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RESEARCH ARTICLE

Protein signalling in response to *ex vivo* dynamic contractions is independent of training status in rat skeletal muscle

Jesper Emil Jakobsgaard[1] | **Frank Vincenzo de Paoli**[2] | **Kristian Vissing**[1]

[1]Exercise Biology, Department of Public Health, Faculty of Health, Aarhus University, Aarhus, Denmark

[2]Department of Biomedicine, Faculty of Health, Aarhus University, Aarhus, Denmark

Correspondence
Kristian Vissing, Department of Public Health, Aarhus University, Dalgas Avenue 4, 8000 Aarhus C, Denmark.
Email: vissing@ph.au.dk

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Abstract

Skeletal muscle training status may influence myofibre regulatory protein signalling in response to contractile activity. The current study employed a purpose-designed *ex vivo* dynamic contractile protocol to evaluate the effect of exercise-accustomization on canonical myofibre protein signalling for metabolic gene expression and for translation initiation and elongation. To this end, rats completed 8 weeks of in vivo voluntary running training versus no running control intervention, whereupon an *ex vivo* endurance-type dynamic contraction stimulus was conducted in isolated soleus muscle preparations from both intervention groups. Protein signalling response by phosphorylation was evaluated by immunoblotting at 0 and 3 h following *ex vivo* stimulation. Phosphorylation of AMP-activated protein kinase α-isoforms and its downstream target, acetyl-CoA carboxylase, as well as phosphorylation of eukaryotic elongation factor 2 (eEF2) was increased immediately following the dynamic contraction protocol (at 0 h). Signalling for translation initiation and elongation was evident at 3 h after dynamic contractile activity, as evidenced by increased phosphorylation of p70 S6 kinase and eukaryotic translation initiation factor 4E-binding protein 1, as well as a decrease in phosphorylation of eEF2 back to resting control levels. However, prior exercise training did not alter phosphorylation responses of the investigated signalling proteins. Accordingly, protein signalling responses to standardized endurance-type contractions may be independent of training status in rat muscle during *ex vivo* conditions. The present findings add to our current understanding of molecular regulatory events responsible for skeletal muscle plasticity.

KEYWORDS
ex vivo contractions, protein signalling, training status

1 | INTRODUCTION

Exercise training constitutes a means to stimulate functional adaptations in skeletal muscle tissue of importance for overall health and locomotive function in humans (Egan & Zierath, 2013; Pedersen & Saltin, 2015; Romanello & Sandri, 2015). To achieve this, a given exercise stimulus introduces systemic and/or intrinsic cues, which then activate distinct myocellular signalling pathways driving

phenotypic alterations (Booth et al., 2015; Drake et al., 2016; Egan & Zierath, 2013). More specifically, the specific modality, volume and intensity of an exercise stimulus dictate elicitation of upstream cues adhering to changes in, for example, mechanical deformation, energy turnover, Ca^{2+} oscillations or reactive oxygen species, to stimulate downstream signalling for myocellular synthetic or degradative events (Hood et al., 2016; Rindom & Vissing, 2016; Tavi & Westerblad, 2011). Upon structured repetitions of exercise sessions over time (i.e., training), these stimulations accumulate to shape properties important for myocellular metabolic and contractile function (Egan & Zierath, 2013; Hood et al., 2016; Perry et al., 2010). To this end, the 5′-AMP-activated protein kinase (AMPK) signalling pathway components, including tumour suppressor p53 and cAMP response element-binding protein (CREB), are considered to constitute a proxy for transcriptional regulation of metabolic genes (Bartlett et al., 2014; Egan & Zierath, 2013; Kjøbsted et al., 2018; McGee & Hargreaves, 2010; Smiles & Camera, 2017). Consequently, signalling pathway components adhering to the Raptor-dependent mechanistic target of rapamycin complex 1 (mTORC1), such as eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), p70 S6 kinase (p70 S6K) and eukaryotic elongation factor 2 (eEF2), represent a proxy for initiation and elongation of protein translation (Browne & Proud, 2002; Figueir et al., 2017; Rindom & Vissing, 2016). Accordingly, in-depth mechanistic knowledge on these, as well as interrelated pathways, is vital to an understanding of plasticity in muscle metabolism, mass and remodelling.

