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Intensive training and reduced volume increases muscle FXYD1 expression and phosphorylation at rest and during exercise in athletes

Martin Thomassen[1], Thomas P Gunnarsson[1], Peter M Christensen[1], Davor Pavlovic[2], Michael J Shattock[2] and Jens Bangsbo[1]

[1]Department of Nutrition, Exercise and Sports, Section of Integrated Physiology, University of Copenhagen, Copenhagen, Denmark; [2]Cardiovascular Division, King's College London, The Rayne Institute, St. Thomas' Hospital, London, United Kingdom

Correspondence to:

Jens Bangsbo

August Krogh Building

Section of Integrated Physiology

Universitetsparken 13

DK-2100 Copenhagen Ø, Denmark

Phone: +45 35 32 16 23

Fax: +45 35 32 16 00

E-mail: jbangsbo@nexs.ku.dk

Running head: Effect of intensified training on exercise muscle signaling

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Abstract

The present study examined the effect of intensive training in combination with marked reduction in training volume on FXYD1 expression and phosphorylation at rest and during exercise. Eight well-trained cyclist replaced their regular training with speed-endurance training (10-12 x ~30-s sprints) 2-3 times per week and aerobic high-intensity training (4-5 x 3-4 min at 90-95% of peak aerobic power output) 1-2 times per week for seven weeks and reduced the training volume by 70%. Muscle biopsies were obtained before and during a repeated high-intensity exercise protocol and protein expression and phosphorylation were determined by western blotting. Expression of FXYD1 (30%), actin (40%), mTOR (12%), PLN (16%) and CaMKII γ/δ (25%) was higher ($P<0.05$) after compared to before the training intervention. In addition, after the intervention non-specific FXYD1 phosphorylation was higher ($P<0.05$) at rest and during exercise, mainly achieved by an increased FXYD1 ser68 phosphorylation, compared to before the intervention. CaMKII thr287 and eEF2 thr56 phosphorylation at rest and during exercise, overall PKCα/β thr638/641 and mTOR ser2448 phosphorylation during repeated intense exercise as well as resting PLN thr17 phosphorylation were also higher ($P<0.05$) after compared to before the intervention period. Thus, a period of high intensity training with reduced training volume increases expression and phosphorylation levels of FXYD1, which may affect Na^+/K^+ pump activity and muscle K^+ homeostasis during intense exercise. Furthermore, higher expression of CaMKII and PLN as well as increased phosphorylation of CaMKII thr287 may have improved intracellular Ca^{2+} handling.

Abbreviations

4E-BP1, eukaryotic initiation factor 4E-binding protein 1; ACC, Acetyl-CoA carboxylase; AMPK, AMP-activated Protein Kinase; CaMK, Ca^{2+}/Calmodulin-dependent Protein Kinase; eEF2, eukaryotic elongation factor 2; FXYD1, phospholemman; mTOR, mammalian target of rapamycin; NaK, Na^+/K^+; p70S6K1, Ribosomal protein S6 p70 Kinase 1; PKC, protein kinase C; PLN, phospholamban; TBST, Tris-buffered Saline including 0.1% Tween-20.

Introduction

Changes in muscle ion homeostasis during intense contraction reduce membrane excitability which may lead to development of fatigue (30). Exercise training improves performance during intense exercise and reduces the accumulation of potassium in both blood (25) and muscle interstitium (32), which has been associated with elevated levels of Na^+/K^+ (NaK) pump subunit expression (25; 31-33). However, training studies have shown improved work capacity without adaptations in the NaK pump content and isoform abundance but with a higher maximal NaK pump activity (3). Thus, factors other than NaK pump subunits expression may affect the capacity of the NaK pump.

Phospholemman (FYXD1) is a regulatory protein associated with the NaK pump and changes in its expression and phosphorylation affect pump activity (7; 13; 27; 35). It is well known that muscle NaK pump activity increases markedly with exercise (9), which may be regulated partly by an increased FXYD1 phosphorylation observed during both moderate intensity (5) and high intensity acute exercise in humans (52). The effect of endurance training on muscle FXYD1 expression and phosphorylation during and after exercise has been examined (5). Ten days of moderate intensity cycle training including 6 x 5 min at 90-100% of an intensity corresponding to $VO_{2\ max}$ did not affect FXYD1 expression or FXYD1 phosphorylation during long-term low intensity exercise in untrained healthy individuals (5). In contrast, a 2-week period of high intensity exercise training elevated resting levels of FXYD1 phosphorylation (54), indicating that intensity during training may be important for the adaptations of FXYD1. However, the effect of intense training on muscle FXYD1 expression and exercise-induced phosphorylation has not been examined. We hypothesize that intensified training does lead to higher expression of FXYD1 and increased FXYD1 phosphorylation during intense exercise, which can explain the finding of a lower femoral venous potassium concentration after intense exercise (23).

Exercise training leads to multiple adaptations in human skeletal muscles as a result of molecular events, including exercise-induced activation of signaling pathways, which regulate changes of muscle structure and function. AMP-activated Protein Kinase (AMPK) is known as a key protein for exercise-mediated muscle adaptations and particular regulation of mitochondrial and GLUT4 biogenesis (44). AMPK content, activity and phosphorylation are markedly regulated during a few weeks of endurance training (17; 29). On the other hand, AMPK thr172 phosphorylation is elevated after high, but not low, intensity exercise (15). Furthermore, AMPK and Acetyl-CoA carboxylase (ACC) phosphorylation are increased after four 30-s bouts of intense exercise (21), indicating that

high intensity exercise training, including training intensities exceeding VO_2 max, may lead to adaptations in the AMPK signaling pathway, but this issue has not been investigated.

Regulation of muscle Ca^{2+} fluxes during exercise does affect the development of fatigue (1). In human skeletal muscles the multifunctional Ca^{2+}/Calmodulin-dependent protein kinase (CaMK) II is the major CaMK and was shown to be activated during low intensity exercise (48). Furthermore, endurance training alters CaMKII cell signaling in human skeletal muscles (47). In contrast, CaMKII thr287 phosphorylation is only elevated after high, and not low, intensity exercise (15). Therefore, high intensity exercise training may induce adaptations in the CaMKII pathway via changes in CaMKII thr287 phosphorylation, which will affect phospholamban (PLN) thr17 phosphorylation and thereby Ca^{2+} fluxes via the SERCA pumps (48).

Mammalian target of rapamycin (mTOR) is part of the multi-protein complex, mTORC1, and plays via e.g. eukaryotic initiation factor 4E-binding protein (4E-BP1) and ribosomal protein S6 p70 kinase 1 (p70S6K1) an essential role in the regulation of muscle mass and protein synthesis (22). Phosphorylation of mTOR ser2448 and activation of mTORC1 have been associated with both atrophy and hypertrophy of skeletal muscles (22; 42). Endurance exercise induces an increased mTOR signaling via phosphorylation of mTOR ser2448 (4) and heavy resistance exercise induces increases in mTOR signaling and protein synthesis (22). On the other hand, four 30-s sprints did not activate mTOR signaling (21), while other studies implementing high intensity exercise do report activation of mTOR signaling (22). Due to the ambiguous findings it is of value to examine whether intense exercise induces mTOR signaling and how intensified training affects mTOR signaling.

Thus, the aim of the present study was to examine the effects of intense training with reduced volume on FXYD1 expression and phosphorylation during repeated high intensity exercise in trained individuals. In addition, to examine the effect of intensified training with a reduced volume on activation of signaling pathways involving mTOR, AMPK and CaMKII in human skeletal muscles.

Materials and Methods

Ethical approval and subjects

The study was approved by the local ethical committee of the capital region of Copenhagen (Region Hovedstaden) and performed in accordance to the principles of the Declaration of Helsinki. The subjects and training intervention were the same as in a study focusing on adaptations of ion transport proteins and ion kinetics (23) and a study focusing on adaptations in oxygen kinetics (8) during repeated high intensity exercise. Eight well trained male cyclists, who had been training and competing on a regular basis for at least 3 years, with an average (mean ± SD) age, weight and maximum oxygen uptake of 33±8 years, 81±8 kg and 59±4 $ml \cdot min^{-1} \cdot kg^{-1}$, respectively, participated in the study. The subjects were informed of any risks and discomforts associated with the experiments before giving their written, informed consent to participate.

Training intervention

A 7-week intensive training intervention including a volume-reduction was performed, as a one-group longitudinal design immediately after the regular cycling season as described in detail previously (8; 23). All training sessions were supervised and performed on public roads and on the subjects' own bikes. Briefly, the subjects replaced all their regular training with 2-3 sessions of speed-endurance training a week performed as 10-12 x ~30-s maximal uphill (~6% gradient) cycle sprinting interspersed by 4.5 min of low intensity exercise and 1-2 sessions a week of aerobic high-intensity training consisting of 4-5 x ~4 min of cycling (2 km flat course) at 90-95% of maximal heart rate interspersed by 2 min of rest with a work-to-rest ratio of ~2:1. During the training intervention subjects reduced the training volume by ~70% (62 vs. 211 km/week).

Experimental design

Subjects carried out two experimental days as well as two performance testing days before and after the 7-week training intervention as described in detail previously (8; 23). Briefly, on the first experimental day subjects arrived at the laboratory in the morning at least 60 min after consumption of a standardized breakfast. After 30 min of supine rest, catheters were inserted into the femoral artery and vein under local anesthesia, using the Seldinger technique. The catheters were used to

measure blood flow and for blood sampling. After 30 min of rest subjects cycled for 6 min at 50% of peak power output on an ergometer bike (Monark, Ergomedic 839E, Vansbro, Sweden), then after 30 min of rest, for 6 min at 70% of peak power output and 60 min later for 6 min at 70% of peak power output. Then, after another 60 min of rest, subjects performed a repeated intense exercise protocol, consisting of 2 min at low intensity (20 W), then intense exercise for 2 min (EX1), followed by 2.5 min of recovery and 2 min of low intensity exercise (20 W), and then another intense exercise bout performed to exhaustion (EX2). The intensity during the intense exercise was 90% of peak aerobic power output (356±6 W). This article focuses on training adaptations and changes in relation to the repeated intense exercise protocol performed at the end of one of the two experimental days (Fig.1).

