DRP1	3.18 ± 3.15*	1.40 ± 0.28	1.16 ± 0.36	1.40 ± 1.20
Positive regulators of proteosomal degradation				
MuRF-1	2.85 ± 2.53*	1.26 ± 1.14	1.15 ± 0.68	0.66 ± 0.44
Atrogin-1	1.11 ± 0.71	0.71 ± 0.27	0.62 ± 0.27*	0.72 ± 0.24*
MUSA1	1.51 ± 0.76*	1.24 ± 0.60	1.14 ± 0.55	1.34 ± 1.61

All data are presented as means ± SD, and display the fold change from baseline at acute experiment 1 and acute experiment 2. ATG7, autophagy related 7; LC3, microtubule-associated protein 1 light chain 3; p62, Sequestosome 1; BNIP3, BCL2/adenovirus E1B 19-kDa interacting protein 3; BNIP3L, BCL2/ adenovirus E1B 19-kDa interacting protein 3 Like; OPA1, mitochondrial dynamin-like GTPase; DRP1, dynamin-related protein 1; MuRF1, muscle RING-finger protein-1; Atrogin-1, muscle atrophy F-Box protein 32, MUSA1, F-Box only protein 30. *$P < 0.05$ vs. baseline within group.

Table 5. Summary of gene and protein expression data at acute experiment 1, acute experiment 2, and following the ten-week intervention (basal levels) in ST+PRO (strength training and protein supplementation) and PRO (protein supplementation only).

		Acute experiment 1		Acute experiment 2		Intervention	
Macro-autophagy	**Gene expression**	**ST+PRO**	**PRO**	**ST+PRO**	**PRO**	**ST+PRO**	**PRO**
	LC3	↑	-	-	-	-	-
	P62	↑	-	-	-	-	-
	ATG7	↑	-	-	-	-	-
	Protein level						
	Cytosolic LC3-I	↑	-	-	-	-	-
	Membrane LC3-II	↓	↓	↓	↓	-	-
	Cytosolic p62	-	-	-	-	-	-
Mitochondrial quality control	**Gene expression**						
	BNIP3	-	-	-	-	-	-
	BNIP3L	↗	-	-	-	-	-
	OPA1	↗	-	-	-	-	-
	DRP1	↑	-	-	-	-	-
Ubiquitin proteasome system	**Gene expression**						
	MuRF-1	↑	-	-	↘	-	-
	Atrogin-1	-	-	↓	↓	-	-
	MUSA1 / Fbox30	↑	-	-	-	-	-
	Protein level						
	Ubiquitinated proteins	↓	-	-	-	-	↗
	Ubiquitin, free	-	-	-	-	-	-
Muscle protein synthesis	**Phosphorylation status**						
	Phospho-p70 S6K / total	↑	↗	↑	↗	-	-
	Phospho 4E-BP1 / total	↗	-	↑	↗	↗	-
	Phospho eEF2 / total	-	-	-	-	-	-

↑ Significant increase from baseline ($P < 0.05$).
↗ Tendency for increase from baseline ($P < 0.1$).
↘ Tendency for decrease from baseline ($P < 0.1$).
↓ Significant decrease from baseline ($P < 0.05$).
- no change from baseline ($P > 0.1$)

FIGURE LEGENDS

Figure 1. Simplified flow chart. For a full account of all subjects, see the previous publication from this trial [1].

Figure 2. Experimental design. An acute experiment was conducted both before and after the ten-week intervention. The acute experiment was similar in the two groups, except for the strength training session in ST+PRO, which was replaced by a rest period in the PRO group. MVC: maximal voluntary contraction, ST: strength training session, REST: rest period.

Figure 3. Representative western blots for anabolic signaling proteins and autophagic markers. AE1: acute experiment 1, conducted before the ten-week intervention. AE2: acute experiment 2, conducted after the ten-week intervention.

Figure 4. Representative western blots for ubiquitinated proteins and unbound (free) ubiquitin. AE1: acute experiment 1, conducted before the ten-week intervention. AE2: acute experiment 2, conducted after the ten-week intervention.

