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Effects of time of day on resistance exercise-induced anabolic signalling in skeletal muscle

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This study examined the effect of morning vs. afternoon exercise on acute responses in phosphorylation of proteins regulating muscle size and metabolism. Twenty-two untrained men, divided into the Morning (n=11) or Afternoon (n=11) group, performed maximal isometric leg extensions before and after resistance loading at 07:30-08:30h and 16:00-17:00h, respectively. Muscle pre- and post-loading biopsies were analyzed for phosphorylated Akt, p70S6K, rpS6, p38MAPK, Erk1/2, and eEF2. Muscle force declined after exercise in both groups ($p<0.001$). p70S6K Thr^{389} ($p<0.05$) and Thr^{421}/Ser^{424} and rpS6 (all $p<0.001$) increased after exercise in both groups. The afternoon, but not morning group showed post-loading decrease ($p<0.05$) and increase ($p<0.01$) in eEF2 and p38MAPK, respectively. Akt and Erk1/2 were statistically unchanged. In conclusion, the time of day did not have an overall effect on protein synthesis signalling, but morning phosphorylated eEF2 and p38 MAPK showed significantly larger between-subject variability in the exercise response compared to the afternoon.

Keywords: diurnal; signalling pathways; phosphoproteins; muscle strength; insulin

Introduction

Time of day has been shown to affect various indices related to neuromuscular performance in both acute responses to a bout of resistance exercise and long-term adaptations to resistance training. For instance, muscle strength is typically lower in the morning compared to the afternoon (for a review see (10)). However, lower neuromuscular performance in the morning can be improved to the afternoon levels by regularly training in the morning hours over the period of several weeks (34,35). Whether the hypertrophic adaptation of skeletal muscle to resistance training also is affected by the time-of-day-specific training, is less studied. To our best knowledge, the

only study performed on humans found a tendency to smaller gains in muscle size when repeatedly training in the morning compared to the late afternoon hours (36). Although statistically insignificant, subjects training in the afternoon hours increased their *m. quadriceps femoris* volume, measured by magnetic resonance imaging, on average 30% more compared to their counterparts in the morning training group (36). One of the possible mechanisms contributing to the above-mentioned time-of-day-dependent training adaptations is signalling pathways involved in the control of protein synthesis and protein degradation.

In general, muscle hypertrophy/atrophy is a net result of an increase in protein synthesis minus protein degradation. A single bout of resistance exercise is a potent stimulus for increasing the post-exercise rate of protein synthesis per se, both in the acute recovery phase and lasting up to 48 hours (24,29). Phosphorylation of specific proteins in protein kinase B/muscle target of rapamycin/p70 ribosomal S6 kinase signalling pathway (Akt/mTOR/p70S6K) and to some extent also in mitogen-activated protein kinases (MAPK) signalling pathway has been shown to positively regulate muscle growth (2,37,40). Further, resistance exercise primarily aimed at increasing muscle hypertrophy is a potent stimulus to increase mTOR and MAPK signalling (9,18,19,40). At least signalling through rapamycin sensitive mTOR complex 1 (mTORC1) is needed to induce protein synthesis after resistance exercise (9). However, there are very limited data available addressing whether and how the activation of these signalling pathways can be influenced by a single bout of exercise or repeated resistance exercise performed at different time of day. To our best knowledge, the only study providing some evidence of time-of-day-dependent responses to a bout of exercise loading was published by Cochran et al. (4). They reported higher phosphorylated p38 MAPK when high-intensity interval cycling exercise was performed in the late morning compared to the afternoon. It must, however, be noted that their study was not primarily designed to address the effect of time of day. Hence, the acute loadings and biopsy samplings were not scheduled to early morning and late afternoon hours, when diurnal minimum and maximum in neuromuscular performance is typically found, respectively (12,26,32,33,35). Furthermore, early morning and late afternoon hours are also when hormonal signalling is dramatically different (e.g., cortisol to testosterone ratio – for a review see (16)). Considering the limited results from the acute studies (4) and from the long-term studies (36), it can be hypothesized that the time of day might influence the response to an acute bout of resistance exercise at the molecular level, e.g. hypertrophic

signalling pathways. Therefore, the main purpose of this study was to examine effects of the time of day on exercise-induced acute responses in phosphorylation levels of important proteins regulating muscle size and metabolism in human subjects.

Materials and methods

Subjects

Forty-eight subjects replied to recruiting flyers placed on several free advertising boards. Out of them, twenty-two men met the following inclusion criteria, collected by questionnaires and a recruiting interview: clinically healthy male with no medication within the last 14 days, non-smokers, regular sleep pattern ranging from 6 to 9 hours per night, age range from 20 to 30 yr, no history of strength training, other regular physical activity not more than once a week during the past 3 years, no history of shift work and classification as "neither type" in the self-assessment questionnaire to determine morningness-eveningness (17).

This study was approved by the Ethics Committee of the Faculty of Physical Education and Sports, Comenius University, Bratislava, Slovakia. An informed consent form was read and signed by each of the subjects prior to the investigation.