One approach to investigation on the influence of exercise stimulation of said pathways involves collection of muscle biopsies during the immediate post-exercise recovery period upon human in vivo exercise (Egan & Zierath, 2013). Although standardization of human exercise protocols can reasonably account for variables like age and sex, human experimental studies are sensitive to genetic heterogeneity (Bouchard et al., 2011; Simoneau & Bouchard, 1989), as well as some rather arbitrary choices of, for example, dietary premise (Kimball & Jefferson, 2006). Another factor that has received relatively little attention is the influence of prior exercise accustomization on myofibre molecular responses to the contractile stress inherent in exercise. Accordingly, the degree to which skeletal myofibres are accustomed to exercise (i.e., training status) may alter (e.g., blunt) exercise-induced signalling responses, and thus challenge interpretation if not carefully addressed. In fact, cross-sectional studies that have compared highly endurance-trained human individuals with untrained counterparts suggest that skeletal myofibres of untrained subjects exhibit greater exercise-induced molecular signalling responses. More specifically, when matching exercise workload by relative standards (i.e., percentage of $\dot{V}_{O_2peak}$), greater post-exercise enzyme activity and/or phosphorylation changes of AMPK α-isoforms (AMPKα) and downstream AMPK substrate acetyl-CoA carboxylase (ACC) has been reported in myofibres from untrained human subjects when compared to those who are highly endurance-trained (Nielsen et al., 2003; Yu et al., 2003). Also highly noteworthy, in a previous study by Coffey et al. (2006), the specificity of training status was highlighted by observations of greater protein signalling responses when long-term endurance and resistance exercise-trained subjects were cross-exposed to matched relative workloads of the exercise modality to which they were unfamiliar (Coffey et al., 2006). However, standardization of exercise intensity may constitute an important factor. In accordance, when applying the same absolute workloads before and after training intervention period, reduced myocellular signalling responses have been reported in the trained/exercise-accustomed state as compared to untrained (Benziane et al., 2008; Granata et al., 2019; McConell et al., 2005; Pilegaard et al., 2003; Stepto et al., 2012). Oppositely, at least one other study has reported similar myofibre protein signalling responses when applying the same relative workload in trained and untrained human subjects (Wilkinson et al., 2008).

Human in vivo exercise models and muscle biopsy sampling applied in the abovementioned studies entail high physiological relevance for the understanding of myofibre remodelling. Yet, such an approach has important inherent limitations that advocate for an supportive *ex vivo* experimental approach. Accordingly, in comparison to in vivo exercise, animal *ex vivo* contraction models on isolated muscle can allow for improved standardization of properties such as homogeneity of the animal subjects and phenotypes of muscle preparations. Furthermore, *ex vivo* electrostimulated contraction allows for more uniform myofibre activation independent of central motor drive (Cairns et al., 2007), as well as standardization of the imposed tensile stress (Rindom et al., 2019). On the other hand, previous *ex vivo*-based studies almost exclusively rely on isometric contraction, which does not ideally resemble in vivo animal activity patterns (e.g., during running locomotion). To our knowledge, no study has previously attempted to evaluate AMPK and mTORC1 protein signalling in response to standardized *ex vivo* dynamic contractile work in exercise-accustomed versus exercise-unaccustomed skeletal muscle phenotypes.

New Findings

- **What is the central question of this study?**
  Are myofibre protein signalling responses to *ex vivo* dynamic contractions altered by accustomization to voluntary endurance training in rats?
- **What is the main finding and its importance?**
  In response to *ex vivo* dynamic muscle contractions, canonical myofibre protein signalling pertaining to metabolic transcriptional regulation, as well as translation initiation and elongation, was not influenced by prior accustomization to voluntary endurance training in rats. Accordingly, intrinsic myofibre protein signalling responses to standardized contractile activity may be independent of prior exercise training in rat skeletal muscle.

The purpose of the present study was therefore to examine the influence of prior exercise training on exercise-induced myocellular AMPK- and mTORC1-related protein signalling. To this end, we applied a translational approach by first using an in vivo voluntary running exercise model to induce an endurance activity-trained muscle phenotype, whereupon a purpose-designed dynamic *ex vivo* endurance exercise protocol model was used to provoke and evaluate protein signalling responses. We hypothesized that hindlimb muscle preparations from endurance trained rats, compared to those of sedentary controls, would exhibit a less pronounced myocellular protein signalling response when exposed to endurance-type dynamic *ex vivo* muscle contractions.