Figure 5. Change in autophagy-related proteins during the acute experiment conducted before (acute experiment 1), and after (acute experiment 2) the ten-week intervention, in ST+PRO (strength training with protein supplementation) and PRO (protein supplementation only). Results show cytosolic LC3-I (panel A-B), membrane LC3-II (panel C-D) and cytosolic p62 (panel E-F). Figures display mean and individual values. AU, arbitrary units. *$P < 0.05$, different from baseline within group.

Figure 6. Change in ubiquitin during the acute experiment conducted before (acute experiment 1), and after (acute experiment 2) the ten-week intervention, in ST+PRO (strength training with protein supplementation) and PRO (protein supplementation only). Results show the level of ubiquitinated proteins (panel A-B) and ubound/free ubiquitin (panel C-D). Figures display mean and individual values. AU, arbitrary units. *$P < 0.05$, different from baseline within group.

Figure 7. Change in phosphorylation ratio of proteins involved in the regulation of muscle protein synthesis during the acute experiment conducted before (acute experiment 1), and after (acute experiment 2) the ten-week intervention, in ST+PRO (strength training with protein supplementation) and PRO (protein supplementation only). Results show the ratio of phosphorylated to total p70S6K (panel A-B), 4E-BP1 (panel C-D), and eEF2 (panel E-F). p70S6K and 4E-BP1 promote muscle protein synthesis when phosphorylated, whereas eEF2 promote synthesis when dephosphorylated. Figures display mean and individual values. AU, arbitrary units. *$P < 0.05$, different from baseline within group.

