

## Protein ingestion preserves proteasome activity during intense aseptic inflammation and facilitates skeletal muscle recovery in humans

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### Abstract

The ubiquitin–proteasome system (UPS) is the main cellular proteolytic system responsible for the degradation of normal and abnormal (e.g. oxidised) proteins. Under catabolic conditions characterised by chronic inflammation, the UPS is activated resulting in proteolysis, muscle wasting and impaired muscle function. Milk proteins provide sulphur-containing amino acid and have been proposed to affect muscle inflammation. However, the response of the UPS to aseptic inflammation and protein supplementation is largely unknown. The aim of this study was to investigate how milk protein supplementation affects UPS activity and skeletal muscle function under conditions of aseptic injury induced by intense, eccentric exercise. In a double-blind, cross-over, repeated measures design, eleven men received either placebo (PLA) or milk protein concentrate (PRO, 4 × 20 g on exercise day and 20 g/d for the following 8 days), following an acute bout of eccentric exercise (twenty sets of fifteen eccentric contractions at 30°/s) on an isokinetic dynamometer. In each trial, muscle biopsies were obtained from the vastus lateralis muscle at baseline, as well as at 2 and 8 d post exercise, whereas blood samples were collected before exercise and at 6 h, 1 d, 2 d and 8 d post exercise. Muscle strength and soreness were assessed before exercise, 6 h post exercise and then daily for 8 consecutive days. PRO preserved chymotrypsin-like activity and attenuated the decrease of strength, facilitating its recovery. PRO also prevented the increase of NF-κB phosphorylation and HSP70 expression throughout recovery. We conclude that milk PRO supplementation following exercise-induced muscle trauma preserves proteasome activity and attenuates strength decline during the pro-inflammatory phase.

**Key words:** Skeletal muscle damage: Protein supplementation: Muscle proteasome: Muscle strength performance: Aseptic inflammation

Strenuous exercise causes skeletal muscle microtrauma, which is characterised by ultrastructural damage of sarcomeres and cell membranes and results in a loss of force-generating capacity and muscle soreness<sup>(1)</sup>. This exercise-induced muscle damage (EIMD) activates an inflammatory response cascade leading to the sequential infiltration of neutrophils and macrophages into the injured muscle tissue, the production of pro-inflammatory cytokines and reactive oxygen species (ROS) at the injury site and elsewhere, as well as a systemic release of leucocytes and cytokines and oxidative stress<sup>(1,2)</sup>. Muscle

damage and inflammation also characterise a number of catabolic diseases such as muscular dystrophies, cancer cachexia and sepsis, although with diverse mechanisms (e.g. different profile of released cytokines)<sup>(2)</sup>. Therefore, eccentric exercise appears to be a valid model to study the cellular and molecular pathways regulating the inflammatory process associated with physical trauma.

During the inflammatory phase, a rapid invasion of ROS-generating immune cells (i.e. neutrophils and macrophages) is crucial for the efficient clearance of cellular debris, as well as

**Abbreviations:** CK, creatine kinase activity; CT-L, chymotrypsin-like; DOMS, delayed onset of muscle soreness; ES, effect size; PC, protein carbonyls; PLA, placebo; ROS, reactive oxygen species; UPS, ubiquitin–proteasome system.

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the subsequent repair and regeneration of muscle fibres. However, ROS produced during this phase by NADPH oxidase (respiratory burst) may also lead to marked perturbations of muscle redox status<sup>(1,3,4)</sup>. Furthermore, damaged myofibres and cytokines released from phagocytes activate ROS-generating enzymes such as xanthine oxidase, cyclo-oxygenase-2 and NADPH oxidase<sup>(5)</sup>. ROS may cause secondary muscle damage of adjacent intact muscle fibres<sup>(1)</sup>, thereby exacerbating the loss of muscle's force-generating capacity<sup>(4)</sup>. It appears that these perturbations of redox status are involved in the regulation of transcriptional pathways such as those of NF- $\kappa$ B, mitogen-activated protein kinases and protein kinase B (Akt)/mammalian target of rapamycin<sup>(4,6-8)</sup>. These cascades mediate the activation and recruitment of immune cells, adhesion molecules, satellite cells and synthesis of antioxidant enzymes<sup>(4,6-8)</sup>.

Increasing evidence suggests that elevated ROS production may down-regulate anabolic signalling and protein synthesis<sup>(9)</sup> and promote oxidative protein modifications and proteolysis via the ubiquitin-proteasome system (UPS) – the main proteolytic system in skeletal muscle<sup>(9-12)</sup>. The 20S proteasome is the ATP-independent proteolytic core, composed by 7  $\alpha$ - and 7  $\beta$ -subunits, three of which ( $\beta_1$ ,  $\beta_2$  and  $\beta_5$ ) are responsible for the specific proteolytic activities of the complex<sup>(12-14)</sup>. Furthermore, the immunoproteasome is also active in muscle under pro-inflammatory conditions. UPS activation in muscle, under pro-inflammatory conditions, is mediated by cytokines (i.e. TNF $\alpha$  and IL-6) and NF- $\kappa$ B signalling<sup>(15,16)</sup>. However, following EIMD, both proteasome- and immunoproteasome-mediated proteolysis promote degradation of damaged and/or unfolding proteins and facilitate myogenesis and recovery of muscle's functional capacity<sup>(17,18)</sup>. Indeed, marked elevation in mRNA and protein expression level of specific E3 ubiquitin ligases persists up to 48 h following a single bout of eccentric exercise<sup>(19)</sup>.

Protein supplementation has been postulated to affect skeletal muscle's remodelling process, by regulating the inflammatory response<sup>(20)</sup>. Whey protein supplementation during recovery from eccentric exercise has been shown to prevent strength decline<sup>(21-22)</sup> and to accelerate satellite cell proliferation<sup>(23)</sup>. Moreover, branched-chain amino acids (BCAA) may also promote the attenuation of EIMD<sup>(24)</sup> and the delayed onset of muscle soreness (DOMS) following eccentric exercise<sup>(25)</sup>. However, protein is also a rich source of sulphur-containing amino acids that act as precursors for GSH synthesis, thereby protecting cells from redox status disturbances<sup>(26)</sup>. Although protein supplementation has been extensively examined in terms of stimulating muscle protein synthesis (MPS) following exercise, its ability to regulate both the inflammatory response and UPS following aseptic muscle injury remains unclear. Thus, we investigated the effects of milk protein concentrate supplementation on muscle damage, inflammatory responses, proteasome activity and muscle strength following muscle-damaging eccentric exercise.

## Methods

### Participants

All participants ( $n$  11) were informed of the purpose of the study, the experimental procedures involved and all the risks,

discomforts and benefits involved, before obtaining written consent. The study was approved by the Institutional Review Board of the University of Thessaly and procedures were in accordance with the 1975 Declaration of Helsinki, as revised in 2000. Participant's characteristics are shown in Table 1. Participants were recreationally active ( $VO_{2max} > 45$  ml/kg per min) and had been engaged in systematic resistance exercise training of  $\geq 3$  times/week for the past 12 months. For inclusion in the study, the criteria for participants were as follows: (1) non-smokers; (2) abstained from any vigorous physical activity during the experimental period; (3) had no consumption of alcohol, caffeine, any type of nutritional supplements and medication before (at least 6 months) and throughout the experimental period; (4) had no allergies or intolerance to milk protein; and (5) had no recent history of muscle lesions, lower limb trauma and metabolic diseases.

