Exercise induces different molecular responses in trained and untrained human muscle

Marcus Moberg¹, Malene E Lindholm²,³, Stefan M Reitzner², Björn Ekblom¹, Carl-Johan Sundberg²,⁴, and Niklas Psilander¹

¹Åstrand Laboratory, Swedish School of Sport and Health Sciences, Stockholm, Sweden, ²Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden ³Department of Medicine, School of Medicine, Stanford University, Stanford, CA, USA, and ⁴Department of Learning, Informatics, Management, and Ethics, Karolinska Institutet, Stockholm, Sweden

Corresponding author:
Marcus Moberg
The Swedish School of Sport and Health Sciences
Box 5626, SE-114 86 Stockholm, Sweden
E-mail: marcus.moberg@gih.se

Running title: Resistance exercise-induced muscle memory

Key words: muscle memory, mTORC1, PGC1α, E3 ligases
Abstract

**Introduction:** Human skeletal muscle is thought to have heightened sensitivity to exercise stimulus when it has been previously trained (i.e., it possesses “muscle memory”). We investigated whether basal and acute resistance exercise-induced gene expression and cell signaling events are influenced by previous strength training history. **Methods:** Accordingly, 19 training naïve women and men completed 10 weeks of unilateral leg strength training, followed by 20 weeks of detraining. Subsequently, an acute resistance exercise session was performed for both legs, with vastus lateralis biopsies taken at rest and 1 h after exercise in both legs (memory and control). **Results:** The phosphorylation of AMPK\(^{Thr172}\) and eEF2\(^{Thr56}\) was higher in the memory leg than in the control leg at both time points. Post-exercise phosphorylation of 4E-BP1\(^{Thr46}\) and Ser65 was higher in the memory leg than in the control leg. The memory leg had lower basal mRNA levels of total PGC1\(\alpha\), and, unlike the control leg, exhibited increases in PGC1\(\alpha\)–ex1a transcripts after exercise. In the genes related to myogenesis (*SETD3*, *MYOD1*, and *MYOG*), mRNA levels differed between the memory and the untrained leg; these effects were evident primarily in the male subjects. Expression of the novel gene *SPRYD7* was lower in the memory leg at rest and decreased after exercise only in the control leg, but SPRYD7 protein levels were higher in the memory leg. **Conclusion:** In conclusion, several key regulatory genes and proteins involved in muscular adaptations to resistance exercise are influenced by previous training history. Although the relevance and mechanistic explanation for these findings need further investigation, they support the view of a molecular muscle memory in response to training.
Introduction

Maintaining or increasing muscle mass and strength is associated with a reduced risk of mobility disability, cardiovascular disease, type 2 diabetes, and cancer (1). In this, strength training has a key role in muscular development and is a critical component of healthy ageing. As skeletal muscle generally becomes more resistant to growth stimulus with age, which is accompanied by a gradual loss of muscle mass and strength (2), it is prompted that strength training should be initiated in early adulthood and subsequently maintained. This notion is exemplified by master athletes being found to have muscular fitness comparable with that of young adults (3). Importantly, it is also argued that if individuals have had a history of strength training before a period of less or no training, muscle mass regrows more rapidly, or to a greater extent, upon new training stimulus (4-6). Data indicate, in other words, that previously trained muscle is more sensitive to new stimuli or possesses a “memory.”

The process of muscle hypertrophy is driven by acute stimulation of transcriptional and translational processes in the muscle fiber after each resistance exercise bout. More specifically this relates to a mechanistic target of rapamycin complex 1 (mTORC1)-dependent stimulation of protein synthesis (7), as well as induced expression of genes related to muscle structure, myogenesis, protein turnover, extracellular matrix, and angiogenesis (8). During hypertrophy, the outcome efficiency and capacity of mRNA translation and gene transcription are also influenced by satellite cell-induced myonuclear addition (9), ribosomal biogenesis (10) and epigenetic modifications (4). With regard to the existence of a “muscle memory,” much attention has been directed to strength training-induced increases in myonuclear content, nuclei which are preserved during atrophy and may enable rapid hypertrophy upon reloading (11-13). However, current scientific evidence of such a memory is limited to animal models and has yet to be shown in humans (14).
Numerous studies have shown that continuous training has a clear effect on the degree of acute exercise-induced cell signaling responses, gene expression, and rate of protein synthesis after both strength and endurance type of exercise (5, 15-22). Although most of these studies have demonstrated an overall attenuation in the acute molecular response, a few studies have shown that some molecular processes can be sensitized. The mechanisms underlying these altered acute responses after a period of training, and the question of whether these alterations are preserved after a period of detraining remains to be determined.

However, epigenetic modifications may play a role. Seaborne et al (4) recently showed that strength training-induced epigenetic modifications are sustained after 7 weeks of detraining and could partially explain the augmented hypertrophic response upon reloading. In contrast, Lindholm et al (23) found no endurance training-induced transcriptome differences between previously trained and untrained legs after a 40 week detraining period.

