The effect of dietary arachidonic acid supplementation on acute muscle adaptive responses to resistance exercise in trained men: a randomized controlled trial

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Abstract

Arachidonic acid (ARA), a polyunsaturated ω-6 fatty acid, acts as precursor to a number of prostaglandins with potential roles in muscle anabolism. It was hypothesized that ARA supplementation might enhance the early anabolic response to resistance exercise (RE) by increasing muscle protein synthesis (MPS) via mammalian target of rapamycin (mTOR) pathway activation, and/or the late anabolic response by modulating ribosome biogenesis and satellite cell expansion. Nineteen men with ≥1 year of resistance training experience were randomized to consume either 1.5 grams daily of ARA or a corn-soy oil placebo in a double-blind manner for 4-weeks. Participants then undertook fasted RE (8 sets each of leg press and extension at 80% 1RM), with *vastus lateralis* biopsies obtained before exercise, immediately post-exercise, and at 2, 4 and 48 hours of recovery. MPS (measured via stable isotope infusion) was not different between groups (P=0.212) over the 4-hour recovery period. mTOR pathway members p70S6K and rpS6 were phosphorylated post-exercise (P<0.05), with no difference between groups. 45S pre-ribosomal RNA (rRNA) increased 48 hours after exercise only in ARA (P=0.012). $NCAM^+$ satellite cells per fibre increased 48 hours after exercise (P=0.013), with no difference between groups (P=0.331). Prior ARA supplementation did not alter the acute anabolic response to RE in previously resistance trained men; however, at 48 of recovery, ribosome biogenesis was stimulated only in the ARA group. The findings do not support a mechanistic link between ARA and short-term anabolism, but ARA supplementation in conjunction with resistance training may stimulate increases in translational capacity.

Key words: omega 6, muscle protein synthesis, resistance training, satellite cells, ribosome biogenesis

New & Noteworthy: 4-weeks of daily arachidonic acid supplementation in trained men did not alter their acute muscle protein synthetic or anabolic signaling response to resistance exercise. However, 48 hours after exercise, men supplemented with arachidonic acid showed greater ribosome biogenesis and a trend towards greater change in satellite cell content. Chronic arachidonic acid supplementation does not appear to regulate the acute anabolic response to resistance exercise, but may augment muscle adaptation in the following days of recovery.

Introduction

Resistance training is a common modality used by athletes in power and aesthetic sports to induce muscle hypertrophy (44) and improve physical performance (57). Muscle hypertrophy is thought to result from repeated stimulation of muscle protein synthesis (MPS) during exercise recovery (49). Resistance exercise also expands the available machinery for protein synthesis (translational capacity) via promoting ribosome biogenesis (3, 18) and recruits muscle stem cells (satellite cells) which are required for myofiber regeneration and myonuclear accretion (3). The rate of muscle hypertrophy and increased physical performance is most rapid at the onset of training in exercise naïve individuals, with adaptations associated with training beginning to plateau after longer training periods (1). Once this occurs, training volume and nutritional intake gain importance to support further adaption (44).

Arachidonic acid (ARA) is a polyunsaturated ω-6 fatty acid (23) which acts as metabolic precursor to bioactive lipid mediators with potential roles in regulating muscle hypertrophy (25, 33). ARA consumed in the diet is incorporated into cell membrane phospholipids (68, 69). In response to mechanical loading of muscle cells, ARA is released to the cytosol by phospholipase A2 (66), where it is converted to prostaglandins (PG) $F_{2\alpha}$ ($PGF_{2\alpha}$) and PGE_2 by the cyclooxygenase (COX) pathway (60, 65, 67). $PGF_{2\alpha}$ signals via the mammalian target of rapamycin (mTOR) and extracellular regulated kinase (ERK) pathways to stimulate MPS (54, 65) and promotes myonuclear accretion via NFATC2-dependent myoblast fusion events (21, 22). PGE_2 also plays a direct and indispensable role in satellite cell expansion in skeletal muscle (20). Free ARA availability and PG biosynthesis both increase in the early hours following a bout of acute resistance exercise (35). Blockade of the exercise-induced PG response with non-steroidal anti-inflammatory drugs (NSAIDs) blunts

muscle protein synthesis (MPS) (64), mTOR/ERK signalling (36) and satellite cell proliferation (30, 39) acutely, and can compromise long-term muscle strength/hypertrophic adaptations in young adults undergoing resistance training (29).

On the basis of the role of the COX/PG pathway in muscle growth and development, it has been suggested that ARA may have potential ergogenic effects. ARA supplementation promotes $PGF_{2\alpha}$ and PGE_2 synthesis (31, 47, 60), stimulates MPS , increases myoblast proliferation (28) and dramatically increases the size and myonuclear content of developing muscle fibres *in-vitro* (7, 31). Two chronic resistance training studies have found that dietary ARA supplementation is able to increase muscle power output in previously trained men (15, 53). However, these studies reported inconclusive results as to the ability of arachidonic acid to induce gains is lean body or muscle mass and strength. Additionally, a single animal study demonstrated that ARA supplementation had only a minor effect of anabolic signalling following a bout of electrical stimulation (15). We have recently shown that dietary ARA supplementation rapidly and safely modulates muscle fatty acid composition and enhances myogenic gene expression in resting muscle (34). It is currently unknown if ARA supplementation influences the anabolic response to an acute bout of resistance exercise in humans *in vivo*.

Therefore, the primary purpose of the present study was to test the effects of 4 weeks of dietary supplementation with ARA or corn/soy oil placebo on acute changes in MPS following a single high volume bout of resistance exercise in previously trained young men. The secondary aims of the study were to characterize muscle anabolic signalling via the mTOR and ERK pathway responses to resistance exercise in this cohort. Lastly, the participants returned to the laboratory 48 hours after exercise in order to test for group differences in $NCAM^+$ satellite cell number and ribosome biogenesis.

Methods

Participants

19 healthy resistance trained young men (Table 1) were recruited via flyers and social media to participate in the study. They were free of neuromuscular or metabolic disease, not taking any medication and had no existing injuries which would limit their ability to exert maximal force. Smokers were also excluded from the study. The use of vitamin/mineral supplements, protein/amino acid supplements or creatine did not preclude participation. However, no consumption of fatty acid supplements such as fish oil were allowed for at least one month prior to the commencement of the trial. All participants had at least one year of experience with lower body resistance training and regularly performed at least one lower body training session per week. The study was conducted in accordance with the most recent version of the Declaration of Helsinki and was approved by the Northern Health and Disability Ethics Committee (New Zealand) (14/NTA/147). The experimental protocol was explained in detail to participants before the commencement of the study and informed written consent was obtained. The study was registered as a clinical trial with the Australian New Zealand Clinical Trials Registry as ACTRN12615000710527.