### Experimental design

A two-trial, cross-over, double-blind, repeated measures design was used in this investigation. Before each trial, participants' body weight and height, RMR and body composition (dual-energy X-ray absorptiometry scan) were measured and they were then provided with accelerometers to measure their habitual physical activity and diet recalls in order to record their dietary intake, over a 7-d period. Thereafter, diet recalls were analysed and participants entered a 3-week adaptive period to ensure that all of them received a standard protein intake of 1 g protein/kg per d, which was considered to be the mean and population-safe protein intake<sup>(27,28)</sup>.

Participants were randomly assigned to consume either milk protein concentrate (PRO) or placebo (PLA), immediately after an acute bout of eccentric exercise on an isokinetic dynamometer and daily for 8 consecutive days post exercise (Fig. 1). A 6-week washout period was adapted between trials. Before each trial, muscle force-generating capacity was tested following familiarisation with the equipment while  $VO_{2max}$  was measured during a graded exercise test to exhaustion using an automated online gas exchange analyser as previously described<sup>(29)</sup>. Participants were asked to refrain from resistance exercise or any other strenuous physical activity for at least 2 weeks before each trial and throughout the experimental period. On each exercise day of each trial, participants arrived at the laboratory after an overnight fast, and baseline blood samples and muscle biopsies were collected. Subsequently, they performed an acute bout of unilateral eccentric contractions on an isokinetic dynamometer. Muscle biopsies and blood samples were also collected at 2 and 8 d after exercise, whereas muscle strength was evaluated at 6 h post exercise and daily for 8 consecutive days. All measurements were performed at the same time of day, in both trials, to prevent the effect of circadian variations.

### Exercise protocol

The exercise protocol applied in the present study has been described to effectively induce myofibrillar disruption, as indicated by electron microscopy and immunohistochemistry<sup>(30)</sup>, and has been efficiently used in a previous study of our group to



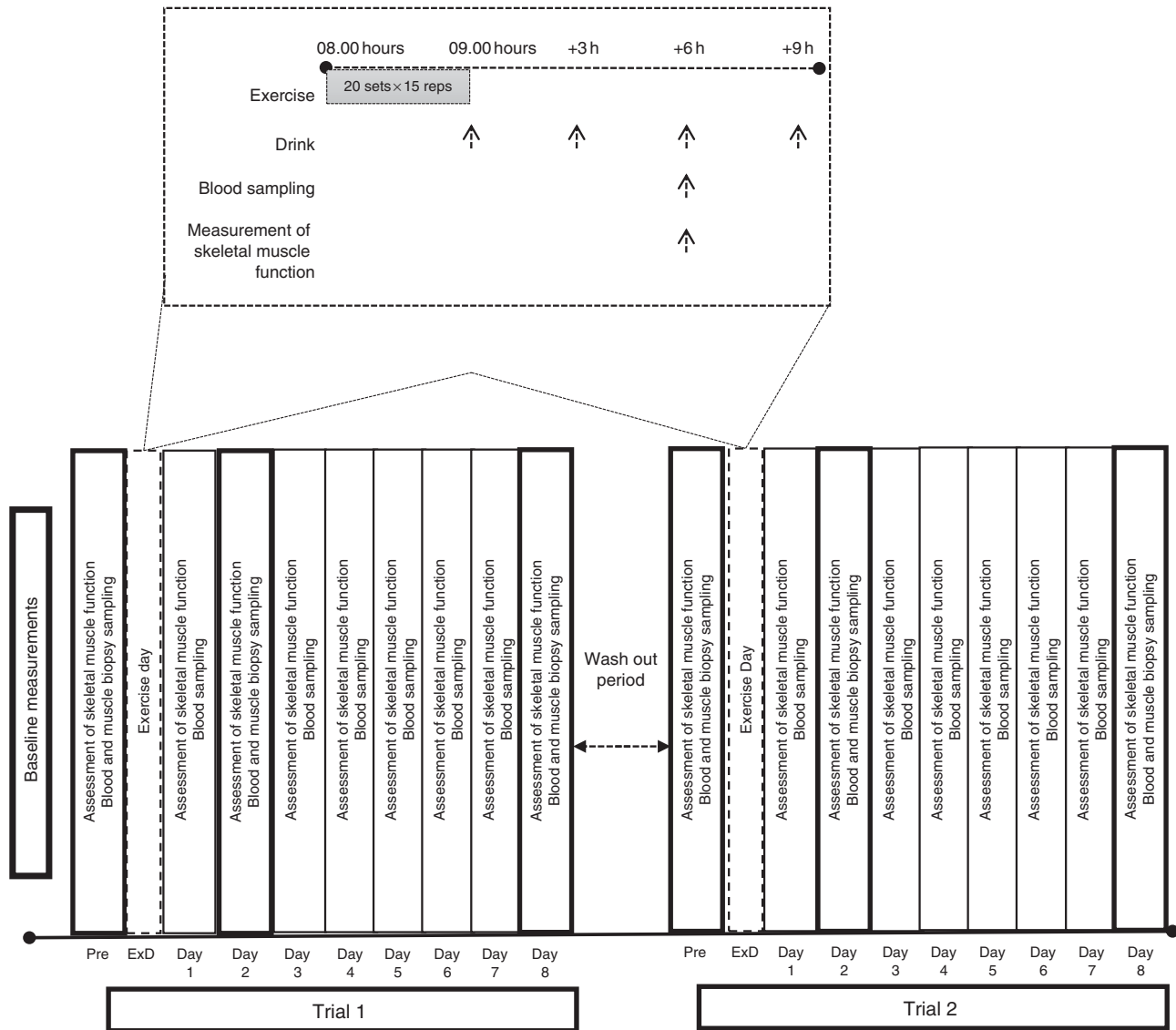


Fig. 1. Experimental design. ExD, exercise day.

induce aseptic inflammation<sup>(8)</sup>. In brief, participants performed 300 eccentric unilateral contractions (twenty sets, fifteen repetitions/set, 30-s rest between sets) of knee extensors on an Iso-force isokinetic dynamometer (TUR GmbH) at a speed of 30°/s.

### Supplementation

On exercise day, participants consumed a protein supplement consisting of 80% protein (80% casein and 20% whey), 1.5% fat and 5% carbohydrates or a matching PLA (containing no protein) as repeated 'pulsed' drinks: immediately post exercise and then one every 3 h on three occasions (+3, +6, +9 h). According to previous reports<sup>(31,32)</sup>, distributing 80 g of protein in 4 x 20 g doses every 3 h is superior to either pulse (8 x 10 g every 1.5 h) or bolus (2 x 40 g every 6 h) feeding in stimulating MPS and maximising whole-body protein balance over a 12-h post-exercise period. On each day of the remaining days, participants consumed a single drink (protein or PLA) with breakfast. A single

dose of 20 g of protein supplemented on non-exercise days is considered the minimum amount of protein required in order to increase protein intake on non-training days<sup>(33,34)</sup>. Each PLA supplement contained 20 g of maltodextrin to keep the two supplements isoenergetic. All drinks were prepared in 250 ml of water and flavoured with banana, to make the contents indistinguishable and non-transparent.

### Muscle performance

To assess muscle performance, maximal knee extensor eccentric peak torque was measured on the same isokinetic dynamometer at 60°/s as described<sup>(30)</sup>. The evaluation was preceded by a familiarisation period (CV for repeated measures was 4.1%). Participants rated their DOMS on a visual analogue scale (1–10) during palpation of the muscle belly and the distal region of relaxed vastus medialis, vastus lateralis and rectus femoris, following three repetitions of a full squat<sup>(35)</sup>.