Experimental design

Familiarization and pre-testing

Five to seven days prior to the actual experiment, subjects underwent one pre-testing session held between 11:00 h and 14:00 h. After familiarization with the testing protocol and apparatus, subjects performed three repetitions of isometric maximum voluntary bilateral leg extension at the knee angle 107º (MVC) on a custom built, computer controlled linear motor-powered leg press dynamometer, described in details elsewhere (6). Briefly, resistance of leg-press pedals is generated, instead of classical weights, by two independent linear electric motors (Kollmorgen IC2022, Danaher, Washington DC, USA), each attached independently to a pedal. A strain gauge (EMS100 5kN, EMSYST, Trencin, Slovakia) is inbuilt to each pedal and connected to a computer. The technical design allows the machine to operate in e.g., isometric and traditional isokinetic mode (both concentric and eccentric). During MVC, participants were seated on the seat with arms placed on the chest. Two adjustable padded bars, connected with the backrest, were placed on the shoulders to prevent any upwards movement during exercise. Knee angle was set to 107º, hip angle to 110 º and ankle

angle to 90º. Participants were instructed to push with maximal effort for 3 – 4 seconds against the footrest platform using both anterior and posterior thigh and hip muscles while keeping firm contact with the seat and backrest. Loud verbal encouragement was given to promote maximal effort. An analogue signal, converted to digital via a 12-bit AD converter was sampled by the computer with the sampling frequency of 1000 Hz and analyzed with custom-made software. Out of three MVC pre testing trials, the trial with the highest peak force was taken for further analyses. Body height and body mass were also measured at this occasion.

Time-of-day-specific test protocol

Subjects were pair-matched based on pre-test MVC and body mass index and the pairs were randomly divided into two time-of-day-specific resistance loading groups: Morning (M, n=11, age 25 ± 4 yrs, body mass 78.4 ± 4.8 kg, height 182 ± 7.9 cm, mean ± SD) and Afternoon (A, n=11, age 24 ± 3 yrs, body mass 77.1 ± 5.2 kg, height 180 ± 8.7 cm, mean ± SD). Both the M and A group underwent an identical strength test protocol differing only with regards to the time of day of testing. The M and A group were tested and exercised between 07:30 - 08:30 h and 16:00 - 17:00 h, respectively. Subjects were instructed to refrain from alcohol, caffeine, sexual and strenuous physical activity 48 hours prior and during the test day. Light- and medium-speed walking shorter than 20 min per a bout was allowed. For travelling longer distances, subjects were asked to use car or public transportation. They were also asked to go to bed between 22:00 and 23:00 h and get up between 05:45 and 06:15 h in the morning of the testing day. Compliance with the requirements for the activity and sleep patterns were visually verified from wrist movement analyzer data (Actiwatch, Cambridge, United Kingdom) worn by subjects during the last 16 – 24 h before the test. All test session were performed during March 2010 with the sunlight of various intensities present in the outdoor environment.

The subjects were scheduled to report at the laboratory at 06:45-07:15 h and at 15:15-16:45 h for the M and A group, respectively. Immediately after arrival to the laboratory, 15 to 20 minutes before the first pre-loading muscle biopsy, subjects of both groups were given carbohydrate rich, low-protein meal consisting of a bun with margarine and strawberry jam. The rationale for this standardized low-protein meal was to diminish possible differences in nutritional status, especially with regards to the amount of protein ingested. Administration of proteins or its containing branched-chain

amino acids have been shown to up regulate skeletal muscle protein synthesis and protein phosphorylation in several of the proteins examined in the present study in both resting and post-loading recovery phase (11,18,23). For the M group, this was a first meal after an overnight fast. For the A group, the standard meal was the third meal during the day after breakfast (07:00 – 07:30, cereals and milk) and lunch (12:00 – 12:30 h, meat with rice or potatoes). Clear written instructions were given to subjects with respect to the amount and contents of the breakfast and lunch.

Subjects then underwent testing procedures in the following order: pre-loading muscle biopsy, pre-loading blood sampling, pre-loading MVC (Pre MVC), acute loading protocol, post-loading MVC (Post MVC), post-loading blood sampling 1 and 2 and post-loading muscle biopsy (Figure 1).

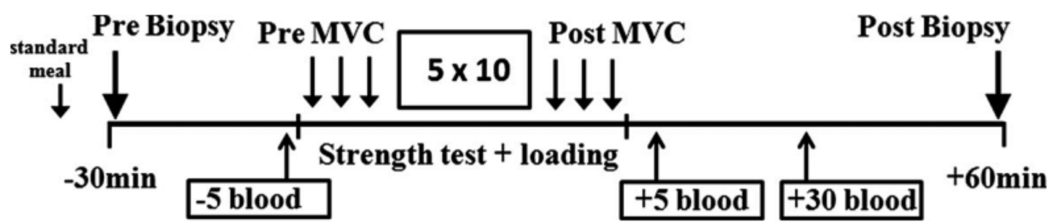

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Figure 1. Chronology of the experiment identical for the morning and afternoon group. Pre and Post – before and after acute heavy resistance loading, respectively, MVC - maximum voluntary contraction, 5x10 – five sets of ten repetitions; -5 blood stands for blood sampling 5 minutes before the first bout of Pre MVC; +5 and +30 blood stands for blood sampling 5 and 30 minutes after the last bout of Post MVC, respectively.