Study design

Participants were randomized to consume 1.5 g/day of ARA or a placebo for 4 weeks in a double blind fashion. Sequences for randomization were generated using www.random.org and allocation was conducted using a locked spreadsheet at a 1:1 ratio. Four weeks prior to the first experiential visit, participants' one repetition maximum (1RM) for the leg press and extension exercise was estimated by having them perform a set of each exercise where momentary muscular failure was achieved in less than six repetitions and then applying the Brzycki equation (8). Prior to the first experimental visit, participants abstained

from lower body resistance training for 72 hours and any vigorous exercise or alcohol consumption for 24 hours. The evening prior they consumed their last meal by 10 pm then arrived at laboratory at ~7 am the following morning in a fasted state. Participants rested in a supine position while a primed constant infusion of L-[ring-$^{13}C_6$] phenylalanine (prime: 2 $\mu mol \cdot kg^{-1}$; infusion: 0.05 $\mu mol \cdot kg^{-1} \cdot min^{-1}$) was commenced and maintained for the remainder of the visit via a cannula in an antecubital vein. A second cannula was inserted into an antecubital vein in the contralateral arm and hourly plasma samples were collected in EDTA coated vacutainers. After two hours of supine rest a muscle biopsy was obtained from the *m. vastus lateralis* under local anesthesia (1% xylocaine) using the suction-modified Bergström technique. Participants then completed two warmup sets of bilateral leg press consisting of 10 repetitions at 50% and 70% of 1RM followed by six sets of 10 repetitions at 80% of 1RM. This was followed by eight sets of 10 bilateral leg extensions at 80% of 1RM. Two minutes of rest was taken between each set and exercise. The last set of each exercise was performed until momentary muscular failure. Within 10 minutes of the completion of exercise a second muscle biopsy was obtained at least 2 cm distal to the first biopsy. Participants then rested in a supine position for the next four hours with water available *ad libitum*. Additional biopsies were obtained at two and four hours after exercise from the non-dominant limb through separate incisions ~ 2 cm apart moving from proximal to distal. 48 hours after the exercise bout participants returned to the laboratory and a final biopsy was collected from the contralateral (dominant) limb.

Supplement

Participants consumed four capsules of either ARA or placebo each day during the supplementation period. Two capsules were consumed in the morning and two in the evening. On training days, participants were instructed to ingest one of these two doses prior to the exercise session. Supplemental ARA was provided as ARASCO® capsules containing

a mixture of oil extracted from the unicellular fungus *Mortierella alpina* and high oleic sunflower oil (HOSO) constituting a total daily dose of 1.58 grams of ARA. The placebo (PLA) capsules (Pbo, Corn-Soy, 1gVegCap) used were indistinguishable from the ARA capsules but contained a soy-corn oil blend (1 g per capsule) lacking ARA. The precise fatty acid composition of the ARA and placebo capsules as determined by GC-MS analysis was reported previously (34).

Muscle protein synthesis

The methods for the calculation of myofibrillar protein synthesis have previously been described in detail , briefly, muscle samples (~40-50 mg) were homogenized using a TissueLyser in a lysis buffer containing protease/phosphatase inhibitor cocktail (HaltTM Protease and Phosphatase Inhibitor Cocktail, Thermo Scientific, cat. 78442). Samples were then centrifuged at 15,000 *g* for 10 min at 4 °C. The supernatant was collected for western blot analysis and the solid pellet was processed as previously described in order create a pellet enriched in myofibrillar proteins. The pellet was hydrolyzed in 6 M HCL at 110 °C overnight. Ion-exchange chromatography was then used to purify the free amino acids followed by conversion to their *N*-acetyl-*N*-propyl ester derivatives for analysis by gas chromatography-combustion-isotope ratio mass spectrometry (IRMS model: Delta Plus XP; Thermo Finnagan) (41). Fractional synthetic rate was calculated by dividing the difference in myofibrillar enrichment between time points by the average plasma enrichment over the same time period and multiplying by the time between biopsies and then multiplying by 100. Briefly, the isotopic enrichment of plasma samples was determined by negative chemical ionization gas chromatography–mass spectrometric analysis of a heptafluorobutyric, *n*-propyl derivative (42). $^{13}C_6$-phenylalanine enrichment was measured using methane negative chemical ionization GC-MS (Agilent 5973 EI/CI MSD with an Agilent 6890 GC). A Phenomenex ZB-1MS capillary column was used to

separate the derivative of phenylalanine. Selected ion chromatograms were obtained by monitoring ions *m/z* 383 and 389 for L-phenylalanine and L-[$^{13}C_6$] phenylalanine, respectively. Isotope enrichment in mole % excess was calculated from peak area ratios at isotopic steady state and baseline. The final value for all determinations was corrected using an enrichment calibration curve. MPS was calculated for the early post exercise period between the immediate post exercise biopsy and the 2h post-exercise biopsy. A late post exercise MPS was also calculated between the 2 and 4 h post exercise biopsies. Total aggregate MPS rate was also calculated between the immediate post exercise biopsy and the 4h post exercise biopsy.

Immunoblotting

Western blotting was conducted as per previously published methods (42). Briefly, protein homogenate concentrations were calculated via a bicinchoninic acid assay as per manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). 20 µg aliquots of total protein were suspended in Laemmli buffer and boiled at 95^0C for 5 minutes. Samples were stored in -80° C for further use. Samples from each participant were loaded in adjacent lanes with one subject from each treatment group loaded on each gel. Samples were subjected to SDS-PAGE and transferred to PVDF membranes using Trans-Blot® Turbo™ Transfer System (Bio-Rad, Hercules, CA, USA), blocked in 5% BSA/TBS (0.1% Tween 20) for 1 h at room temperature followed by overnight 4° C incubation in primary antibodies (1:1000) (p-p70S6K $^{(Thr389)}$ (Cell Signalling, 9205S), p-p70S6K $^{(Thr421/Ser424)}$ (Cell Signalling, 9204S), p-rpS6 $^{(Ser235/236)}$ (Cell Signalling, 4865S), p-rpS6 $^{(Ser240/244)}$ (Cell Signalling, 2215S) and p-p38MAPK $^{(Thr180/Tyr182)}$ (Cell Signalling, 4511S); (1:2000) p-ERK1/2 $^{(Thr202/Tyr204)}$ (Cell Signalling, 4370S) and p-p90RSK $^{(Ser380)}$ (Cell Signalling, 11989S); and (1:1000) UBF (Santa Cruz biotechnology, 13125), with (1:5000) GAPDH (Abcam, ab9485) with gentle agitation. The following morning membranes were washed in TBST for 25 min and probed with HRP

conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature. Membranes were washed with TBST for a further 25 min and visualised using ECL Select Western blotting detection reagent (Amersham, UK) with chemiluminescent signals captured with a ChemiDoc™ MP Imaging System (Bio-Rad, Hong Kong, China). Protein band densitometry was quantified using ImageJ software. Abundance of proteins of interest was normalised for protein loading by stripping and re-probing membranes for GAPDH.

RNA Extraction

Total RNA was extracted from approximately 20 mg of frozen muscle tissue using the AllPrep® DNA/RNA/miRNA Universal Kit (QIAGEN GmbH, Hilden, Germany) as previously described (13). RNA concentration was measured using a NanoPhotometer® N60 (IMPLEN, Inc., Westlake Village, CA, USA). RNA (1 μg) was reverse transcribed using a High Capacity RNA-to-cDNA Kit (Life Technologies, Carlsbad, CA, USA).