While the studies of Lindholm et al (23) and Seaborne et al (4) have provided important data regarding training-induced muscle memory in resting human skeletal muscle, no researchers have explored the potential of a muscle memory concerning acute exercise-induced gene expression and cell signaling response. Accordingly, in this study, young and completely untrained women and men underwent a 10 week unilateral leg strength training program followed by a 20 week detraining period. This period was followed by an acute strength training session involving both the previously trained and untrained (control) legs. Skeletal muscle biopsy samples were collected at rest and 1 h after exercise to determine both basal and exercise-induced gene expression, protein content, and phosphorylation status of proteins known to respond acutely to strength training stimuli. We hypothesized that the previously trained and untrained legs would show differences indicating long-lasting qualitative changes in the molecular machinery regulating muscle adaptations to resistance exercise.
**Methods**

**Subjects**

Nineteen healthy, inactive subjects (10 women and 9 men) who had never been engaged in any regular sport or physical activity volunteered to participate in this study. Their mean age was 25 years (±1 year); mean weight, 71 kg (±4 kg); and mean height, 175 cm (±8 cm). The subjects were carefully informed about the experimental design and possible risks related to the project and signed a written consent form before entering the project. The study was approved by the Regional Ethics Committee of Stockholm, Sweden (DNR 2015/211-31/4) and was performed in accordance with the Declaration of Helsinki.

**Experimental protocol**

Figure 1 is a schematic illustration of the experimental design. Subjects underwent a 10 week unilateral strength training period, followed by 20 weeks of detraining, during which no training was allowed. Only the memory leg was trained during the unilateral training period. The exercises included were leg presses and leg extensions, and training usually took place three times per week. Both moderate (70% to 75% of one repetition maximum (1-RM)) and heavy loading (80% to 85% of 1-RM) were performed in an undulating, periodized manner. During weeks 4 and 8, low-load, blood flow–restricted exercise was performed as well. The purpose of this strength training design was to maximize hypertrophy, satellite cell activation and fusion with the aim of stimulating possible lasting effects in the trained leg. To ensure optimal protein intake and to stimulate muscle growth, subjects consumed 25 g of whey protein concentrate (One Whey, Fitnessguru Sweden AB, Stockholm, Sweden) immediately after each training session. A more detailed description of the training protocol was published by Psilander et al (14).
The detraining period was followed by a bilateral exercise session (three sets of leg presses and three sets of leg extension) performed at approximately 75% of 1-RM until volitional failure. The legs were exercised one at a time, alternating between sets. A bilateral 1-RM test was performed in the leg press and leg extension before the exercise session, and the relative load (75%) was calculated from this test. Details of the 1-RM test protocol are available in the paper by Psilander et al (14). Biopsy samples were obtained from both legs before and approximately 1 h after exercise. The subjects reported to the laboratory between 8:30 AM and 3:30 PM in a nonfasted state. Only water was allowed during the 1 h post-exercise period.

**Muscle biopsies**

The muscle biopsies were collected under local anesthesia (2% Carbocain, AstraZeneca, Södertälje, Sweden) from the mid-part of m. vastus lateralis, proximally separated by at least 3 cm. An incision was made in the skin and the fascia before the biopsies were obtained using a Weil–Blakesley conchotome. The typical yield was 50 to 100 mg of muscle tissue. The tissue obtained was rapidly frozen in liquid nitrogen and stored at −80°C. The frozen samples were thereafter freeze-dried; powdered; dissected free of blood, fat, and connective tissue; and stored at −80°C for later determination of DNA/mRNA content and immunoblotting.

**RNA extraction and cDNA synthesis**

Total RNA was extracted from approximately 3 mg of freeze-dried skeletal muscle tissue with the TRizol® (Invitrogen) method. Briefly, the skeletal muscle sample was homogenized in TRizol with a bead beater and subsequently mixed with chloroform. After centrifugation, the aqueous phase was mixed with an equal volume of isopropanol to cause RNA precipitation. After centrifugation, the RNA pellet was washed in 75% ethanol, air-dried, and
resuspended in ultra-pure RNA water. To completely dissolve the RNA, the pellet was
incubated at 55°C for 10 min, and concentration was subsequently measured on a NanoDrop
Spectrophotometer. One microgram of RNA was used for cDNA conversion with the High-
Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) in a
total volume of 20 µL, in accordance with the manufacturer’s specifications.

Gene expression

Quantitative real-time polymerase chain reaction (PCR) was performed for gene expression
analysis of ABRA, ANGPT2, ANGPTL2, AXIN1, FBXO32, MSTN, MYF6, MYOD1, MYOG,
PPARGC1A–exon 1a, PPARGC1A–exon 1b, PPARGC1A total (PGC1α), SETD3, SPRYD7,
TGFBI, TRAF1, TRIM63, and UBR5. β2-Microglobulin was used as a housekeeping control,
and expression levels were calculated with the 2^ΔΔC_T method (24). All samples were run in
duplicates on a C1000 Touch thermal cycler (Bio-Rad Laboratories, Richmond, CA, USA)
using the SsoAdvanced Universal SYBR Green Supermix (model 1725272; Bio-Rad
Laboratories). PCR primers and assays were synthesized by Eurofins Genomics
(Luxembourg), Sigma-Aldrich (St. Louis, MO, USA), and Qiagen (Hilden, Germany). Primer
sequences and assay information are listed in Supplemental Table 1.