2 | METHODS

2.1 | Ethical approval

All experimental procedures were approved by the Danish Animal Experimental Inspectorate (No. 2021-15-0201-01028), and complied with Danish Animal welfare legislation and the Directive 2010/63/EU of the European Parliament and the Council of 22 September 2010. The experiments conformed to the principles and standards of reporting in animal experiments of *Experimental Physiology* (Grundy, 2015).

2.2 | Animals

Female wild-type Wistar rats (Janvier Labs, Le Genest-Saint-Isle, France) were used in the present study. In total, 18 rats were used. Upon arrival at 5 weeks of age, rats were acclimatized for 1 week in the animal facilities, and enrolled in the study at 6 weeks of age (starting weight 168.4 ± 11.9 g, mean ± SD). All housing was in a thermostatically controlled environment at 21°C room temperature, with a 12/12 h light–dark cycle and ad libitum access to food and water. Killing of the animals at the end of the intervention period was performed at 14 weeks of age by an intraperitoneal injection of sodium pentobarbital (200 mg kg^{-1}) containing 10% lidocaine.

2.3 | Study design

Following acclimatization, rats were randomly allocated to either (1) a voluntary running group (runners) or (2) a sedentary control group (sedentary), for a total duration of 8 weeks. The voluntary running training protocol was designed as previously described by Broch-Lips et al. (2011). Rats allocated to the running group were housed individually in a case with ad libitum access to a 35 cm-diameter unloaded running wheel (Tecniplast, Buguggiate, Italy). Running wheels were equipped with a magnet-based odometer (BC 9.16; Sigma-Elektro GmbH, Neustadt, Germany) by which weekly running distance was registered. To reduce between-rat running variability, only rats that achieved a running distance of >50 km during the initial 2 weeks of the intervention period were included for the remaining 6 weeks. In total, 8 of 9 rats allocated to the running group displayed adequate running willingness, and were thus included for the remainder of the intervention. Rats allocated to the control group were likewise housed individually, but without access to a running wheel, allowing only for normal sedentary behaviour of the animals. Since only rats in the running group were selected on the basis of running willingness, this could in theory introduce a phenotypic bias in this intervention group in the interpretation of myocellular analyses. However, given that group allocation was randomized, and all but one rat reached the inclusion criteria in the running group, we argue that such an issue is of minimal concern. Following the 8-week intervention period, rats were killed at 14 weeks of age with the purpose of harvesting skeletal muscle samples for subsequent *ex vivo* experiments. Running wheels were locked 24 hours prior to killing.

2.4 | Preparation of muscle samples

Following killing, upon confirmation of cessation of circulation, soleus muscles from both hindlimbs were quickly dissected out. More specifically, an incision was made in the skin just proximal to the calcaneus, by which the hindlimb was skinned and the triceps surae was exposed. A clamp was fixed to the most distal part of the achilles tendon, near the insertion on the calcaneus, from which the tendon was cut. The triceps surae was then elevated from the hindlimb using the clamp. Subsequently, the soleus muscle was isolated from the gastrocnemius muscle at the achilles tendon, and then completely excised by isolating it from its origin on a small piece of bone from the fibula. During the entire procedure, an ice-cold NaCl solution (154 mM) was used to wet the exposed muscle tissue. Soleus was chosen as the muscle type of interest, as it has been widely demonstrated to exhibit phenotypic adaptations to endurance-type exercise training in rodents (Bahreinipour et al., 2018; Beleza et al., 2019; Broch-Lips et al., 2011; Hyatt et al., 2019; Rodnick et al., 1989; Sexton & Laughlin, 1994; Zheng et al., 2020). To minimize potential issues of tissue perfusion related to muscle thickness during incubation in organ baths, each soleus muscle was split longitudinally from tendon to tendon into two muscle strips of approximately equal size to reduce cross-sectional area. The use of muscle strips allowed for a doubling of the yield of experimental muscle preparations, thus minimizing the overall number of animals necessary for the study purpose. A total of 18 and 16 soleus muscle preparations were harvested yielding 36 and 32 muscle strip preparations from sedentary controls and runners, respectively. Immediately following splitting, each muscle strip was suspended tendon to tendon in an organ bath containing a Krebs–Ringer–bicarbonate buffer consisting of (in mmol l^{-1}): 122 NaCl, 25 $NaHCO_3$, 2.8 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.3 $CaCl_2$ and 5.0 D-glucose. The buffer was kept at a temperature of 30°C and perfused with a carbogen gas mixture (95% O_2–5% CO_2). One end of the muscle strip was attached to a motorized force-transducer lever arm (model 305, Aurora Scientific, Aurora, ON, Canada), controlled by computer software (DMC 5.50, Aurora Scientific) that allowed for recording of force and length output at 1000 Hz. Additionally, hearts were also excised from the rats, followed by removal of connective tissue and blood, blotting on paper and weighing.