Real-Time polymerase chain reaction (RT-PCR)

RT-PCR was performed on a LightCycler 480 (Roche Applied Science, Penzberg, Germany) using SYBR™ Green I Master Mix (Roche Applied Science). Results are expressed as each participant's fold change in mRNA expression from their respective baseline sample following normalization to an endogenous control using the $2^{-\Delta\Delta Ct}$ method. The geometric mean of human *CHMP2A*, *HPRT* and *VCP* mRNA expression was used as an endogenous control. Primers (Table 2) for the mRNA analysis were obtained from Invitrogen (Life Technologies, Carlsbad, CA, USA). For pre-rRNA and rRNA analysis, primers were designed by QIAGEN, using RT^2 Profiler PCR Arrays (QIAGEN). These primers have been described in details elsewhere (38).

Immunohistochemistry

Muscle biopsies were cryosectioned at 8 μm, air dried at room temperature and stored at -80 °C. Before immunostaining, frozen sections were air-dried and subsequently fixed with 1% PFA for 7 min. Sections were permeabilized with 0.2% Triton-X in PBS for 20 min and blocked with 1% bovine serum albumin/20% goat serum/1 % dry milk/0.2% Triton-X in PBS for 1 hr at room temperature. Sections were incubated with primary antibodies at 4°C overnight and secondary antibodies for 1 hr at room temperature. The following primary antibodies were used: rabbit anti-laminin (1:100; Dako, Z0097) and mouse anti-NCAM (1:100; Abcam, no. AB55494). Secondary antibodies used were: goat anti-mouse (1:200; Alexa Fluor 488, Invitrogen) or goat anti-rabbit (1:200; Alexa Fluor 594, Invitrogen). Nuclei were co-stained with 4',6-diamidino-2-phenylindole (DAPI) (ProLong Gold Antifade Reagent with DAPI, P36935, Invitrogen). Sections were imaged using an Axi-ocam camera (Zeiss, Oberkochen, Germany) mounted on an Axioskop-2 light microscope (Zeiss). Satellite cells were identified by DAPI staining surrounded by NCAM staining inside the laminin ring and are presented as the number of positive cells per fiber.

Statistical analysis

The study was powered at ten participants per group using the predefined primary outcome of post exercise MPS based on a 0.038%/h difference between groups with a standard deviation of 0.03%/h (12), 80% power and alpha set at 5%. Differences in satellite cell number, MPS and anabolic signalling were assessed using two-way ANOVA with group as a between subject factor and time as repeated factor. The holm-sidak post hoc procedure was used where appropriate. An independent sample T-test was used to test for differences in post exercise aggregate MPS. Normality was assessed using the Shapiro–Wilk test and data

were log transformed if required. All analysis was conducted using SigmaPlot 13 (SYSTAT Software, San Jose, CA, USA). Alpha as set at $P<0.05$ and means are shown as ± S.E.M.

Results

Muscle protein synthesis

Myofibrillar protein synthesis was not different between groups in the early (0-2 h) post or late (2-4 h) post exercise period (Figure 1A). Furthermore, there were no between group differences in the aggregate rate of myofibrillar protein synthesis for the full four-hour post exercise period (Figure 1B). The plasma precursor pool enrichment of $^{13}C_6$-phenylalanine was stable throughout the full measurement period and was not different between groups (Figure 2).

Anabolic signalling

p70S6K $^{(Thr389)}$ was increased immediately post exercise ($P<0.001$) and at four hours post exercise ($P=0.007$), with no differences between groups (group × time interaction $P=0.478$) (Figure 3A). p70S6K$^{(Thr421/Ser424)}$ followed the same pattern, but was also elevated at two hours post exercise also ($P=0.001$), with no between group differences (group × time interaction $P=0.954$) (Figure 3B). rpS6 $^{(Ser240/244)}$ phosphorylation was increased immediately following exercise ($P<0.001$) and at four hours post exercise ($P=0.009$), with no difference between groups (group × time interaction $P=0.630$) (Figure 3C). rpS6 $^{(Ser235/236)}$ phosphorylation was elevated immediately post, two and four hours after exercise ($P<0.001$) with no difference between groups (group × time interaction $P=0.326$) (Figure 3D).

Phosphorylation of ERK1/2$^{(Thr202/Tyr204)}$ was not significantly increased at any time point following exercise, but there was a tendency for increased phosphorylation immediately post exercise (main time effect $P=0.089$), with no difference between groups (group × time interaction $P=0.810$) (Figure 4A). p90RSK $^{(Ser380)}$ increased at all post exercise time points ($P<0.001$), but was not different between groups (group × time interaction $P=0.355$) (Figure

4B). p38MAPK$^{(Thr180/Tyr182)}$ phosphorylation following exercise was not elevated above baseline (P=0.094), but was greater immediately post compared to two hours post exercise (P=0.005). The post-exercise change in p38MAPK$^{(Thr180/Tyr182)}$ phosphorylation did not differ between groups (group × time interaction p=0.743) (Figure 4C). Representative Western blot images are shown in Figure 5

Satellite cells

A representative satellite cell image is shown in Figure 6A. The absolute number of NCAM^{+} cells per 100 fibres was increased 48 hours after exercise (main effect P=0.013) with no differences between groups (group × time interaction P=0.212) (Figure 6B). The percentage change in NCAM^{+} cells from pre-exercise showed a statistical trend for a group by time interaction (p=0.066), with a greater % change in the ARA group compared to placebo at 48 h (p=0.017) (Figure 6C). *PAX7* gene expression was not altered acutely by exercise in either group (main time effect P=0.842, group × time interaction P=0.022), but was greater 48 hours post exercise in the ARA group compared to placebo (P=0.002) (Figure 7A). *NCAM* gene expression was increased from baseline at 48 hours post-exercise (P=0.038), with no difference between groups (group × time interaction P=0.245). However, there was a trend towards greater *NCAM* expression in the ARA compared with placebo group at 2 hours post-exercise (P=0.070) (Figure 7B). mRNA expression of *MYOG* was increased at 2 hours post-exercise (P=0.004). Although overall differences between groups were apparent (group × time interaction P=0.127), *MYOG* mRNA expression was higher in the placebo compared to the ARA group at 4 hours post exercise (P=0.014) (Figure 7C).

Ribosome biogenesis

Analysis of muscle expression of precursor and mature ribosomal RNA (pre-rRNA and rRNA, respectively) are shown in Figure 8. There was a group by time interaction effect (P=0.012) for expression of the 45S pre-rRNA such that expression increased 48 hours post exercise only in the ARA group (P=0.007) (Figure 8A). ITS+28S also showed a group by time interaction (P=0.016) and was increased 48 hours post-exercise only in the ARA group (P=0.001) (Figure 8B). ITS+5.8S displayed a group × time interaction effect (P=0.016), increasing in the ARA group only (P=0.001) at 48 h post-exercise (Figure 8C). Resistance exercise had no effect on rRNA expression of the 28S rRNA (time effect P=0.457, Figure 8D). There was a time by group interaction effect for 5.8S rRNA (P=0.019) such that 5.8S rRNA expression increased at 48 hours post-exercise only in the ARA group (P=0.002) (Figure 8E). The 18S rRNA was not altered with exercise or supplementation (time effect P=0.351), Figure 8F). Ribosomal RNA5S (5S rRNA) expression exhibited a non-significant trend towards an increase at 48 h post-exercise (P=0.066), with no difference between groups (group × time interaction P=0.214) (Figure 8G). The fold-change in muscle rRNA expression at 48 h post-exercise was greater in the ARA group compared to placebo for 45S (Figure 8A P=0.001), ITS+28S (Figure 8B, P=0.001), ITS+5.8S (Figure 8C, P=0.001), and 5.8S (Figure 8E, P=0.002).

