Endurance-type exercise increases bulk and individual mitochondrial protein synthesis rates in rats

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ABSTRACT (244 words)

Physical activity increases muscle protein synthesis rates. However, the impact of exercise on the coordinated up and/or downregulation of individual protein synthesis rates in skeletal muscle tissue remains unclear. We assessed the impact of exercise on mixed muscle, myofibrillar and mitochondrial protein synthesis rates as well as individual protein synthesis rates *in vivo* in rats. Adult Lewis rats either remained sedentary (n=3) or had access to a running wheel (n=3) for the last 2 weeks of a three-week experimental period. Deuterated water (^2H_2O) was injected and subsequently administered in drinking water over the experimental period. Blood and soleus muscle were collected and used to assess bulk mixed muscle, myofibrillar and mitochondrial protein synthesis rates using gas chromatography mass spectrometry and individual muscle protein synthesis rates using liquid chromatography mass spectrometry (i.e., dynamic proteomic profiling). Wheel running resulted in greater myofibrillar (3.94±0.26 vs 3.03±0.15%·d^{-1}; P<0.01) and mitochondrial (4.64±0.24 vs 3.97±0.26%·d^{-1}; P<0.05), but not mixed muscle (2.64±0.96 vs 2.38±0.62%·d^{-1}; P=0.71) protein synthesis rates, when compared with the sedentary condition. Exercise impacted the synthesis rates of 80 proteins, with the difference from the sedentary condition ranging between -64 and +420%. Significantly greater synthesis rates were detected for F1-ATP synthase, ATP synthase subunit alpha, hemoglobin, myosin light chain-6, and synaptopodin-2 (P<0.05). The skeletal muscle protein adaptive response to endurance-type exercise involves upregulation of mitochondrial protein synthesis rates, but is highly coordinated as reflected by the up- and down-regulation of various individual proteins across different bulk sub-cellular protein fractions.

INTRODUCTION

Skeletal muscle adaptation is regulated by the balance between protein synthesis and protein breakdown rates. Muscle protein fractional synthesis rates (FSR) can be determined by administration of stable isotope labeled amino acids and the subsequent measurement of their incorporation into muscle protein. In the 1990s, investigators first applied basic extraction techniques to show differences between bulk myofibrillar and mitochondrial protein synthesis rates in resting skeletal muscle tissue (Rooyackers et al., 1996). This approach was subsequently applied to demonstrate that, for example, endurance-type exercise more robustly increases mitochondrial protein synthesis rates (Wilkinson et al., 2008), whereas resistance-type exercise strongly increases myofibrillar protein synthesis rates (Moore et al., 2009; Burd et al., 2010a; Burd et al., 2010b). Even more detailed insight into individual protein translational responses has been obtained by performing 2-dimensional gel electrophoresis before measuring labeled amino acid incorporation (Balagopal et al., 1997a; Balagopal et al., 1994). Using this approach, synthesis rates of key myofibrillar (Balagopal et al., 1997b; Balagopal et al., 1997a; Hasten et al., 1998) and several mitochondrial proteins (Hesketh et al., 2016b; Jaleel et al., 2008) have been determined. However, the separation technique is labor intensive (13-15 h for one sample), technically challenging, and label quantification can require relatively large tissue samples. These limitations have restricted wide-spread application of individual protein FSR measurements.

We and others have applied deuterated water (^2H_2O) to assess *in vivo* muscle protein synthesis rates over several days or weeks (Holwerda et al., 2018b; Holwerda et al., 2018a; Murphy et al., 2018; Robinson et al., 2011; Wilkinson et al., 2014). The endogenous labeling of nearly all (non-essential) amino acids increases total label incorporation into newly synthesized proteins, which improves analytical detectability. Recently, investigators have combined deuterated water administration with high-throughput analytical techniques of liquid chromatography-

47 mass spectrometry to determine deuterium enrichment in hundreds of tissue-derived peptides
48 (Hesketh et al., 2016b; Kasumov et al., 2011; Price et al., 2012; Shankaran et al., 2016b; Wang
49 et al., 2014; Zangarelli et al., 2006). Based upon shifts in mass spectra over time, the synthesis
50 rates of several proteins may be assessed simultaneously (Price et al., 2012; Shankaran et al.,
51 2016a). Within the last couple of years, this *dynamic proteome profiling* (DPP) approach has
52 been applied in human and rodent studies to assess *in vivo* synthesis rates of several proteins in
53 skeletal muscle tissue (Camera et al., 2017; Shankaran et al., 2016a). Simultaneous application
54 of DPP and assessment of bulk mixed muscle, myofibrillar, and mitochondrial protein FSR in
55 the same tissue is warranted to reveal individual protein synthetic responses that may be masked
56 when assessing bulk protein synthesis rates. This is apparent from earlier work demonstrating
57 that myosin heavy chain synthesis rates are ~30% lower when compared with mixed muscle
58 protein synthesis rates (Balagopal et al., 1997a). Furthermore, a quantitative comparison
59 between the methods is required to further establish DPP as an effective approach to evaluate
60 skeletal muscle adaptive responses. Therefore, in the present study we assessed the impact of
61 exercise on bulk mixed muscle, myofibrillar, and mitochondrial protein synthesis rates and
62 applied DPP to assess individual muscle protein synthesis rates over a 3-week period in rat
63 skeletal muscle tissue. We hypothesized 1) that protein synthesis rates of individual proteins
64 will span a broader range in comparison with bulk protein synthesis rates, 2) that exercise will
65 induce both negative and positive differences in individual protein synthesis rates, and 3) that
66 the grouped average FSR of all individual proteins would not differ from bulk muscle FSR.
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METHODS

Experimental animals

Male Lewis rats (*n*=7, 7±1 months, 363±11 g, Charles River Laboratories, USA) were housed in cages and maintained on 12 h light/12 h dark cycles. Rats were sacrificed after an intraperitoneal injection of sodium pentobarbital (150 $mg \cdot kg^{-1}$ body mass). All procedures were approved by the Animal Experiments Committee at Maastricht University and were in accordance with the *Code of Conduct of the Central Animal Experiments Committee*.

Experimental protocol

Six rats received standard chow diet and water *ad libitum* and underwent the three-week deuterated water protocol. During the first week, all rats remained sedentary to acclimatize to the experimental setting, including deuterated water administration. Starting after week 1, 3 rats received cage access to a running wheel, whereas the other 3 rats remained sedentary. Lewis rats voluntarily perform wheel running for distances of 4,500-10,000 m per day (Werme et al., 1999; Makatsori et al., 2003). After the 3-week experimental period, the animals were sacrificed and blood and muscle samples were collected and stored as previously described (Holwerda et al., 2018b). A separate rat, housed under identical (sedentary) conditions, was sacrificed to provide unlabeled control soleus muscle for LC-MS/MS analysis.

Deuterated water dosing protocol

The deuterated water dosing protocol was adopted from previous study (Neese et al., 2002) and consisted of one intraperitoneal injection of 70% deuterium oxide (Cambridge Isotopes Laboratories, USA) at 0.02 $mL \cdot g^{-1}$ body mass and access to 4% deuterium-enriched drinking water.