Muscle biopsies

Muscle biopsies were obtained 30 minutes before the first bout of pre-loading MVC and 60 minutes after the last bout of post-loading MVC. Based on the literature, significant changes in phosphorylation of the studied proteins have been previously reported when biopsy samples were taken 60 minutes after resistance exercise (for review see (43)). Biopsy samples were taken from the m. vastus lateralis muscle with a 5-mm Bergström biopsy needle and with manual suction, midway between the upper region of patella and greater trochanter. The pre-loading and post-loading biopsy sample was always taken from the right and left vastus lateralis, respectively. Muscle biopsy specimens were

cleaned of any visible connective and adipose tissue as well as blood, frozen immediately in liquid nitrogen and stored at -80°C until assay.

Strength test and acute loading protocol

Thirty minutes after pre-loading biopsy subjects performed a standardized warm-up protocol consisting of brief dynamic stretching movements, fifteen unloaded parallel squats and one trial of approximately 50% MVC in testing conditions. Subsequently, three trials of Pre MVC were measured in the same manner as described above. After a 2 minute-rest period, an acute loading protocol consisting of five sets of ten repetitions of isokinetic bilateral leg extension was performed on the dynamometer with rest intervals of 1.5 minutes between sets. This type of exercise strongly activates both right and left vastus lateralis muscles by means of electromyographic activity (unpublished observation). Movements of the pedals started from 90º in knee joint and ended approximately 0.08 m before legs were fully extended. Speed of the pedals was 0.2 $m.s^{-1}$. Subjects were required to push with a maximal voluntary effort both in the eccentric and concentric phase of all movement cycles. Loud verbal encouragement was given during each repetition. Two minutes after the acute loading exercise protocol 3 trials of MVC were carried out again (Post MVC).

Blood samples were drawn from the antecubital vein using a needle and syringe 5 minutes before the first Pre MVC and 5 and 30 minutes after and the last bout of Post MVC (Figure 1), respectively. Subjects were resting in a supine position between the 5 and 30 minutes Post loading samplings.

Tissue and blood processing

Muscle specimens were later homogenized in ice-cold buffer (20mM HEPES, pH 7.4, 1mM EDTA, 5mM EGTA, 10mM MgCl2, 100mM b-glycerophosphate, 1mM Na3VO4, 2mM DTT, 1% Triton X-100, 0.2% sodium deoxycholate, and 1% phosphatase inhibitor cocktail (P 78443; Pierce, Rockford, USA)) at a dilution of 15 ml/mg of wet weight muscle. Homogenates were rotated for 30 min at 4°C, centrifuged at 10 000 g for 10 min at 4°C to remove cell debris, and stored at -80°C. Total protein content was determined using the bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, USA) in triplicates with an automated KoneLab device (Thermo Scientific, Vantaa, Finland).

Western immunoblot analyses

Aliquots of muscle lysate were solubilized in Laemmli sample buffer and heated at 95°C to denaturise proteins. Samples containing 30 µg of total protein were separated by SDS-PAGE for 60 min at 200 V using 4–20 % gradient gel on Criterion electrophoresis cell (Bio-Rad Laboratories, Richmond, CA). Both samples from each subject were run on the same gel. Proteins were transferred to PVDF membranes at 300 mA constant current for 2 h on ice at 4°C. The uniformity of protein loading was checked by staining the membrane with Ponceau S and by re-probing the membrane with an antibody against α-actin (Sigma, Saint Louis). Membranes were blocked in TBS with 0.1 % Tween 20 (TBS-T) containing 5 % non-fat dry milk for 1 h and then incubated overnight at 4°C with commercially available rabbit polyclonal primary phosphospecific antibodies. Antibodies recognized phosphorylated (p-) protein kinase B (Akt) at Ser^{473}, p70 ribosomal S6 kinase (p70S6K) at Thr^{389} and Thr^{421}/Ser^{424}, ribosomal protein S6 (rpS6) at $Ser^{240/244}$, p38 mitogen-activated kinase (p38 MAPK) at Thr^{180}/Tyr^{182}, extracellular signal-related kinase 1/2 (p44/p42) (Erk1/2) at Thr^{202}/Tyr^{204}, and eukaryotic elongation factor 2 (eEF2) at Thr^{56} (Cell Signaling Technology, USA).

The primary antibodies were diluted in TBS-T containing 2.5% non-fat dry milk 1:1000-1:2000 except for p-eEF2, which was diluted 1:5000. Membranes were then washed in TBS-T, incubated with secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG; Cell Signaling Technology, USA) diluted 1:25 000 in TBS-T with 2.5 % milk for 1h followed by washing in TBS-T.