Before the repeated intense exercise protocol, a muscle biopsy (n=7 as one subject did not have biopsies taken) was obtained from the m. vastus lateralis (6) under local anesthesia (1 ml of lidocaine, 20 mg/ml without epinephrine) and incisions were made as preparation for the following three biopsies. A biopsy was collected immediately after EX1, just prior to the low intensity exercise before EX2 and at exhaustion in EX2 within 10 seconds of exercise cessation with the subjects still placed on the bike (Fig. 1). All muscle samples were immediately frozen in liquid N_2 and stored at -80°C until analyses were initiated.

Protein expression in muscle homogenate lysates

Protein expression was determined as described previously (54). In short, samples of approximately 2.5 mg freeze dried human muscle tissue were dissected free from blood, fat and connective tissue. Samples were homogenized for 1 min at 28,5 Hz (Qiagen Tissuelyser II, Retsch GmbH, Haan, Germany) in a fresh batch of ice-cold buffer containing (in mM): 10% glycerol, 20 Na-pyrophosphate, 150 NaCl, 50 HEPES (pH 7.5), 1% NP-40, 20 β-glycerophosphate, 2 Na_3VO_4, 10 NaF, 2 PMSF, 1 EDTA (pH 8), 1 EGTA (pH 8), 10 µg/ml Aprotinin, 10 µg/ml Leupeptin and 3 Benzamidine, afterwards rotating for 1 hour at 4 °C and centrifuged at 18,320 G for 20 min at 4 °C to exclude non dissolved structures. The supernatant (lysate) was collected and used for further analysis. Total protein concentration in each sample was determined by a BSA standard kit (Thermo Scientific, USA) and samples were mixed with 6 x Laemmli buffer (7 ml 0.5 M Tris-base,

3 ml glycerol, 0.93 g DTT, 1 g SDS and 1.2 mg bromophenol blue) and ddH_2O to reach equal protein concentration before protein expression were determined by western blotting.

Western blotting

Equal amount of total protein were loaded in each well of pre-cast gels (Bio-Rad Laboratories, USA). All samples from each subject were loaded on the same gel. Proteins were separated according to their molecular weight by SDS page gel electrophoresis and semi-dry transferred to a PVDF membrane (BioRad, Denmark). The membranes were blocked in either 2% skimmed milk or 3% BSA in Tris-buffered Saline including 0.1% Tween-20 (TBST) before an overnight incubation in primary antibody at 4 °C and a subsequent 1 hour incubation in horseradish-peroxidase conjugated secondary antibody at room temperature. The bands were visualized with ECL (Millipore) and recorded with a digital camera (ChemiDoc MP Imaging System, Bio-Rad Laboratories, USA). Densitometry quantification of the western blot band intensity was done using Image Lab version 4.0 (Bio-Rad Laboratories, USA) and determined as the total band intensity adjusted for background intensity. Representative blots are shown in Figure 2.

Antibodies

The primary antibodies used in the present experiment were optimized by use of mixed human muscle standard lysates to ensure that the protein amount loaded would result in band signal intensities localized on the steep and linear part of a standard curve. To determine total and phospho-specific protein expression the antibodies included in Table 1 were used with the localization of the quantified signal noted. The phospho-specific Acetyl-CoA carboxylase (ACC) α ser79 antibody (#07-303, Millipore) was previously shown to recognize the equivalent ser221 in human ACC β (45; 57) and therefore used to determine ACC β ser221 phosphorylation. The secondary antibodies used were horseradish-peroxidase conjugated rabbit anti-sheep (P-0163), rabbit anti-goat (P-0449), goat anti-mouse (P-0447, DAKO, Denmark) and goat anti-rabbit IgM/IgG (4010-05 Southern Biotech).

193 *FXYD1 antibody phospho-specificity*

194 All of the FXYD1 antibodies used in the present study were previously shown to detect FXYD1 in
195 human skeletal muscle (5; 52) as well as FXYD1 in other tissues (18; 41; 50). In order to interpret
196 the data meaningfully, it should be noted, that AB_FXYD1 recognizes mainly unphosphorylated
197 FXYD1, however, phosphorylation at ser63, ser68 and thr69 reduces the AB_FXYD1 signal
198 intensity, as the antibody epitope is located in the C-terminal region of FXYD1 protein, where the
199 phosphorylation sites are also located (5; 41; 50; 53). This was confirmed in the present study by
200 dephosphorylation of the membrane proteins (43) after the original western blot analysis with
201 AB_FXYD1: The original PVDF membrane was first reactivated in ethanol and afterwards
202 incubated in TBST. Then the membrane was incubated in a stripping buffer (0.5 M Tris-HCL; pH
203 6.7, 2% SDS and 100 mM 2-Mercaptoethanol) at 50 °C for 2 hours. After 3 x 10 min washing in
204 TBST in another container, the membrane was blocked with TBST including 2% skimmed milk in
205 15 min and incubated in secondary antibody for 1 hour. Membranes were then washed again for 3 x
206 15 min and the stripping procedure was confirmed by exposure of the membrane. When the entire
207 primary antibody was removed by the stripping protocol, the dephosphorylation protocol was
208 conducted by incubating membranes for 2 hours at 37 °C in the dephosphorylation buffer (50 mM
209 Tris-HCL, 0.1 mM Na_2EDTA, 5 mM DTT, 0.01% Brij 35 and 2 mM $MnCl_2$; pH 7.5) including 500
210 U/ml lambda protein phosphatase (P07535, New England BioLabs). Then the membrane was
211 blocked with TBST including 2% skimmed milk, incubated overnight in AB_FXYD1, washed 2 x 5
212 min in TBST, incubated for 1 hour in secondary antibody and exposed by ECL. Following this
213 procedure, total FXYD1 expression (using AB_FXYD1 on dephosphorylated proteins) was shown
214 to be significantly increased (0.91±0.05 vs. 1.04±0.06) after compared to before the training
215 intervention. A similar result (30% increase) was obtained with the total FXYD1 antibody (Table
216 2), raised against the N-terminal region of the FXYD1, confirming the AB_FXYD1 phospho-
217 specificity. For clarity purposes, data obtained with AB_FXYD1 is inverted and shown as
218 1/AB_FXYD1, thus an increase on the figure (Fig. 3A) represents an increase in non-specific
219 FXYD1 phosphorylation.

220 AB_FXYD1ser68 (originally named CP68) is phospho-specific for ser68 residue in humans (52),
221 although it should be noted that the affinity for ser68 residue is affected by the phosphorylation
222 status of the adjacent thr69. Thus, the amount of ser68 phosphorylation, as determined by
223 AB_FXYD1ser68 (Fig. 3C), can be underestimated if thr69 is phosphorylated (18). Similarly,

FXYD1 thr69 phosphorylation (Fig. 3E) can be affected by the phosphorylation status of the ser68 residue.

Furthermore, a new batch of FXYD1 phospho-specific antibodies: FXYD1ser63, FXYD1ser68 and FXYD1thr69 (developed by Will Fuller and Michael Shattock), have also been used. These antibodies were used in mouse and rat ventricular myocytes, where FXYD1 is poorly phosphorylated at thr69 (16), however, in vitro phosphorylation data indicates (18) that the FXYD1ser68 and FXYD1thr69 antibodies are affected to the similar extent as the older generation of antibodies, AB_FXYD1ser68 and AB_FXYD1. Indeed, in our study, FXYD1 ser68 phosphorylation data obtained by the FXYD1ser68 and AB_FXYD1ser68 antibodies were similar and thus, for simplicity, only data obtained using AB_FXYD1ser68 are included in the results section.

In order to take into account the phospho-specificity and -sensitivity of the used antibodies, AB_FXYD1ser68/AB_FXYD1 ratio (Fig 3D) was used as an alternative to determine ser68 phosphorylation (Fig. 3C), as done in the past (54), whereas, FXYD1thr69/AB_FXYD1 (Fig. 3F) was used as alternative to determine thr69 phosphorylation (Fig. 3E). These ratios may overcome that the determination of FXYD1 ser68 and thr69 phosphorylation probably are affected by simultaneously phosphorylation at the two sites located next to each other. Data obtained from the ratio FXYD1ser68/AB_FXYD1 were similar to AB_FXYD1ser68/AB_FXYD1 and not included.

Data treatment

For each muscle sample, protein expression and phosphorylation was determined in duplicate (except for three muscle samples where only one measure was performed due to limited muscle tissue) and the average intensities were calculated. Values for all the individual time points were compared with the average resting value before the training intervention.

Training induced changes in total protein expression and phosphorylation are shown in relation to the total expression of the same protein, where both are determined, e.g. mTOR phosphorylation/mTOR expression. Determination of the specific phosphorylation level and total protein expression was performed on separate membranes in separated analyses.

253 *Statistics*

254 Changes in protein phosphorylation and expression were evaluated by a Two-way repeated measure
255 ANOVA. If overall significant main effects were observed, a Student-Newman-Keul post-hoc
256 analysis was conducted to identify differences in protein phosphorylation within specific time
257 points (SigmaPlot 11.0). $P < 0.05$ was chosen as the level of significance.

Results

Effect of the training intervention on protein expression

Total expression of muscle FXYD1, CaMKII γ/δ, PLN, mTOR and actin was 30% ($P<0.01$), 25% ($P<0.01$), 16% ($P<0.01$), 12% ($P<0.05$) and 40% ($P<0.05$) higher after than before the training intervention. The expression of 4E-BP1 was 24% lower ($P<0.05$) after than before the training intervention. CaMKII β_M expression tended ($P=0.072$) to be higher after compared to before the training intervention, whereas the expression of AMPKα2, eEF2 and p70S6K1 was not changed with the training intervention (Table 2).