Figure 1

Click here to access/download;Figure;Figure 1 (Flow chart).tif

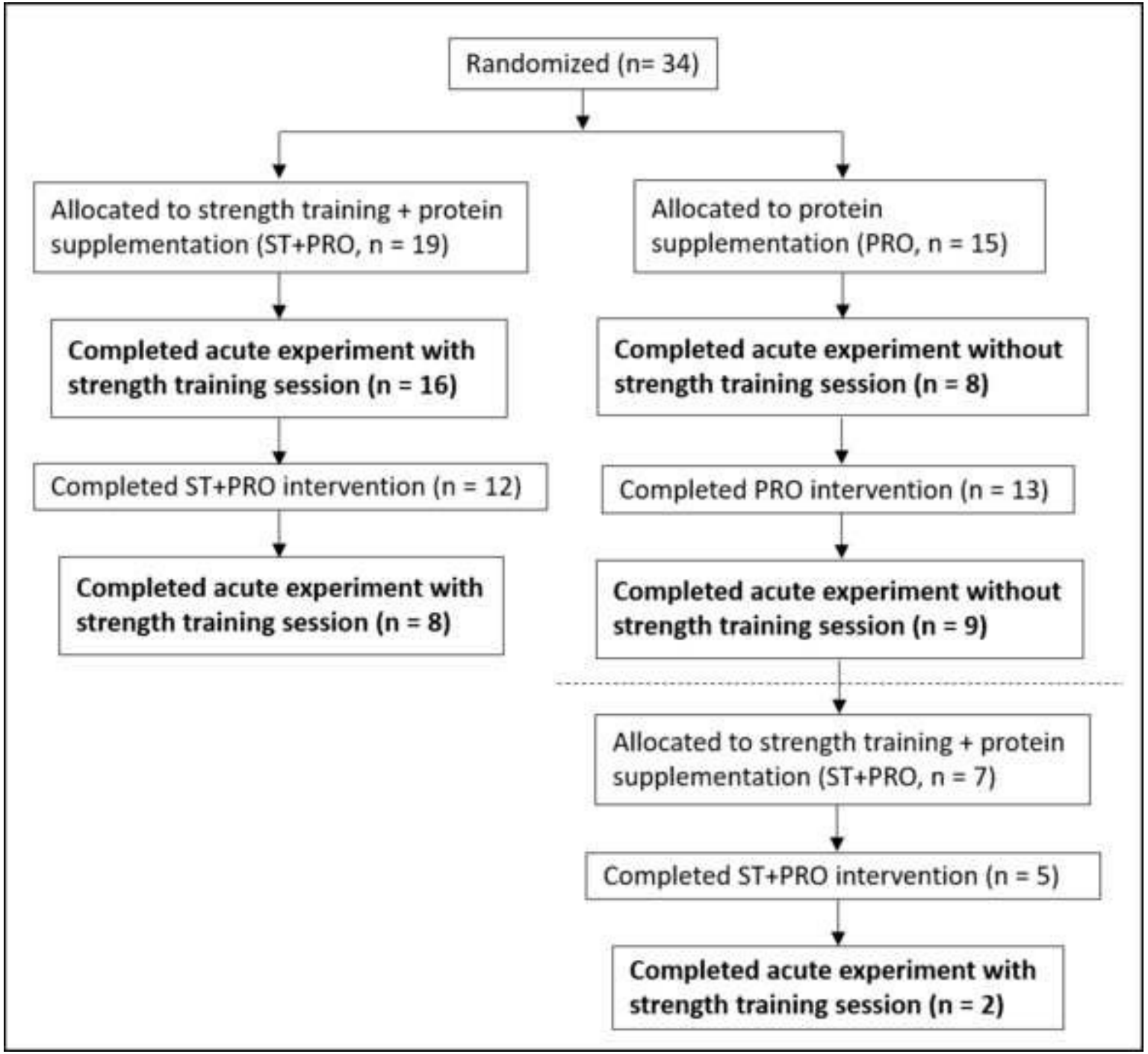


Figure 2