Phosphorylated proteins were visualized by ECL according to the manufacturer's protocol (SuperSignal West femto maximum sensitivity substrate, Pierce Biotechnology, Rockford, USA) and quantified (band intensity x volume) using a Chemi Doc XRS in combination with Quantity One software (version 4.6.3. Bio-Rad Laboratories). The results were normalized to Ponceau S. Our earlier experiments confirmed a proportional linear relationship between protein loaded and, especially, Ponceau S in quantification between 5 and 60 µg of total protein loaded (20).

The membranes described above were incubated in Restore Western blot stripping buffer (Pierce Biotechnology) for 30 min and reprobed with appropriate antibodies for detection of the total expression levels of Akt, rpS6, p38 and Erk 1/2 (Cell Signaling Technology) and p70S6K (Santa Cruz Biotechnology) by immunoblot analysis as described above. The rationale was to especially verify whether the total

protein content of part of the phosphospecific proteins analyzed of signalling proteins significantly changes from pre- to post-loading condition.

Blood analyses

Blood samples were centrifuged at 2500 x g for 15 min at 4°C. Plasma was aspirated and kept at -80 ^{o}C until measurement. Plasma insulin was measured by a commercial insulin-specific immunoradiometric assay (Immunotech, Prague, Czech Republic) according to the manufacturer instructions. All samples were measured in one assay and the sensitivity limit was 1.04 μIU/ml.

Statistical analyses

Standard descriptive statistics (mean ± SD) were calculated. Data at each time-point were analyzed for normality using the Shapiro-Wilk test. Except for MVC and Post p-eEF2, all variables at both the Pre and Post time points showed serious violation of normality. Thus for the phosphoprotein data, non-parametric related-samples Wilcoxon signed ranks test (Wilcox.) was used to examine Pre-to-Post changes with groups merged together (acute loading effect only) and within the groups separately. To compare percentage changes between the groups in the phosphoprotein data, Mann-Whitney U test for independent samples (Mann-Whitney) was used. The differences in MVC between group, Pre-Post loading effect and their interaction were analyzed by two factor ANOVA model (ANOVA). Insulin concentration data were logarithmically transformed for reducing the skew and examined by the general linear model with repeated measures (GLM). A Group was included as a between-subject factor in GLM. Pearson’s correlation coefficients were calculated between the insulin levels (Pre loading levels, % change from Pre to Post 5 min and Post 30 min) and phosphorylation levels of the selected proteins (% change from Pre to Post loading) and between the phosphorylation levels of the selected proteins, protein-to-protein. In addition, the Levene’s test was used assess the equality of variances (data homogeneity) between the M and A group at the Pre and Post loading state.

The level of significance was set at $p<0.05$.

Results

Muscle force at baseline was similar in both groups and declined significantly ($p<0.001$) after the acute resistance loading with no statistical difference between

morning and afternoon groups (-39 % and -38 % in the M and A group, respectively) (Table 1).

Table 1. Absolute values of maximum voluntary isometric strength during bilateral leg extension and plasma insulin concentrations before (Pre loading) and after (Post loading) acute heavy resistance loading between 07:30 - 08:30 h (Morning group) and 16:00 - 17:00 h (Afternoon group).

	MVC (N)		Insulin (μIU/ml)		
	Preloading	Postloading	Preloading	5 min post	30 min post
Morning group	3372 ± 983	2062 ± 372***	25.8 ± 13.7§	14.7 ± 9.3	15.8 ± 10.7
Afternoon group	3447 ± 1202	2147 ± 693***	12.9 ± 11.7	10.5 ± 5.4	16.2 ± 13.3

Note: MVC - maximum voluntary isometric strength *** - significantly lower compared to Pre loading ($p<0.001$). § - significantly higher compared to the same time point of the afternoon group ($p<0.05$)

There were no differences between the morning and afternoon group in the resting state of phospho- or total proteins. When groups were merged together, increased phosphorylation after exercise compared to the pre-exercise phosphorylation was found in p70S6K at Thr^{389} ($p<0.05$), p70S6K at Thr^{421}/Ser^{424} ($p<0.001$), rpS6 at $Ser^{240}/^{244}$ ($p<0.001$, Figure 2) and p38 at $Thr^{180}/Tyr^{182,}$ analyzed as the two pooled bands ($p<0.001$, ANOVA, Pre-Post loading-effect, Figure 3), respectively. Originally, p38 MAPK was analyzed also separately for the upper and lower band, purportedly isoform bands alpha/beta and gamma. However, these two isoform bands yielded very similar statistical results compared to pooled data and also both bands were statistically highly correlated ($p<0.001$, $r=0.756$ between Pre-loading levels of p38 α/β and Pre-loading p38 γ isoform and $p<0.001$, $r=0.949$ between Post-loading levels of p38 α/β and Post-loading p38 γ isoform).