Effect of the training intervention on protein phosphorylation during intense exercise

Non-specific FXYD1 phosphorylation was higher ($P<0.05$) at all time-points during the repeated intense exercise, compared to rest. After the training intervention period, non-specific FXYD1 phosphorylation was higher ($P<0.05$) after EX1 and before EX2, than before the training intervention (Fig. 3A).

FXYD1 ser63 phosphorylation was not altered during the repeated intense exercise, nor was it changed with the training intervention (Fig. 3B).

FXYD1 ser68 phosphorylation was higher ($P<0.001$) at the end of EX1, compared to rest, decreased ($P<0.001$) after EX1, and then increased ($P<0.05$) after compared to before EX2 (Fig. 3D). Furthermore, FXYD1 ser68 phosphorylation was higher ($P<0.05$) at rest and throughout the repeated intense exercise protocol after compared to before the training intervention (Fig. 3C and 3D).

FXYD1 thr69 phosphorylation was higher ($P<0.05$) after EX1, before and after EX2 compared to rest, while the training intervention did not affect FXYD1 thr69 phosphorylation (Fig. 3F).

PKCα/β thr638/641 phosphorylation

PKCα/β thr638/641 phosphorylation did not change during the repeated intense exercise, but after the training intervention, it was higher ($P<0.01$) before EX2 compared to rest (Table 3). After the

training intervention PKCα/β thr638/641 phosphorylation was higher at the end of EX1 ($P<0.05$) and before EX2 ($P<0.01$) compared to before the training intervention.

CaMKII thr287, PLN thr17 and eEF2 thr56 phosphorylation

Neither CaMKII β_M nor γ/δ subunit thr287 phosphorylation was altered during the repeated high intensity exercise. After the training intervention CaMKII γ/δ thr287 phosphorylation was higher ($P<0.01$) at rest and both CaMKII β_M and γ/δ thr287 phosphorylation were higher ($P<0.01$) before and after EX2, compared to before the training intervention (Table 3).

After the training intervention Phospholamban (PLN) thr17 phosphorylation, was higher at rest ($P<0.01$) compared to before the intervention. Furthermore, before the training intervention, PLN thr17 phosphorylation was higher before EX2 compared to rest, while there were no changes in PLN thr17 phosphorylation with exercise after the training intervention (Table 3).

Another CaMKII downstream target, eukaryotic elongation factor 2 (eEF2) thr56 phosphorylation, was increased at rest ($P<0.01$) and after EX2 ($P<0.05$) after the training intervention compared to before. Before the training intervention eEF2 thr56 phosphorylation after EX2 was higher ($P<0.05$) than at rest, while after the intervention the eEF2 thr56 phosphorylation after EX2 was higher ($P<0.05$) than at all other time points (Table 3).

mTOR ser2448, p70S6K1 thr389 and 4E-BP1 thr37/46 phosphorylation

Phosphorylation of mTOR ser2448 tended ($P=0.064$) overall to change during the exercise bouts. After the training intervention mTOR ser2448 phosphorylation was higher before EX2 ($P<0.01$) and after EX2 ($P<0.05$), compared to before the training intervention (Table 3).

Before the training intervention mTORC1 activity determined by p70S6K1 thr389 phosphorylation at all time points was higher ($P<0.05$) compared to rest. After the training intervention p70S6K thr389 phosphorylation was higher ($P<0.05$) after EX1 compared to rest (Table 3).

The mTOR substrate eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) thr37/46 phosphorylation was not changed with neither exercise nor training (Table 3).

312

313 *AMPKα thr172 and ACC β Ser221 phosphorylation*

314 Before the training intervention, AMPKα thr172 phosphorylation was higher ($P<0.01$) after EX2
315 compared to the other time points. After the training intervention AMPKα thr172 phosphorylation
316 after EX2 was lower ($P<0.01$) than before the training intervention (Table 3).

317 As a downstream target of AMPK, the ACC β ser221 phosphorylation was higher after EX1
318 ($P<0.001$) and before EX2 ($P<0.01$) compared to rest, and was further increased ($P<0.05$) at
319 exhaustion, but was not affected by the training intervention (Table 3).

320

321

Discussion

The main findings of the present experiment were that seven weeks of intensive training, with a reduced training volume, increased the total expression of FXYD1 and elevated the resting non-specific FXYD1 phosphorylation level in endurance trained cyclist. In addition, repeated intense exercise after the training intervention induced a higher level of non-specific FXYD1 phosphorylation than before the intervention. This was dominated by higher phosphorylation at FXYD1 ser68 residues. Other important findings were that the training intervention elevated the expression of actin, mTOR, PLN and CaMKII γ/δ and lowered the 4E-BP1 expression. Furthermore, the resting PLN thr17 phosphorylation, the overall PKCα/β thr638/641 and mTOR ser2448 phosphorylation during repeated intense exercise as well as CaMKII thr287, and eEF2 thr56 phosphorylation at rest and during exercise was higher after compared to before the training intervention.

Total FXYD1 expression was higher after compared to before the intensified training period, with no change in NaK pump α- and β-isoform expression (NaKα1: -11%, NaKα2: -8%, NaKβ1: -3%; (23). In contrast, no change in total FXYD1 expression, but elevated NaK pump α1-, α2- and β1-isoform protein expressions were shown after 10 days of moderate intensity (75-100% of $VO_{2\ peak}$) cycle training in recreationally active subjects (5). Thus, it appears that the intensity of training and/or the training status of the subjects are important for adaptation of muscle FXYD1. In support of the first notion, sprint training in rats induced higher muscle FXYD1 levels, while endurance training did not have any effect on FXYD1 expression (38). Treadmill running with a 10%-grade, 5 days a week for 45 min in about 14 weeks, elevated FXYD1 expression in rat skeletal muscles (40). The different effect of the various training forms may have been caused by the degree of the FT muscle fiber stimulation, as FT muscle fibers are expected to be more activated during the intense training. In agreement, it has been demonstrated in humans, that the exercise (5 min cycling at 95% of $VO_{2\ max}$) induced change in FXYD1 phosphorylation is more pronounced in type II fibers than in type I fibers (51).

In the resting state, non-specific FXYD1 phosphorylation and ser68 phosphorylation was higher after compared to before the training intervention. In agreement, a higher level of FXYD1 ser68 phosphorylation at rest was observed after two weeks of intensified training in soccer players (54). In contrast, 10 days of moderate intensity exercise training did not induce changes in the resting

352 FXYD1 phosphorylation level (5), indicating that exercise intensity is also important for the
353 training adaptations of FXYD1 phosphorylation at rest.

354 During the repeated intense exercise the non-specific FXYD1 phosphorylation increased due to
355 greater ser68 and thr69 phosphorylation, which is also observed during exercise with moderate
356 intensity (52). On the other hand, FXYD1 ser63 phosphorylation did not change during the short
357 and intense repeated exercise protocol as shown after 20-30 min of moderate intensity exercise (5;
358 52). This may be explained by the lack of increase in PKCα/β thr638/641 phosphorylation level, as
359 ser63 phosphorylation is PKC mediated (7; 36). The duration of the repeated intense exercise
360 protocol may have been too short or the intensity too high to induce ser63 phosphorylation. FXYD1
361 thr69 phosphorylation increased after EX1 and stayed elevated during the repeated intense exercise
362 protocol, while ser68 phosphorylation increased during both exercise bouts and decreased in
363 recovery from EX1. These marked increases in FXYD1 phosphorylation levels during exercise
364 suggest that FXYD1 phosphorylation may play a crucial role in regulation of the NaK pump, and
365 hence, K^+ regulation during and after intense exercise, where K^+ fluxes are pronounced (24; 28).
366 Thus, in the same study it was observed that the average venous K^+ concentration during the first 2
367 min of recovery from the intense exercise bouts was lower ($P<0.05$) after compared to before the
368 training intervention (4.2±0.2 vs. 4.9±0.2 and 4.3±0.2 vs. 5.1±0.1 mM), suggesting an enhanced
369 muscle K^+ reuptake, without changes in the expression of NaK pumps subunits (20). Furthermore,
370 performance during repeated intense exercise was improved with the training intervention (256 vs.
371 217 s) (23).

372 After the training intervention non-specific FXYD1 phosphorylation was higher at the end of EX1
373 and before EX2, due to higher FXYD1 ser68 phosphorylation, compared to before the intervention.
374 The training intervention did not affect FXYD1 thr69 phosphorylation, which is in agreement with
375 findings after a period of moderate intensity training (5). The training induced increase in PKCα/β
376 thr638/641 phosphorylation may have contributed to the elevated FXYD1 phosphorylation, since
377 PKCα activity has been shown to be required for contraction induced FXYD1 phosphorylation in
378 mouse skeletal muscles (52) and other tissues (7; 18; 35).

379 The higher expression of FXYD1 and FXYD1 phosphorylation after compared to before the
380 training intervention may have affected the NaK pump activity and, hence, muscle potassium
381 reuptake at rest and during contractions (10). In rat skeletal muscles around 30% of the α-subunits
382 were co-expressed with FXYD1 (39), and the finding of a larger amount of FXYD1 may suggest a

higher degree of NaK pumps found as α/β/FXYD1 or a higher pool of free FXYD1 proteins. It has been shown in Xenopus oocytes, that the affinity for potassium (K^+) and especially sodium (Na^+) is lower for α/β/FXYD1 pumps compared to α/β pumps (both α1/β1 and α2/β1) without differences in the maximal pump activity (13). Thus, at rest a potential higher amount of α/β/FXYD1 pumps after compared to before the training intervention may *per se* lower the NaK pump activity, but it may also have been counterbalanced by an increased Na^+ affinity expected from a higher resting FXYD1 phosphorylation (7; 35).