Methylation analysis

The Gentra Puregene DNA purification kit (Qiagen, #158667) with Proteinase K (Qiagen,
#158918) and RNase A solution (Qiagen, #158922) were used to extract genomic DNA from
the freeze-dried skeletal muscle by bead homogenization. The EpiTect Fast DNA Bisulfite kit
(Qiagen, #59824) was used to perform bisulfite transformation with 500 µg of genomic DNA
as starting material. The PyroMark PCR kit (Qiagen, #978703) with 10 ng of bisulfite-
transformed DNA was used to amplify the transformed DNA. PCR primers were synthesized
by Eurofins Genomics (see Supplemental Table 2). Assays were designed in the genomic
environment of the PGC1α-ex1a with the selected region based on the H3K4me3 mark
annotation of the UCSC genome browser (GRCh37/hg19 assembly). Pyrosequencing was
performed with the PyroMark Q96 ID device (Qiagen), PyroMark Gold Q96 pyrosequencing
reagents (Qiagen, #972804) and with sequencing primers synthesized by Eurofins Genomics
(see Supplemental Table 2). For bias control, bisulfite-transformed control DNA from the
EpiTect PCR Control DNA Set (Qiagen #59695) was used. CpG pyrosequencing was
analyzed with PyroMark Q96 software (Qiagen).

Protein extraction and Western blot
Lyophilized muscle samples (approximately 3 mg) free from blood and connective tissue
were homogenized by a Bullet Blender (Next Advance, Troy, NY, USA) in ice-cold buffer
(100 µL/mg dry weight) containing 2 mM of HEPES buffer (pH 7.4), 1 mM of EDTA, 5 mM
of EGTA, 10 mM of MgCl2, 50 mM of β-glycerophosphate, 1% Triton X-100, 1 mM of
Na3VO4, 2 mM of dithiothreitol, 1% phosphatase inhibitor cocktail (Sigma P-2850), and 1%
(v/v) Halt Protease Inhibitor Cocktail (Thermo Scientific, Rockford, IL, USA). After
homogenization, the samples were rotated for 30 min at 4°C and subsequently cleared from
myofibrillar and connective tissue debris by centrifugation at 10,000 g for 10 min at 4°C, and
the resulting supernatant was collected.

Protein concentration of the supernatants was determined in aliquots diluted 1:10 in
distilled water with the Pierce™ 660 nm protein assay (Thermo Fisher Scientific). Samples
were diluted in 4x Laemmli sample buffer (Bio-Rad Laboratories) and homogenizing buffer
to obtain a final protein concentration of 1.25 µg/µL. All samples were then heated at 95°C
for 5 min to denature the proteins and subsequently kept at −20°C until further separation in
SDS-Page.
For protein separation, 18.75 µg of protein from each sample were loaded on 26-well Criterion TGX gradient gels (4% to 20% acrylamide; Bio-Rad Laboratories), and electrophoresis was performed on ice at 300 V for 30 min. Next, gels were equilibrated in transfer buffer (25 mM Tris base, 192 mM glycine, and 10% methanol) for 30 min at 4°C, after which proteins were transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories) at a constant current of 300 mA for 3 h at 4°C. To confirm equal loading and transfer, the membranes were stained with MemCode™ Reversible Protein Stain Kit (Thermo Fisher Scientific). For each target proteins, all samples from each subject were loaded on the same gel, and gels for all subjects were run simultaneously.

Blocking of membranes was performed for 1 h at room temperature in Tris-buffered saline (TBS; 20 mM of Tris base and 137 mM of NaCl; pH 7.6) containing 5% nonfat dry milk and followed by overnight incubation with commercially available primary antibodies diluted in TBS supplemented with 0.1% Tween-20 containing 2.5% nonfat dry milk (TBS-TM). Membranes were washed free from primary antibody with TBS-TM and then incubated for 1 h at room temperature with secondary HRP-conjugated antibodies. Next, the membranes were washed with TBS-TM (twice for 1 min, three times for 10 min), followed by four washes for 5 min with TBS only. Finally, to visualize the target proteins, SuperSignal™ West Femto Chemiluminescent Substrate (Thermo Fisher Scientific) was applied to the membranes, and a ChemiDoc™ XRS molecular imaging system was used for detection. The detected bands were quantified using the contour tool in the Quantity One® version 4.6.3 software (Bio-Rad Laboratories).

Before blocking, membranes from each gel were cut in strips for each target protein and assembled. Accordingly, all samples were exposed to the same blotting conditions. After visualization, the membranes were stripped of the phospho-specific antibodies by Restore Western Blot Stripping Buffer (Thermo Fisher Scientific) for 30 min at 37°C, after which the
membranes were washed and reprobed with primary antibodies for each respective total protein, as described previously. All phospho-proteins were normalized to their corresponding total protein. For MuRF-1, SPRYD7, GAPDH, COX IV, and rpS6 values were normalized against the total protein stain obtained with the MemCode™ kit.