Plasma free ^{2}H-alanine and body water ^{2}H enrichments

Plasma amino acid enrichments were determined by GC-MS as described previously (Holwerda et al., 2018b). Body water ^{2}H enrichments were determined by dividing plasma ^{2}H-alanine enrichments by 3.7, which is the labeling factor between body water and alanine (Holwerda et al., 2018b; Wilkinson et al., 2014).

Bulk protein-bound ^{2}H-alanine enrichment

Bulk mixed muscle, myofibrillar, and mitochondrial protein fractions were isolated from muscle samples and protein-bound ^{2}H-alanine enrichments were measured using GC-MS, as described previously (Holwerda et al., 2016; Churchward-Venne et al., 2019; Holwerda et al., 2018b).

Liquid chromatography–mass spectrometry analysis

A separate piece of muscle underwent a trypsin digestion protocol as previously described (Qiao et al., 2019) to cleave peptides. The peptide mixture was loaded onto an LC-MS/MS, configured as described previously (Qiao et al., 2019; Vogel et al., 2019).

Label-free quantitation of protein abundances

Progenesis Quantitative Informatics for proteomics (Waters, USA) was used to perform label-free quantitation as described previously (Bowden-Davies et al., 2015; Burniston et al., 2014; Camera et al., 2017; Sollanek et al., 2017). Log-transformed MS data were normalized by inter-sample abundance ratio, and relative protein abundances (RA) were calculated using non-conflicting peptides only. Spectra generated from Mass Spectrometer 2 (MS2) were exported in Mascot generic format and searched against the Swiss-Prot database (2018.7) using a locally

implemented Mascot server (v.2.2.03; www.matrixscience.com). The Mascot output, restricted to non-homologous protein identifications (False discovery rate, FDR: <1%), was recombined with MS profile data in Progenesis QI (Burniston et al., 2014).

Fractional synthetic rate calculations – Bulk muscle protein fractions

Bulk mixed muscle, myofibrillar, and mitochondrial protein fractional synthetic rates (FSR) were calculated over the 3-week deuterium water administration period using a first-order kinetics equation (Shankaran et al., 2016a):

$$FSR(\% \cdot d^{-1}) = \frac{-\ln(1-f)}{t}$$

We applied a first-order kinetics equation to account for the prolonged labeling period and potential differences in the synthetic rates between protein fractions and individual proteins. f is the cumulative fractional synthesis, which was determined by dividing the protein-bound ^{2}H-alanine enrichment in the 3-week muscle samples by the free ^{2}H-alanine enrichment in the 3-week plasma samples. t represents the time (21 d).

Fractional synthetic rate calculations – Dynamic proteomic profiling

Protein synthesis rates were calculated from peptide mass isotopomer abundance data extracted from spectra generated from Mass Spectrometer 1 (MS1) using Progenesis Quantitative Informatics (Waters, USA), as described previously (Camera et al., 2017). The rate of decay (*k)* of the molar fraction (MF0) of the monoisotopic peak (M0) was calculated as a first-order exponential spanning the beginning (*t0*) to end (*t*) of the experimental period.

$$MF0 = \frac{M0}{M0 + M1 + M2 + M3}$$

$$k = \frac{1}{t - t0} \bullet - \ln\left(\frac{\mathrm{MF0}_t}{\mathrm{MF0}_{t0}}\right)$$

Fractional synthesis rate (FSR, %·d^{-1}), was derived by dividing *k* by body water ^{2}H enrichment (*p*) multiplied by the number (*n*) of ^{2}H exchangeable H-C bonds present in each peptide. *n* was calculated for each peptide using existing data on amino acid ^{2}H labeling in humans (Price et al., 2012).

$$FSR(\% \cdot d^{-1}) = \frac{k}{(n \cdot p)}$$

Statistical analysis

Data are expressed as means±SD. *Independent two-tailed Student's t-tests* were used to compare plasma ^{2}H-alanine enrichments, estimated body water ^{2}H enrichments, average bulk protein synthesis rates, and grouped (*i.e.*, total, mitochondrial, myofibrillar) individual protein synthesis rates. A *one-way between-group ANOVA* was used to compare individual protein differences between the sedentary and exercise conditions. Statistical significance was set at $P < 0.05$. The FDR Q-value was calculated to control multiple testing. Unless otherwise stated, calculations were performed using Excel or SPSS 21.0 (IBM, USA). Pearson's *r* product moment correlation analysis was used to examine the linear relationship between bulk mixed muscle, myofibrillar and mitochondrial FSRs with grouped averages of the individual protein FSRs, which were assigned to sub-cellular protein fraction (i.e., total, myofibrillar, and mitochondrial).

RESULTS

Deuterated water precursor labeling

The deuterated water dosing protocol resulted in plasma free ^{2}H-alanine enrichments of 8.16±0.09 and 8.04±0.05 mole percent excess (MPE) at day 21 in the sedentary and exercise group, respectively (P=0.10). The corresponding body water deuterium enrichments averaged 2.21±0.02 and 2.17±0.02 % in the sedentary and exercise group, respectively (P=0.10).

Fractional synthetic rates of bulk mixed muscle, myofibrillar, and mitochondrial protein

Bulk mixed muscle protein-bound ^{2}H-alanine measured by GC-MS averaged 3.37±0.93 MPE in the exercise group and did not differ (P=0.79) from 3.18±0.66 MPE in the sedentary group. Bulk myofibrillar protein-bound ^{2}H-alanine enrichments averaged 4.52±0.19 MPE in the exercise group, which was significantly (P<0.05) greater than 3.84±0.18 MPE in the sedentary group. Bulk mitochondrial protein-bound ^{2}H-alanine enrichments averaged 5.01±0.12 MPE in the exercise group, which was significantly (P<0.05) greater than 4.62±0.21 MPE in the sedentary group. Mixed muscle protein FSRs (**Figure 1**) did not differ between the exercising (2.64±0.96 %·d^{-1}) and sedentary rats (2.38±0.62 %·d^{-1}; P=0.71). Myofibrillar muscle protein FSR (**Figure 1**) was ~30% greater in the exercise group (3.94±0.26 %·d^{-1}) when compared to the sedentary group (3.03±0.15 %·d^{-1}; P<0.01). Mitochondrial muscle protein FSR (**Figure 1**) was ~17% greater in the exercise group (4.64±0.24 %·d^{-1}) when compared to the sedentary group (3.97±0.26 %·d^{-1}; P<0.05).

Individual muscle protein abundances

Label-free profiling was performed on 256 proteins that had at least 1 unique peptide detected in each animal. The dynamic range of the label-free analysis spanned 6 orders of magnitude.

The most abundant muscle protein was myosin light chain 3 (MYL3) (RA = 9.89E+08) and the least abundant muscle protein detected was myosin heavy chain 9 (MYH9) (RA = 3.61E+04). The normalized abundances of 3 proteins [Musculoskeletal embryonic nuclear protein 1 (MUSTN1), Adenylate kinase isoenzyme 1 (KAD1), and 60S ribosomal protein L32 (RPL32)] were statistically (P<0.05) greater in the exercise compared to sedentary control samples, though with a high False Discovery Rate (FDR = 0.48).