Incubation of rat muscle tissue homogenates with an anti-FXYD1 antibody lowered the NaK enzymatic activity by more than 50% compared to samples with no treatment (40), indicating that more FXYD1 increases the activity of NaK pumps in muscles through a higher amount of NaK pumps found as α/β/FXYD1. In addition, a higher pool of free FXYD1 after compared to before the training intervention, may have elevated the NaK pump activity during contractions. Indeed, FXYD1 has been suggested to translocate from an intracellular pool to the sarcolemma membrane during contractions, concomitant with an increased association between FXYD1 and the α1-subunit and a higher pump activity in the sarcolemma membrane fraction (39). Furthermore, the higher FXYD1 phosphorylation after the training intervention may have improved the pump activity through both a higher Na^+ affinity (27) and a higher V_{max} (34; 35). Thus, during exercise both the higher FXYD1 expression and phosphorylation may have contributed to an increased NaK pump activity after the training intervention compared to before. Unfortunately, the maximal NaK pump activity could not be determined due to lack of muscle tissue. Nevertheless, a higher activity of the NaK pump during and after exercise may explain the observation of lowered femoral venous plasma K^+ concentration in the first 2 min of recovery after EX1 and EX2 as a result of the training intervention (23).

An increased exercise-induced extracellular K^+ concentration has been linked to depolarization of the muscle membranes, decreased excitability and muscle fatigue. Therefore higher muscle K^+ reuptake is expected to improve performance. Improved K^+ handling and exercise performance has been related to higher NaK pump content after a period of training (25; 31-33). On the other hand, high intensity training has augmented maximal pump activity despite unchanged total pump content and protein isoform expression (3). FXYD1 expression and phosphorylation were not determined in either of these studies and adaptations in the FXYD1 proteins may be the missing link explaining increased NaK pump activity without changes in pump content or isoform expression (3).

Concomitant adaptations in the NaK pump α2-subunit and FXYD1 phosphorylation have previously been demonstrated after intensified training (54). Thus, the adaptations in FXYD1 expression and FXYD1 phosphorylation shown here may have improved K^+ handling during exercise, despite no changes in NaK pump subunit expression. It is interesting to hypothesize that these adaptations in the FXYD1 protein may be the cause of the improved performance during repeated high intensity exercise of already trained athletes after the intensified training intervention with reduced training volume, as observed in the present study (23).

An improved performance as a result of the training intervention (23) may also have been related to an improved intracellular Ca^{2+} handling (20). The high intensity training intervention with reduced volume induced increases in the CaMKII γ/δ isoforms while the CaMKII β_M tended to be higher. The elevated CaMKII expression was associated with a higher expression of PLN and a higher resting phosphorylation of the substrate phospholamban thr17, which relieves the phospholamban inhibition on SERCA, allowing a higher Ca^{2+} affinity and, thus, a higher rate of Ca^{2+} uptake (48). A higher content of PLN with the same degree of thr17 phosphorylation would most likely lead to better Ca^{2+} homeostasis in the trained muscle (47), as observed previously in rats (26). It should be noted, however, that the changes in CaMKII expression in the present study were less pronounced than with 10 days of endurance training (4) and three weeks of one-legged endurance exercise training, which doubled the CaMKII activity, CaMKII kinase isoform expression and CaMKII autophosphorylation in resting muscles (47). On the other hand, the changes in PLN expression and thr17 phosphorylation at rest as well as in CaMKII thr287 phosphorylation (up to 8-fold increases) at rest and throughout the repeated intense exercise protocol shown after the intense training intervention, were either not seen after 10 days of endurance training (4) or were less pronounced after three weeks of endurance training (47), even though the subjects in the present study were trained before the intervention period. Thus, adaptations in PLN expression and CaMKII thr287 phosphorylation seem to be intensity dependent. CaMKII phosphorylation accelerates ATP provision via glycogenolysis and glycolysis during contractions (48) and may explain why higher muscle lactate levels were observed during exercise after the training intervention (23).

AMPK thr172 phosphorylation at exhaustion was lower after the intervention period. In accordance, 10 days of endurance exercise training abolished a 9-fold increase in AMPK α2 activity, observed during prolonged exercise before the training period (29). On the other hand, in the present experiment the downstream target of AMPK, ACCser221 phosphorylation was not affected by the

training intervention, which was observed after a period of endurance training (4; 29). These findings indicate that high intensity training has an impact on AMPK signaling, but the effect is less pronounced than seen after endurance training. When the energy sensing and signaling protein AMPK is activated, it increases ATP production by stimulation of glucose uptake and fatty acid oxidation. Furthermore activation of AMPK inhibits ATP consuming processes such as protein synthesis (56). The observed decrease in the exercise induced AMPK thr172 phosphorylation after the training intervention may indicate an abolished AMPK activity during high intensity exercise even though other factors are involved. A decrease in AMPK activity will improve the ability for ATP consuming processes in the muscle cell, such as an increased NaK pump activity, which may contribute to improved K^+ handling and the improved performance. In support for a link between AMPK and NaK pump activity, repeated treatment of mice with the AMPK activator AICAR increased FXYD1 phosphorylation and affected the NaK pump activity by increasing the Na^+ affinity (27).

AMPK may be involved in the regulation of mTOR, as elevated AMPK signaling lowers mTOR signaling in mouse skeletal muscles (14), while it is presently unclear whether it also occurs in humans (19). Thus, the abolished AMPK phosphorylation after the training intervention may have caused the increased expression of mTOR as well as mTOR ser2448 phosphorylation. These increases in mTOR and ser2448 phosphorylation were similar to the adaptations seen after moderate intensity training (4) and appear not to be intensity dependent. The mTOR signaling pathway is involved in many processes in the muscle cell including pathways controlling protein synthesis and muscle hypertrophy (12; 22). The increased actin expression may indicate muscle hypertrophy. It is supported by a training induced decrease in 4E-BP1 expression, which may have reduced eIF4E/4E-BP1 binding and elevated translation initiation (22). The mTORC1 readout p70S6K1 thr389 phosphorylation was in the present study higher during 2 x 2-4 min of high intensity exercise, which is in contrast to shorter high intensity exercise bouts (11; 21). During the training intervention both 30-s and 4-min bouts were performed, thus mTORC1 may have been activated during the training and may have induced hypertrophy. On the other hand, both exercise and training induced an increase in the downstream target of CaMKII, eEF2 thr56 phosphorylation (19; 37), which is expected to lower protein synthesis by lowering the eEF2 interaction with the ribosome and, thereby, impairing the elongation rate (37). Likewise, acute endurance exercise and endurance exercise training intervention, where hypertrophy is not expected, do lead to higher eEF2 thr56 phosphorylation levels (55). The higher eEF2 thr56 phosphorylation observed at rest after the

training intervention is expected to blunt the overall muscle protein synthesis (49) and does not indicate hypertrophy. In support, mean or peak power output during the initial sprint was not changed with the training intervention (23). Thus, it is unclear whether the intervention did lead to mTORC1 induced muscle hypertrophy and further studies are warranted to examine whether high intensity exercise training can lead to hypertrophy in already endurance trained individuals.

In summary, seven weeks of high intensity training with reduced training volume in endurance trained cyclist increased FXYD1 expression and FXYD1 phosphorylation levels and may have caused the improved K^+ reuptake during the intense repeated exercise, thus possibly contributing to the improved performance. Furthermore, the intense training intervention induced adaptations in CaMKII and PLN expression as well as CaMKII phosphorylation that may improve intracellular Ca^{2+} handling during exercise, which may potentially contribute to the improved performance.

Perspectives and Significance

The present study showed that high intensity exercise training in combination with a reduced training volume can induce significant adaptations in already endurance trained cyclists. It also demonstrated that it is important to examine changes in muscle protein phosphorylation and signaling during acute exercise before and after a training intervention. Higher FXYD1 expression and phosphorylation as well as CaMKII signaling may have elevated K^+ reuptake (23), via increased NaK pump activity (13; 27; 34; 35), and improved Ca^{2+} handling (26; 47; 48), respectively, but these effects need to be examined and possible links to improved excitation-contraction coupling should be investigated. Further studies are also warranted to clarify the effects of high intensity exercise training with reduced training volume on muscle hypertrophy and the signaling mechanisms regulating protein synthesis.

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505 *Disclosures*

506 No conflicts of interest are declared by the authors.

507

508 *Author contributions*

509 The experiment was performed at the Department of Nutrition, Exercise and Sports, University of
510 Copenhagen. All authors contributed to the conception and design of the experiment and to the
511 interpretation of the data. Collection and analysis of data were performed by MT, TPG, PMC and
512 JB. All authors contributed to drafting the article or revising it critically for important intellectual
513 content and approved the final version of the manuscript.

514

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679 **Figure and table legends**

680 **Figure 1**

681 A Schematic illustration of the protocol performed on the experimental day. Muscle biopsies were
682 obtained at the time points indicated by solid arrows. A fifth biopsy was as well obtained at rest in
683 the morning, indicated by the dashed arrow, but data from this biopsy is not included in the article.
684 The present article only includes data related to the repeated intense exercise protocol performed at
685 the end of the experimental day. iPPO, incremental peak power output.

686

687 **Figure 2**

688 Representative western blots, including the molecular weight of band migration. 4E-BP1:
689 eukaryotic initiation factor 4E-binding protein 1; ACCβ Ser221 phos: Acetyl-CoA carboxylase β
690 serine 221 phosphorylation; AMPKα2: AMP-activated Protein Kinase α2; CaMKII:
691 Ca^{2+}/Calmodulin-dependent Protein Kinase II; eEF2: Eukaryotic elongation factor 2; FXYD1:
692 phospholemman; mTOR: mammalian target of rapamycin; PKCα/β Thr638/641 phos: protein
693 kinase Cα/β threonine 638/641 phosphorylation; p70S6K1: Ribosomal protein S6 p70 Kinase 1;
694 PLN: Phospholamban.

695

696 **Figure 3A**

697 Muscle protein non-specific FXYD1 phosphorylation at rest and during repeated intense exercise
698 (EX1 and EX2) before (PRE) and after (POST) 7 weeks of high-intensity training in combination
699 with a reduced training volume in trained cyclist (n=7). Data are normalized to mean at rest before
700 the intervention period (PRE) and expressed as means ± SEM. The overall statistical effects – Acute
701 exercise: P>0.001, Training: P=0.012 and Interaction: P=0.232. * Post higher than Pre. # Rest lower
702 than all other time points. $ Rest lower than all other time points after IT (Post). ¤ End of EX1 and
703 EX2 higher than rest before IT (Pre) and ¤¤ End of EX2 higher than before EX2 before IT (Pre).