For immunoblotting, primary antibodies against mTOR (Ser\textsubscript{2448}, #2971; total, #2983), S6K1 (Thr\textsuperscript{389}, #9234; total #2708), 4E-BP1 (Ser\textsuperscript{65}, #9456; total, #9644), eEF2 (Thr\textsuperscript{56}, #2331; total, #2332), AMPK (Thr\textsuperscript{172}, #4188; total, #2532), S6 (Ser\textsuperscript{235/236} #2211; total, #2217), COX IV (#4850) and GAPDH (#5174) were purchased from Cell Signaling Technology (Beverly, MA, USA). Primary antibody against 4E-BP1Thr\textsuperscript{46} (#sc-271947), MuRF-1 (#sc-32920), SPRYD7 (#sc-514533) antibody was purchased from Santa Cruz Biotechnology (Heidelberg, Germany).

All primary antibodies were diluted 1:1000 except for phospho-eEF2, COX IV, GAPDH, and S6 total, which were diluted 1:2000, and 4E-BP1Thr\textsuperscript{46}, which was diluted 1:200. Secondary anti-rabbit (#7074; 1:10,000) and secondary anti-mouse (#7076; 1:10,000) were purchased from Cell Signaling Technology.

**Statistical analysis**

Data are presented as mean ± SEM. Statistics for gene expression, as well as for protein content and phosphorylation were calculated by repeated-measures ANOVA together with Fisher’s LSD post hoc test and Bonferroni’s Multiple Comparison Test on group level (control: n=19, memory: n=19), as well as on males (n=9) and females (n=10) separated using Statistica 13.3 (TIBCO Software, Inc.). Statistics for methylation analysis was calculated by two-way ANOVA with Prism 7.05 (GraphPad, San Diego, CA, USA). Statistical significance was determined at \( p < 0.05 \).
Results

I-RM test and loading during the exercise session

The memory leg was significantly stronger than the control leg after 20 weeks of detraining in both the leg press (126 ± 10 kg vs. 98 ± 9 kg, respectively; \( p < 0.05 \)) and the leg extension (45 ± 3 kg vs. 41 ± 3 kg, respectively; \( p < 0.05 \)) exercise. There were however no differences between legs in mean muscle fiber cross-sectional area (CSA) at this stage that contributed to the difference in strength, memory leg 4385 \( \mu \text{m}^2 \) (range 2478 – 6187) and control leg 4237 \( \mu \text{m}^2 \) (range 2997 – 6235), see Psilander et al (14) for details. The absolute load used during the exercise session was therefore higher for the memory leg than for the control leg (94 ± 8 kg vs. 74 ± 7 kg, respectively, for the leg press and 34 ± 2 kg vs. 31 ± 2 kg, respectively, for the leg extension; \( p < 0.05 \)). The average number of repetitions performed during the exercise session was similar for the memory leg (10.5 ± 0.3 repetitions) and control leg (10.7 ± 0.2 repetitions) in the leg press exercise. However, a small difference between the legs was observed for the leg extension exercise (9.6 ± 0.4 repetitions for the memory leg and 9.0 ± 0.4 repetitions for the control leg; \( p < 0.05 \)).

Gene expression

Total expression of PGC1α mRNA was 18% lower in the memory leg at baseline and decreased significantly after exercise only in the control leg (\( p < 0.05 \) for time and leg interaction; Fig. 2A). These effects on total PGC1α at group level were primarily mediated by the male subjects, whereas the female subjects had similar levels at baseline and exhibited no change after exercise (Fig. 2B). PGC1α–ex1a mRNA was affected by exercise only in the memory leg and increased by approximately 60% at group level (\( p < 0.05 \) for time and leg interaction; Fig. 2C). In sex-specific gene expression analysis, PGC1α–ex1a expression was increased only in the male subjects (Fig. 2D).
SPRYD7 mRNA was unaffected by exercise in the memory leg but decreased by approximately 35% in the control leg and was expressed at lower levels in the memory leg at baseline ($p < 0.05$ for time and leg interaction; Fig. 2E). The expression of ANGPTL2 mRNA increased after exercise in both legs ($p < 0.05$ for time; Fig. 2G), but sex-specific analysis demonstrated this increase only in the female subjects ($p < 0.05$ for time; Fig. 2H). Moreover, the male subjects exhibited a 13% to 32% higher expression of ANGPTL2 mRNA in the memory leg, independent of time ($p < 0.05$ for leg; Fig. 2H).

MYOG mRNA was 15%–65% higher in the memory leg independent of time, with the expression also being reduced after exercise in both legs ($p < 0.05$ for time and leg; Fig. 3A). The expression of MYOD1 mRNA was not different between legs and was not altered by exercise on group basis (Fig. 3C). However, a decrease in MYOD1 mRNA content was noted among the male subjects after exercise in the control leg only; the expression in the control leg was also significantly different from that in the memory leg at that time point ($p < 0.05$ for time and leg interaction; Fig. 3D).