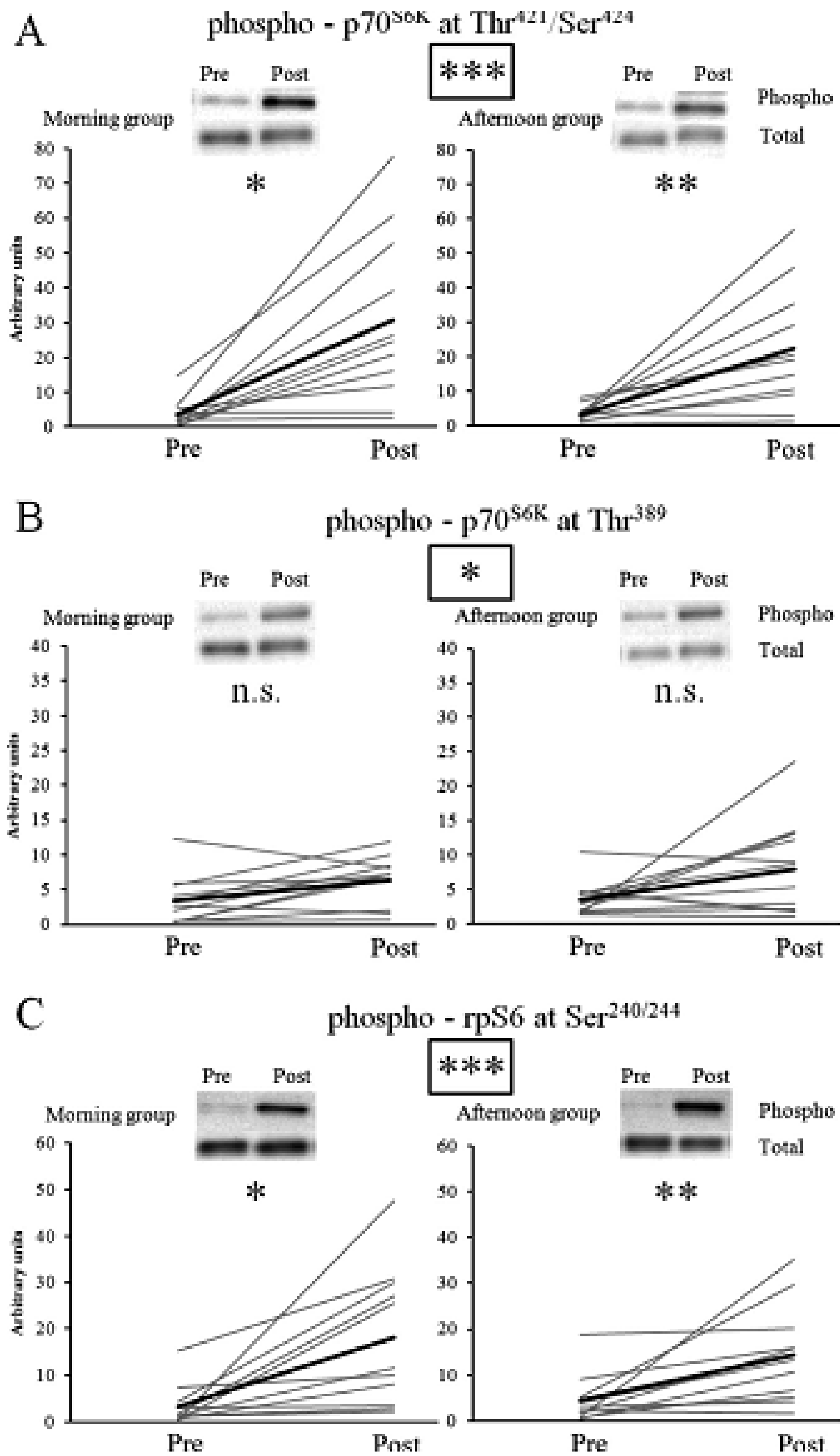

Figure 2. Change in phosphorylation of A) p70S6K at Thr^{389}, B) p70S6K at Thr^{421}/Ser^{424}, C) rpS6 at Ser^{240}/^{244} before (Pre) and after (Post) acute heavy resistance loading between 07:30 - 08:30 h (Morning group) and 16:00 - 17:00 h (Afternoon group). Thick black lines – average of

the group, thin grey lines – individual values n.s. – not significant. *, **, *** - significant at $p<0.05$, $p<0.01$, $p<0.001$, respectively, [***] - significance when groups merged together

When examined group by group, both groups increased post-loading phosphorylation of p70S6K at Thr^{421}/Ser^{424} and rpS6 at Ser^{240}/^{244} ($p<0.05$ in the M and $P<0.01$ in the A group, Wilcox). The relative post vs. pre changes between A and M conditions did not reveal statistical significance in the studied phosphoproteins. However, there was a tendency in eEF2 at Thr^{56} for the between-group difference ($p=0.058$, Mann-Whitney). In addition, the A group, but not the M group, showed statistically different post loading status of phosphorylation also for eEF2 at Thr^{56} showing a decrease ($p<0.05$) and increase in p38 MAPK at Thr^{180}/Tyr^{182} ($p<0.01$, Wilcox, Figure 3). Importantly, there was a statistically significant difference in data homogenity of p38 MAPK at Thr^{180}/Tyr^{182} with higher between-subject variation in the M group compared to the A group (Levene's test, $p<0.05$).