704

705

Figure 3B

Muscle protein FXYD1 ser63 phosphorylation at rest and during repeated intense exercise (EX1 and EX2) before (PRE) and after (POST) 7 weeks of high-intensity training in combination with a reduced training volume in trained cyclist (n=7). Data are normalized to mean at rest before the intervention period (PRE) and expressed as means ± SEM. The overall statistical effects – Acute exercise: P=0.359, Training: P=0.938 and Interaction: P=0.165.

Figure 3C

Muscle protein FXYD1 ser68 phosphorylation at rest and during repeated intense exercise (EX1 and EX2) before (PRE) and after (POST) 7 weeks of high-intensity training in combination with a reduced training volume in trained cyclist (n=7). Data are normalized to mean at rest before the intervention period (PRE) and expressed as means ± SEM. The overall statistical effects – Acute exercise: P=0.053, Training: P=0.046 and Interaction: P=0.520. * Post higher than Pre.

Figure 3D

Muscle protein FXYD1 ser68 phosphorylation, considering antibody phospho-sensitivity, at rest and during repeated intense exercise (EX1 and EX2) before (PRE) and after (POST) 7 weeks of high-intensity training in combination with a reduced training volume in trained cyclist (n=7). Data are normalized to mean at rest before the intervention period (PRE) and expressed as means ± SEM. The overall statistical effects – Acute exercise: P<0.001, Training: P=0.004 and Interaction: P=0.920. * Post higher than Pre. # End of EX1 higher than all other time points. ## End of EX2 higher than Rest and before EX2. $ End of EX1 higher than all other time points after IT (Post). ¤ End of EX1 higher than Rest and before EX2 before IT (Pre).

Figure 3E

Muscle protein FXYD1 thr69 phosphorylation at rest and during repeated intense exercise (EX1 and EX2) before (PRE) and after (POST) 7 weeks of high-intensity training in combination with a

reduced training volume in trained cyclist (n=7). Data are normalized to mean at rest before the intervention period (PRE) and expressed as means ± SEM. The overall statistical effects – Acute exercise: P=0.824, Training: P=0.001 and Interaction: P=0.937. * Post lower than Pre.

Figure 3F

Muscle protein FXYD1 thr69 phosphorylation, considering antibody phospho-sensitivity, at rest and during repeated intense exercise (EX1 and EX2) before (PRE) and after (POST) 7 weeks of high-intensity training in combination with a reduced training volume in trained cyclist (n=7). Data are normalized to mean at rest before the intervention period (PRE) and expressed as means ± SEM. The overall statistical effects – Acute exercise: P=0.006, Training: P=0.071 and Interaction: P=0.723. # Rest lower than all other time points. ¤ End of EX1 and End of EX2 higher than Rest before IT (Pre).

Table 1

Antibody overview

4E-BP1: eukaryotic initiation factor 4E-binding protein 1; ACCβ Ser221 phos: Acetyl-CoA carboxylase β serine 221 phosphorylation; AMPKα2: AMP-activated Protein Kinase α2; CaMKII: Ca^{2+}/Calmodulin-dependent Protein Kinase II; eEF2: Eukaryotic elongation factor 2; FXYD1: phospholemman; mTOR: mammalian target of rapamycin; PKCα/β Thr638/641 phos: protein kinase Cα/β threonine 638/641 phosphorylation; p70S6K1: Ribosomal protein S6 p70 Kinase 1; PLN: Phospholamban.

Table 2

Muscle protein expression at rest, before and after 7 weeks of high-intensity training in combination with a reduced training volume in trained cyclist

4E-BP1: eukaryotic initiation factor 4E-binding protein 1; AMPKα2: AMP-activated Protein Kinase α2; CaMKII: Ca^{2+}/Calmodulin-dependent Protein Kinase II; eEF2: Eukaryotic elongation

760 factor 2; FXYD1: phospholemman; mTOR: mammalian target of rapamycin; p70S6K1: Ribosomal
761 protein S6 p70 Kinase 1; PLN: Phospholamban. Values are means ± SE in arbitrary units; n = 7.
762 The main statistical P-values obtained from a Two-way RM ANOVA statistical analysis are
763 expressed. Protein expression is different after compared to before the training intervention * $P <$
764 0.05, and ** $P < 0.01$. Protein expression tended to be different after compared to before the
765 training intervention # $P < 0.10$.

766

767 **Table 3**

768 **Changes in protein phosphorylation at rest and during the repeated intense exercise protocol**
769 **before and after 7 weeks of high-intensity training with a reduced training volume in trained**
770 **cyclist**

771 4E-BP1: eukaryotic initiation factor 4E-binding protein 1; ACCβ: Acetyl-CoA carboxylase β;
772 AMPKα2: AMP-activated Protein Kinase α2; CaMKII: Ca^{2+}/Calmodulin-dependent Protein Kinase
773 II; eEF2: Eukaryotic elongation factor 2; mTOR: mammalian target of rapamycin; PKCα/β: protein
774 kinase C α/β; p70S6K1: Ribosomal protein S6 p70 Kinase 1; PLN: Phospholamban. E: Acute
775 exercise, T: Training, I: Interaction, End of EX1: After the first intense exercise bout lasting 2min,
776 Before EX2: Before the second exercise bout and End of EX2: after the second high intensity
777 exercise bout performed to exhaustion. Data are expressed as means±SE. * PRE higher than POST.
778 ** POST higher than PRE. $ Higher than Rest within PRE or POST, $$ Higher than all other time
779 points within PRE or POST, # Higher than Rest, ## Higher than all other time points, ¤ Higher than
780 before EX2.

781 **Table 1** Antibody overview

Protein target	Ab cat. number or name	Company or donor	Ab source	Migration MW
4E-BP1	#9452	Cell Signaling Technology	rabbit	15-20 kDa
4E-BP1 Thr37/46 phos	#2855	Cell Signaling Technology	rabbit	15-20 kDa
ACCβ Ser221 phos	#07-303	Millipore	rabbit	259 kDa
Actin	A2066	Sigma Aldrich	rabbit	42 kDa
AMPKα2	AMPK α2	Dr. J. Birk, University of Copenhagen	sheep	63 kDa
AMPKα Thr172 phos	#2531	Cell Signaling Technology	rabbit	63 kDa
CaMKII	611293	BD Transduction Laboratories	mouse	55-75 kDa
CaMKII Thr286 phos	#3361	Cell Signaling Technology	rabbit	55-75 kDa
eEF2	ab130187	Abcam	mouse	95 kDa
eEF2 Thr56 phos	#2331	Cell Signaling Technology	rabbit	95 kDa
FXYD1	13721-1-AP	Proteintech	rabbit	12 kDa
FXYD1 unphosphorylated	AB_FXYD1 – C2	Dr. J. Randall Moorman, University of Virginia	rabbit	12 kDa
FXYD1 Ser68 phos	AB_FXYD1ser68 – CP68	Dr. D. Bers, Loyola University	rabbit	12 kDa
FXYD1 Ser63 phos	FXYD1ser63 phos	Professor M. Shattock, King's College London	rabbit	12 kDa
FXYD1 Ser68 phos	FXYD1ser68 phos	Professor M. Shattock, King's College London	rabbit	12 kDa
FXYD1 Thr69 phos	FXYD1thr69 phos	Professor M. Shattock, King's College London	sheep	12 kDa
mTOR	#2972	Cell Signaling Technology	rabbit	289 kDa
mTOR Ser2448 phos	#2971	Cell Signaling Technology	rabbit	289 kDa
p70S6K1	#2708	Cell Signaling Technology	rabbit	70 kDa
p70S6K1 Thr389 phos	#9234	Cell Signaling Technology	rabbit	70 kDa
PKCα/β Thr638/641 phos	#9375	Cell Signaling Technology	rabbit	80-82 kDa
PLN	PA5-19351	Pierce – ThermoScientific	goat	6 kDa
PLN Thr17 phos	Sc-17024	Santa Cruz Biotechnology	rabbit	6 kDa

782 4E-BP1: eukaryotic initiation factor 4E-binding protein 1; ACCβ Ser221 phos: Acetyl-CoA carboxylase β serine 221 phosphorylation; AMPKα2: AMP-
783 activated Protein Kinase α2; CaMKII: Ca^{2+}/Calmodulin-dependent Protein Kinase II; eEF2: Eukaryotic elongation factor 2; FXYD1: phospholemman;
784 mTOR: mammalian target of rapamycin; PKCα/β Thr638/641 phos: protein kinase Cα/β threonine 638/641 phosphorylation; p70S6K1: Ribosomal
785 protein S6 p70 Kinase 1; PLN: Phospholamban.

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Table 2 Muscle protein expression before and after 7 weeks of high-intensity training in combination with a reduced training volume in trained cyclist.