Fractional synthetic rates of individual muscle proteins

Based on our selection criteria we were able to assess the fractional synthesis rates of 108 out of 256 identified proteins (42%), which resided in the muscle cytosolic, mitochondrial, myofibrillar, and nuclear fractions, as well as in residual blood (**Table 1**). The weighted average FSR of all individual proteins in both groups was 2.59±0.69 %·d^{-1}, which was not statistically different from bulk mixed muscle protein FSR measured by GC-MS analysis (P=0.91). The rank order of the synthesis rates for these 108 proteins is displayed in **Figure 2**. Fractional synthetic rate of the detected individual proteins was lowest in Histone H2A type 1-C (H2A1C; 0.48±0.09 %·d^{-1}) and highest in Alpha-2-HS-glycoprotein (FETUA; 9.25±0.38 %·d^{-1}). The average FSR across individual mitochondrial proteins demonstrated a strong, positive correlation with bulk mitochondrial FSR (P=0.03, r=0.73, **Figure 3C**).

Individual protein synthetic responses to exercise.

Exercise appeared to impact the synthesis rates of the majority of identified proteins. Eighty proteins demonstrated a difference in FSR greater than +10% or less than -10% between muscles of sedentary and exercising animals, and the percent difference of myofibrillar and mitochondrial proteins relative to sedentary conditions ranged from -64 to +420% (**Figure 4**). When all detected proteins were averaged, exercise did not result in significantly higher protein

synthesis rates when compared to sedentary (3.30±1.22 vs 2.69±0.62 %·d^{-1}, respectively, *P*=0.09). When proteins were grouped into sub-cellular protein fraction, exercise did not result in significantly higher mitochondrial protein synthesis rates when compared to sedentary (3.34±1.05 vs 2.51±0.52 %·d^{-1}, respectively, *P*=0.07). At the individual protein level, exercise resulted in greater synthesis rates (*P*<0.05) of F1-ATP synthase (AT5F1), ATP synthase subunit alpha (ATPA), Hemoglobin (HBB2), Myosin light chain 6 (MYL6) and Synaptopodin-2 (SYNP2) (**Figure 5**).

DISCUSSION

We observed that bulk myofibrillar and mitochondrial protein synthesis rates were greater in the soleus muscle tissue of exercising rats when compared to their sedentary littermates. Exercise impacted the synthesis rates of 80 out of 108 detected proteins, with the response ranging between -64 to +420% when expressed relative to the sedentary condition.

Bulk myofibrillar and mitochondrial protein synthesis rates were respectively ~30% higher and ~17% higher in the exercising rats when compared to the sedentary rats (**Figure 1**). Our findings align with previous studies in rodents that have applied a variety of isotope-based methods to demonstrate the robust anabolic impact of chronic and acute exercise on muscle protein synthesis rates (Gasier et al., 2010; Kubica et al., 2005; Mosoni et al., 1995; Munoz et al., 1994; Wong & Booth, 1990). However, exercise did not result in significantly higher mixed muscle protein synthesis rates when compared to the sedentary rats (**Figure 1**). This finding is in contrast with some (Gasier et al., 2010; Kubica et al., 2005; Mosoni et al., 1995; Munoz et al., 1994; Wong & Booth, 1990; Ogasawara et al., 2016; Ogasawara et al., 2014), but not all (Katzeff et al., 1995; Wang et al., 2017) studies that have assessed the impact of exercise on mixed muscle protein synthesis rates in rats. The absence of a response may be explained by a relatively low (or negative) synthetic response of proteins residing outside of the myofibrillar and mitochondrial sub-fractions. Non-myofibrillar and mitochondrial proteins have been estimated to comprise 30-40% of all muscle proteins (Balagopal et al., 1996). It has also been demonstrated that synthesis of myosin heavy-chain, one of the most abundant muscle proteins (~25%), only contributes ~18% to mixed muscle protein synthesis rates (Balagopal et al., 1997a). Theoretically, a ~30% increase in myosin heavy-chain synthesis would only increase mixed muscle protein synthesis by ~5-6%, which could be masked by the co-occurring responses of other proteins (Balagopal et al., 1997a).

We performed DPP by measuring the mass spectra of hundreds of muscle-derived peptides using high-throughput tandem mass spectrometry. Individual protein synthesis rates in the present study align well with absolute individual protein synthesis rates reported in a recent study that applied a comparable DPP approach in rat muscle (Shankaran et al., 2016b). The individual protein synthesis rates spanned a broader range than the absolute bulk protein synthesis rates (**Figure 2B**). We observed that the synthetic rates of key mitochondrial proteins, such as cytochrome-c oxidase (2.77±0.40 %·d^{-1}) and succinate dehydrogenase (3.30±0.35 %·d^{-1}), were 4-fold higher when compared to key myofibrillar proteins, such as α-actin (0.77±0.28 %·d^{-1}) and α-actinin-1 (1.55±0.11 %·d^{-1}), which aligns with previous literature (Jaleel et al., 2008). The FSR of proteins assigned to "mitochondrion" had a strong, positive correlation with bulk mitochondrial protein FSR (**Figure 3C**). However, we did not detect a correlation in the mixed muscle or myofibrillar protein fractions (**Figure 3A, 3B**). The discrepancy may be explained by the greater detection of mitochondrial proteins (*n*=27) in comparison with myofibrillar proteins (*n*=14). Furthermore, certain highly abundant proteins (e.g., collagen) were not detected using DPP, but were likely captured in the bulk mixed protein isolation. Strict categorization of some proteins known to reside in more than one cellular compartment or translocate between compartments (e.g., heat shock proteins) may have also contributed to the absence of a correlation between methods for myofibrillar proteins.

Exercise seemed to impact the synthesis rates of the majority of detected proteins (80/108), with the response ranging from -64 to +420% relative to sedentary conditions. Notably, we reveal that exercise resulted in significantly greater synthesis rates of F1-ATP synthase and ATP synthase subunit alpha when compared to the sedentary group (**Table 1, Figure 5**). Furthermore, several proteins within the TCA cycle and electron transport system demonstrated 35-140% higher protein synthesis rates in the exercise group when compared with the sedentary group (**Figure 4A**). Increased synthesis rates of these proteins, and ATP synthase in particular,

likely contribute to the increase in muscle oxidative capacity observed over more prolonged endurance-type exercise training (Burniston & Hoffman, 2011; Hesketh et al., 2016a; Holloszy et al., 1970). However, we did not detect significantly greater abundances of these proteins in the present study, which is likely due to low subject number or the mere 2-week exercise intervention. We chose to assess protein synthetic responses in the soleus muscle primarily due to the high type I muscle fiber content and aerobic nature of wheel running exercise. As soleus muscle may possess a greater sensitivity to induce transcriptional and/or protein synthetic responses to physical (in)activity when compared to other muscles (e.g., plantaris or tibialis anterior) (Miller et al., 2019), we should be careful when translating these findings to other muscle groups.