At group level the mRNA content of FBXO32 was unaffected by exercise and expressed to a similar degree in both legs (Fig. 3E). However, in male subjects only, FBXO32 mRNA expression was 30% lower in the memory leg at baseline and decreased by 37% in the control leg after exercise ($p < 0.05$ for time and leg interaction; Fig. 3F), with no differences noted in the female subjects. The expression pattern of SETD3 mRNA was similar to that of FBXO32 mRNA, with no differences noted a group level but a similar interaction between time and leg for the male subjects (Fig. 3G and H). The mRNA content of ABRA, AXIN1, MYF6, PGC1α–ex1b, TGFB1, and TRIM63 increased acutely following exercise and, to a similar extent, in both legs ($p < 0.05$ for time; Table 1), whereas that of UBR5, TRAF1, and MSTN showed no changes in all biopsy samples.
Methylation of key promoter regions of the PGC1α-ex1a isoform

As stated previously, there was a significant difference in the expression level of the PGC1α-ex1a isoform between the memory leg (60%) and the control leg (no increase). A bisulfite methylation assay was performed to investigate whether this could be explained by differences in the methylation level of key promoter regions of the PGC1α-ex1a isoform.

Seven CpG sites located in association with exon 1a were included in the analysis; methylation levels ranged from 2.2% to 18.6%. Statistical testing across time points for each site showed no significant difference between methylation of target site in control and memory legs.

Protein phosphorylation and content

The phosphorylation of 4E-BP1Thr46 was reduced by 13% in the control leg after exercise; the phosphorylation was 18% higher in the memory leg at that time point ($p < 0.05$ for time and leg interaction; Fig. 4A). These effects at group level were present in the male subjects, whereas the female subjects exhibited a 15% increase in phosphorylation in the memory leg only ($p < 0.05$ for time and leg interaction; Fig. 4B). The phosphorylation of 4E-BP1Ser65 exhibited a similar pattern to that of 4E-BP1Thr46 without an exercise-induced increase in the memory leg for the women (data not shown). The phosphorylation of mTORSer2248, S6K1Thr389, S6Ser235/236 increased by approximately 30%, 6-fold, 15-fold, and 15-fold, respectively, after exercise in both the control and memory legs ($p < 0.05$ for time; Table 2), with no differences in response between the two legs.

The phosphorylation of eEF2Thr56 was 16% to 19% lower in the control leg, regardless of time point ($p < 0.05$ for leg; Fig 4C), and after exercise, phosphorylation was reduced by approximately 40% in both legs ($p < 0.05$ for time; Fig. 4C). These effects were apparent in both male and female subjects (Fig. 4D). In conformity with eEF2Thr56, the phosphorylation of
AMPK\textsuperscript{Thr172} was 10% to 17% lower in the control leg at both time points (\(p < 0.05\) for leg; Fig 4E) but increased to a similar extent in both legs as a result of the exercise (\(p < 0.05\) for time). The sex-specific analysis revealed a difference between legs only in the women (\(p < 0.05\) for leg; Fig 4E), with insufficient power to detect an increase over time.

Total protein levels of SPRYD7 was similar in both legs and not altered by exercise at group level (Fig 4G), but the male subjects exhibited 23% to 57% higher SPRYD7 protein levels in the memory leg (\(p<0.05\) for leg; Fig 4G). Total protein levels of MuRF-1 were increased after exercise, detected as a main effect of time in the statistical analysis, with a 10% increase in the control leg and a 3% increase in the memory leg (\(p < 0.05\) for time; Table 2). Protein levels of GAPDH and COX IV (Table 2), as well as total protein content of the proteins probed for phosphorylation status were not altered by exercise and did not differ between legs at baseline before exercise.

Discussion

In this study, using a model in which previously strength-trained and untrained muscles were subjected to an acute bout of resistance exercise, we obtained novel data showing that basal and exercise-induced specific gene expression and cell signaling are modified by previous training history. More specifically, we showed that the previously trained memory leg had lower pre exercise levels of total PGC1\(\alpha\) mRNA and that exercise-induced increases in PGC1\(\alpha\)-ex1a transcripts occurred only in that leg. Moreover, post-exercise phosphorylation of 4E-BP\(1\)\textsuperscript{Thr46} and 4E-BP\(1\)\textsuperscript{Ser65} was higher in the memory leg, as was overall phosphorylation of AMPK\textsuperscript{Thr172} and eEF\(2\)\textsuperscript{Thr56}. We also found that previous training history modified both basal and exercise-induced mRNA expression of the novel gene SPRYD7. Finally, we show that differences in mRNA expression between the memory and control legs were apparent for ANGPTL2, MYOG, MYOD1, FBXO32, and SETD3. Overall, our data suggest that the
regulation of transcriptional and translational processes in skeletal muscle in relation to exercise can be both reduced and augmented by previous training history. The alteration, which was both suppressive and stimulatory, conforms to findings in previous research that a continuous training period induces a diverse adaptive response in related molecular processes (5, 15-20), which emphasizes the necessity of gene- and protein-specific evaluations with regard to training status/history.