2.5 | Electrical stimulation and dynamic muscle contractions

Electrical stimulation of the muscle preparations was performed as previously reported (Jakobsgaard et al., 2021). Muscle contractions were evoked through field stimulation applied with supramaximal constant voltage (16 V cm^{-1}), through two platinum plate electrodes running parallel on each side of the muscle strip. The pulse duration was 0.2 ms. Prior to the main stimulation protocol, muscles preparations incubated for 60 min in the organ baths, during which optimal suspension length (L_o) was established and maximal tetanic force capacity (P_o) was measured. During the latter 30 min of this period, P_o was evaluated every 10 min to ensure stabile contractile function of the muscle strip. All muscle strip preparations displayed a stable P_o during this period (CV within 1.63%). The main stimulation protocol consisted of dynamic stretch–shortening contractions evoked by low-frequency electrical stimulation, delivered in intermittent trains every 2 s for 40 min. The duration of each pulse train was 250 ms with a pulse frequency of 30 Hz, corresponding to six pulses per train. Simultaneously, the muscle tendon unit was length-manipulated in a sine wave pattern comprising an initial 2 mm stretch followed by a 4 mm shortening and finally 2 mm stretch back to L_o. Accordingly, contraction velocity was kept as the independent variable, leaving force output as the dependent. The electrical pulse train was delivered during the initial 75% of this stretch–shortening sine wave, corresponding to the initial stretch and the following shortening. Thirty-second averages for peak force development of the contraction cycles, as well as total force–time integral, was calculated for the 40 min main stimulation protocol, using custom-designed PC software (SVX 2.2.6, Department of Public Health, Aarhus University). For each muscle strip preparation pair, one strip was subjected to the main stimulation protocol, whereas the other served as a paired resting control, exposed to an identical period of incubation in the organ bath. Either immediately (0 h) or at 3 h after termination of the main stimulation protocol, muscle preparations were rapidly removed from the organ baths, rinsed in isotonic saline and blotted on paper, followed by removal of tendons and connective tissue, weighing and subsequently snap-freezing in liquid nitrogen. To evaluate contractile fatigue as a response to the main stimulation protocol, P_o was measured again immediately after stimulation prior to the resting period in muscle strips allocated to the 3 h time point. Muscle preparations harvested immediately after stimulation (time point 0 h) were divided longitudinally into two parts, one allocated for immunoblotting analyses, and one for measures of glycogen content. All muscle samples were stored at −80°C for later analysis.

2.6 | Muscle homogenization

Initially, muscle samples were powdered under liquid nitrogen using a mortar and pestle. Subsequent protein extraction of the samples was commenced with steel-bead homogenization in an ice-cold buffer containing (in mM): 50 NaCl, 20 Tris, 250 sucrose, 50 NaF, 5 $Na_4P_2O_7$ and 2 dithiothreitol, supplemented with 1% Triton X-100 and 1% Halt protease inhibitor cocktail (cat. no. 78430, Thermo Fisher Scientific, Waltham, MA, USA), using a TissueLyser LT homogenizer (Qiagen, Hilden, Germany) by 2 × 2 min cycles of 50 Hz. Subsequently, the samples were mixed at 700 rpm, 4°C, for 15 min using a thermomixer (Eppendorf, Hamburg, Germany), followed by centrifugation at 16,000 *g*, 4°C, for 20 min. The supernatant was transferred to a new tube and the protein concentration of this fraction was determined using Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA).