Protein expression of the rDNA upstream binding factor (UBF) is shown in (Figure 9). There were group by time interaction effects for total UBF (Figure 9A, P=0.040) and trends towards interactions for UBF 1 (Figure 9B) P=0.052) and UBF 2 (Figure 9C, P=0.081). Total UBF (P=0.011) and UBF 1 (P=0.021) were increased at 48 hours post exercise only in the ARA group. There was a trend for UBF 2 to increase at 48 hours post-exercise also (P=0.076). Compared to placebo, the ARA group showed greater fold change in muscle abundance of total UBF (P=0.007), UBF 1 (P=0.021) and UBF 2 (P=0.025).

Discussion

Four weeks of ARA supplementation did not alter the early myofibrillar MPS response following an acute bout of resistance exercise. Whilst there was an expected effect of resistance exercise to stimulate translation initiation signalling via the mTOR and ERK pathways during the first four hours post-exercise, this response was also not influenced by ARA supplementation. Following 48 hours of recovery, however, markers of ribosome biogenesis were increased to a greater extent in men receiving ARA supplementation compared to placebo. Similarly, the % change in muscle satellite cell number 48 hour after exercise tended to be greater with ARA than placebo. Therefore, ARA supplementation does not appear to modulate early anabolic responses to resistance exercise performed in a fasted state, but there remains the possibility that it may modulate the degree of tissue remodelling during the latter stages of exercise recovery via positive effects on myofiber translational capacity and satellite cell kinetics.

Myofibrillar protein synthesis measured by a primed constant infusion of $^{13}C_6$ phenylalanine was not different between groups in the four-hour recovery period following resistance exercise. The study design used did not allow for a direct measurement of the degree of increase in MPS from baseline or for the identification of potential between group differences in basal rate of MPS. However, numerous previous studies have established that an acute bout of resistance exercise stimulates MPS, even in the fasted state (24, 50). The participants were well accustomed with habitual resistance training which may have slightly attenuated their absolute post exercise MPS rates compared to exercise naïve individuals (24, 62). Nevertheless, based on the robust stimulation of phosphorylation of the downstream mTOR targets p70S6K and rpS6, the participants were still able to mount an anabolic response to the high volume exercise stimulus (26). Previous work has shown that pharmacological blockade of the COX pathway via oral ingestion of non-selective NSAIDs

(e.g. ibuprofen) depresses resistance exercise induced PG production , which in turn blunts early anabolic signalling (36) and delayed mixed muscle MPS at 24 h of recovery (67). *In vitro* models suggest that provision of ARA substrate can increase COX dependent PG synthesis/release (31, 47, 60), resulting in increased phosphorylation of p70S6K (7), muscle protein turnover and myotube hypertrophy (31). In particular, $PGF_{2\alpha}$ which is released by muscle cells in response to mechanical loading (60, 65, 67), appears to directly mediate ERK/p70S6K/rpS6 phosphorylation (32) and MPS (54, 65). Therefore, we hypothesised that MPS and muscle anabolic signalling would be enhanced early post-exercise in men receiving prior ARA supplementation compared with placebo. The present results could suggest that despite increasing muscle ARA concentrations above placebo levels (34) dietary supplementation did not result in sufficient muscle ARA concentrations to stimulate PG production, possibly due length of the supplementation period. It is also possible COX/PGs behave differently *in vivo*, or that resistance exercise induced increases in PG production are sufficient to maximally stimulate anabolic signalling/MPS following exercise in the absence of ARA supplementation. Our results agree with a recent rodent study conducted by De Souza et al. which showed an equivalent increase in anabolic signalling and MPS following electrical stimulation in rats after ARA supplementation on control feeding.

A strength of this study was that participants were studied in a fasted state in order to isolate the effects of prior ARA supplementation on muscle responses to resistance exercise alone. The lack of feeding also make the results more comparable to the study by De Souza et al. (15) where the animals were fasted prior to electrical stimulated muscle contractions. This design introduces a potential limitation, however, because in practice protein containing meals are often consumed in the hours following resistance exercise , which is well established to result in further stimulation of both the mTOR pathway (14) and MPS (43). It is possible that since resistance exercise performed in a fasted state results in net muscle

protein catabolism , that the combination of resistance exercise and protein feeding would have been required in order for ARA to exert an effect on MPS or anabolic signalling. Indeed, *in-vitro* ARA treatment has been shown to enhance muscle insulin sensitivity (10, 11, 56, 59) and insulin stimulated MPS has been reported to be dependent on conversion of ARA to PGs (e.g. blocked by NSAIDs) (46, 51). Thus, it is possible that ARA supplementation may influence acute MPS responses to feeding or pharmacologically induced hyperinsulinemia, similar to that reported recently for n-3 PUFA supplementation (27, 58, 59). However, it is also possible that consuming a bolus dose of protein after exercise would have maximized the acute anabolic response in both groups and thus could have prevented the detection of an ARA effect in the hours following exercise. Future studies may extend our findings by investigating the influence of prior ARA supplementation on changes in muscle protein turnover induced by nutrient intake alone and post-exercise nutrient ingestion. The present study only measured MPS in the hours following exercise, whereas it is known that elevated MPS especially in response to feeding can persist for days following resistance exercise (9). It is possible that using a different approach such as a deuterium oxide tracer to measure longer term MPS would have yielded an effect of ARA on MPS 24-48 hours post exercise when ribosome biogenesis was increased.

Participants were not provided with an acute dose of ARA on the day of the experimental trial prior to tracer infusion or during the early exercise recovery period. This is because it was hypothesized that incorporation of ARA into muscle lipids following a period of prior dietary supplementation, rather than acute ARA ingestion per se, would influence anabolic signalling and MPS in response to a subsequent bout of resistance exercise. Nevertheless, provision of a single dose of free ARA substrate has been found to modulate acute rates of protein turnover in rodent skeletal muscle *ex-vivo* (47, 54, 60). Therefore, we cannot discount the possibility that ingestion of an acute dose of ARA within close proximity

to the bout of resistance exercise may have had an additional influence on MPS. Future studies may therefore investigate the effect of acute ARA ingestion on basal or exercise-induced MPS in humans.