The participants in this study had no previous experience in sports or physical activity ensuring that the untrained leg served as a true training naïve and intra-individual control. The 20 week detraining period ensured proper reversal of previous training-induced hypertrophy, and the unilateral training model enabled control of engagement in any spontaneous physical activities during detraining. The unilateral training model also enabled control of confounding factors such as genetics, environmental stress, and diet as well as acute exercise-induced systemic factors such as hormones, myokines, and lactate levels. Observed differences between the control and memory legs are thus probably attributable to previous training per se. Moreover it is worth noticing that satellite cell and myonuclear content in these subjects did not change during the initial training period or during detraining (14). There were also no differences in muscle fiber CSA between the legs at this point and we could not find any correlations between gene expression or protein content/phosphorylation and muscle fiber CSA. Therefore, observed differences could not be ascribed to altered nuclei number but were more likely to result from sustained epigenetic modifications, acetylase/deacetylase activity, phosphorylase/dephosphorylase activity, or other preserved structural adaptations.

Continuous strength training has been shown to alter both resting and exercise-induced rates of protein synthesis. Although previous findings are somewhat disparate, taken together, they suggest that the basal synthesis rate is increased, but exercise-induced synthesis magnitude is reduced with improved training status (15, 21, 22). Whether and how rapidly
detraining alters this response is unknown, but it is evident that physical inactivity or muscle
disuse rapidly reduces muscle protein synthesis rates (25, 26). With regard to mTORC1-
signaling, Wilkinson et al (15) found no changes in basal or exercise-induced mTORC1-
signaling after 10 weeks of strength training. In contrast, Ogasawara et al (5) demonstrated
that mTORC1-signaling is attenuated during chronic strength training but sensitized after
subsequent detraining in rat skeletal muscle. As in previous data (27-29), mTORC1-signaling
was clearly induced by the acute resistance exercise bout in this study, but, interestingly, post-
exercise phosphorylation of 4E-BP1^{Thr46} and 4E-BP1^{Ser65} was higher in the memory leg. The
fact that no differences between the control and memory legs was noted for mTOR^{Ser2448},
S6K1^{Thr389} and S6^{Ser235/236} suggests that upstream stimulatory mechanisms did not differ
between the legs and that differences in 4E-BP1^{Thr46} and 4E-BP1^{Ser65} phosphorylation
between the legs could be attributed to a process such as modified phosphatase activity (30).

Moreover, we found higher pre exercise and post-exercise phosphorylation status of
eEF2^{Thr56} and AMPK^{Thr172} in the memory leg, which could indicate a general reduction in
translational capacity (31, 32). This is, however, unlikely or has only minor physiological
relevance, inasmuch as muscle mass and fiber size did not differ between the legs after the
detraining period or after the subsequent 5 weeks of reloading in these subjects (14). One
obvious potential explanation for the observed differences in phosphorylation of AMPK,
eEF2 and also 4E-BP1 for that matter, is altered specific total protein content. However, no
differences in total protein content between legs were noted for any of the analyzed signaling
proteins. It is therefore possible, although speculative, that differences in eEF2^{Thr56} and
AMPK^{Thr172} between the legs are attributable to sustained training-induced alterations in
upstream kinase activity or phosphatase action.

Of note was that higher levels of AMPK^{Thr172} in the memory leg were observed only in
the female subjects. Women have lower resting levels of AMPK^{Thr172} than do men (33), which
is ascribed to the higher type II fiber content in men, inasmuch as resting AMPKThr172 has been shown to be higher in type II fibers than in type I fibers and has also been shown to increase to a similar extent in both fiber types after a short intensified training period (34). In this study, although the female subjects had a lower proportion of type II fibers than did the male subjects (data not shown), there were no differences in fiber type composition between legs. This argues that the higher AMPKThr172 phosphorylation in the memory leg is attributable not to fiber type differences per se but rather to a training-induced elevation that is preserved in a sex-specific manner.

We found quite variable effects with regard to PGC1α transcription; total levels were lower in the memory leg at baseline, and an exercise-induced reduction was noted in the control leg. At the same time, the PGC1α-ex1a isoform was induced after exercise only in the memory leg and its levels also tended to be lower in that leg at baseline. To explore potential mechanisms for the differences between legs, we performed a targeted epigenetic analysis of methylated CpG sites within the PGC1α proximal promoter region, which is associated with exon 1a, and found no differences in any of the seven analyzed CpG sites (three located in the immediate transcription start site (TSS) and coding sequence region, three located upstream and one downstream of TSS) that exhibited sufficient methodological quality. This does, however, not contradict the idea that the training history-induced differences in PGC1α transcription result from epigenetics modifications, inasmuch as there are a total of 49 CpG sites related to exon 1a (between 1300 bp upstream and 1500 bp downstream of the transcription start site of exon 1a; selection based on the H3K4me3 annotation mark track from the UCSC Genome Browser GRCh37/hg19 assembly), and the possibility of histone modifications must also be acknowledged.