Click here to access/download;Figure;Figure 2 (Design).tif

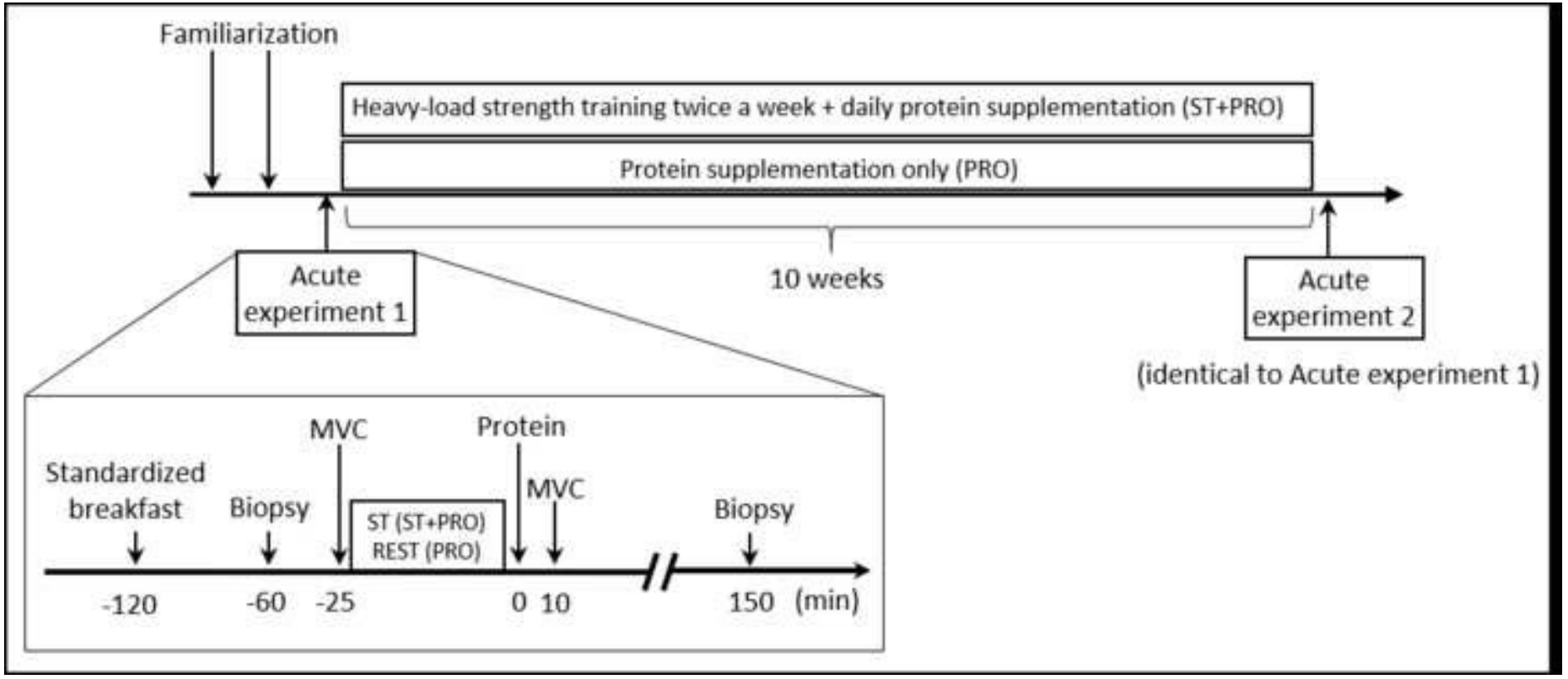


Figure 3