### Diet records

In the beginning of the study, participants were taught (by a trained dietitian) how to estimate food servings and sizes and how to record their regular dietary intake. Then, participants completed 7-d diet recalls to evaluate their protein and energy intake. On the basis of diet analysis, participants were provided with a dietary plan providing 1.0 (SD 0.02) g protein/kg/day over a 3-week period (adaptive period). During the first trial (8-d testing period), as well as during the washout period and the second trial (8-d testing period), participants completed again 7-d diet recalls to record their daily intake to verify that they followed the same diet practice as they did in the preliminary adaptive period. Analysis of diet recalls collected throughout the study indicated a similar diet composition (total energy: 2581.7 (SD 105.4)%, carbohydrate intake: 56.3 (SD 3.1)%, fat intake: 28.1 (SD 3.1)%, protein intake: 15.6 (SD 1.5)%) of participants during all phases of the study (adaptive period, trial 1, washout period, trial 2). Science Fit Diet 200 A (Science Technologies) was used for the analysis of diet records.

### Blood sampling and assays

Resting blood samples were collected at 07.00 hours on the exercise day, after an overnight fast. All samples were collected via a 20-gauge disposable needle equipped with a Vacutainer tube holder (Becton Dickinson) from an antecubital arm vein, while subjects were in a seated position. For serum separation, blood samples were allowed to clot at room temperature first and then centrifuged (1500 **g**, 4°C, 15 min). The supernatant was dispensed in multiple aliquots (into Eppendorf tubes) that were stored at -80°C for later analysis of creatine kinase activity (CK). A blood portion was collected into tubes containing EDTA and centrifuged (1370 **g**, 4°C, 10 min). Plasma was collected into Eppendorf tubes and stored at -80°C, and it was used for the measurement of insulin and glucose. The remaining plasma erythrocytes were then lysed and the resultant lysate was collected as described<sup>(36)</sup> and used for analysis of protein carbonyls<sup>(37)</sup>. Another blood portion (2 ml) was immediately collected in EDTA tubes and were used for the determination of leucocyte count using an automated haematology analyzer (BC-5500; Shenzhen Mindray). CK and glucose concentrations were measured using a Clinical Chemistry Analyser Z1145 (Zafiroopoulos Diagnostica) using commercially available kits (Zafiroopoulos). Insulin was measured with a commercially available insulin (Human) ELISA kit (Abnova<sup>R</sup> corporation). Protein carbonyls (PC) were assayed spectrophotometrically (Hitachi UV/VIS; Hitachi Instruments Inc.) at 375 nm, as described<sup>(35)</sup>. Plasma and serum samples were stored in multiple aliquots at -80°C and thawed only once before analysis. All assays were performed in duplicate. Inter- and intra-assay coefficients of all assays ranged from 1.8 to 8.2% and from 2.9 to 8.6%, respectively.

### Muscle biopsy sampling

Muscle samples were obtained using the standard Bergström needles, with suction, from the middle portion of vastus lateralis

of the exercising leg under local anaesthesia (xylocaine 1%)<sup>(38)</sup>. The pre-exercise sample was obtained 25 cm from mid-patella, 30 min before exercise. Subsequent biopsies (at 2 and 8 d post exercise) were obtained from the same muscle, 5 cm distal to each previous biopsy site. A different limb (randomly selected) was used for eccentric exercise and biopsy sampling in each trial. Adipose tissue and blood were carefully removed from muscle samples that were rapidly frozen in N<sub>2</sub> and stored at -80°C for further analysis of protein phosphorylation, proteasome activity and expression of its catalytic subunits.

### Proteasome peptidase assay

This method allows the direct quantification of proteasome activities and is performed as previously described<sup>(39,40)</sup>. In brief, tissue samples were homogenised in proteasome activity lysis buffer (1 M Tris-HCl, pH 7.6, 100 mM ATP, 3 M KCl, 0.1 M EDTA, 1 M dithiothreitol, 0.2% NP-40, 10% glycerol, 10 µg/ml aprotinin and 10 mM phenylmethylsulfonyl fluoride (PMSF)). Chymotrypsin-like (CT-L), caspase-like (C-L) and trypsin-like (T-L) activities were assayed with hydrolysis of the fluorogenic peptide LLVY-AMC, LLE-AMC and LSTR-AMC, respectively (Enzo Life Sciences), for 30 min at 37°C. Specific activity was determined in the presence of 20 µM MG132-specific proteasome inhibitor. Fluorescence was measured in the Safire<sup>2</sup> Multi-detection Microplate Reader (Tecana).

### Immunoblot analysis

Immunoblot analysis was used to detect protein expression levels of proteasome ( $\beta 5$ ,  $\beta 2$  and  $\beta 1$ ) and immunoproteasome ( $\beta 5i$ ,  $\beta 2i$  and  $\beta 1i$ ) subunits. A portion of the tissue samples that were homogenised during the proteasome peptidase assay was boiled in non-reducing Laemmli buffer (NRLB) for 10 min, as previously described<sup>(39)</sup>. SDS-PAGE and immunoblotting were then contacted with specific antibodies against 20S, 19S, proteasome complexes and immunoproteasome. GAPDH was used as a loading control. Each immunoblot was repeated at least twice.

### Antibodies

Polyclonal NF- $\kappa$ B p65<sup>Ser536</sup> was purchased from Cell Signaling Technology Inc. GAPDH was purchased from Santa Cruz Biotechnology Inc. Anti-rabbit (IgG, horseradish peroxidase linked) secondary antibody was also purchased from Signaling Technology Inc. Antibodies against  $\beta 2$ ,  $\beta 1$ ,  $\beta 5$ ,  $\beta 2i$ ,  $\beta 1i$  and  $\beta 5i$  proteasome subunits were purchased from Enzo Life Sciences and antibody against HSP70 was purchased from Santa Cruz Biotechnology.

### Measurement of intracellular-signalling-related proteins

Changes in the phosphorylation of NF- $\kappa$ B p65<sup>Ser536</sup> and protein expression of HSP70 were analysed by immunoblotting. Briefly, muscle samples (approximately 40–50 mg) were homogenised in activity lysis buffer and then centrifuged at 13 000 rpm at 4°C

for 10 min and the supernatant was collected. Total protein concentration was measured (Bradford Protein Assay; Bio-Rad) to prepare working samples of equal concentration in NRLB. Equal amounts of protein (20 µg) were loaded onto 8% or gradient precast gels (Mini-PROTEAN TGX Gels; Bio-Rad) and separated by SDS-PAGE electrophoresis. Immediately after, proteins were transferred to nitrocellulose membrane, blocked with 5% non-fat milk/Tris-buffered saline for 1 h and incubated with primary antibody overnight at 4°C. Membranes were washed with Tris-buffered saline-Tween (TBS-T) and incubated with secondary antibody for 1 h at room temperature. Subsequently, membranes were washed again with TBS-T, exposed, visualised by chemiluminescence and quantified by densitometry. Phosphorylation status of NF-κB and HSP70 expression was then expressed relative to a corresponding GAPDH from the equivalent sample lysate.

**Statistics**

On the basis of a preliminary power analysis, a sample size of ten to twelve subjects was suggested in order to detect differences between repeated measurements with a power of 80% at a significance level of 5% (α error). Accordingly, fourteen healthy, trained male volunteers were recruited in the present study, but data from eleven volunteers have been analysed (for three participants, biopsy samples were not collected at all time points or the quantity of muscle tissue was not adequate for analysis).