			Main statistical P-values for a Two-way RM ANOVA		
Protein / Antibody	Before	After	Training	Acute exercise	Interaction
4E-BP1	0.99 ± 0.06	0.75 ± 0.05*	**0.013**	0.896	0.850
Actin	0.86 ± 0.05	1.26 ± 0.09*	**0.018**	0.399	0.828
AMPKα2	1.00 ± 0.03	1.04 ± 0.04	0.325	0.285	0.965
CaMKII β_M	0.96 ± 0.07	1.19 ± 0.13#	0.072	0.563	0.771
CaMKII γ/δ	0.92 ± 0.07	1.17 ± 0.11**	**0.006**	0.382	0.179
eEF2	0.74 ± 0.05	0.80 ± 0.06	0.357	0.143	0.051
FXYD1	0.98 ± 0.05	1.28 ± 0.08**	**0.005**	0.215	0.081
mTOR	0.95 ± 0.05	1.07 ± 0.06*	**0.015**	0.630	0.211
p70S6K1	0.87 ± 0.03	0.88 ± 0.04	0.570	0.106	**0.030**
PLN	1.06 ± 0.05	1.22 ± 0.06**	**0.007**	0.470	0.328

4E-BP1: eukaryotic initiation factor 4E-binding protein 1; AMPKα2: AMP-activated Protein Kinase α2; CaMKII: Ca^{2+}/Calmodulin-dependent Protein Kinase II; eEF2: Eukaryotic elongation factor 2; FXYD1: phospholemman; mTOR: mammalian target of rapamycin; p70S6K1: Ribosomal protein S6 p70 Kinase 1; PLN: Phospholamban. Values are means ± SE in arbitrary units; n = 7. The main statistical P-values obtained from a Two-way RM ANOVA statistical analysis are expressed. Protein expression is different after compared to before the training intervention * $P < 0.05$, and ** $P < 0.01$. Protein expression tended to be different after compared to before the training intervention # $P < 0.10$.

798 **Table 3. Changes in protein phosphorylation at rest and during the repeated intense exercise protocol before and after 7 weeks of**
799 **high-intensity training in combination with a reduced training volume in trained cyclist**

Target	Main effects ANOVA P-values	Time	Rest	End of EX1	Before EX2	End of EX2
PKCα/β thr638/641	E: P=0.240 **T: P=0.036** **I: P=0.029**	PRE POST	1.00±0.12 0.81±0.09	0.75±0.09 1.03±0.20**	0.88±0.09 1.27±0.16**$	0.93±0.07 1.05±0.16
CaMKIIβ thr287	E: P=0.156 **T: P=0.014** I: P=0.337	PRE POST	1.00±0.24 6.03±1.04	4.37±2.12 8.95±2.34	1.08±0.15 8.61±2.23**	2.45±0.68 11.32±3.77**
CaMKIIγ/δ thr287	E: P=0.279 **T: P=0.004** I: P=0.263	PRE POST	1.00±0.27 5.48±1.21**	3.81±1.38 6.10±1.37	1.06±0.13 6.22±1.52**	1.96±0.35 7.30±2.09**
PLN thr17	E: P=0.117 T: P=0.235 **I: P=0.039**	PRE POST	1.00±0.16 1.42±0.07**	1.37±0.05 1.31±0.07	1.52±0.12$ 1.44±0.14	1.14±0.10 1.21±0.09
eEF2 thr56	**E: P=0.007** **T: P=0.002** I: P=0.086	PRE POST	1.00±0.19 3.16±0.64**	2.90±0.37 2.87±0.47	2.75±0.32 2.60±0.51	3.52±0.24##$ 5.29±0.99**##$$
mTOR ser2448	E: P=0.064 **T: P=0.018** I: P=0.139	PRE POST	1.00±0.13 1.32±0.22	1.63±0.25 1.59±0.19	0.99±0.13 1.62±0.30**	1.28±0.15 1.72±0.14**
p70S6K1 thr389	**E: P=0.021** T: P=0.524	PRE POST	1.00±0.12 1.47±0.29	2.36±0.39#$ 2.60±0.36#$	1.96±0.34$ 2.09±0.46	2.27±0.26$ 1.95±0.23

	I: P=0.178					
4E-BP1 thr37/46	E: P=0.271 T: P=0.197 I: P=0.296	PRE POST	1.00±0.16 1.01±0.25	0.67±0.11 0.73±0.11	0.82±0.09 0.93±0.17	0.59±0.10 0.88±0.18
AMPKα thr172	**E: P=0.003** T: P=0.210 **I: P=0.047**	PRE POST	1.00±0.09 0.90±0.10	0.74±0.08 0.65±0.14	0.74±0.09 0.79±0.11	1.46±0.19*$^{\$\$\#\#}$ 1.03±0.10$^{\#\#}$
ACCβ ser221	**E: P<0.001** T: P=0.182 I: P=0.558	PRE POST	1.00±0.18 1.00±0.15	3.25±0.76$^{\#\$}$ 2.51±0.37$^{\#\$}$	2.98±0.84$^{\#\$}$ 1.84±0.37$^{\#}$	3.99±0.86$^{\#\$¤}$ 3.00±0.60$^{\#\$¤}$

4E-BP1: eukaryotic initiation factor 4E-binding protein 1; ACCβ: Acetyl-CoA carboxylase β; AMPKα2: AMP-activated Protein Kinase α2; CaMKII: Ca^{2+}/Calmodulin-dependent Protein Kinase II; eEF2: Eukaryotic elongation factor 2; mTOR: mammalian target of rapamycin; PKCα/β: protein kinase C α/β; p70S6K1: Ribosomal protein S6 p70 Kinase 1; PLN: Phospholamban. E: Acute exercise, T: Training, I: Interaction, End of EX1: After the first intense exercise bout lasting 2min, Before EX2: Before the second exercise bout and End of EX2: after the second high intensity exercise bout performed to exhaustion. Data are expressed as means±SE. * PRE higher than POST. ** POST higher than PRE. $^{\$}$ Higher than Rest within PRE or POST, $^{\$\$}$ Higher than all other time points within PRE or POST, $^{\#}$ Higher than Rest, $^{\#\#}$ Higher than all other time points, ¤ Higher than before EX2.

Figure 1

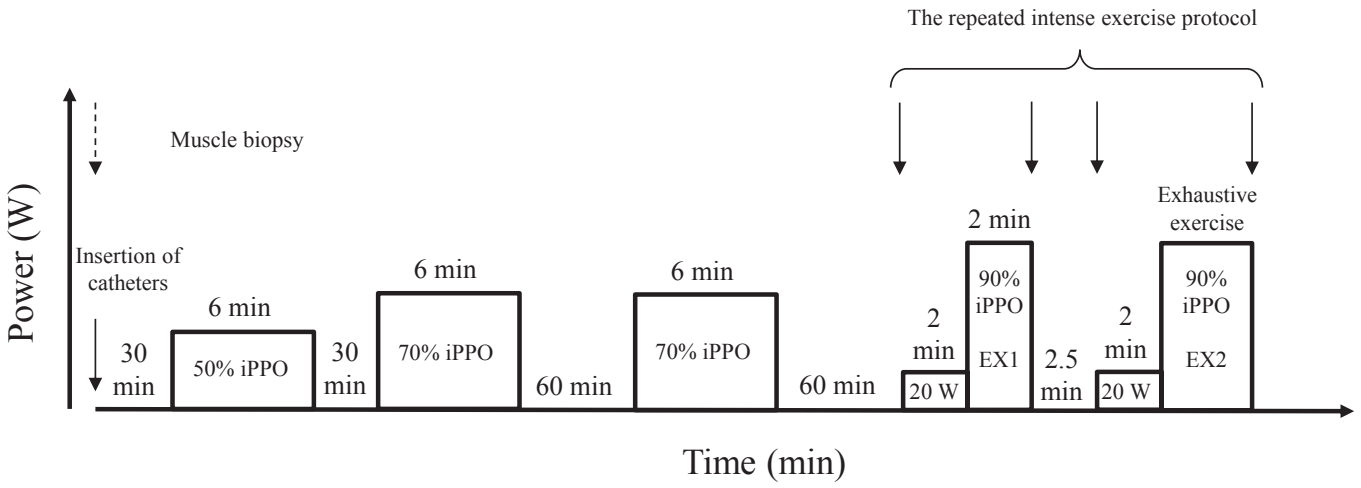

The repeated intense exercise protocol
Muscle biopsy
Insertion of catheters
Power (W)
30 min
6 min
50% iPPO
30 min
6 min
70% iPPO
60 min
6 min
70% iPPO
60 min
2 min
20 W
2 min
90% iPPO
EX1
2.5 min
2 min
20 W
Exhaustive exercise
90% iPPO
EX2
Time (min)