2.7 | Immunoblotting

Immunoblotting procedures were performed as previously described (Jakobsgaard et al., 2021). Twenty-five micrograms of protein for each sample was separated by gel electrophoresis on pre-cast Stain-Free 4–15% gels (Bio-Rad). Subsequently, the proteins were transferred by electroblotting onto a polyvinylidene difluoride membrane using a Trans-blot Turbo system (Bio-Rad) and then blocked for 1.5 h at room temperature with 5% BSA in a Tris-buffered saline solution containing 0.1% Tween-20 (TBST). Membranes were then incubated overnight at 5°C in a primary antibody diluted in TBST containing 5% BSA. The following primary antibodies (all Cell Signaling Technology, Danvers, MA, USA) and concentrations were applied: p-4E-BP1$^{Thr36/45}$ (cat. no. 2855, RRID AB_560835, conc. 1:1000), p-ACC^{Ser79} (cat. no. 3661, RRID AB_330337, conc. 1:1000), p-AMPKα1/2^{Thr172} (cat. no. 2535, RRID AB_331250, conc. 1:1000), p-CREB^{Ser133} (cat. no. 9198, RRID AB_2561044, conc. 1:500), p-eEF2^{Thr56} (cat. no. 2331, RRID AB_10015204, conc. 1:1000), p-p53^{Ser15} (cat. no. 9284, RRID AB_331464, conc. 1:1000), and p-p70 S6K^{thr389} (cat. no. 9234, RRID AB_2269803, conc. 1:500). Secondary antibody incubation was performed for 1 h at room temperature with a horseradish peroxidase-conjugated goat anti-rabbit antibody (cat. no. 7074, RRID AB_2099233, Cell Signaling Technology) with a concentration of 1:5000 in 1% BSA, except for p-AMPK and p-p70 S6K for which a concentration of 1:3000 in 1% BSA was used. Protein bands were visualized by chemiluminescence (Thermo Fisher Scientific) using a ChemiDoc MP imaging system (Bio-Rad). Arbitrary band intensity was measured by software using Image Lab v6.1.0 (Bio-Rad). Initial normalization of the raw band intensity was performed against the internal standard, followed by normalization to the total amount of protein loaded in the corresponding lane as visualized by Stain-Free technology (Gurtler et al., 2013).

2.8 | Muscle glycogen content

Determination of total muscle glycogen content was performed with a spectrophotometer (UV-1600 PC; VWR International LLC, Darmstadt, Germany). After freeze-drying, approximately 2 mg muscle sample was boiled in 0.5 ml 1 M HCl for 2.5 h, followed by cooling on ice, whirl mixing, and centrifugation at 3500 *g* for 10 min at 4°C. A reagent solution containing (in M) 1 Tris-buffer, 0.1 ATP, 1 $MgCl_2$, 0.1 NADP-

glucose 6-phosphate dehydrogenase and Milli-Q water was prepared from which 500 μl was mixed with 20 μl sample. The reaction process was started by adding 5 μl of diluted hexokinase. Absorbance was recorded for 60 min at 340 nm wavelength, and glycogen content was calculated using a standard curve of known concentrations from the same analysis batch. Calculations are expressed as mmol glycogen (kg muscle dry weight)$^{-1}$.

2.9 | Statistical analyses

Normal distribution of the data and model validation were evaluated by visual inspection of QQ plots as well as testing for equal standard deviations. Protein expression data for p-p70 S6K did not exhibit normal distribution. However, on log-transformed data, identical results were observed, wherefore non-transformed data is likewise presented for p-p70 S6K. For analysis of the effect of electrostimulation on P_o, glycogen content and protein expression, a linear mixed-effects model was applied to evaluate main effects and related interactions, with appropriate fixed effects for the given dependent variable, and muscle strip pairs as random effects. *Post hoc* analysis of significant main effects was performed by pairwise comparisons, whereas significant interactions were evaluated by linear comparison analysis. A non-paired Student's *t*-test was used to test for differences in body weight, muscle and heart characteristics, force–time integral of stimulated muscles, as well as basal control-level protein phosphorylation expression between intervention groups. α-Level was set to $P < 0.05$. All statistical analyses were performed using STATA 15.1 (StataCorp, College Station, TX, USA). Figures were created using GraphPad Prism 9.0 (GraphPad Software Inc., San Diego, CA, USA). All values are displayed as means ± SD, unless otherwise specified.