All data are presented as means and standard deviations. A two-way (trial (PRO and PLA) × time (before exercise, and 2 and 8 d post exercise)) repeated measures ANOVA with planned contrasts on different time points was used to determine the effects of treatment and time on dependent variables. Bonferonni test was used for *post hoc* analysis, when a significant main effect was detected. Significance was set at  $P < 0.05$ . Effect sizes (ES) were also calculated on results of CT-L proteasome activity. We also conducted bivariate Pearson's correlation analysis to estimate the relation of changes in skeletal muscle performance (isometric peak torque) and DOMS with proteasome function (activity and expression level of different β-subunits). Correlation coefficients of  $r < 0.2$ ,  $0.2 < r < 0.7$  and  $r > 0.7$  were characterised as small, moderate and high, respectively. The level of statistical significance was set at  $P < 0.05$ . All analyses were performed using the SPSS 20.0 software (IBM SPSS Statistics).

**Results**

*Participants' physical characteristics and dietary profile*

Participants' characteristics are presented in Table 1. The dietary profile was similar among trials, with the exception of protein intake (Table 2). Baseline values of muscle performance variables were indistinguishable between trials (Fig. 2), indicating that the 6-week washout period was efficient in eliminating the systemic inflammatory responses induced by the first trial.

**Table 1.** Participants' characteristics in each trial\* (Mean values and standard deviations)

	Placebo trial		Protein trial	
	Mean	SD	Mean	SD
Age (years)	22	1.4	22	1.4
Body mass (kg)	79.4	11.1	78.9	10.3
Height (m)	1.78	0.06	1.78	0.06
BMI (kg/m <sup>2</sup> )	25.06	2.85	24.92	2.63
Body fat (%)	15.31	1.77	15.21	1.67
VO <sub>2max</sub> (ml/kg per min)	56.47	6.82	56.1	6.86
Resting metabolic rate (kJ/d)	7930.3	635.1	8092.2	763.2
Resting metabolic rate (kcal/d)	1895.4	151.8	1934.1	182.4

\* Participants demonstrated comparable anthropometric profile and physical conditioning status at baseline (n 11). One-way (time) repeated measures ANOVA was used. VO<sub>2max</sub>, maximal O<sub>2</sub> uptake.

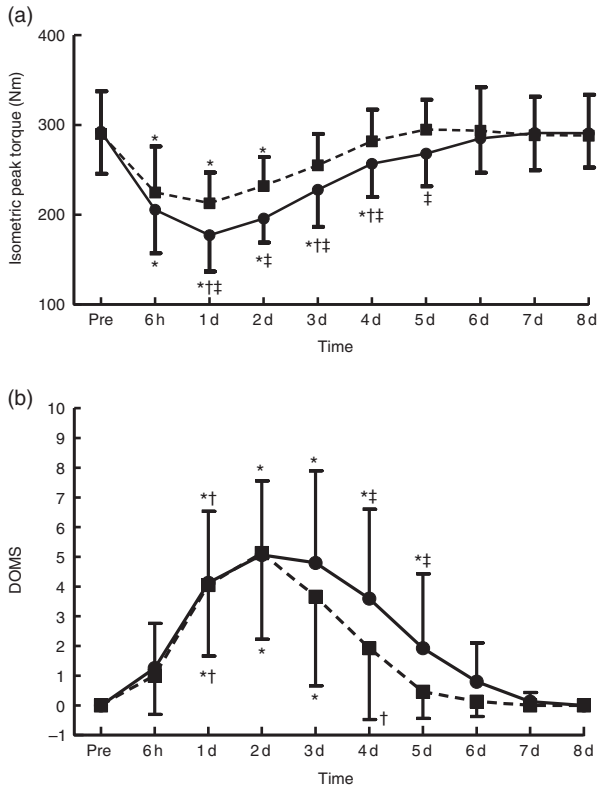
**Table 2.** Daily total dietary energy intake and macronutrient consumption during the course of placebo (PLA) and protein (PRO) trials (protein supplement not included) (Mean values and standard deviations)

	PLA		PRO	
	Mean	SD	Mean	SD
Daily energy intake (kJ)	11 588.8	557.3	11 671.3	592.5
Daily energy intake (kcal)	2769.8	133.2	2789.5	141.6
Daily carbohydrate intake (% of total energy content)	56.2	4.2	56.4	3.9
Daily fat intake (% of total energy content)	27.3	3.5	27.5	3.8
Daily protein intake (% of total energy content)	16.5	2.4	16.1	2.2
Protein intake on exercise day (g)	1.02	0.2	2.05*	0.5
Protein intake during recovery days (g/kg per d)	1.02	0.2	1.29*	0.3

\* Significant difference between trials ( $P < 0.05$ ).

*Skeletal muscle performance*

With PLA, peak torque (Fig. 2(a)) decreased at 6 h post exercise and remained below baseline until day 4 (−39 to −12%,  $P < 0.05$ ), reaching its lowest value at 1 d of recovery. With PRO, peak torque remained below baseline until day 2 (−26.5 to −20%,  $P < 0.005$ ) and recovered thereafter. Supplementation with PLA resulted in greater decline of isometric peak torque during the first 5 d of recovery compared with PRO (day 1:  $P = 0.02$ , ES: −0.91; 95% CI −1.79, −0.03; day 2:  $P = 0.001$ , ES: −1.18; 95% CI −2.09, −0.27; day 3:  $P = 0.006$ , ES: −0.69; 95% CI −1.55, 0.17; day 4:  $P = 0.003$ , ES: −0.67; 95% CI −1.52, 0.19; day 5:  $P = 0.001$ , ES: −0.74; 95% CI −1.61, 0.12). The online Supplementary Fig. S1 demonstrates percent changes between baseline and two dimensional in muscle strength of all participants. DOMS (Fig. 2(b)) in both trials increased at 6 h post exercise until day 7 (6 h: 4.1-fold in PLA *v.* 3.8-fold in PRO,  $P = 0.000$ ; day 1: 6.8-fold in PLA *v.* 5.6-fold in PRO,  $P = 0.000$ ; day 2: 9.3-fold in PLA *v.* 8.1-fold in PRO,  $P = 0.000$ ; day 3: 8-fold in PLA *v.* 6.9-fold in PRO,  $P = 0.000$ ; day 4: 6.2-fold in PLA *v.* 5.2-fold in PRO,  $P = 0.000$ ; day 5: 4.5-fold in PLA *v.* 3.8-fold in PRO,  $P = 0.000$ ; day 6: 2.7-fold in PLA *v.* 2.2-fold in PRO,  $P = 0.000$ ; day 7: 1.4-fold in PLA *v.* 1.1-fold in PRO,  $P = 0.002$ ) and