Figure 2

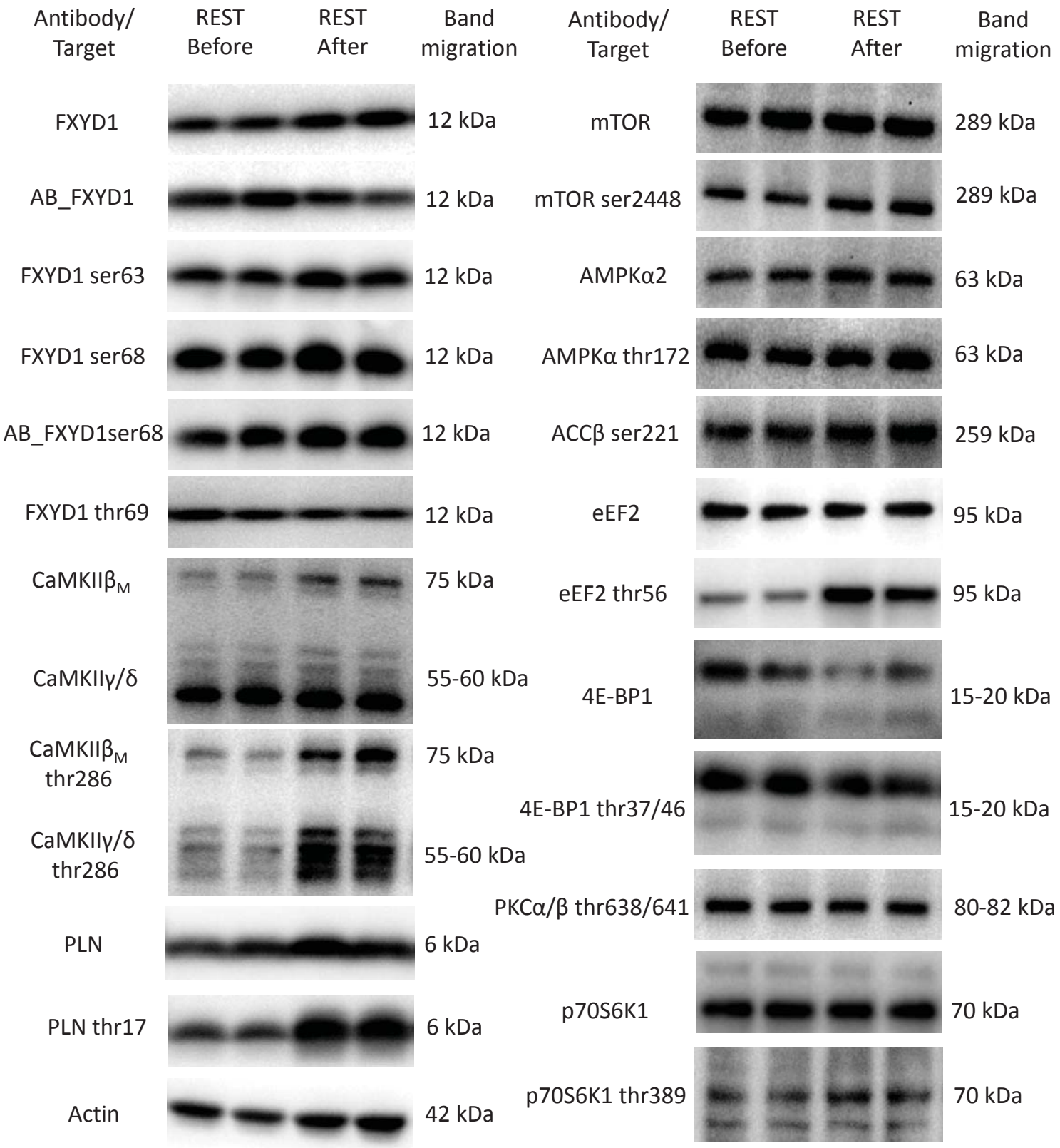

Antibody/ Target
REST Before
REST After
Band migration
FXYD1
12 kDa
AB_FXYD1
12 kDa
FXYD1 ser63
12 kDa
FXYD1 ser68
12 kDa
AB_FXYD1ser68
12 kDa
FXYD1 thr69
12 kDa
CaMKIIβM
75 kDa
CaMKIIγ/δ
55-60 kDa
CaMKIIβM thr286
75 kDa
CaMKIIγ/δ thr286
55-60 kDa
PLN
6 kDa
PLN thr17
6 kDa
Actin
42 kDa
Antibody/ Target
REST Before
REST After
Band migration
mTOR
289 kDa
mTOR ser2448
289 kDa
AMPKα2
63 kDa
AMPKα thr172
63 kDa
ACCβ ser221
259 kDa
eEF2
95 kDa
eEF2 thr56
95 kDa
4E-BP1
15-20 kDa
4E-BP1 thr37/46
15-20 kDa
PKCα/β thr638/641
80-82 kDa
p70S6K1
70 kDa
p70S6K1 thr389
70 kDa

Fig 3A

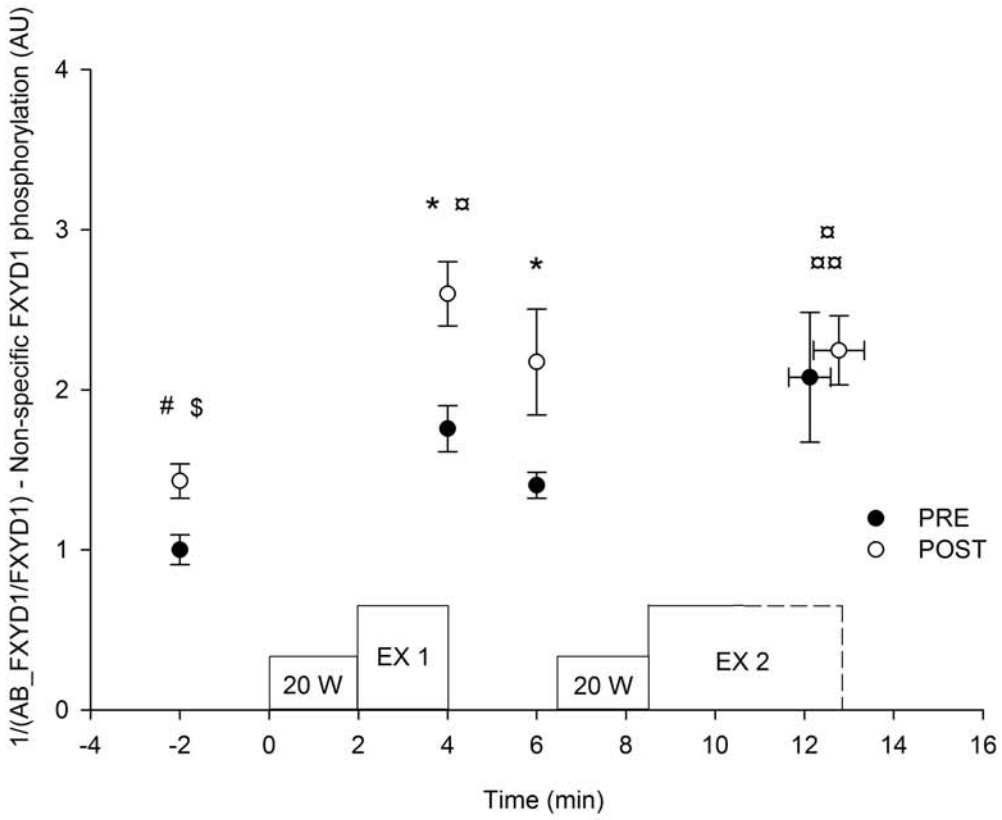

1/(AB_FXYD1/FXYD1) - Non-specific FXYD1 phosphorylation (AU)
4
3
2
1
0
-4
-2
0
2
4
6
8
10
12
14
16
Time (min)
$
* ¤
*
¤
¤¤
20 W
EX 1
20 W
EX 2
PRE
POST

Fig 3B

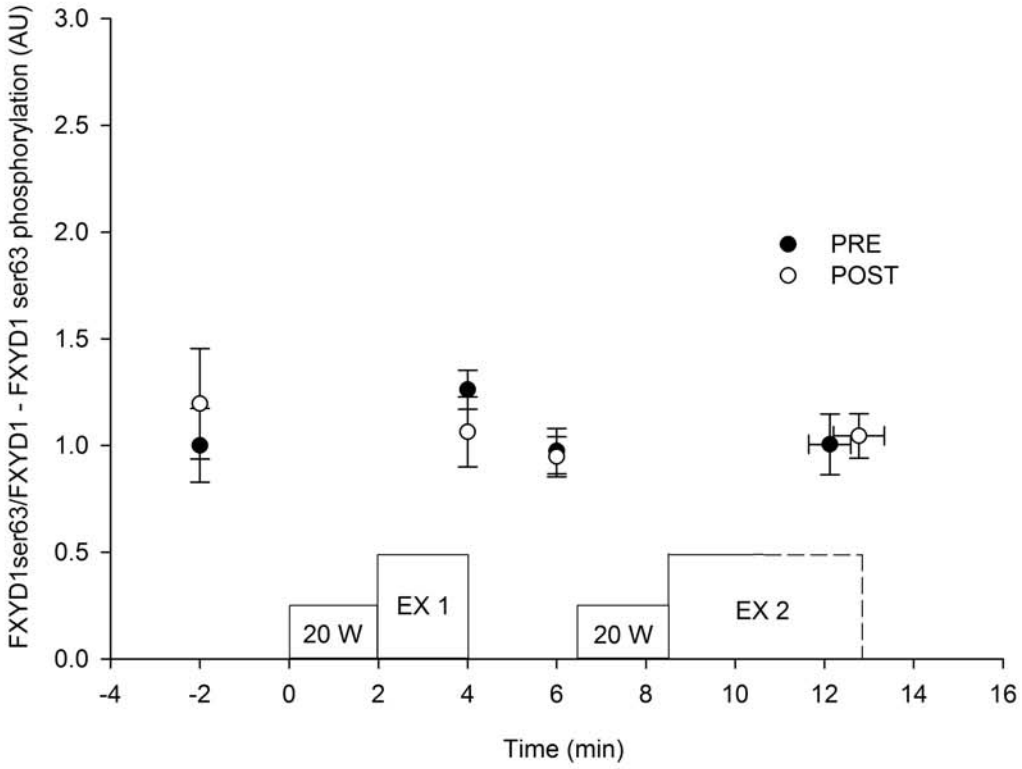

FXYD1ser63/FXYD1 - FXYD1 ser63 phosphorylation (AU)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
PRE
POST
20 W
EX 1
20 W
EX 2
-4
-2
0
2
4
6
8
10
12
14
16
Time (min)