Bulk protein FSR assessment and DPP provide distinct advantages and disadvantages and can complement each other. For example, DPP allows identification and assessment of protein synthesis rates within sub-cellular protein fractions, which is valuable for resolving more specific physiological issues, such as characterizing how key mitochondrial proteins (*e.g.*, ATP synthase) respond to physical (in)activity, aging, and/or disease. Characterizing the coordinated regulation of individual mitochondrial proteins is of particular relevance given the gap in our understanding of mitochondrial protein adaptation due to the challenges of isolating a pure mitochondrial fraction from muscle tissue (Burd et al., 2015). Despite this advantage, we and others have been able to assess the synthesis rates of only 100-150 individual proteins in skeletal muscle tissue. This number is equivalent to merely 2% of the approximately 5500 proteins present in the skeletal muscle proteome (Gonzalez-Freire et al., 2017). Although the top-100 detectable muscle proteins have been suggested to represent 85% of total muscle protein (Geiger et al., 2013), the detectable proteins cannot currently be localized to the different muscle fiber types or to non-muscle tissue (e.g., fat, connective tissue), which likely respond differently to stimuli. The methodological approach (e.g., sample preparation, instrument

sensitivity) may be modified to detect targeted peptide/protein families, which would be useful for resolving some of these issues in the future. On the other hand, bulk protein FSR assessment provides an average representation of all proteins residing in the isolated fraction. Therefore, bulk protein FSR assessment can be applied to reveal the regulation of bulk muscle protein abundance. Acquiring this information can deepen our understanding of skeletal muscle mass regulation to physical (in)activity, nutrition, aging, and disease. Considering the high cost of DPP and the rather limited number of proteins detected in muscle tissue, it is evident that, in most cases, the assessment of bulk protein FSR provides sufficient insight into the muscle protein synthetic response to various interventions.

Limitations of the current study must be acknowledged. Firstly, the relatively low subject numbers may have resulted in type II errors among comparisons between bulk mixed muscle protein FSR and grouped individual protein FSR data as well as the lack of a difference in mixed muscle protein FSR between muscle tissue collected from the sedentary and exercising rats. Secondly, we allowed the exercising rats to run with no restriction but did not quantitate exercise duration or intensity.

In conclusion, bulk mitochondrial, but not mixed muscle or myofibrillar, protein synthesis rates align well with the averages of grouped individual protein FSRs from the same sub-cellular fraction. The impact of exercise on individual muscle proteins is highly coordinated as reflected by the simultaneous up- and down-regulation of several individual proteins residing in different muscle protein fractions.

DISCUSSION

A.M.H., F.G.B., M.N., and L.J.C.L. designed the research; A.M.H. and M.N. conducted the research; F.G.B., J.G.B., P.W., J.K., and A.P.G. analyzed the data; A.H.M. and J.G.B. performed the statistical analysis; All authors contributed to data interpretation; and A.M.H., F.G.B., J.G.B., E.C.C.M., and L.J.C.L. wrote the paper and hold primary responsibility for the final content. All authors read and approved the final manuscript.

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DECLARATIONS

The authors declare no conflicts of interest.

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Table 1. Protein enrichments and fractional synthetic rates of 108 individual proteins across blood, sarcoplasmic, mitochondrial, myofibrillar, and nuclear sub-cellular protein fractions in soleus muscle of sedentary and exercising rats[1]

Sub-cellular protein fraction	Accession	Protein description	Unique peptide sequences identified (*n*)	FSR (%·d^{-1})		Difference (%)	P-value	Q-value
				Sedentary	Exercise			
Blood	A1I3	Alpha-1-inhibitor 3	2	3.364±0.394	3.052±0.695	-9	0.535	0.781
Blood	ALBU	Serum albumin	28	4.507±0.357	5.024±0.531	11	0.235	0.643
Blood	ANXA2	Annexin A2	3	1.922±0.797	2.430±0.540	26	0.412	0.665
Blood	ANXA5	Annexin A5	1	3.684±0.577	3.883±0.126	5	0.592	0.818
Blood	CALR	Calreticulin	2	6.725±0.449	6.780±2.005	1	0.965	0.993
Blood	CAVN1	Caveolae-associated protein 1	2	3.143±0.831	2.526±0.425	-20	0.316	0.643
Blood	FETUA	Alpha-2-HS-glycoprotein	2	8.514±0.445	9.253±0.379	9	0.094	0.643
Blood	HBA	Hemoglobin subunit alpha-1/2	7	1.754±0.351	1.921±0.665	10	0.720	0.907
Blood	HBB2	Hemoglobin subunit beta-2	5	4.740±0.701	1.716±1.456	-64	0.032	0.643
Blood	HEMO	Hemopexin	1	2.296±0.391	2.470±0.971	8	0.787	0.933
Blood	KACB	Ig kappa chain C region, B allele	1	1.471±0.480	2.252±0.669	53	0.176	0.643
Blood	PDLI5	PDZ and LIM domain protein 5	6	0.892±0.388	3.136±3.098	252	0.281	0.643
Blood	PGS2	Decorin	3	3.338±0.213	4.079±0.937	22	0.252	0.643
Blood	PTMS	Parathymosin	1	2.153±0.448	3.310±1.095	54	0.166	0.643
Blood	TAGL2	Transgelin-2	2	4.904±2.697	4.731±5.561	-4	0.963	0.993
Blood	TRFE	Serotransferrin	5	0.773±0.520	1.263±1.391	63	0.598	0.818
Blood	VTDB	Vitamin D-binding protein	1	8.584±2.013	8.566±6.576	0	0.997	0.997
Average values, blood proteins (n = 17)				3.692±0.709	3.905±1.595	6	0.140	-