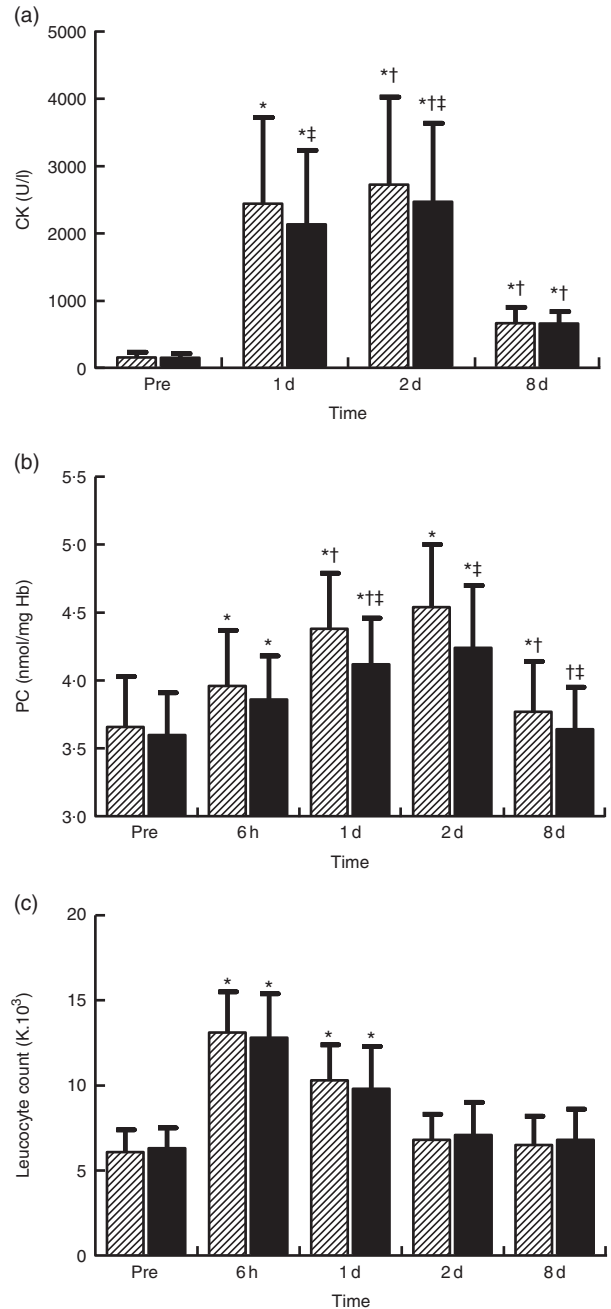


**Fig. 2.** Changes of muscle performance in response to protein and placebo administration. (a) Isometric peak torque and (b) delayed onset of muscle soreness (DOMS) at baseline (Pre) and throughout recovery in the two trials. Values are means and standard deviations. —●—, Placebo; -■-, protein. \* Significantly different from baseline ( $P < 0.05$ ). † Significantly different from the previous time point ( $P < 0.05$ ). ‡ Significant difference between placebo and protein ( $P < 0.05$ ).

recovered on day 8. The rise in DOMS was of a lower magnitude in PRO compared with PLA on day 1 through day 5 of recovery (day 1:  $P = 0.000$ , ES: 1.54; 95% CI 0.58, 2.49; day 2:  $P = 0.000$ , ES: 1.79; 95% CI 0.80, 2.77; day 3:  $P = 0.000$ , ES: 1.17; 95% CI 0.26, 2.07; day 4:  $P = 0.002$ , ES: 1.13; 95% CI 0.23, 2.03; day 5:  $P = 0.046$ , ES: 0.62; 95% CI -0.23, 1.48).

**Muscle damage and inflammatory responses**

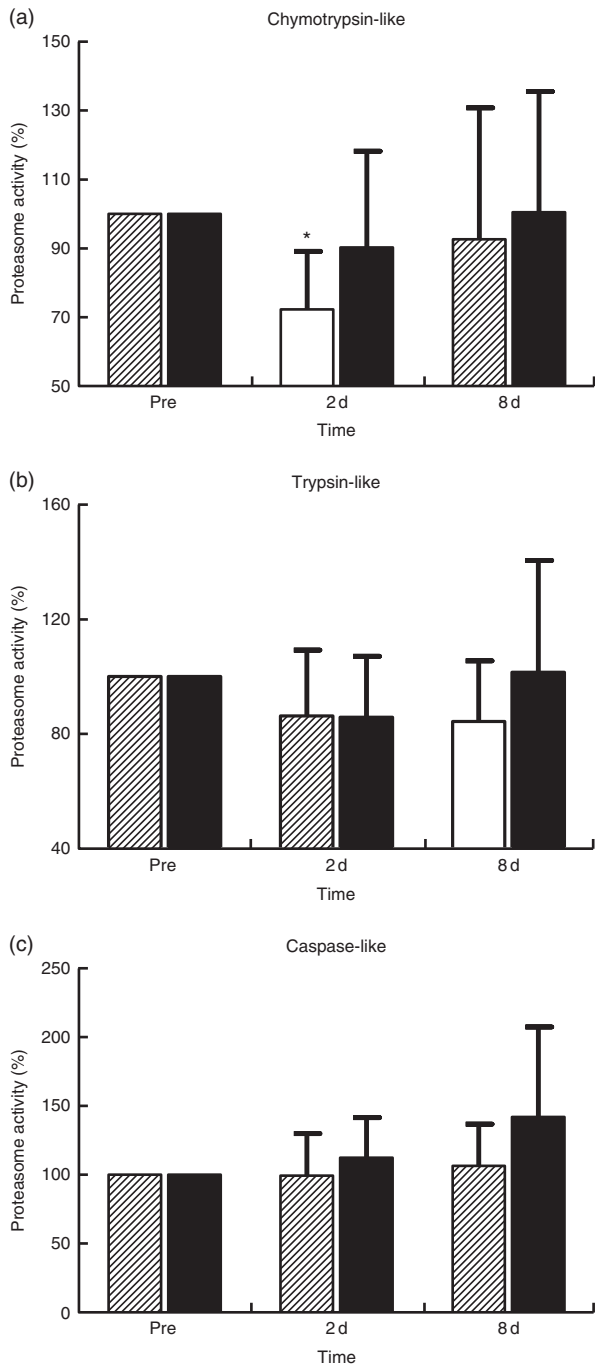
CK (Fig. 3(a)) increased throughout recovery in both trials ( $P = 0.000-0.001$ ). PRO exhibited a rise of lower magnitude than PLA at 1 d ( $P = 0.001$ ; ES 0.25; 95% CI -0.59, 1.08) and 2 d ( $P = 0.001$ ; ES 0.2; 95% CI -0.64, 1.04). PC (Fig. 3(b)) remained above baseline throughout recovery in PLA, demonstrating a peak on day 2 (3–24%,  $P = 0.000-0.002$ ). PC in PRO remained elevated until day 2 (7–18%,  $P = 0.000$ ), demonstrating a peak on day 2 and recovered thereafter. PC rise was greater in PLA than that in PRO at 1 d ( $P = 0.002$ ), 2 d ( $P = 0.034$ ; ES: 0.62; 95% CI -0.2, 1.44) and 8 d ( $P = 0.037$ ; ES: 0.36; 95% CI -0.45, 1.16). In both trials, leucocyte counts (Fig. 3(c)) increased ( $P < 0.05$ ) 6 h post exercise, and were maintained above baseline values at 1 d of recovery and were normalised thereafter. However, there were no significant differences at any time point between PLA and PRO.



**Fig. 3.** Changes of muscle damage and inflammatory markers in response to protein and placebo administration. Changes in (a) creatine kinase activity (CK), (b) protein carbonyls (PC) and (c) leucocyte count during the two trials. Values are means and standard deviations represented by vertical bars. ▨, Placebo; ■, protein. \* Significantly different from baseline ( $P < 0.05$ ). † Significantly different from the previous time point ( $P < 0.05$ ). ‡ Significant difference between placebo and protein ( $P < 0.05$ ).