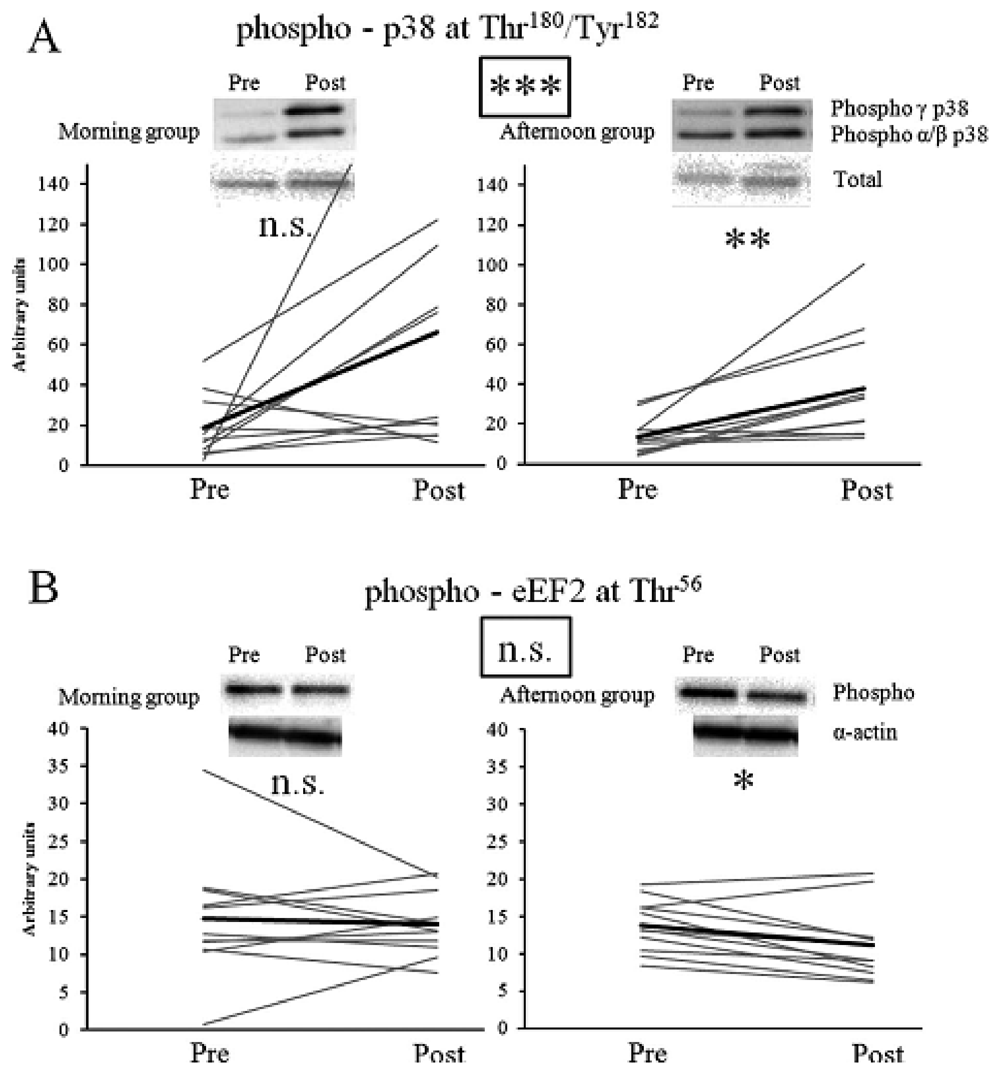

Figure 3. Change in phosphorylation of A) p38MAPK at Thr^{180}/Tyr^{182}, B) eEF2 at Thr^{56} before (Pre) and after (Post) acute heavy resistance loading between 07:30 - 08:30 h (Morning group) and 16:00 - 17:00 h (Afternoon group). Thick black lines – average of the group, thin grey lines – individual values. n.s. – not significant. *, **, *** - significant at $p<0.05$, $p<0.01$, $p<0.001$, respectively, [***] - significance when groups merged together

Phosphorylation of Akt and Erk1/2 did not statistically change due to exercise or the time of day (Figure 4). There was no change in the total protein expression of p70S6K, p38, Erk 1/2 and rpS6 (see blot images in Figures 2, 3, and 4).

Blood insulin levels were statistically higher in the M group at the pre-loading state compared to the A group ($p<0.05$, Pre-Post loading x Group interaction, Table 1), but did not differ between the groups in the post-loading state. No significant correlation was found between insulin levels and phosphorylation levels of the

measured proteins.