3 | RESULTS

3.1 | Voluntary running training

Figure 1 depicts mean weekly running distance performed by rats allocated to the running group. On average, runners ran 119.4 ± 48 km during the initial 2-week period. Over the course of the entire training period, runners covered a mean daily running distance of 11.4 ± 1.8 km. While no differences in body weight was observed between control and running rats, hearts and soleus muscles weighed ~25 and ~39% more, respectively, in running compared to sedentary control rats ($P < 0.0001$, Table 1).

3.2 | Force development and changes in maximal tetanic force

Figure 2 displays force parameters in response to 40 min of low frequency electrical stimulated dynamic contractions. Relative to the initial 30 s, average peak force decreased to 73.1 ± 6.5% and 72.5 ± 7.2% after 5 min, 55.6 ± 9.9% and 55.5 ± 8.6% after 20 min, and remained at 57.4 ± 11.0% and 52.9 ± 11.3% after 40 min of stimulation in muscle preparations from sedentary and runners, respectively (Figure 2a). On average throughout the 40-min stimulation protocol, peak force relative to P_o was 34.5 ± 5.8% and 34.1 ± 6.0% in sedentary and running trained muscles, respectively (Figure 2b). No differences in force–time integral between the groups was observed ($P = 0.436$ and 0.635, Figure 2c, d). After the main stimulation protocol, maximal tetanic force (P_o) decreased to 54.0 ± 4.9% and 62.2 ± 3.1% of pre in muscle samples from sedentary and runners, respectively ($P < 0.0001$, Figure 2e). This decrease in P_o was greater by an average of 8.2 percentage points in muscle samples from sedentary rats as compared to runners ($P < 0.0001$).

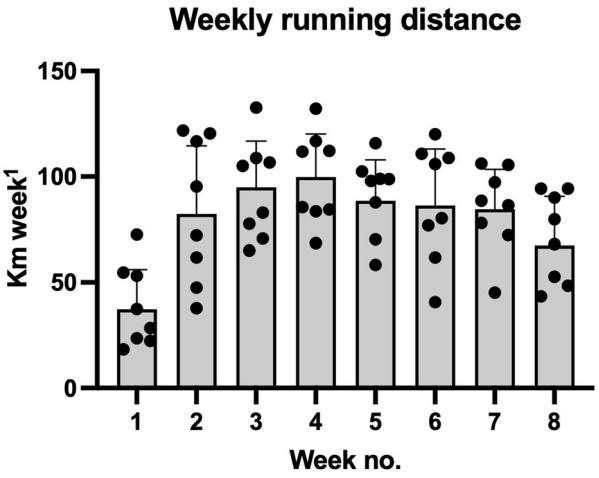

FIGURE 1 Weekly running distance performed by rats allocated to voluntary running training group. Bars represent means ± SD, $n = 8$

3.3 | Muscle glycogen content

Control muscle strip preparations from runners displayed ~21% higher glycogen content as compared to those from sedentary rats (167.5 ± 15.3 vs. 137.9 ± 23.9 mmol (kg dry weight)$^{-1}$, respectively, $P = 0.008$, Figure 2f). Compared to control samples, muscle glycogen content was 31.7 ± 17.4% ($P = 0.001$) and 38.6 ± 14.1% ($P = 0.002$) lower in stimulated samples from sedentary and runners, respectively, with no difference in stimulation–control contrasts between groups (interaction $P = 0.985$).