Cytosolic	1433E	14-3-3 protein epsilon	1	2.228±0.432	3.231±0.727	45	0.109	0.643
Cytosolic	AATC	Aspartate aminotransferase, cytoplasmic	7	2.107±0.107	1.926±0.748	-9	0.700	0.907
Cytosolic	ALDOA	Fructose-bisphosphate aldolase A	21	2.234±0.186	2.647±0.194	18	0.056	0.643
Cytosolic	ANXA6	Annexin A6	4	3.504±0.051	4.162±1.321	19	0.438	0.685
Cytosolic	CAH3	Carbonic anhydrase 3	24	1.933±0.087	2.542±0.828	32	0.274	0.643
Cytosolic	DOPD	D-dopachrome decarboxylase	3	3.014±1.690	2.409±0.383	-20	0.578	0.818
Cytosolic	DPYL2	Dihydropyrimidinase-related protein 2	2	5.524±4.594	5.116±2.265	-7	0.897	0.993
Cytosolic	EF2	Elongation factor 2	5	1.911±0.243	2.538±1.138	33	0.403	0.665
Cytosolic	ENOB	Beta-enolase	17	1.276±0.258	1.887±0.542	48	0.153	0.643
Cytosolic	ESTD	S-formylglutathione hydrolase	2	1.824±1.361	3.829±3.017	110	0.353	0.645
Cytosolic	FABP4	Fatty acid-binding protein, adipocyte	5	5.161±0.601	5.050±2.468	-2	0.943	0.993
Cytosolic	FKB1A	Peptidyl-prolyl cis-trans isomerase	1	2.099±0.902	2.704±0.261	29	0.327	0.643
Cytosolic	G3P	Glyceraldehyde-3-phosphate dehydrogenase	20	1.714±0.297	2.896±1.680	69	0.296	0.643
Cytosolic	G6PI	Glucose-6-phosphate isomerase	5	0.756±0.271	2.919±3.102	286	0.295	0.643
Cytosolic	GPDA	Glycerol-3-phosphate dehydrogenase	3	2.382±1.000	3.366±1.854	41	0.464	0.696
Cytosolic	GPX1	Glutathione peroxidase 1	1	1.747±0.409	2.394±0.236	37	0.077	0.643
Cytosolic	GSTA3	Glutathione S-transferase alpha-3	2	2.877±0.481	3.229±1.178	12	0.657	0.865
Cytosolic	HS90B	Heat shock protein HSP 90-beta	2	4.498±0.767	3.531±1.868	-21	0.453	0.689
Cytosolic	HSP7C	Heat shock cognate 71 kDa protein	11	3.287±0.304	4.001±0.623	22	0.149	0.643
Cytosolic	HSPB6	Heat shock protein beta-6	4	2.175±0.089	3.346±1.366	54	0.212	0.643
Cytosolic	KAD1	Adenylate kinase isoenzyme 1	9	1.174±0.035	2.232±0.767	90	0.075	0.643
Cytosolic	KCRB	Creatine kinase B-type	2	4.195±1.085	4.250±0.899	1	0.950	0.993
Cytosolic	KCRM	Creatine kinase M-type	28	1.632±0.311	1.533±0.533	-6	0.794	0.933
Cytosolic	KPYM	Pyruvate kinase PKM	19	2.501±0.445	2.634±0.558	5	0.763	0.926
Cytosolic	LDHA	L-lactate dehydrogenase A chain	3	2.713±0.146	4.233±1.049	56	0.068	0.643
Cytosolic	MDHC	Malate dehydrogenase, cytoplasmic	8	1.964±0.076	3.951±3.133	101	0.334	0.643
Cytosolic	MYG	Myoglobin	8	1.820±0.084	1.782±0.378	-2	0.872	0.992

Cytosolic	NTF2	Nuclear transport factor 2	1	1.129±0.919	5.875±7.078	420	0.314	0.643
Cytosolic	PARK7	Protein/nucleic acid deglycase DJ-1	3	1.338±0.686	2.029±0.428	52	0.213	0.643
Cytosolic	PDLI1	PDZ and LIM domain protein 1	7	3.944±0.314	5.129±1.140	30	0.157	0.643
Cytosolic	PFKAM	ATP-dependent 6-phosphofructokinase	2	1.946±0.717	1.484±1.174	-24	0.592	0.818
Cytosolic	PGAM1	Phosphoglycerate mutase 1	1	2.617±0.458	3.128±0.629	20	0.318	0.643
Cytosolic	PGAM2	Phosphoglycerate mutase 2	6	1.224±0.270	1.943±0.720	59	0.181	0.643
Cytosolic	PGK1	Phosphoglycerate kinase 1	14	1.826±0.037	2.866±1.492	57	0.294	0.643
Cytosolic	PGM1	Phosphoglucomutase-1	5	1.707±0.535	2.575±2.129	51	0.531	0.781
Cytosolic	PIMT	Protein-L-isoaspartate	1	1.382±0.394	2.312±0.445	67	0.053	0.643
Cytosolic	PRDX2	Peroxiredoxin-2	3	4.360±1.726	3.298±0.744	-24	0.384	0.665
Cytosolic	PRDX6	Peroxiredoxin-6	1	1.593±0.337	1.636±0.195	3	0.857	0.985
Cytosolic	PROF1	Profilin-1	3	3.127±0.291	3.271±1.765	5	0.897	0.993
Cytosolic	PYGM	Glycogen phosphorylase	7	3.055±0.801	4.734±1.905	55	0.232	0.643
Cytosolic	RLA1	60S acidic ribosomal protein P1	1	1.958±0.763	2.392±0.148	22	0.388	0.665
Cytosolic	SODC	Superoxide dismutase [Cu-Zn]	2	1.730±0.152	2.156±0.623	25	0.313	0.643
Cytosolic	TERA	Transitional endoplasmic reticulum ATPase	1	3.884±0.895	3.609±0.987	-7	0.739	0.907
Cytosolic	TPIS	Triosephosphate isomerase	11	1.416±0.240	2.303±1.012	63	0.213	0.643
Average values, cytosolic proteins (n = 44)				2.420±0.587	3.070±1.267	27	0.200	-
Mitochondrial	ACADL	Long-chain specific acyl-CoA dehydrogenase	6	2.520±0.432	2.876±0.533	14	0.419	0.665
Mitochondrial	ACADV	Very long-chain specific acyl-CoA dehydrogenase	2	2.902±0.664	3.785±1.321	30	0.359	0.645
Mitochondrial	ALDH2	Aldehyde dehydrogenase	1	7.085±1.031	6.859±0.238	-3	0.730	0.907
Mitochondrial	AT5F1	ATP synthase F1	2	2.155±0.581	3.522±0.579	63	0.045	0.643
Mitochondrial	ATP5H	ATP synthase subunit d	2	0.791±0.510	2.181±1.309	176	0.162	0.643
Mitochondrial	ATPA	ATP synthase subunit alpha	21	1.856±0.074	2.380±0.303	28	0.044	0.643
Mitochondrial	ATPD	ATP synthase subunit delta	2	1.867±0.282	2.322±0.402	24	0.184	0.643
Mitochondrial	CATA	Catalase	2	5.002±0.700	4.967±0.664	-1	0.953	0.993