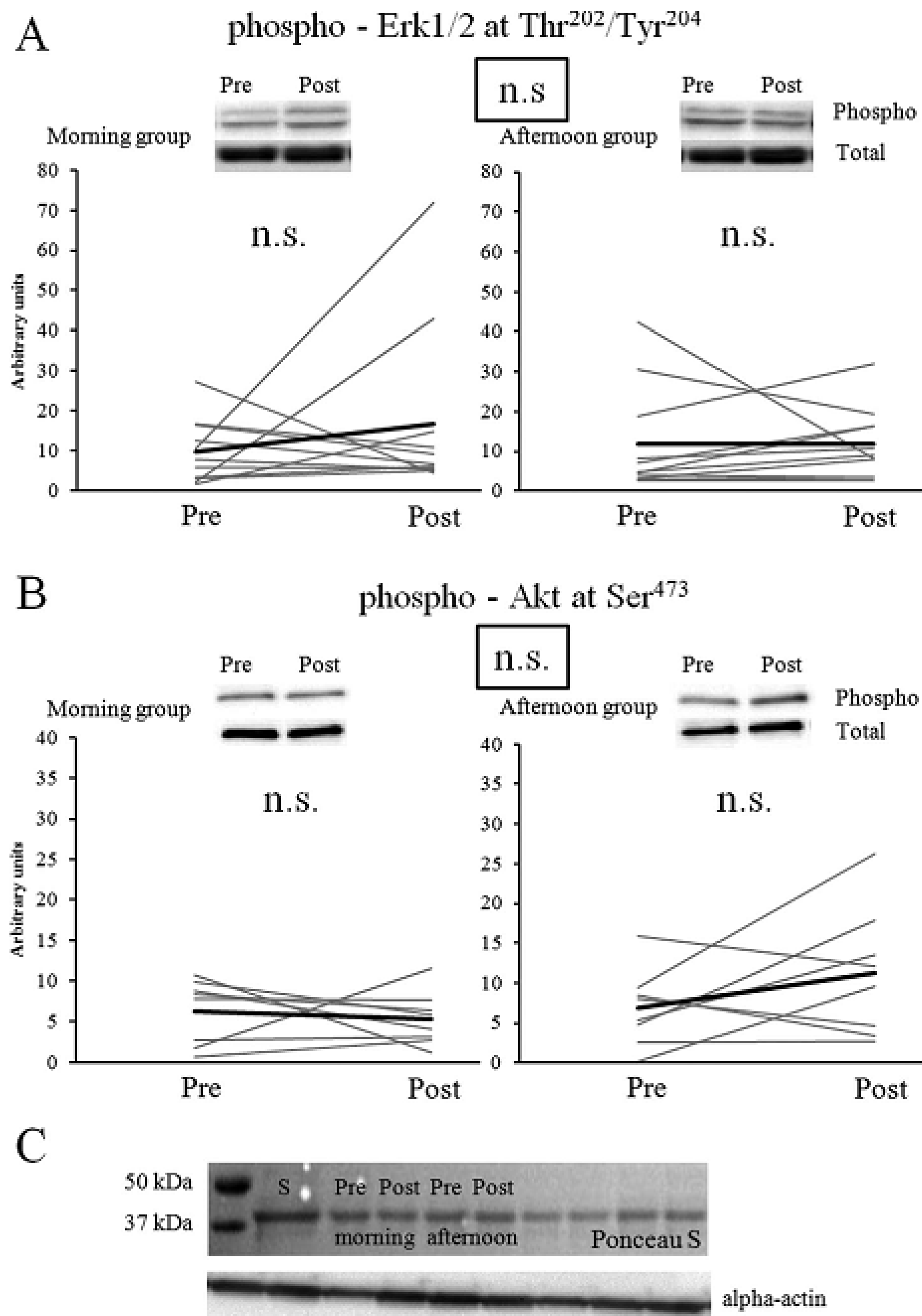

Figure 4. Change in phosphorylation of A) Erk1/2 (p44/p42) at Thr^{202}/Tyr^{204}, B) Akt at Ser^{473} before (Pre) and after (Post) acute heavy resistance loading between 07:30 - 08:30 h (Morning group) and 16:00 - 17:00 h (Afternoon group). Thick black lines – average of the group, thin grey lines – individual values. n.s. – not significant. C) Ponceau S staining shows an equal protein loading in an example membrane in which lane 1 is a molecule marker (Precision Plus Protein™ Dual Color Standards, Biorad) and lane 2 pooled standard (S - 15g of total protein). The strongest band at ~42 kDA is probably largely due to abundant skeletal α-actin. Blotting against α-actin is shown below Ponceau S.

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Discussion

The main finding of the present study was that the time of day did not have a clear overall effect on anabolic signalling in skeletal muscle, but phosphorylated eEF2 and especially p38 MAPK showed significantly larger between-subject variability in the exercise response in the morning group compared to more consistent exercise response in the afternoon group.

eEF2 mediates peptide-chain elongation (22) and its activity is decreased by its phosphorylation via eEF2 kinase, which is controlled and blocked e.g., by the upstream mTOR pathway (31). In human subjects, resistance exercise has been shown to decrease eEF2 phosphorylation (increases eEF2 activity) from 15 min up to 2 hrs post exercise by more than 40% in some (8,25), but not all studies (18). In the present study, phospho-eEF2 decreased significantly after exercise only in the afternoon group. However, the reason for somewhat larger inter-individual variation in the eEF2 response in the morning group compared to the afternoon group, resulting in significant strong trend in between-group difference (p=0.058), remains unknown (Fig 3 B) but can be speculated to be a result from more amino acids available in the afternoon for the translation elongation.