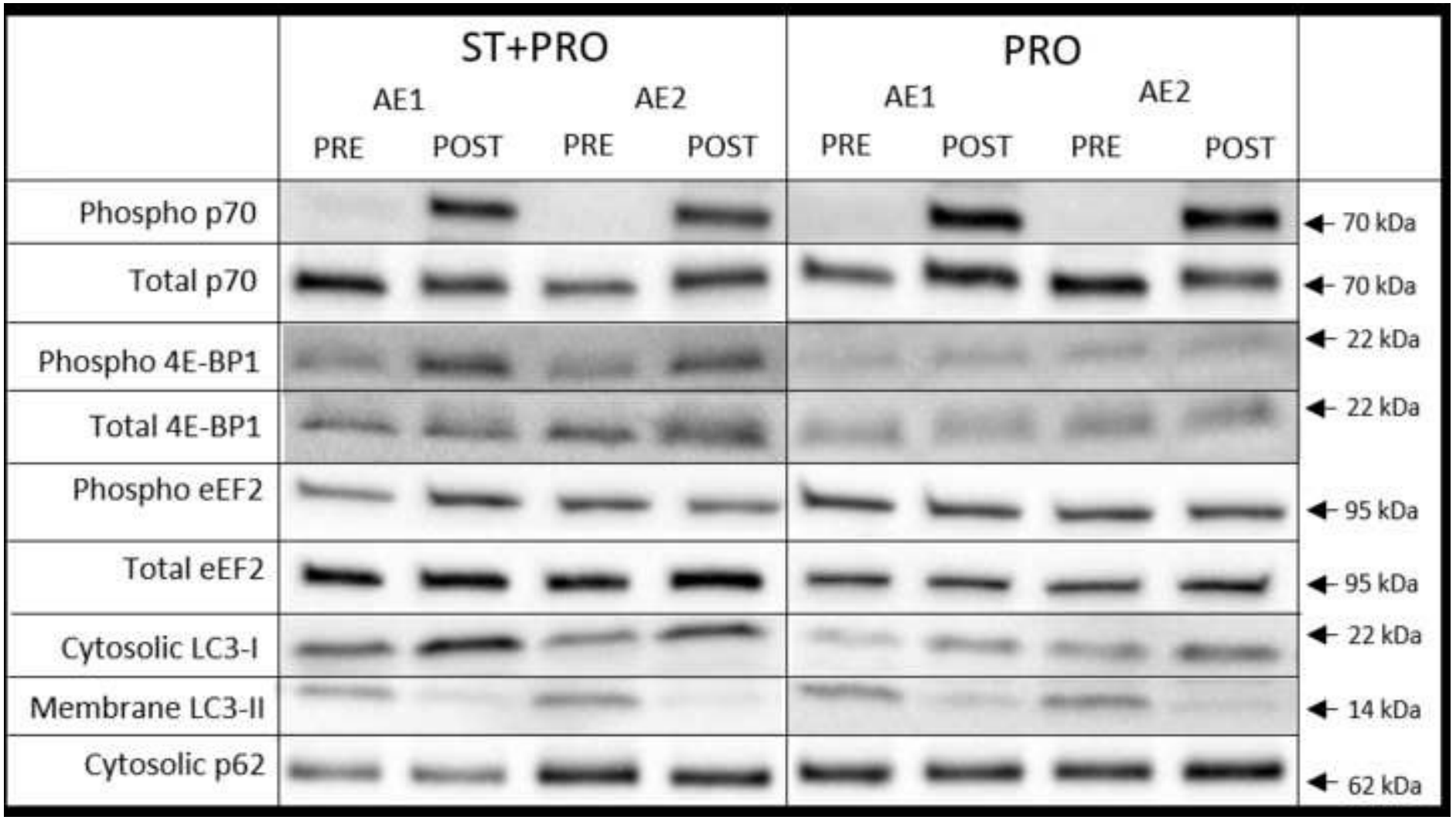


Figure 4

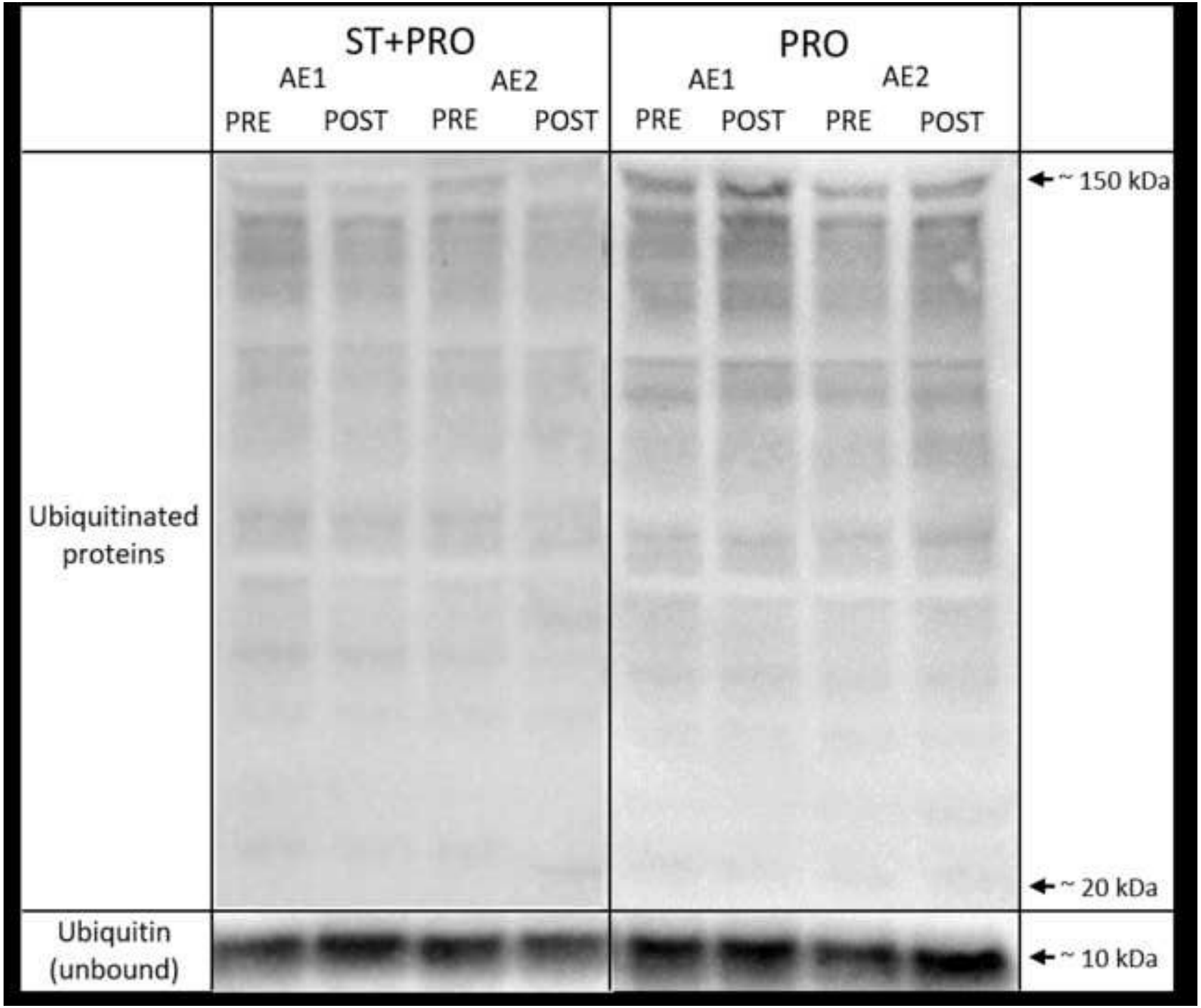


Figure 5

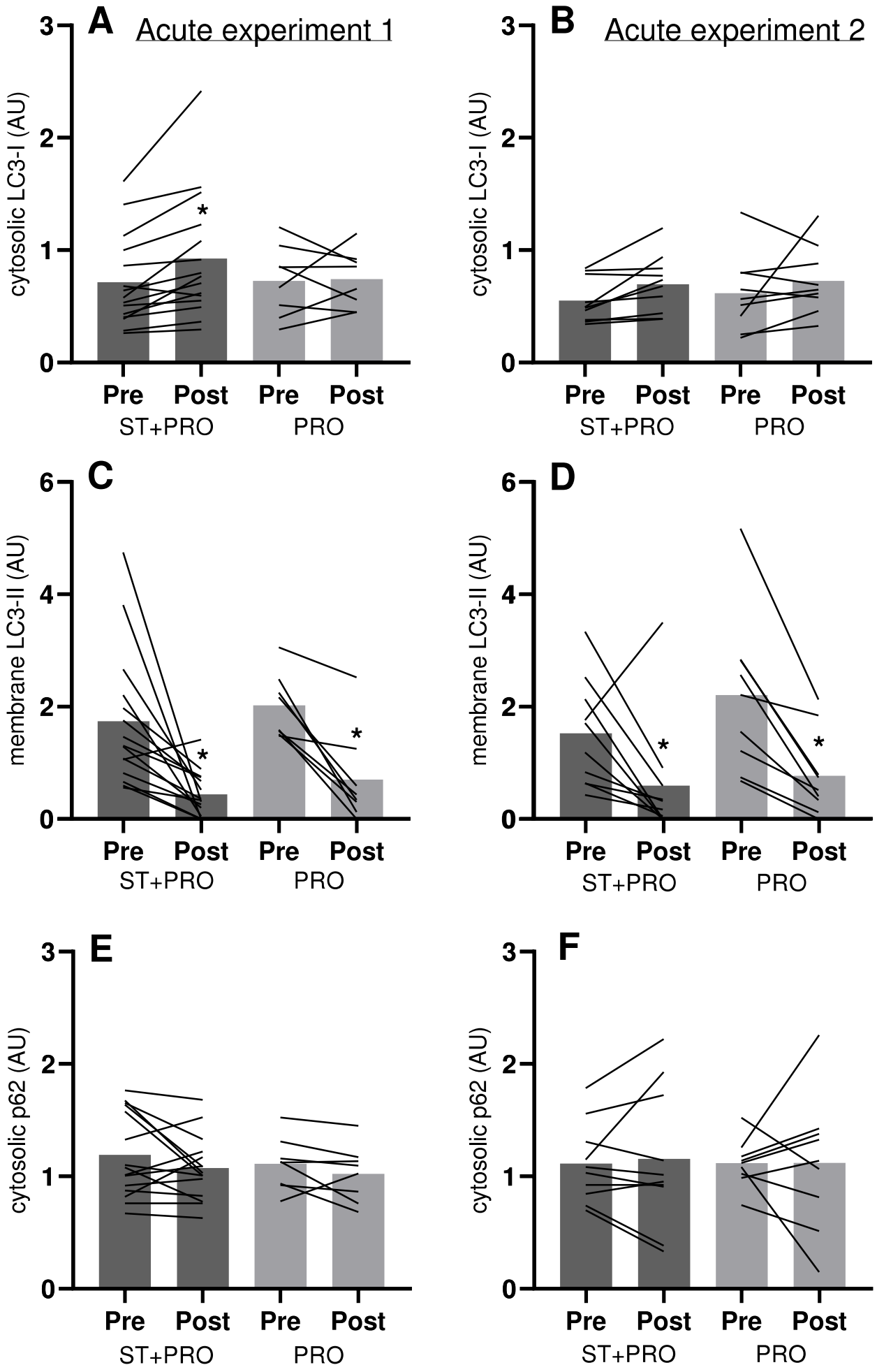

A
Acute experiment 1
B
Acute experiment 2
cytosolic LC3-I (AU)
membrane LC3-II (AU)
cytosolic p62 (AU)
Pre
Post
ST+PRO
PRO
*