Fig 3C

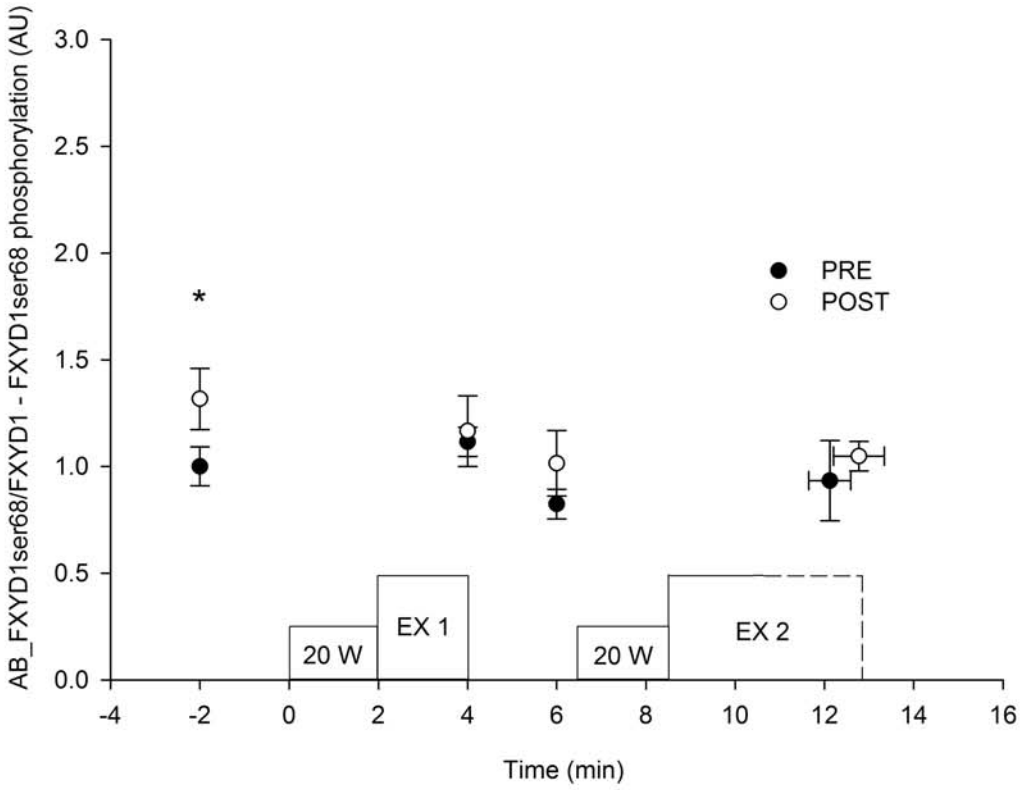

AB_FXYD1ser68/FXYD1 - FXYD1ser68 phosphorylation (AU)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-4
-2
0
2
4
6
8
10
12
14
16
Time (min)
PRE
POST
*
20 W
EX 1
20 W
EX 2

Fig 3D

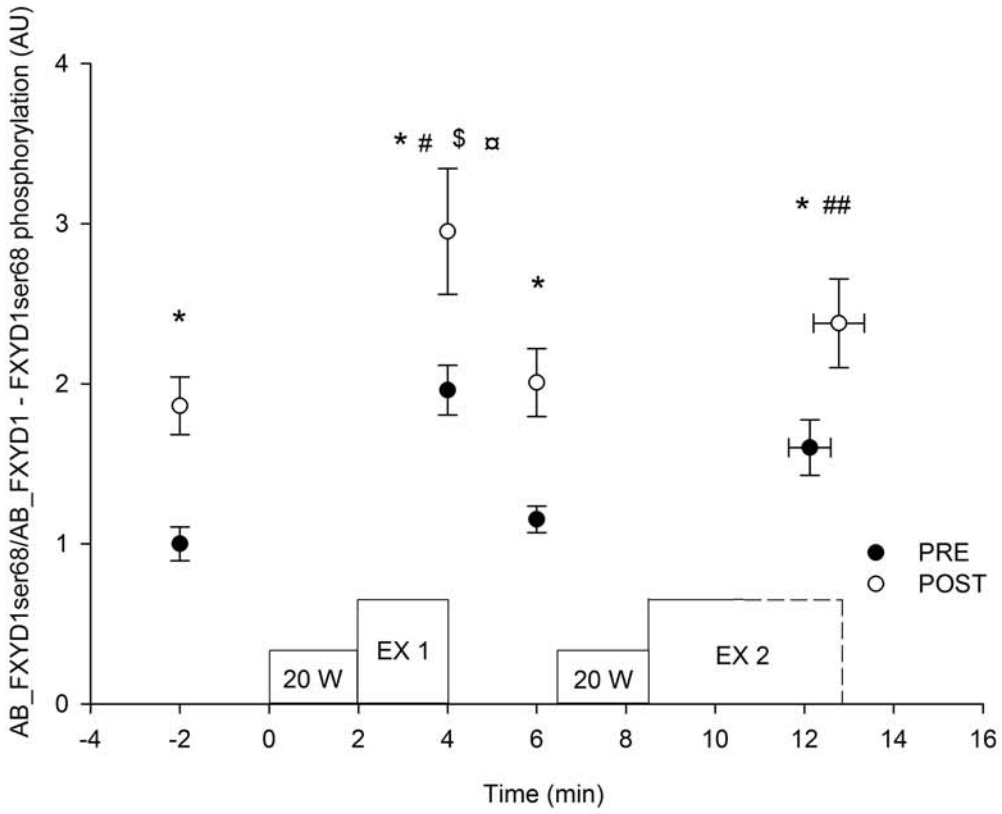

AB_FXYD1ser68/AB_FXYD1 - FXYD1ser68 phosphorylation (AU)
Time (min)
20 W
EX 1
20 W
EX 2
PRE
POST
* # $ ¤
* ##
*
*

Fig 3E

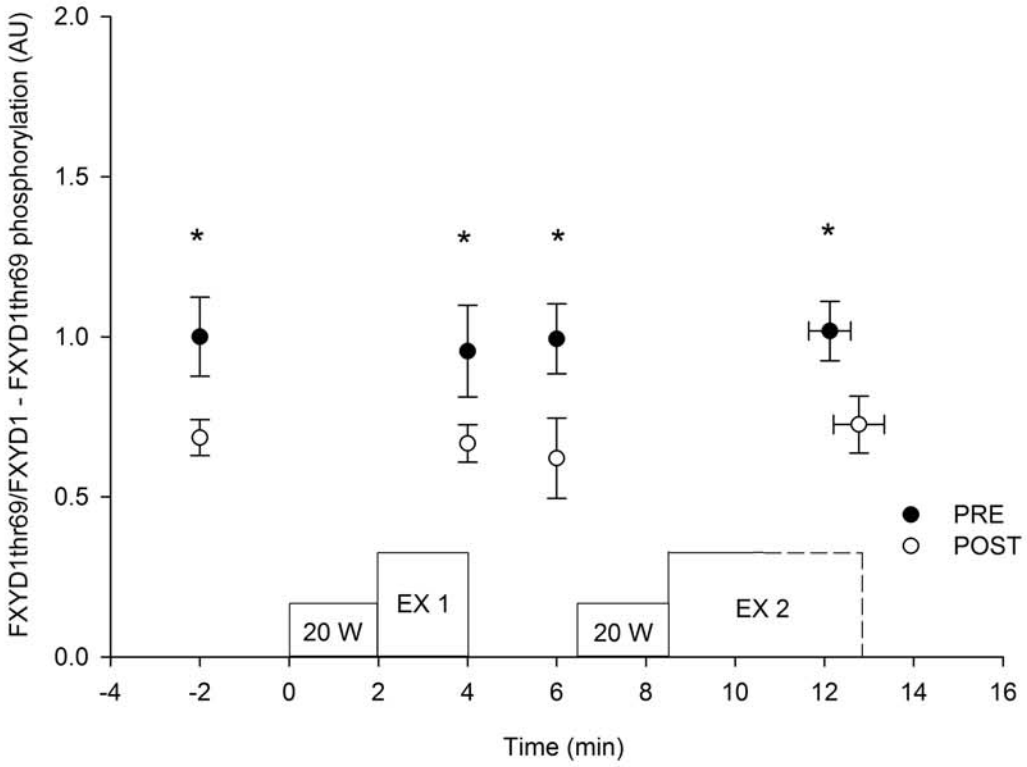
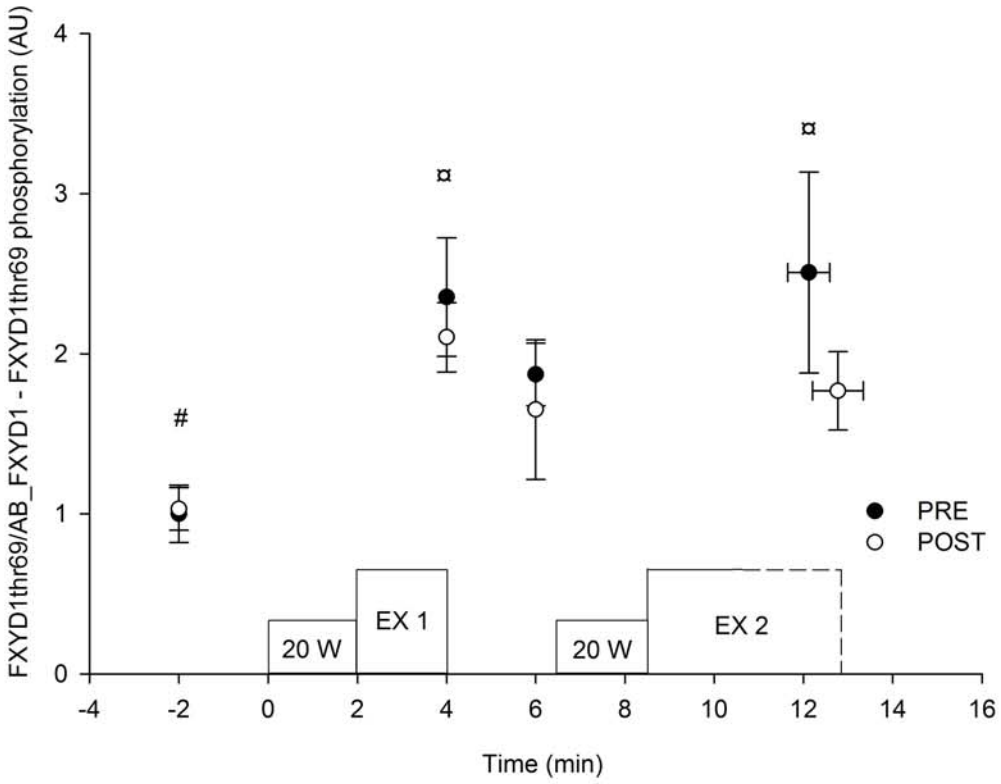

FXYD1thr69/FXYD1 - FXYD1thr69 phosphorylation (AU)
2.0
1.5
1.0
0.5
0.0
-4
-2
0
2
4
6
8
10
12
14
16
Time (min)
*
*
*
*
20 W
EX 1
20 W
EX 2
PRE
POST

Fig 3F


FXYD1thr69/AB_FXYD1 - FXYD1thr69 phosphorylation (AU)
Time (min)
PRE
POST
20 W
EX 1
20 W
EX 2
#
¤
¤