**Proteasome activity and protein levels**

Proteasome’s CT-L activity declined in PLA on day 2 (30%,  $P = 0.009$ ) but not in PRO (Fig. 4(a)). Furthermore, the two trials differed in CT-L proteasome activity at 2 d ( $P = 0.09$ , ES: -0.75; 95% CI -1.61, 0.12). The online Supplementary Fig. S2 demonstrates individual percentage changes (eleven



**Fig. 4.** Changes of proteasome activities in response to protein and placebo administration. Percentage change of (a) chymotrypsin-like activity, (b) trypsin-like activity and (c) caspase-like activity during the two trials. Values are means and standard deviations represented by vertical bars. ▨, Placebo; ■, protein. \* Significantly different from baseline ( $P < 0.05$ ).

participants) in CT-L activity between baseline and 2d. No changes were observed in T-L (Fig. 4(b)) and C-L (Fig. 4(c)) activities, in both trials. None of the  $\beta$ -subunit (catalytic proteasomal  $\beta$ -subunits  $\beta 1$ ,  $\beta 2$  ( $n 9$ ) and  $\beta 5$  and the corresponding  $\beta 1i$ ,  $\beta 2i$  ( $n 8$ ) and  $\beta 5i$  subunits of immunoproteasome) protein expression levels measured demonstrated any alterations following exercise in both trials (Fig. 5). As a quality control,

blood levels of glucose and insulin were measured because increased insulin may lead to Akt phosphorylation and subsequent proteasome inhibition through the *forkhead box O* (*FoXO*) transcription factors<sup>(41)</sup>. Glucose and insulin remained unchanged throughout recovery in both trials (online Supplementary Fig. S3).

### Intracellular signalling proteins

NF- $\kappa$ B phosphorylation (Fig. 6(a),  $n 8$ ) in PLA increased by approximately 45% at 2d ( $P=0.036$ ) and by approximately 111% at 8d ( $P=0.05$ ). In PRO, although NF- $\kappa$ B phosphorylation increased by 18% at 2d and by 130% at 8d, no statistical significance was detected for both time points. The online Supplementary Fig. S4 demonstrates individual percentage changes (eight participants) of NF- $\kappa$ B phosphorylation between baseline and 2d. Immunoblot analysis for HSP70 ( $n 8$ ; Fig. 6(b)) was also performed as it is considered to be a potent activator of proteasome. In PLA, although HSP70 increased at 2d by approximately 162% and at 8d by approximately 194%, only the later change was found to be statistically meaningful ( $P=0.069$ ). HSP70 remained unaffected in PRO throughout recovery (2d: 18%; 8d: 85%). The online Supplementary Fig. S5 illustrates individual percentage changes (eight participants) of HSP70 between baseline and 2d.

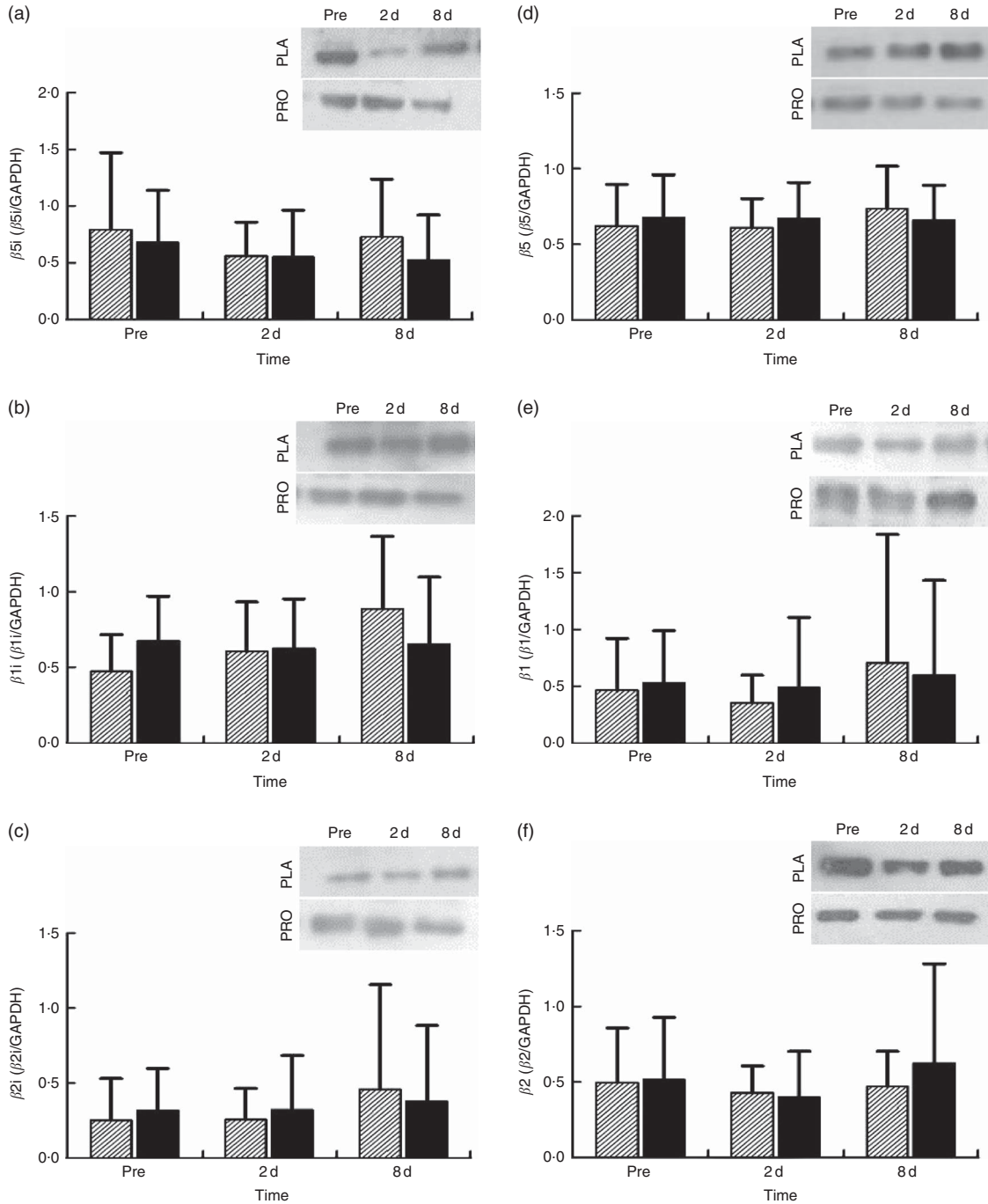
### Correlations

Changes in isometric peak torque were not correlated with changes in proteasome activity (CT-L, C-L and T-L) or changes in the protein expression of  $\beta$ -( $\beta 5$ ,  $\beta 2$  and  $\beta 1$ ) and  $\beta i$ -subunits ( $\beta 5i$ ,  $\beta 2i$  and  $\beta 1i$ ) at 2 and 8d. Similarly, no significant correlation was observed between DOMS and either proteasome activity or protein expression of  $\beta$ - and  $\beta i$ -subunits throughout recovery.

### Discussion

In this study, we investigated the effect of exercise-induced aseptic inflammation and protein ingestion on UPS, muscle damage, inflammatory responses, as well as muscle function. The primary findings of this investigation suggest that milk protein concentrate supplementation may preserve the status of proteasome CT-L activity, as well as accelerate the rate of skeletal muscle strength recovery, under conditions of intense aseptic inflammation.