The finding that exercise increased specific isoforms in the memory leg, without increasing total PGC1α mRNA, was somewhat unexpected because the unspecific primers
utilized in this study have previously been used to detect robust increases after both resistance
and endurance exercise (35, 36). One apparent explanation for this is that the 1 h post exercise
biopsy might have been too early to detect significant increases. Furthermore, exon 1a and
especially exon 1b transcripts are only a fraction of total PGC1α transcripts and that increased
expression of one specific isoform could be masked by unaltered or reduced expression of
other isoforms in the evaluation of total PGC1α.

On the basis of the data of Seaborne et al (4), we performed a targeted gene expression
analysis of TRAF1, AXIN1, SETD3, and UBR5. The first two genes were reported to be
hypomethylated with induced expression after loading, an effect that was preserved after
detraining. We found no differences in the expression of TRAF1 and AXIN1 between the
control and memory legs at any time point, but we did detect an acute increase after exercise
in both legs. It remains a possibility that our initial loading period did not sufficiently alter
TRAF1 and AXIN1 expression, but this seems less likely as the first training period induced
significant hypertrophy, and an acute increase in expression was confirmed upon reloading. It
is thus possible that a detraining period 13 weeks longer than what we used might have
reversed potential initial changes in AXIN1 and TRAF1 expression. Furthermore, Seaborne et
al reported that UBR5 and SETD3 hypomethylation and gene expression were increased after
loading, reversed after detraining, and then elevated in an augmented manner upon reloading,
which indicates the existence of an epigenetic memory in these genes. We found no effects on
UBR5 expression, and instead of an augmented effect of training on SETD3, its expression in
the memory leg was lower before exercise and an attenuating effect of exercise was observed
in the control leg in the male subjects. In C2C12 cells, SETD3, together with MYOD1, been
reported to control the expression of MYOG (37). Of interest was that we observed reduced
expression of all these genes after exercise in the control leg, as well as different expression
levels between the control and memory legs; this indicates that there are training history-
induced differences in myogenic capacity between the legs.

Although no “muscle memory” effects were noted for the E3 ligase UBR5, we found
lower basal expression of \textit{FBXO32} (the protein MAFbx) in the memory leg in the male
subjects, and only the control leg displayed increased expression after exercise. We also
found strong indications that \textit{TRIM63} (the protein MuRF-1) had lower basal expression in the
memory leg ($p = 0.07$ for leg and time interaction in the ANOVA), and 16 of the 19 subjects
exhibited lower \textit{TRIM63} expression in the memory leg than in the control at baseline. Baehr
et al (38) showed that \textit{TRIM63} and \textit{FBXO32} mRNA expression increases early during
functional overload in mice and that the expression then decreases when hypertrophy
becomes pronounced. These data do not conform to those of Léger et al (39), which in human
skeletal muscle showed a pronounced increase in both \textit{TRIM63} and \textit{FBXO32} mRNA after
8 weeks of strength training, which subsequently was completely reversed after a detraining
period. In our study, we observed more than just a reversed expression after detraining,
inasmuch as the memory leg displayed lower \textit{TRIM63} and \textit{FBXO32} mRNA levels than did
the control leg. Together, our data indicate that training-induced adaptations in the ubiquitin-
proteasome pathway could be preserved after detraining.

From a training memory perspective, notable effects were detected for the expression of
the novel gene \textit{SPRYD7}. This gene is expressed highly in skeletal muscle and heart muscle
and encodes for a protein termed “chronic lymphocytic leukemia deletion region gene 6
protein” (CLLD6). \textit{SPRYD7} (chronic lymphocytic leukemia deletion region gene 6 protein;
CLLD6) is a conserved gene enriched in skeletal muscle tissue (40), and was therefore chosen
as novel candidate gene that has not been investigated in skeletal muscle in association to
exercise before. The function of \textit{SPRYD7} is currently unknown, but according to a genome-
wide association study, it is linked to body mass (41). We found that mRNA expression of
SPRYD7 was lower in the memory leg at baseline and was reduced after exercise only in the control leg. Although protein levels of SPRYD7 did not change acutely after exercise, the lower mRNA levels in the memory leg coincided with higher protein levels in the male subjects ($p = 0.09$ for leg at group level, $p = 0.01$ for leg in male subjects only). It is therefore possible that the higher protein levels in the memory leg resulted in reduced mRNA expression in a negative feedback manner. These findings warrant further investigation to determine the potential role of SPRYD7 in muscle adaptations to strength training.