Mitochondrial	CH60	60 kDa heat shock protein	7	3.443±0.212	3.412±0.733	-1	0.948	0.993
Mitochondrial	COQ8A	Atypical kinase COQ8A	1	3.468±1.453	3.516±0.903	1	0.964	0.993
Mitochondrial	COX5B	Cytochrome c oxidase subunit 5B	1	2.768±0.397	5.035±3.379	82	0.313	0.643
Mitochondrial	DLDH	Dihydrolipoyl dehydrogenase	5	1.820±0.817	2.290±0.264	26	0.397	0.665
Mitochondrial	ECHA	Trifunctional enzyme subunit alpha	8	1.419±0.208	1.805±0.211	27	0.087	0.643
Mitochondrial	ECHM	Enoyl-CoA hydratase	2	3.024±0.718	3.094±0.586	2	0.903	0.993
Mitochondrial	ES1	ES1 protein homolog	2	1.277±0.195	1.285±0.367	1	0.974	0.993
Mitochondrial	ETFA	Electron transfer flavoprotein subunit alpha	7	2.295±0.344	3.572±1.354	56	0.189	0.643
Mitochondrial	FUMH	Fumarate hydratase	4	0.777±0.134	1.031±0.889	33	0.650	0.865
Mitochondrial	MDHM	Malate dehydrogenase	6	1.914±0.077	2.899±0.619	51	0.052	0.643
Mitochondrial	NDUS1	NADH-ubiquinone oxidoreductase 75 kDa subunit	3	3.380±0.804	4.845±0.754	43	0.083	0.643
Mitochondrial	NDUS6	NADH dehydrogenase iron-sulfur protein 6	1	3.270±0.418	3.596±0.530	10	0.450	0.689
Mitochondrial	ODPB	Pyruvate dehydrogenase E1 component subunit beta	2	1.676±0.266	2.569±0.685	53	0.103	0.643
Mitochondrial	PHB2	Prohibitin-2	2	2.086±1.412	3.685±1.282	77	0.220	0.643
Mitochondrial	PRDX5	Peroxiredoxin-5	4	1.253±0.428	3.658±4.048	192	0.364	0.645
Mitochondrial	QCR2	Cytochrome b-c1 complex subunit 2	2	0.927±0.603	2.498±2.371	169	0.328	0.643
Mitochondrial	QCR6	Cytochrome b-c1 complex subunit 6	1	3.018±0.628	4.188±1.109	39	0.187	0.643
Mitochondrial	SDHA	Succinate dehydrogenase flavoprotein subunit	4	3.298±0.353	4.467±1.138	35	0.164	0.643
Mitochondrial	THIL	Acetyl-CoA acetyltransferase	7	2.338±0.267	3.732±1.750	60	0.244	0.643
*Average values, mitochondrial proteins (*n = *27)*				2.505±0.519	3.336±1.049	33	0.068	-
Myofibrillar	ACTC	Actin, alpha cardiac muscle 1	2	1.002±0.499	1.61±0.463	61	0.197	0.643
Myofibrillar	ACTN1	Alpha-actinin-1	7	1.554±0.109	3.603±2.365	132	0.208	0.643
Myofibrillar	ACTS	Actin, alpha skeletal muscle	5	0.765±0.28	1.884±1.428	146	0.254	0.643
Myofibrillar	CALD1	Non-muscle caldesmon	2	3.078±0.971	3.090±0.641	0	0.987	0.996
Myofibrillar	CALM1	Calmodulin-1	1	6.185±2.286	5.372±1.449	-13	0.630	0.851
Myofibrillar	CAPZB	F-actin-capping protein subunit beta	5	2.887±0.238	3.522±0.604	22	0.165	0.643

Myofibrillar	ENOB	Beta-enolase	17	1.276±0.258	1.887±0.542	48	0.153	0.643
Myofibrillar	MYL1	Myosin light chain 1/3, skeletal muscle isoform	14	3.676±2.090	4.110±1.324	12	0.776	0.932
Myofibrillar	MYL6	Myosin light polypeptide 6	3	2.514±0.329	3.319±0.379	32	0.050	0.643
Myofibrillar	PROF1	Profilin-1	3	3.127±0.291	3.271±1.765	5	0.897	0.993
Myofibrillar	TBB4B	Tubulin beta-4B chain	5	1.989±0.271	2.218±0.558	12	0.556	0.801
Myofibrillar	TNNT3	Troponin T, fast skeletal muscle	2	3.056±1.607	1.858±0.943	-39	0.328	0.643
Myofibrillar	TPM1	Tropomyosin alpha-1 chain	7	1.249±0.230	2.136±1.041	71	0.223	0.643
Myofibrillar	TPM3	Tropomyosin alpha-3 chain	1	2.249±0.101	3.664±1.452	63	0.167	0.643
Average values, myofibrillar proteins (n=14)				2.967±1.068	2.472±0.683	20	0.168	-
Nuclear	CAPG	Macrophage-capping protein	2	3.137±2.015	5.558±3.239	77	0.333	0.643
Nuclear	CSRP1	Cysteine and glycine-rich protein 1	2	4.957±1.699	5.251±1.987	6	0.855	0.985
Nuclear	H2A1C	Histone H2A type 1-C	2	0.476±0.093	0.676±0.32	42	0.358	0.645
Nuclear	H2B1	Histone H2B type 1	2	0.946±0.275	1.136±0.798	20	0.716	0.907
Nuclear	HMGB1	High mobility group protein B1	1	0.749±0.296	0.828±0.249	11	0.739	0.907
Nuclear	ROA2	Heterogeneous nuclear ribonucleoproteins A2/B1	1	6.819±0.731	7.335±0.64	8	0.410	0.665
Nuclear	ROA3	Heterogeneous nuclear ribonucleoprotein A3	1	3.469±0.282	3.856±0.55	11	0.340	0.643
Nuclear	SYNP2	Synaptopodin-2	3	3.074±0.277	3.665±0.111	19	0.027	0.643
Average values, nuclear proteins (n=8)				2.953±0.709	3.538±0.987	20	0.349	-
Average values, total proteins (n= 108)				2.692±0.617	3.298±1.220	23	0.091	-

[1]Average values for each protein sub-fraction are expressed as means±SDs. n = 3 for sedentary rats and n = 3 for exercising rats. FSR: fractional synthetic rate. Data were analyzed using *between-group ANOVA on* each protein. Statistical significance was set at $P < 0.05$.

FIGURE LEGENDS

Figure 1. Mixed muscle, myofibrillar, and mitochondrial protein fractional synthetic rates (FSR, $\%\cdot d^{-1}$) assessed in the soleus muscle of Lewis rats that either had access to a cage running wheel (Exercise, $n = 3$) or were restricted to sedentary conditions (Sedentary, $n = 3$) for the last two weeks of a three-week assessment period. Values are presented as means+SD. *Significantly different from the sedentary condition ($P < 0.05$).

Figure 2. Rank order of individual protein synthesis rates in rat soleus muscle. Panel A displays the mean rate constant (k) for the synthesis of each protein ranked from lowest to highest during the 3-week experimental period. Labels indicate the UniProt Knowledgebase identifiers for the detected proteins. Panel B displays boxplots presenting the average (median and IQR) rate constant of synthesis of proteins that were analyzed using dynamic proteome profiling (DPP) and the bulk mixed muscle protein fraction (BULK) during the three-week experimental period. Independent two-tailed Student's t-test found no significant ($P = 0.91$) difference in the average rate of synthesis of the grouped individual proteins ($n = 108$) when compared with BULK.

Figure 3. Correlations between the averages of grouped individual protein FSRs and the corresponding bulk mixed muscle (A), myofibrillar (B), and mitochondrial (C) protein fractions FSRs. The solid line indicates the linear regression line of best fit, and the dashed lines the 95% confidence interval. A significant positive correlation was observed between methods for mitochondrial proteins ($P = 0.03$, $R^2 = 0.73$).

Figure 4. Percent difference in fractional synthetic rates (FSR) of individual mitochondrial (A) and myofibrillar (B) proteins assessed in the soleus muscle of Lewis rats that either had access

to a cage running wheel (Exercise, $n = 3$) or were restricted to sedentary conditions (Sedentary, $n = 3$) over a 3-week experimental period. Presented proteins demonstrated a difference in FSR greater than +10% or less than -10% between Sedentary and Exercise rats. Values are presented as the average percent difference (%) of FSR in the Exercise group compared with the Sedentary group. *Significantly different in Exercise vs Sedentary ($P<0.05$).