The extreme model of eccentric exercise applied in this study resulted in severe muscle injury and aseptic inflammation, as indicated by the increased levels of CK, leucocytes, PC and NF- $\kappa$ B phosphorylation. Furthermore, in agreement with previous work, we observed a pronounced decline in force-generating capacity along with a remarkable elevation in DOMS<sup>(8,30)</sup>. Although the rise observed in CK exceeded 2000 U/l during the first 2d of recovery, it was of smaller magnitude than that previously observed in response to the same protocol<sup>(8)</sup>. This difference may be attributed to the higher fitness status of participants in this investigation ( $VO_{2max}$ : 56.4 (SD 6.8) *v.* 47.6 (SD 4.5) ml/kg per min), indicating a superior level of



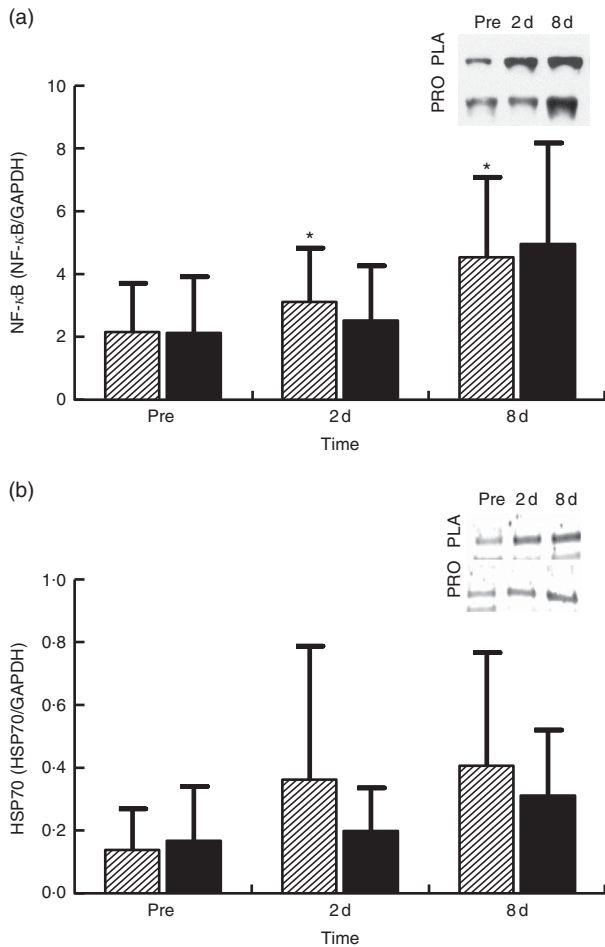
**Fig. 5.** Changes in the expression of proteasome subunits in response to protein (PRO) and placebo (PLA) administration. Changes in PRO levels of (a)  $\beta 5i$ , (b)  $\beta 1i$ , (c)  $\beta 2i$ , (d)  $\beta 5$ , (e)  $\beta 1$  and (f)  $\beta 2$  proteasome subunits during the two trials. Values are means and standard deviations represented by vertical bars. ▨, PLA; ■, PRO.

cardiovascular conditioning, which has been shown to positively affect muscle damage responses<sup>(42)</sup>.

The main proteolytic sites of UPS are hosted in the  $\beta 1$ ,  $\beta 2$  and  $\beta 5$  subunits that exhibit C-L, T-L and CT-L activities, respectively<sup>(14)</sup>. Although changes in the mRNA and protein expression of *muscle atrophy F-box (MAFbx)* and *muscle ring finger-1 (MuRF1)* have been extensively examined in response to different types and loads of exercise alone<sup>(43–46)</sup> or in

combination with nutritional supplements<sup>(47–49)</sup>, alterations in proteasome activities and expression of the  $\beta$ -subunits have been investigated to a lesser extent in response to muscle inflammation. To the best of our knowledge, this is the first study examining the responses of  $\beta$ -subunits,  $\beta i$ -subunits (related to the immunoproteasome) and their activities to aseptic inflammation induced by extremely damaging exercise and in combination with protein supplementation.





**Fig. 6.** Changes of molecules regulating proteasome activity in response to protein (PRO) and placebo (PLA) administration. Changes in PRO levels of (a) phosphorylated NF- $\kappa$ B and (b) HSP70 during the two trials. Values are means and standard deviations represented by vertical bars. ▨, PLA; ■, PRO. Representative immunoblots are shown in panels. \* Significantly different from baseline ( $P < 0.05$ ).

Although the UPS proteolytic pathway is upregulated under inflammatory conditions<sup>(50,51)</sup>, it seems that it contributes to the remodelling of skeletal muscle following muscle injury induced by exercise<sup>(18)</sup>. In previous studies, both mRNA and protein levels of ubiquitin ligases (*MuRF1* and *MAFbx/Atrogin-1*) and the 20S proteasome have been repeatedly shown to be upregulated following eccentric exercise for as long as 24–48 h<sup>(43,45,52)</sup>. Moreover, increased CT-L activity has been observed at 14 d following downhill running<sup>(55)</sup>, further supporting the fundamental role of UPS in skeletal muscle remodelling<sup>(18,19)</sup>. In contrast, we observed a 30% decrease in CT-L activity at 2 d post exercise that was not accompanied by any change in protein levels of 20S proteasome and immunoproteasome  $\beta$ -subunits and  $\beta$ i-subunits, respectively. It is characteristic that all participants demonstrated a decline of CT-L activity of proteasome at 2 d, suggesting that there was limited inter-individual variability on this outcome. Interestingly, our data corroborate previous findings, showing decreased CT-L activity (by 21%) in response to extreme exercise such as a 200-km run<sup>(54)</sup>. The authors

assumed that an inhibitory mechanism results in the decline of proteasomal activity in response to increased load of oxidative stress<sup>(54)</sup>. This assumption may also be valid in our case, as the 300 eccentric contractions protocol performed in our present study resulted in pronounced oxidative stress (40–95%) and inflammatory responses that persisted throughout the recovery phase, especially in PLA, a response also reported previously<sup>(8)</sup>. Likewise, a 200-km run, another form of excessive exercise resulting in EIMD, induced a marked increase in metallothionein 1F, metallothionein 1H and NADPH oxidase by approximately 519, 666 and 162%, respectively<sup>(54)</sup>. Therefore, reduced proteasomal activity has been observed only in response to cases of extreme exercise models (e.g., ultra-marathons, high-volume eccentric exercise) that predispose skeletal muscle to excessive ROS production and redox status disturbances. In agreement with this theory, an excessive amount of protein carbonylation in SH-SY5Y cells has been shown to cause a substantial decrease in proteasome S6 ATPase activity and 26S proteasome-mediated degradation<sup>(55)</sup>.

On the other hand, protein supplementation prevented the decline of proteasome activity. Preservation of UPS activity, and as such clearance of oxidised proteins, might have also contributed to the accelerated recovery of the skeletal muscle following damaging exercise. Interestingly, individual changes of CT-L proteasomal activity at 2 d are very similar, with eight out of eleven subjects exhibiting either maintained or enhanced CT-L proteasomal activity and only three participants exhibiting reduced CT-L proteasomal activity in PRO. To explain this response, we measured changes in HSP70 expression and NF- $\kappa$ B phosphorylation. The molecular chaperone HSP70 protein is considered to directly activate the 26S proteasome activity, thereby preventing the accumulation of unfolding proteins and aggregates<sup>(56,57)</sup>. HSP70 increased markedly at 2 and 8 d post exercise, but this rise was more pronounced in PLA compared with PRO. Indeed, it has been reported that HSP70 exhibits an intensity-dependent response to exercise<sup>(58)</sup>. Furthermore, its expression has been shown to increase under conditions of elevated oxidative stress (as in this case) to promote degradation of oxidised proteins through the 20S proteasome following its dissociation with 26S<sup>(56,59)</sup>. Interestingly, HSP70 has been shown to mediate the dissociation and re-association of the 26S proteasome in response to oxidative damage induced by an acute rise of ROS<sup>(59)</sup>. Therefore, the lower HSP70 response in PRO compared with that observed in PLA may have contributed to the lack of an adverse exercise effect on proteasome activity in PRO. Although the two trials demonstrated HSP70 changes of considerable difference in magnitude, no statistically meaningful differences were detected. However, when individual responses are observed, it is obvious that the vast majority of participants (six out of eight exhibited a reduced response in PRO, one had a similar response in the two trials and one had a more pronounced response in PRO) demonstrated a reduced HSP70 response in PRO compared with PLA.