3.4 | Protein signalling in response to dynamic contractions

For all protein targets, no differences were observed between sedentary and runners in control levels of myofibre phosphorylation-specific protein expression within each time point (Table 2). Adjusting for running volume in protein expression measures derived from

TABLE 1 Body weight, muscle and heart characteristics

	Sedentary	Runners	*n*	*P*
Body weight (g)	252.3 ± 13.7	269.2 ± 20.5 (+6.7 ± 8.2%)	9/8	0.0624
Soleus wet weight (mg)	122.6 ± 8.2	170.5 ± 21.7 (+39.1 ± 17.7%)	18/16	<0.0001
Soleus wet weight relative to body weight (mg g^{-1})	0.485 ± 0.025	0.639 ± 0.082 (+31.8 ± 16.9%)	18/16	<0.0001
100 Hz tetanic force (P_o) relative to muscle strip weight (g mg^{-1})	1.27 ± 0.19	0.99 ± 0.19 (−22.1 ± 14.9%)	36/32	<0.0001
Heart wet weight (mg)	735.6 ± 47.2	916.3 ± 45.3 (+24.6 ± 6.2%)	9/8	<0.0001
Heart wet weight relative to body weight (mg g^{-1})	2.92 ± 0.14	3.43 ± 0.32 (+17.5 ± 10.9%)	9/8	0.0004

Values are means ± SD. %-value in Runners-column is relative difference vs. Sedentary. *n* represents the sample size of each outcome analysed in sedentary/runners intervention group.