Figure 5. Volcano plot presenting the average fold-changes in synthesis rates of individual proteins of the Exercise group compared with the Sedentary group. P-values were calculated using between-subject ANOVA. Proteins are represented in black if they exhibited a statistically significant ($P<0.05$, all FDR = 0.64) change when compared to the sedentary condition. AT5F1: F1-ATP synthase, ATPA: ATP synthase subunit alpha, HBB2: hemoglobin, MYL6: myosin light chain-6, and SYNP2: synaptopodin-2.

Figure 1

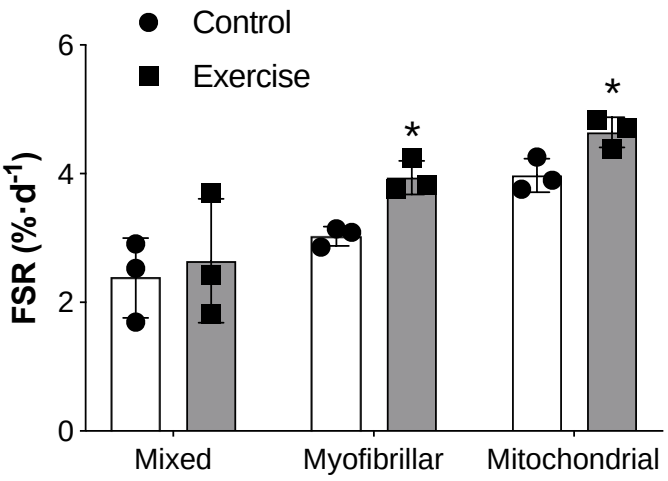

Control
Exercise
FSR (%·d-1)
6
4
2
0
*
*
Mixed
Myofibrillar
Mitochondrial

Figure 2

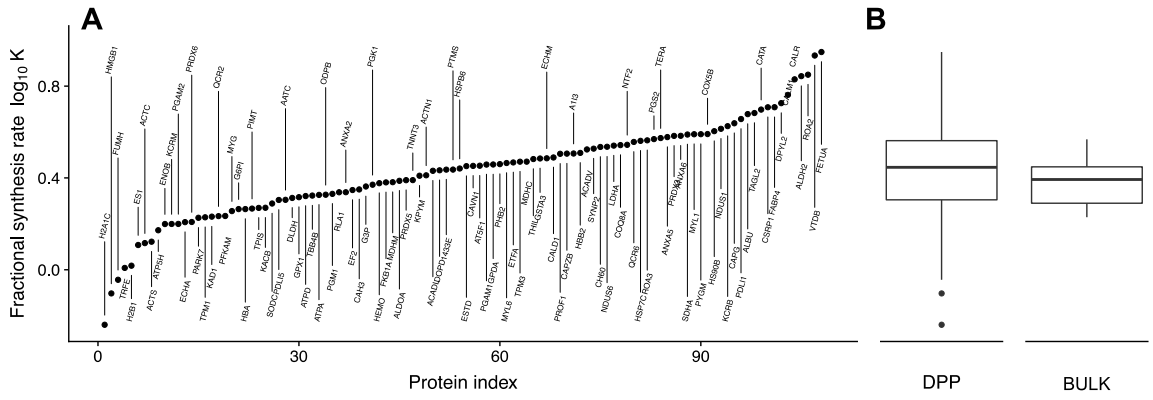

A
B
Fractional synthesis rate log10 K
0.0
0.4
0.8
0
30
60
90
Protein index
DPP
BULK

Figure 3

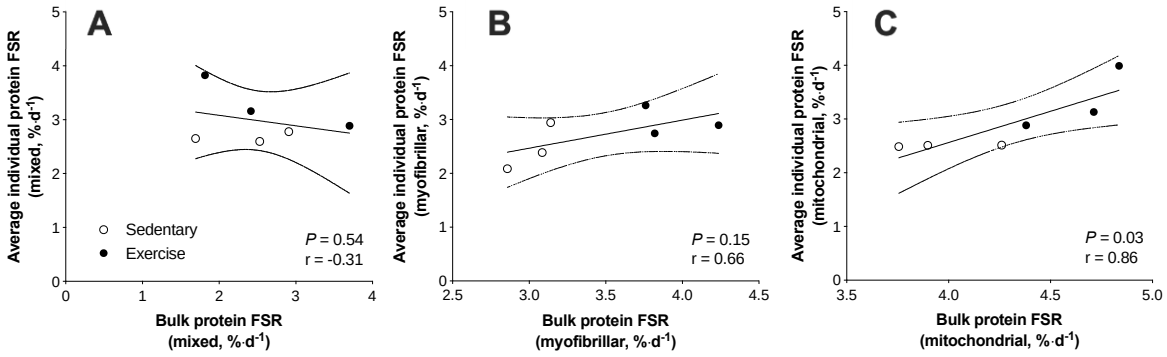

A
Average individual protein FSR (mixed, %·d-1)
Bulk protein FSR (mixed, %·d-1)
Sedentary
Exercise
P = 0.54
r = -0.31
B
Average individual protein FSR (myofibrillar, %·d-1)
Bulk protein FSR (myofibrillar, %·d-1)
P = 0.15
r = 0.66
C
Average individual protein FSR (mitochondrial, %·d-1)
Bulk protein FSR (mitochondrial, %·d-1)
P = 0.03
r = 0.86