This study had a few limitations. First, experiments were not performed in conditions of overnight fasting and were performed during different times of day (approximately 8 AM to 3 PM). This was because all 19 subjects underwent the training study during the same weekly period, and we could not logistically fit 19 acute session in a time-standardized manner within a limited time frame. For nutrition, we know that phosphorylation of both S6K1 and S6 is very sensitive to nutritional (amino acid) stimuli, and basal phosphorylation of these proteins was in general very low or barely detectable, which indicates that it was influenced little by prior nutrient intake. The unilateral leg design in which subjects are their own control also reduced the potential confounding influence of nutrition, hormone levels, and circadian rhythm. Furthermore, as the memory leg had preserved some of its initial strength gains despite 20 weeks of detraining and reversal of hypertrophy (the memory leg was approximately 10% stronger than the control leg), there was a difference is absolute but not relative load during the acute resistance exercise session. Finally, we are limited by the single post-exercise biopsy that was collected 1 h into recovery. It is reasonable to assume that we missed some effects on protein phosphorylation and gene expression that may have peaked earlier or later during recovery. One or two additional biopsies, enabling a time course evaluation, would have been of great benefit for the interpretation. Nonetheless, we did find exercise-induced changes for the majority of genes and proteins analyzed.
In summary, we demonstrated that both basal and exercise-induced gene expression and cell signaling that are important for muscle adaptations to strength training can be altered by previous training history and that some of the changes seem to be sex-dependent. We found training history–sensitive factors relating to translation initiation/elongation, myogenesis, oxidative metabolism, angiogenesis, and the ubiquitin-proteasome pathway. It is difficult to conclude whether the effect of training history represents a general augmentation or reduction, inasmuch as the genes and proteins studied exhibited both a sensitized and repressed response. This notion is supported by previous data showing that molecular processes in trained muscle are both upregulated and downregulated and emphasizes that the effect of training history must be evaluated in a gene- and protein-specific manner. Altogether, our results indicate that some of the molecular hallmarks of strength-trained muscle can be preserved after 20 weeks of detraining. The practical relevance of these findings, as well as the molecular mechanisms explaining the sustained alterations, clearly warrants further investigation.

Acknowledgements

We would like to thank MSc Sebastian Edman for his help in carefully dissecting the skeletal muscle biopsies. This project has been funded by grants to Dr. Psilander from the Swedish National Centre for Research in Sports (#2016-0134) and The Swedish School of Sport and Health Sciences. Dr. Moberg is funded through an Early Career Research Fellowship from the Swedish National Centre for Research in Sports (#D2017-0012).

The authors declare that the results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation, and statement that results of the present study do not constitute endorsement by ACSM. The authors declare no conflict of interest.
References


38. Baehr LM, Tunzi M, Bodine SC. Muscle hypertrophy is associated with increases in proteasome activity that is independent of MuRF1 and MAFbx expression. Front Physiol. 2014;5:69.


Figure Legends

Figure 1. Schematic illustration of the experimental protocol. Only one leg (the memory leg) was trained during the unilateral training period, whereas both legs were trained during the acute exercise session after the detraining period. A one-repetition maximum (1-RM) test was performed individually for each leg after the detraining period to ensure same relative load in the acute exercise session. Skeletal muscle biopsy samples from the vastus lateralis were taken before and 1 h after completion of the acute exercise session. Each exercise session consisted of three sets of leg presses, followed by three sets of leg extension at 75% of 1-RM. In the acute exercise session, the legs were exercised one at a time, alternating between sets.

Figure 2. Effect of the resistance exercise session on (A, B) PGC1α-total, (C, D) PGC1α-ex1a, (E, F) SPRYD7, and (G, H) ANGPTL2 mRNA content in the vastus lateralis muscle before (Pre) and 1 h after (Post) exercise. Control; leg without a history of strength training. Memory; leg that had previously undergone strength training for 10 weeks. N = 19. Values are reported as the mean ± SEM. aDifference between pre-exercise and post-exercise values was significant, p < 0.05. bDifference between memory leg and control leg was significant, p < 0.05, at the indicated time point. Letters above lines indicate a main effect in the ANOVA, while letters above single bars indicate an interaction between leg and time.

Figure 3. Effect of the resistance exercise session on (A, B) MYOG, (C, D) MYOD1, (E, F) FBOX32, and (G, H) SETD3 mRNA content in the vastus lateralis muscle before (Pre) and
1 h after (Post) exercise. Control; leg without a history of strength training. Memory; leg that had previously undergone strength training for 10 weeks. $N = 19$. Values are reported as the mean ± SEM. $^a$Difference between pre-exercise and post-exercise values was significant, $p < 0.05$. $^b$Difference between memory leg and control leg was significant $p < 0.05$, at the indicated time point. Letters above lines indicate a main effect in the ANOVA, while letters above single bars indicate an interaction between leg and time.

**Figure 4.** Effect of the resistance exercise session on phosphorylation of (A, B) 4E-BP1$^{Thr46}$, (C, D) eEF2$^{Thr56}$, and (E, F) AMPK$^{Thr172}$ as well as (G, H) SPRYD7 protein content in the vastus lateralis muscle before and 1 h after exercise. Control; leg without a history of strength training. Memory; leg that had previously undergone strength training for 10 weeks. $N = 19$. Values are reported as the mean ± SEM. $^a$Difference between pre-exercise and post-exercise values was significant, $p < 0.05$. $^b$Difference between memory leg and control leg was significant, $p < 0.05$, at the indicated time point. Letters above lines under indicate a main effect in the analysis of variance; letters over single bars indicate an interaction between leg and time. (I) A panel of representative blots from both male and female subjects for the protein data (phosphorylated, total and loading control) presented.