Satellite cells play an important role in repair of damaged muscle, tissue remodelling (19) and may be regulate extreme muscle hypertrophy (48). In the present study, the number of cells in muscle staining positive for the satellite cell marker NCAM was increased 48 hours after exercise in both groups. However, there was a tendency for a greater percentage increase in $NCAM^+$ cells in the ARA group (84%) compared with placebo (16%). NCAM mRNA expression also tended to increase to a greater extent in the ARA group than placebo at 2 hours after exercise. Additionally, mRNA expression of the satellite cell marker PAX7 was greater in the ARA group compared with placebo at 48 hours after exercise. Together these data suggest that ARA may exert a small but positive effect on the resistance exercise induced muscle satellite cell proliferation *in vivo*. Previous cell culture work has shown satellite cell proliferation (28), myoblast differentiation (7) and myonuclear accretion/myotube hypertrophy (31) are all enhanced *in vitro* with increasing supplemental ARA concentrations Moreover, pharmacological blockade of the COX pathway by NSAID administration has been shown to blunt both exercise induced satellite cell activity *in vivo* (30, 39), as well proliferation, differentiation and fusion of satellite cells cultured *in vitro* (31, 38). Although, less pronounced than cell culture based studies, prior dietary ARA supplementation appears to result in a small increase in satellite cell proliferation following a subsequent bout of resistance exercise in trained men.

Ribosome biogenesis has emerged recently as an important regulator of muscle mass (17). Synthesis of the 45S pre-ribosomal RNA (pre-rRNA), a bottleneck for ribosome biogenesis, increases following acute resistance exercise, an effect that persists for at least 24 or 48 h into recovery (18, 61). 45S pre-RNA is subsequently processed to mature 28S, 18S,

and 5.8S rRNAs, which together with the independently transcribed 5S rRNA, comprise the small (18S rRNA) and large (28S, 5.8S, 5S rRNA) ribosomal subunits (17). In the current study, we assessed the effect of ARA supplementation on pre- and mature rRNA expression at 48 h following resistance exercise. As expected, and consistent with previous work (18), expression of the mature rRNAs 28S and 18S were unaffected following an acute bout of resistance exercise irrespective of ARA supplementation. Since rRNAs are the most abundant cellular RNA, multiples bouts of exercise (e.g. resistance training) are likely to be required before changes in pre-rRNA synthesis can be observed at mature rRNA level (16). However, expression of 5.8S rRNA increased only in participants receiving the ARA supplement. This was unexpected as mature 18S, 28S or 5S (transcribed independently of 45S) levels were not increased. It is possible that the smaller size of the 5.8S rRNA could allow for greater sensitivity and earlier post exercise increases in expression. However, given that the two largest mature rRNAs are not increased at 48 hours following exercise we conclude that a single bout of resistance exercise irrespective of ARA supplementation is insufficient to induce increases in the capacity for protein translation.

To quantify rDNA transcription, three different primer sets, targeting sequences spanning the internal or external transcribed spacer (respectively IST and ETS) and mature rRNA regions of the 45S pre-RNA, including ITS+5.8S, ITS+28S and 5'ETS (often referred to as simply 45S) were used (16). Contrary to our previous study (18), the placebo group did not exhibit any increase in 45S pre-rRNA abundance. This may have been due to subject training status, which is a key determinant of the ribosome biogenesis response to RE (45). Despite similar inclusion criteria, differences in training status between the current and our previous studies (18, 52) may have blunted the RE response. This is supported by a ~11kg higher lean body mass in participants in the current study with no differences in height. The observed blunted response may have also been due to differences in the RE bout in each

study. For example, the present study had fewer exercises and a slightly lower exercise volume. In contrast to placebo, expression of 45S pre-rRNA was increased at 48 h post exercise in the ARA group, by all three independent primer sets (5'ETS, ITS+5.8S, ITS+28S). Although it is currently unknown how ARA supplementation may have promoted ribosome biogenesis, an inflammatory response could have impacted in the pre-rRNA synthesis. A potential link between ARA supplementation and ribosome biogenesis is via the inflammatory cytokine IL-6 which has been shown to promote pre-rRNA synthesis in cancer cells (5, 6). ARA supplementation upregulates IL-6 expression in other cell types in-vitro (2, 4). Moreover, ARA supplementation increased muscle UBF protein levels suggesting that ARA supplementation may enhance ribosome biogenesis via increased expression of components of the pre-initiation complex (PIC), leading to increased capacity for Polymerase I activity (55). Clearly, the potential mechanism by which ARA supplementation enhances the muscle ribosome biogenesis response to resistance exercise warrants further work.

Two prior human trials have tested the effects of long term ARA supplementation on resistance training mediated muscle hypertrophy and performance in resistance trained men (15, 53). ARA supplementation increased muscle power output in both studies, but conflicting results were found for muscle mass (15, 53). Whereas Roberts et al. (53) showed a similar increase in lean mass with resistance training regardless of ARA supplementation, De Souza et al. (15) observed muscle hypertrophy only in those participants consuming the ARA supplement. The results of the current study are not directly comparable to long-term changes in training induced muscle hypertrophy. However, MPS (37), anabolic signalling (40, 63) and ribosome biogenesis (16) have all been found to be positively correlated with resistance training induced muscle hypertrophy. Therefore, although acute MPS and anabolic signalling were not influenced by ARA supplementation, short term differences in ribosome biogenesis

and possibly satellite cell activity may support greater muscle hypertrophy in men participating in resistance training with regular ARA supplementation.

In conclusion, four weeks of prior ARA supplementation did not alter the acute MPS or anabolic signalling response to a single bout of fasted resistance exercise in trained men. Despite this, following 48 hours of recovery there was increased ribosome biogenesis and a greater % change in the number of $NCAM^+$ satellite cells in the muscle of the ARA group. Therefore, ARA supplementation does not appear to alter the muscle anabolic response which occurs in the early hours of recovery, but may regulate muscle remodelling and translational capacity in the days following exercise.

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Figure 1. Myofibrillar Protein Synthesis. Early and late post exercise myofibrillar protein synthesis response (A) and aggregate 0-4 hour post exercise myofibrillar protein synthesis response (B). Error bars represent means ± S.E.M.

Figure 2. Plasma Precursor Pool Enrichment. Tracer to tracee ratio of $^{13}C_6$ phenylalanine to unlabelled phenylalanine in plasma. Error bars represent means ± S.E.M.

Figure 3. mTOR Pathway Activation. Phosphorylation of downstream mTOR targets p70S6K$^{(Thr389)}$ (A), p70S6K$^{(Thr421/Ser424)}$ (B), rpS6 $^{(Ser240/244)}$ (C), and rpS6 $^{(Ser235/236)}$ (D). Error bars represent means ± S.E.M. Horizontal lines represent main effects for time. * Represents a difference from pre training at a given time point ($P<0.05$).

Figure 4. MAPK Pathway Activation. Phosphorylation of MAPK pathway targets ERK1/2$^{(Thr202/Tyr204}$), (A), p90RSK $^{(Ser380)}$ (B), and p38MAPK$^{(Thr180/Tyr182)}$ (C). Error bars represent means ± S.E.M. Horizontal lines represent main effects for time. Ψ represents a difference between the immediately post and 2 hour post exercise time points ($P<0.05$).

Figure 5. Representative Western blots.