Figure 6

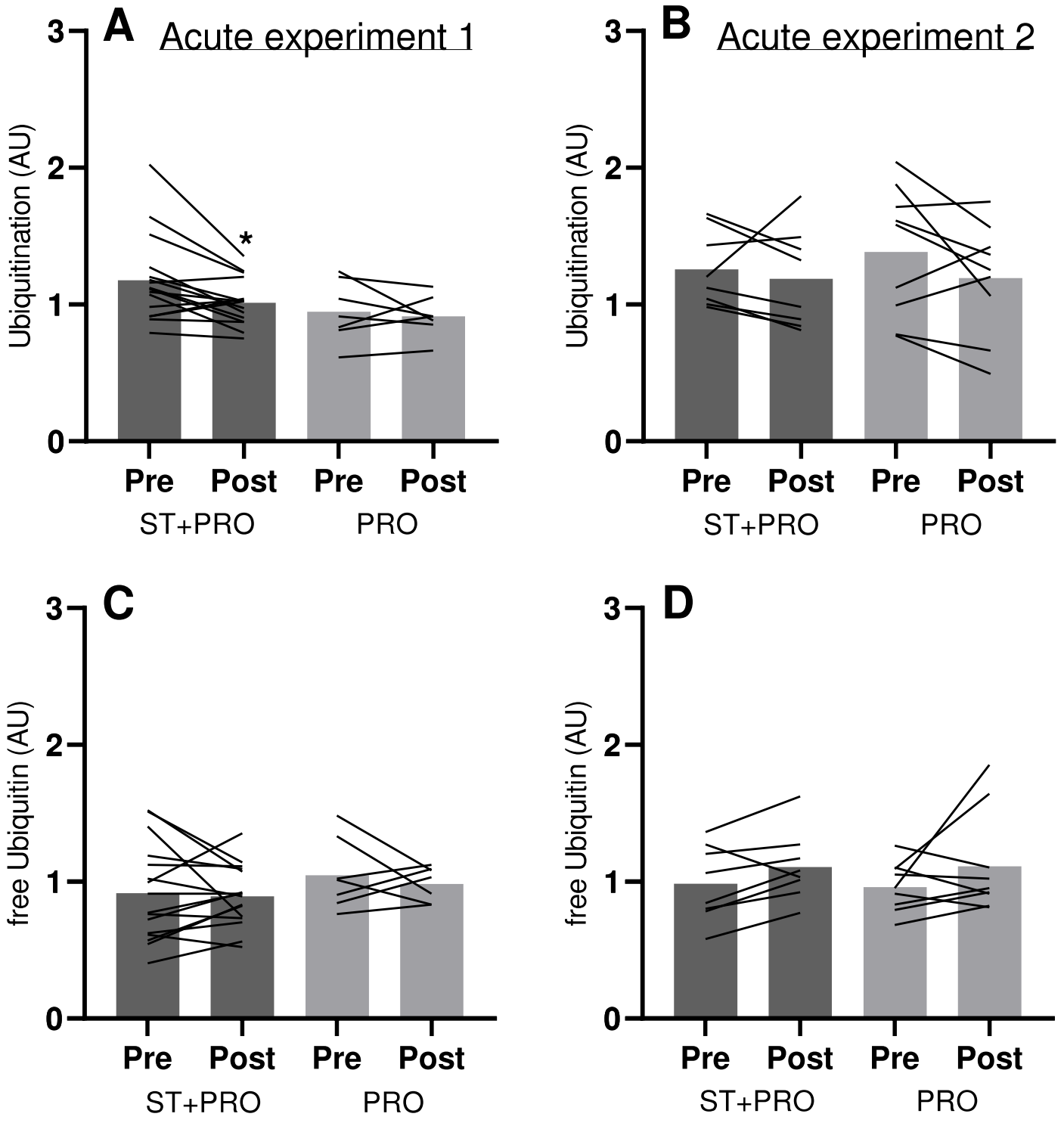
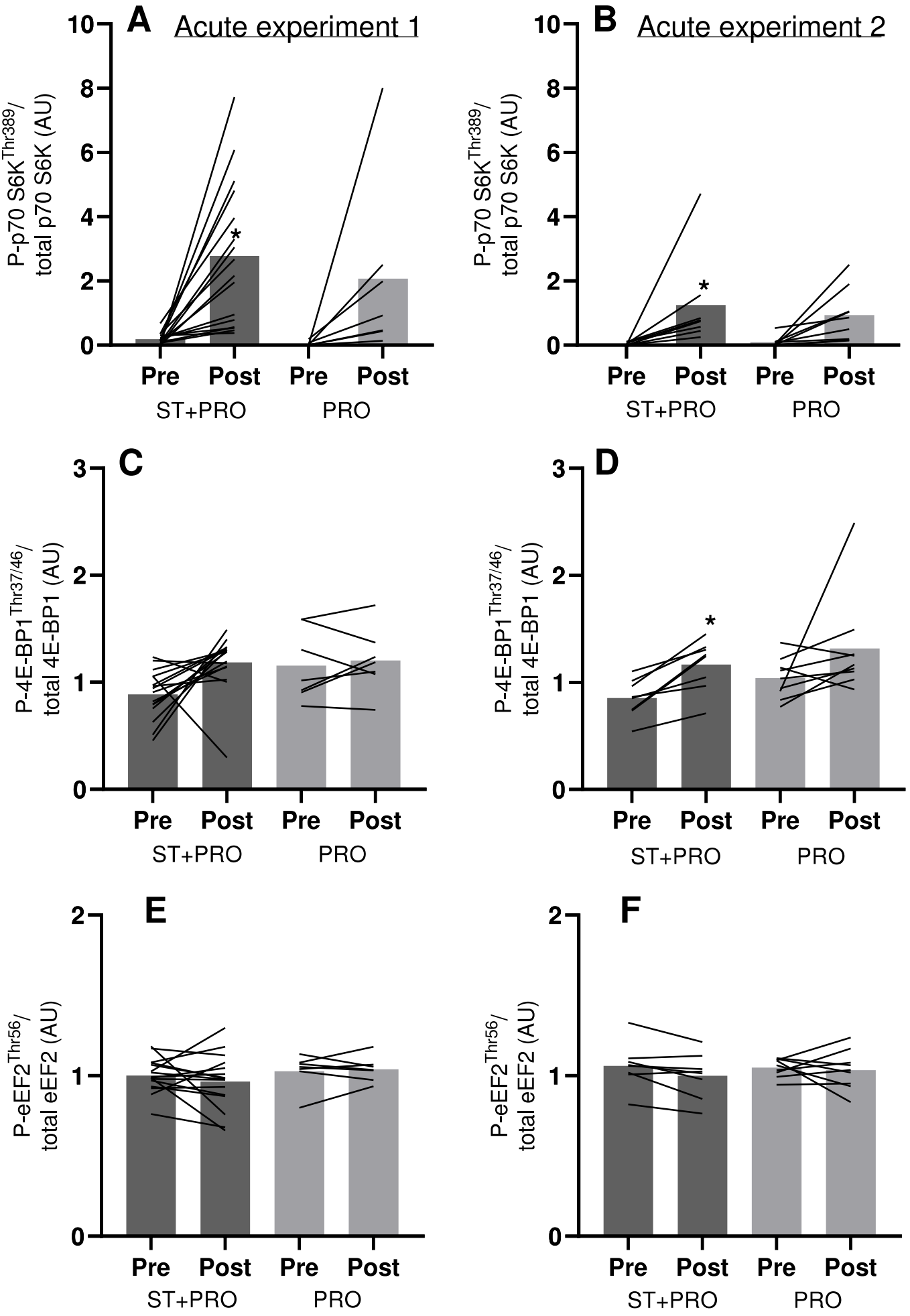

A
Acute experiment 1
Ubiquitination (AU)
3
2
1
0
*
Pre
Post
Pre
Post
ST+PRO
PRO
B
Acute experiment 2
Ubiquitination (AU)
3
2
1
0
Pre
Post
Pre
Post
ST+PRO
PRO
C
free Ubiquitin (AU)
3
2
1
0
Pre
Post
Pre
Post
ST+PRO
PRO
D
free Ubiquitin (AU)
3
2
1
0
Pre
Post
Pre
Post
ST+PRO
PRO

Figure 7


A
Acute experiment 1
B
Acute experiment 2
P-p70 S6K^Thr389/ total p70 S6K (AU)
C
D
P-4E-BP1^Thr37/46/ total 4E-BP1 (AU)
E
F
P-eEF2^Thr56/ total eEF2 (AU)
Pre
Post
ST+PRO
PRO