p38 MAPK, one of the four major subfamilies in MAPK signalling, has been shown as an important signalling protein for muscle metabolic adaptations via activating transcription factors and also other groups of kinases (3). p38 MAPK is responding to both anabolic (e.g., muscle cell stretch, functional overload) and catabolic stimuli (e.g., tumor necrosis factor α) (21,27,44). In the present study, phosphorylated p38 MAPK was elevated after the resistance loading in line with previous reports in humans (4,19,39,41). Interestingly, these changes, when expressed as groups' means were greater in the morning compared to the afternoon in the present study. Similar observation was made by Cochran et al. (4) but their sampling time points were at the later morning and early afternoon hours and exercise was high-intensity interval cycling, so direct comparison cannot be made. However, the increase in phosphorylated p38 MAPK was statistically significant in the A group but not in the M group in the present study. This was apparently caused by a larger, and statistically significant, inter-individual variation in the M group, which may be an effect of the time of day per se, e.g. via distinct diurnal differences in some stimuli triggering p38 MAPK phosphorylation (e.g., hormones, growth factors). In other words, resistance loading in

the afternoon hours produced a more homogenous elevation in phosphorylated p38 MAPK. In the morning hours, on the other hand, more pronounced increases in some subjects, compared to the afternoon, but also decreases in other subjects, were observed (Figure 3A, 3B).

Similar type of a trend for more consistent exercise response in the afternoon existed also in p-eEF2, as stated above. Therefore, conducting exercise in the morning may not provide optimal stimulus for some individuals but that statement needs more of both acute and long-term studies.

Another of the major subfamilies within the MAPK signalling is Erk 1/2. The Erk 1/2 pathway is associated with e.g. enhanced protein synthesis (38) and can be involved, in part, in mediating hypertrophic effect of extracellular signals like IGF-1 (13), but can also have catabolic effects on muscle in some situations (28). No significant effects of resistance loading and the time of day were observed in the present study. Tannerstedt et al. (39) and Hulmi et al. (19) found significant exercise-induced elevations in phospho-Erk 1/2 but their timing of post-loading biopsy was closer to cessation of exercise.

Both p70S6K and rpS6 phosphorylation has been repeatedly shown to be elevated in response to acute resistance loading in untrained subjects (1,8,18,40,41). An interesting observation was a clear inter-individual variation, independent of the time-of-day factor and neuromuscular fatigue after the acute loading, best seen in p70S6K Thr^{421}/Ser^{424} phosphorylation change after resistance loading.

Levels of phosphorylated protein kinase B (Akt) did not change statistically after resistance loading, although the afternoon group showed somewhat larger post-exercise values (p= 0.08). Previous reports on humans have shown increases (e.g., (8), decreases (7,40) but majority of studies reported no change in Akt phosphorylation after exercise (1,11,18,39,41). It can be speculated that low muscle glycogen levels decrease resistance exercise response of Akt phosphorylation (5), and may be thus the reason for the lack of Akt responses in some individuals in the morning in the present study.

An interesting finding was a significantly larger insulin response to the standard meal in the morning group compared to the afternoon group, but with no diurnal difference in the post-loading concentrations. Partial explanation for higher morning postprandial insulin concentrations could come from lower resting insulin clearance rate in the morning compared to the afternoon hours (42). The present pre- and post-loading insulin concentrations of the morning loading group were in line with previously

published data on humans, e.g. (4). The present findings on Akt/mTOR pathway phosphorylation could not be probably explained by the observed differences in its upstream regulator insulin (e.g. (30)), as there was no significant correlation between insulin and Akt/mTOR pathway proteins. Indeed, insulin-Akt -pathway has been suggested earlier to be less important for muscle protein synthesis response to muscle adaptations (14).

Regarding muscle voluntary strength, the acute loading caused a prominent and consistent decrease in maximum voluntary isometric strength in all subjects (by 39 % and 38 % in the morning and afternoon group, respectively). From a physiological point of view, the fatigue induced clearly suggest that the loading protocol was sufficient to produce changes in the phospohorylation state and comparable to commonly used resistance loading protocols in this area of research (for a review see (43)). Similar decrease in voluntary strength was also in accordance with an observation of Nicolas and collaborators (26) reporting no difference in torque production between morning and afternoon hours when only the second half of the 50 consequent voluntary contractions is considered. These very consistent post-loading decreases in voluntary force in both groups clearly suggest that any between-subject (e.g., in p70S6K Thr^{421}/Ser^{424}) and/or time-of–day-related (p38 MAPK and eEF2) changes in protein signalling were not due to possible effect of the loading protocol itself. Nevertheless, it remains unknown whether selecting extreme morning and/or evening chronotypes or individuals accustomed to morning and afternoon training could have give different (more pronounced) results not only in voluntary muscle strength but also in signalling pathways.

In conclusion, timing of exercise throughout the day may have a significant role in training studies focused on hypertrophic muscle signalling using resistance loading protocols. If possible, morning exercise loading before 09:00 h should be carefully taken into consideration, both in training and also in studies when consistent responses would be desirable. We found that early morning may induce significantly higher between-subject variation in some muscle growth- or metabolism-related signalling pathways compared to the same loading later in the day. This daily variation muscle signalling brings redundant variability to data.

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