Figure 6. Satellite cell number. A representative histology image (A) show an NCAM+ satellite cells indicated with a white arrow, NCAM is shown as green, nuclei (DAPI) and laminin shown in red. The number of NCAM+ satellite cells expressed pre 100 muscle fibres in the main panel (B) and as a percentage change from baseline in the inset (C). Error bars represent means ± S.E.M. Horizontal lines represent main effects for time. * Represents a difference from pre training at a given time point ($P<0.05$).

Figure 7. Myogenic Gene expression. The expression of *PAX7* (A), *NCAM* (B) and *MYOG* (C) mRNA as a fold change from pre exercise normalized to the geometric mean of 3 housekeeping genes. Error bars represent means ± S.E.M. Horizontal lines represent main effects for time. * Represents a difference from pre training at a given time point ($P<0.05$). #

764 represents a difference between ARA and Placebo at a given time point (P<0.05). Where no
765 significant effects apparent P values for trends towards between group differences at a given
766 time point are indicated directly on the graph.

767 **Figure 8. Ribosomal RNA.** Expression of 45S (A), ITS+28S 8S (Internal transcribed
768 spacer) (B), ITS+5.8 (C), 28S (D), 5.8S (E), 18S (F) and 5S (G) ribosomal RNA fold change
769 from pre exercise normalized to the geometric mean of 3 housekeeping genes. Error bars
770 represent means ± S.E.M. * Represents a difference from pre training at a given time point
771 (P<0.05). # represents a difference between ARA and Placebo at a given time point
772 (P<0.05).

773 **Figure 9. UBF protein.** Total UBF (upstream binding factor) (A) and UBF 1 (B) and 2 (C).
774 Error bars represent means ± S.E.M. * Represents a difference from pre training at a given
775 time point (P<0.05). # represents a difference between ARA and Placebo at a given time
776 point (P<0.05).

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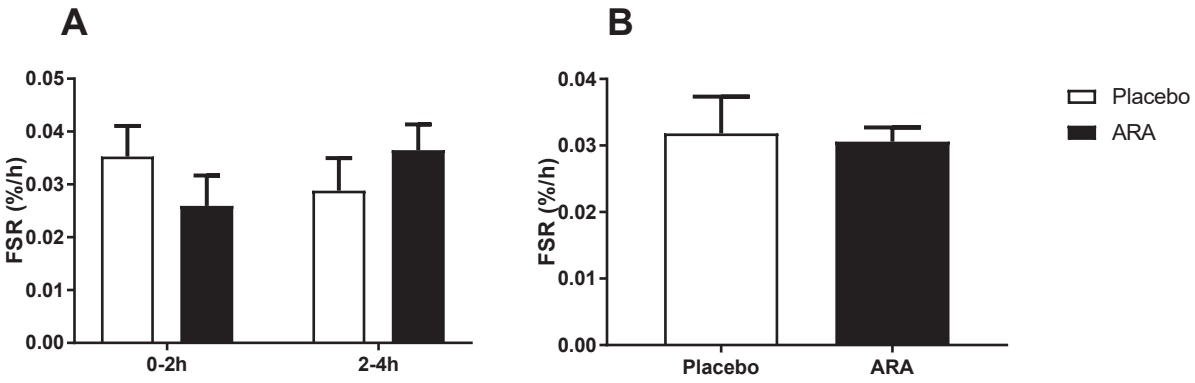