Figure 4

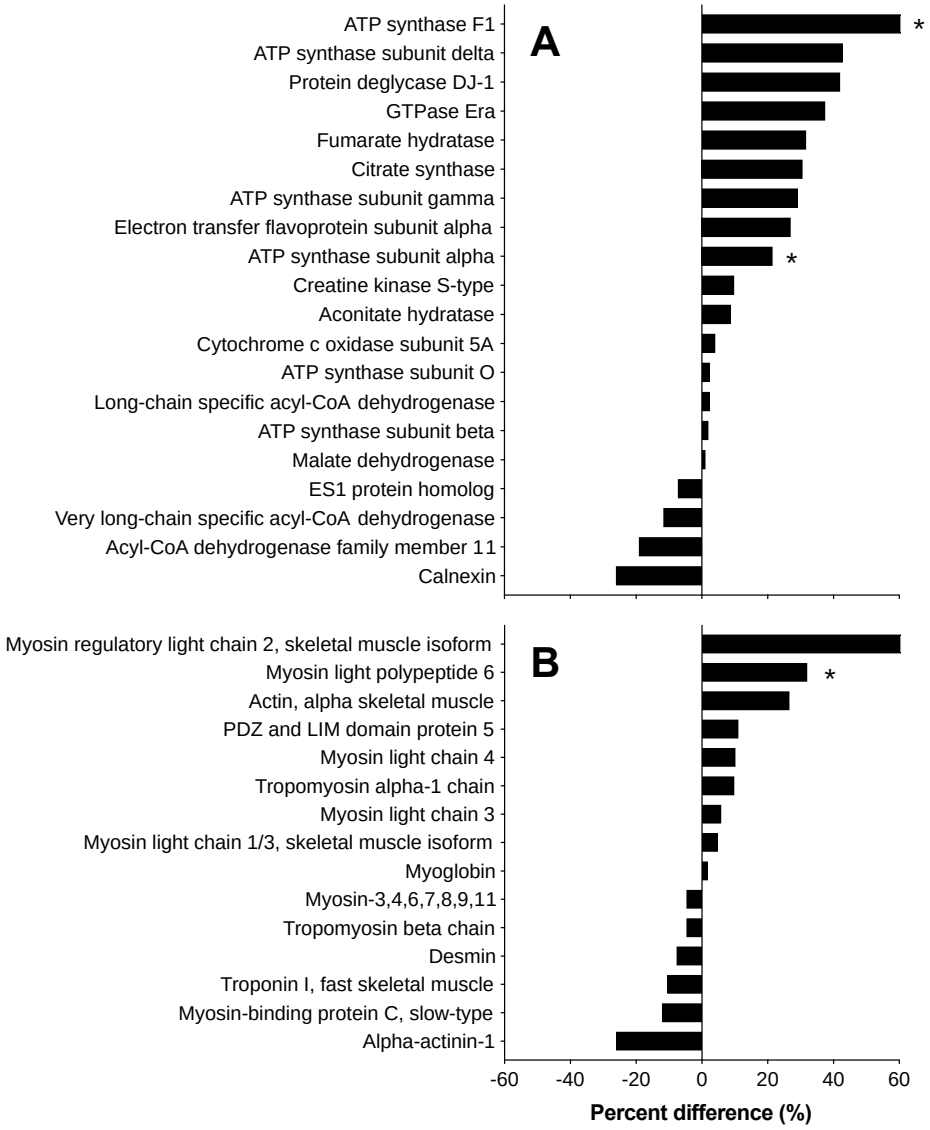
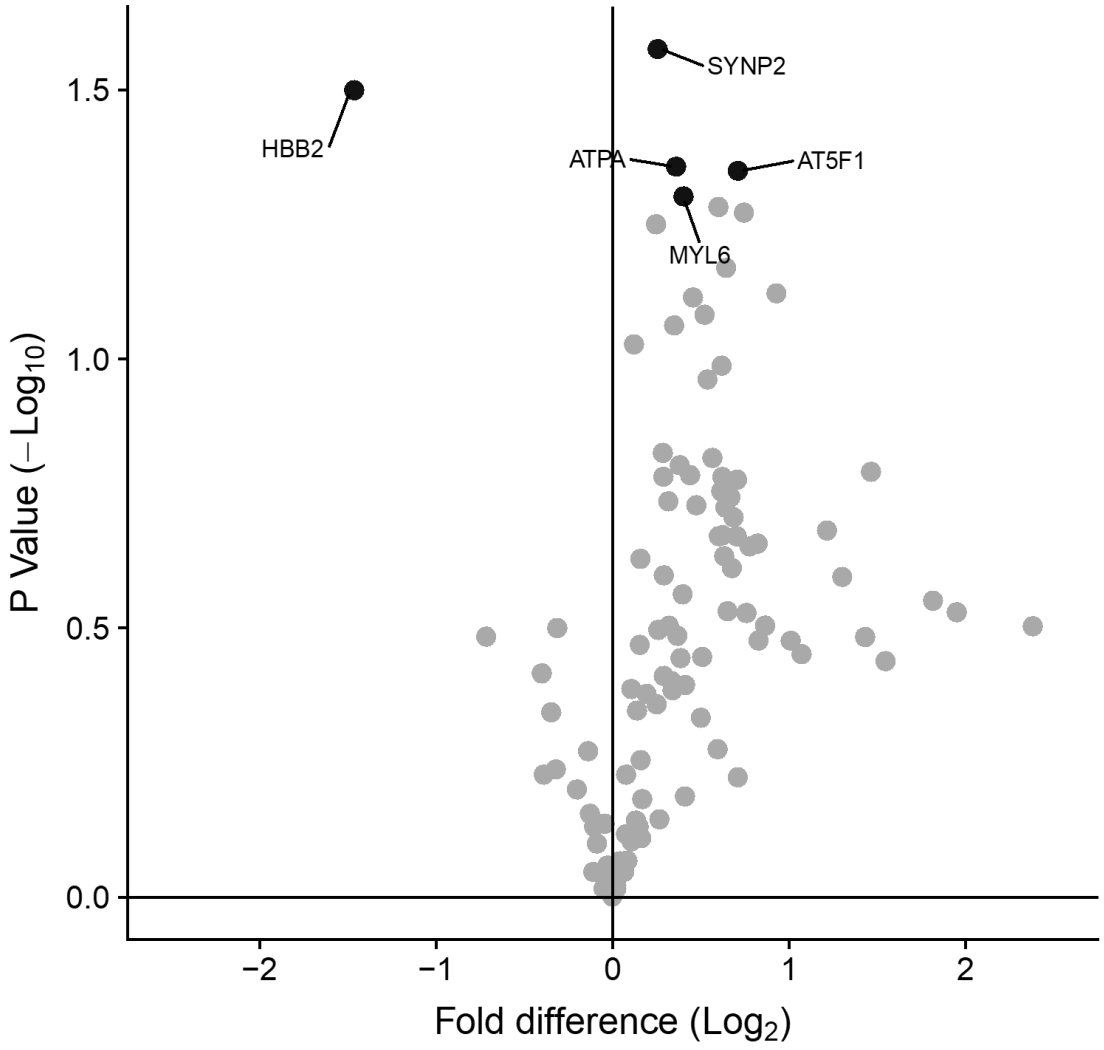

A
ATP synthase F1
ATP synthase subunit delta
Protein deglycase DJ-1
GTPase Era
Fumarate hydratase
Citrate synthase
ATP synthase subunit gamma
Electron transfer flavoprotein subunit alpha
ATP synthase subunit alpha
Creatine kinase S-type
Aconitate hydratase
Cytochrome c oxidase subunit 5A
ATP synthase subunit O
Long-chain specific acyl-CoA dehydrogenase
ATP synthase subunit beta
Malate dehydrogenase
ES1 protein homolog
Very long-chain specific acyl-CoA dehydrogenase
Acyl-CoA dehydrogenase family member 11
Calnexin
*
*
B
Myosin regulatory light chain 2, skeletal muscle isoform
Myosin light polypeptide 6
Actin, alpha skeletal muscle
PDZ and LIM domain protein 5
Myosin light chain 4
Tropomyosin alpha-1 chain
Myosin light chain 3
Myosin light chain 1/3, skeletal muscle isoform
Myoglobin
Myosin-3,4,6,7,8,9,11
Tropomyosin beta chain
Desmin
Troponin I, fast skeletal muscle
Myosin-binding protein C, slow-type
Alpha-actinin-1
*
-60
-40
-20
0
20
40
60
Percent difference (%)

Figure 5


SYNP2
HBB2
ATPA
AT5F1
MYL6
1.5
1.0
0.5
0.0
P Value (−Log10)
−2
−1
0
1
2
Fold difference (Log2)