The NF- $\kappa$ B pathway is one of the main redox-sensitive signalling pathways that regulates inflammatory and oxidative stress responses in human skeletal muscle<sup>(1,5,60)</sup>. It may be activated in response to elevated pro-inflammatory mediators and ROS, but it also controls the expression of genes including

cytokines such as TNF- $\alpha$ , IL-6 and IL-1 $\beta$ , suggesting a vicious cycle between NF- $\kappa$ B activation, inflammatory and oxidative stress responses<sup>(15,61)</sup>. The increased phosphorylation of NF- $\kappa$ B observed following eccentric exercise was mitigated by PRO (although it was not found to be statistically meaningful, NF- $\kappa$ B phosphorylation was lower in PRO compared with PLA by approximately 26% at 2 d and approximately 23% at 8 d). When individual responses are taken into account at 2 d, it appears that 50% of participants showed an attenuated NF- $\kappa$ B response in PRO and 50% did not. Similar perturbations of NF- $\kappa$ B signalling following aseptic muscle injury have been observed with ingestion of thiol-based and other antioxidants<sup>(8,62)</sup>.

Because insulin levels may also interfere with UPS through the IGF1-Akt-FoxO signalling pathway<sup>(11,48)</sup>, we measured glucose and insulin levels during recovery. Both glucose and insulin remained unaltered throughout recovery, suggesting that they had no impact on proteasome activity. This also indicates that the reduction observed in CT-L activity at 2 d was clearly induced by the exercise stimulus *per se*.

In our present study, protein supplementation resulted in the attenuation of markers of muscle damage as evidenced by CK and DOMS values during the post-exercise recovery. Specifically, the elevation of both plasma CK and reported DOMS was greater in the PLA group compared with the PRO group. This effect might be explained by the high BCAA content of milk protein<sup>(63)</sup>, which has been shown to attenuate DOMS following damaging exercise when consumed in adequate amounts<sup>(24,64)</sup>. However, a number of studies reported no effect in response to BCAA supplementation<sup>(65,66)</sup>. This discrepancy among studies may be attributed to differences in the length of the supplementation period, as well as the amount of BCAA administered. For instance, in the study by Nosaka *et al.*<sup>(65)</sup>, it was clearly shown that in contrast to acute supplementation (before and after exercise) long-term supplementation (before and after exercise and at eight more occasions) with amino acids during exercise recovery results in lower plasma CK and muscle soreness.

Soreness develops because of sensitisation of nociceptors at the level of the perimysium and epimysium by various inflammatory stimuli<sup>(64,67)</sup>. In accordance with the above, in our present study, we observed an increase in CK, PC, NF- $\kappa$ B in PLA, whereas PRO resulted in attenuated inflammatory responses. Therefore, it is likely that protein supplementation reduces DOMS via the attenuation of specific inflammatory markers. An alternative mechanism explaining this protein effect on DOMS may be related to feeding *per se*, but a mechanism relating these two is lacking<sup>(24,25)</sup>.

In line with others, protein suppressed the exercise-induced decline in force-generating capacity and resulted in a faster strength recovery compared with PLA<sup>(21,22)</sup>. Although the molecular mechanisms are still obscure, recent evidence suggests that amino acids may promote muscle repair and remodelling by attenuating the excessive inflammatory responses following injury<sup>(68–70)</sup>. In fact, administration of a leucine-enriched protein supplement (70 g protein/15 g leucine) following high-intensity endurance exercise activated a pro-inflammatory transcriptome at 30 min and promoted an anti-inflammatory and pro-myogenic transcriptome at 4 h, characterised by decreased leucocyte migration<sup>(69)</sup>. The transition from a pro- to an anti-inflammatory phenotype, mediated by the progressive shift of M1 macrophages

to M2, is pivotal for muscle regeneration and growth<sup>(60)</sup>. In this study, PRO attenuated exercise-induced inflammatory response, as evidenced by the reduced PC and CK, as well as the unaltered phosphorylation levels of NF- $\kappa$ B at 2 and 8 d. Thus, PRO may have resulted in a faster transition to an anti-inflammatory phenotype and consequently an accelerated recovery of muscle function. NF- $\kappa$ B activation may inhibit myogenesis and increase the expression of many pro-inflammatory cytokines (e.g. TNF- $\alpha$ , IL-6 and IL-1 $\beta$ ) that are able to amplify the inflammatory response<sup>(60)</sup>. A recent animal study reported decreased IL-6 expression and enhanced muscle performance after damaging exercise following supplementation with leucine-enriched essential amino acids<sup>(68)</sup>. Moreover, during phagocytosis, infiltrating neutrophils generate ROS through the NADPH oxidase, a phenomenon known as oxidative burst, which induces secondary damage in skeletal muscle<sup>(71)</sup>. The lower PC levels observed in PRO may suggest, among others, reduced ROS generation that indicates reduced oxidative burst and as such smaller secondary muscle damage. This mechanism may also explain the attenuated reduction of force-generating capacity in PRO during recovery, as previously shown with thiol-based antioxidant supplementation in humans<sup>(8,72)</sup>.

Proteasome activity though was not correlated with peak isometric torque and DOMS, suggesting that neither the former nor the latter is related directly to UPS. In contrast to these findings, the study of Pereira *et al.*<sup>(70)</sup> provided evidence that leucine supplementation in rats, after induction of muscle damage through cryolesion, improved muscle function via suppression of protein ubiquitination and decreased activation of FOX3a at days 3 and 10 of recovery. The discrepancy with that study may be partly explained by the fact that Pereira *et al.*<sup>(70)</sup> measured protein ubiquitination and expression of FOXO family transcription factors that lead to activation of E3 ligases, whereas we evaluated the direct proteasome activity and expression of specific catalytic subunits. Moreover, transcription factors other than FOXO1 and FOXO3a are probably responsible for the activation of E3 ubiquitin ligases in humans (e.g. MuRF1 and MAFbx/Atrogin-1) as compared with animals<sup>(73)</sup>.

## Conclusions

This is the first investigation showing that under severe aseptic inflammation proteasomic activity may be severely impaired. Protein supplementation may not only prevent the attenuation of proteasome activity possibly via the expression of HSP70, reduced oxidative stress and altered NF- $\kappa$ B phosphorylation but also results in reduced muscle discomfort and an acceleration of skeletal muscle strength recovery. These results suggest that PRO may provide a viable nutritional intervention to enhance muscle remodelling and maintain muscle function under conditions of increased inflammation induced by exercise.

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A. A., M. P., A. Z. J. and I. G. F. contributed to study implementation. D. D., N. C., A. C., A. S., A. A., M. L., M. P., C. K. D., L. G. K. and I. G. F. performed the sample analysis and data interpretation. D. D., N. C., L. G. K. and I. G. F. wrote the first version of the manuscript. All authors contributed to the paper, reviewed it and approved the final version of the manuscript.

The authors declare that there are no conflicts of interest.

### Supplementary material

For supplementary material/s referred to in this article, please visit <https://doi.org/10.1017/S0007114517001829>

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