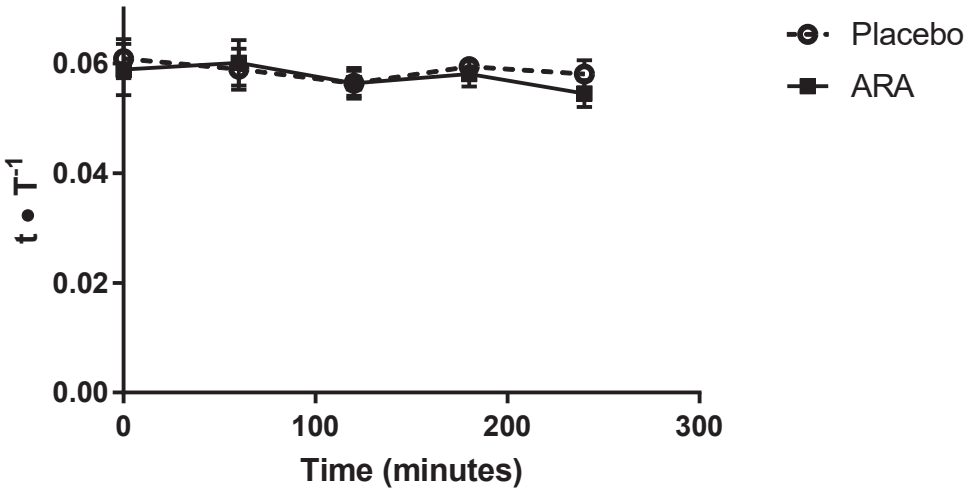

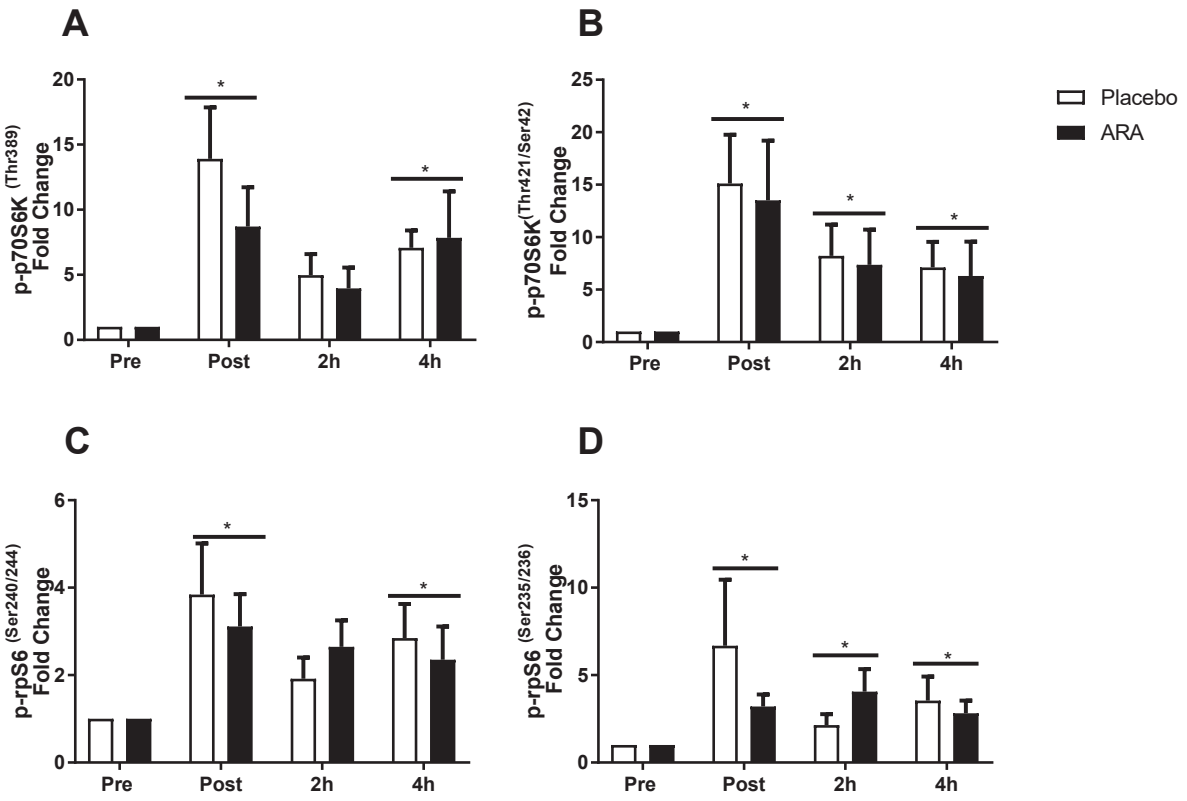

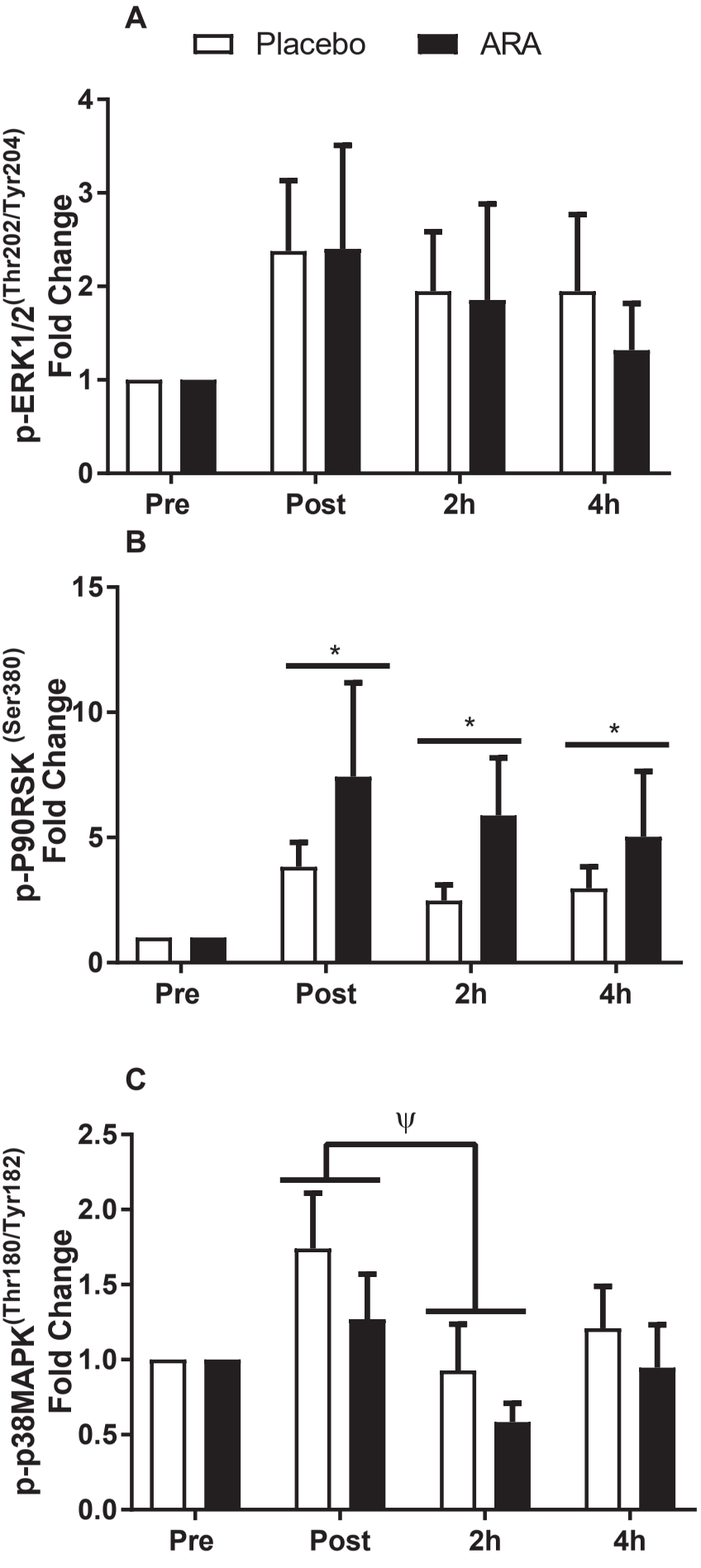

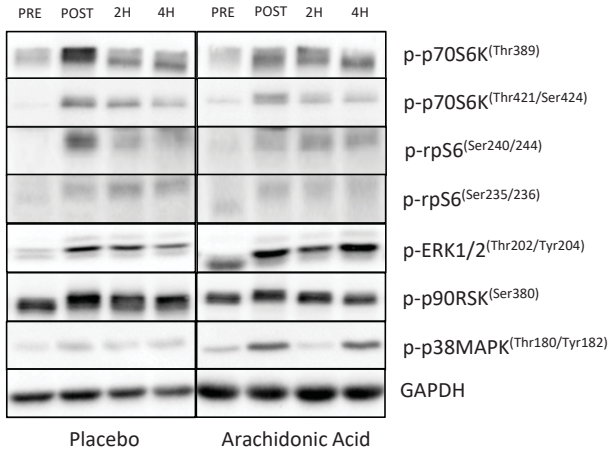

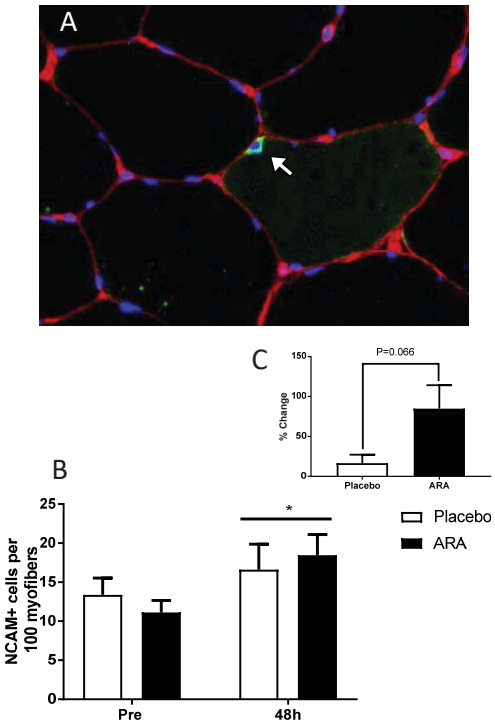

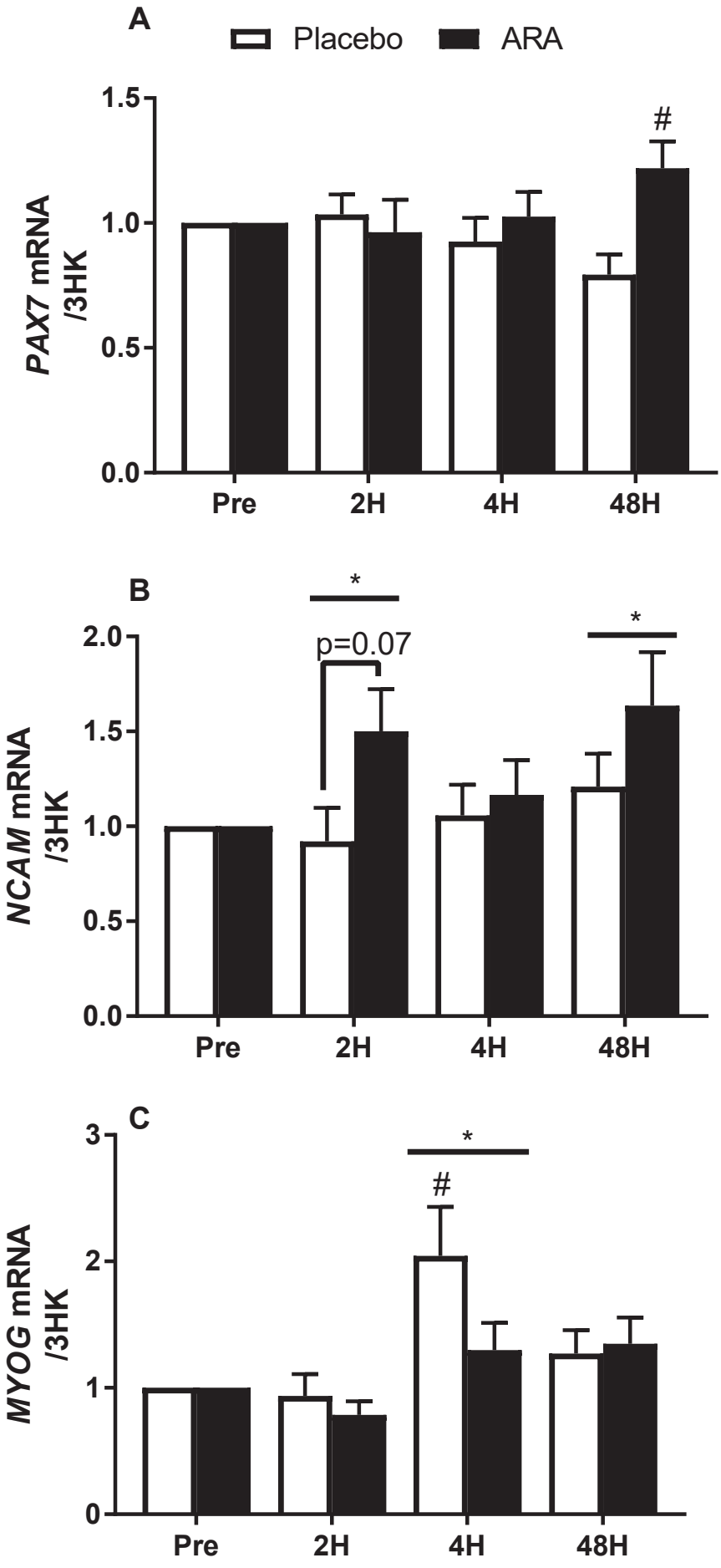

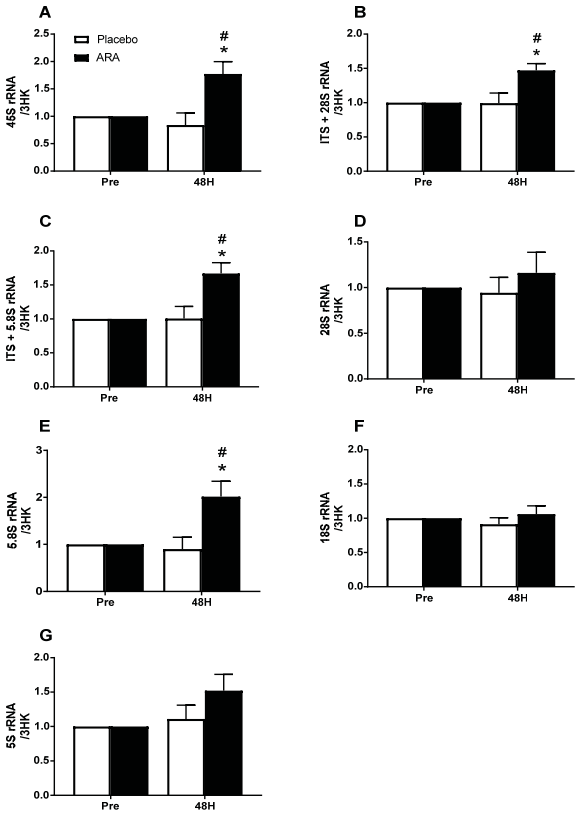

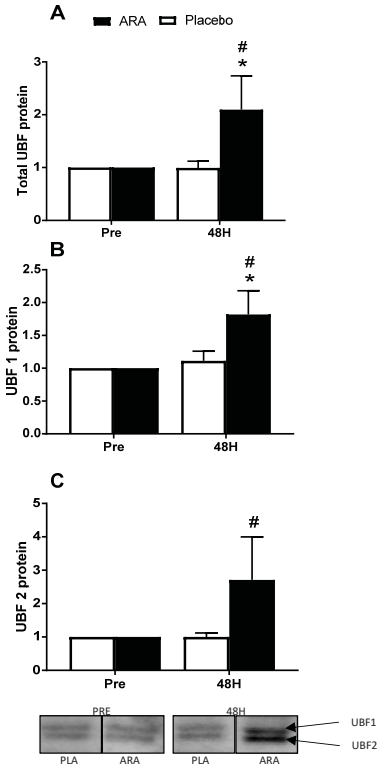

Table 1. Subject Characteristics

	Placebo (n=10)	ARA (n=9)
Age (years)	24.6 ± 4.9	26.4 ± 4.1
Height (cm)	181.0 ± 8.8	180.2 ± 9.2
Weight (kg)	92.0 ± 10.6	89.6 ± 10.5
BMI (kg/m^2)	28.1 ± 3.3	27.6 ± 1.8
Body fat (%)[1]	18.0 ± 6.4	19.0 ± 4.4
Protein supplement use (n)	6	5
Creatine monohydrate use (n)	2	3

Means ± SD. [1]determined using dual-energy X-ray absorptiometry.

Table 2. Forward and reverse mRNAs sequences of analysed genes

Gene	Sequence
PAX 7 (Forward)	CCTTTGGAAGTGTCCACCCC
PAX 7 (Reverse)	TCGCCCATTGATGAAGACCC
MYOG (Forward)	GGCCAAACTTTTGCAGTGAATATT
MYOG (Reverse)	TCGGATGGCAGCTTTACAAACAAC
NCAM 1 (Forward)	GCAGCGAAGAAAAGACTCTGG
NCAM 1 (Reverse)	GCAGATGTACTCTCCGGCAT
VCP (Forward)	AAACTCATGGCGAGGTGGAG
VCP (Reverse)	TGTCAAAGCGACCAAATCGC
CHMP2A (Forward)	CGCTATGTGCGCAAGTTTGT
CHMP2A (Reverse)	GGGGCAACTTCAGCTGTCTG
HPRT (Forward)	CCTGGCGTCGTGATTAGTGAT
HPRT (Reverse)	TCGAGCAAGACGTTCAGTCC