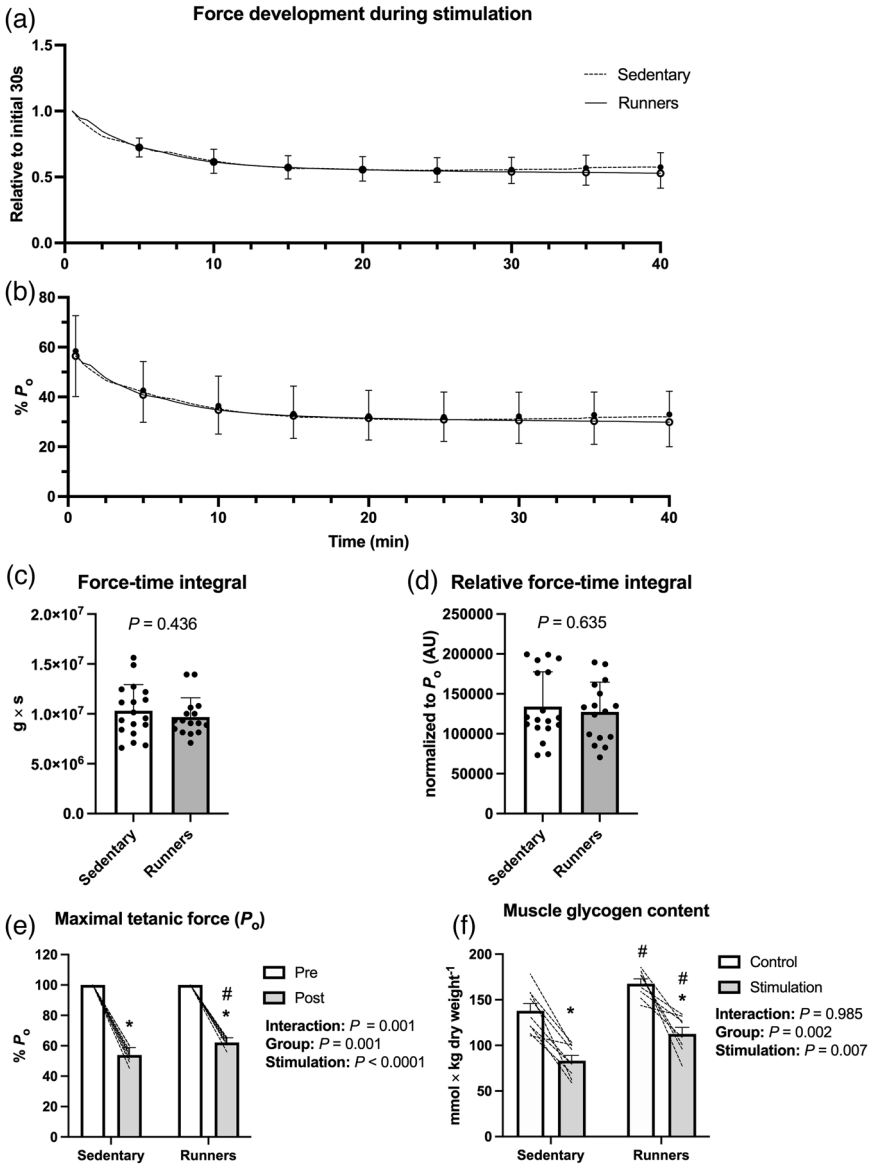

FIGURE 2 Force development and changes in maximal tetanic force and glycogen content in response to electrostimulation-evoked dynamic muscle contraction cycles. (a, b) Force development during the 40-min main stimulation protocol, relative to initial 30 s and maximal tetanic force (P_o), respectively, with filled and open circles representing means ±SD for every 5 min in muscle preparations from sedentary ($n = 18$) and runners ($n = 16$), respectively. (c, d) Summed force–time integral for the 40-min main stimulation protocol (c), and the same normalized to P_o (d) ($n = 18$ and 16 in sedentary and runners, respectively). *P*-values are derived from an unpaired Student's *t*-test. (e) Relative changes in P_o from pre- to post-stimulation ($n = 9$ and 8 in sedentary and runners, respectively). $^*P < 0.0001$ vs. pre within group. $\#P < 0.0001$ vs. post in sedentary. (f) Total muscle glycogen content in control and stimulated muscle strip preparations from sedentary ($n = 9$ for each condition) and runners ($n = 8$ for each condition), $^*P < 0.0001$ and 0.002 vs. control condition within group in sedentary and runners, respectively, and $\#P = 0.002$ and 0.006 vs. control or stimulation condition in sedentary, respectively. Bars are means ± SD; dashed lines represent individual paired data

TABLE 2 Control levels of protein phosphorylation expression for included protein targets

	0 h		3 h		
	Sedentary	Runners	Sedentary	Runners	*P*
p-AMPK^{Thr172}	0.52 ± 0.12	0.69 ± 0.22	0.46 ± 0.11	0.58 ± 0.18	0h: 0.146 3h: 0.427
p-ACC^{Ser79}	0.65 ± 0.16	0.83 ± 0.40	0.60 ± 0.23	0.69 ± 0.23	0h: 0.506 3h: 0.884
p-p53^{Ser15}	0.80 ± 0.26	0.82 ± 0.16	0.80 ± 0.28	0.80 ± 0.22	0h: 0.998 3h: 0.999
p-CREB^{Ser133}	0.95 ± 0.31	1.28 ± 0.37	0.83 ± 0.32	1.18 ± 0.62	0h: 0.389 3h: 0.339
p-4E-BP1$^{Thr36/45}$	1.26 ± 0.50	1.35 ± 0.36	1.05 ± 0.31	1.31 ± 0.32	0h: 0.964 3h: 0.499
p-p70 S6K^{Thr389}	1.25 ± 1.35	1.43 ± 1.32	0.56 ± 0.22	0.40 ± 0.26	0h: 0.981 3h: 0.989
p-eEF2^{Thr56}	0.87 ± 0.27	0.99 ± 0.35	0.81 ± 0.17	0.97 ± 0.14	0h: 0.717 3h: 0.533

Values are means ± SD. Data are measured in resting control muscle preparation samples not subjected to the main stimulation protocol. *P*-values are derived from pairwise comparison testing adjusted for multiple comparisons (Tukey). $n = 9$ and 8 for each condition and time point in sedentary and running trained group, respectively, except for $n = 7$ for p-p70 S6K at 3 h time point in Runners, due to exclusion of one outlier. 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1; ACC, acetyl-CoA carboxylase; AMPKα, 5′-AMP-activated protein kinase subunit α-1/2; CREB, cAMP-response element-binding protein; eEF2, eukaryotic elongation factor 2; p53, tumour suppressor protein p53; p70 S6K, p70 S6 kinase.

muscle preparations in runners did not alter data variation, as evaluated by testing for equal standard deviations. Accordingly, all protein expression data are presented as unadjusted.

Representative blots for post-stimulation signalling after intervention are depicted in Figure 3.

3.5 | Signalling related to transcriptional regulation of metabolic genes

An effect of stimulation on phosphorylation of both AMPKα and ACC was observed in both groups at 0 h. Compared to control condition, p-AMPKα increased 4.7 ± 1.2-fold ($P = 0.001$, Figure 4a) and 4.5 ± 2.0-fold ($P = 0.001$, Figure 4b) at 0 h after stimulation in stimulated muscle samples from sedentary and runners, respectively. At the same time point, p-ACC increased 3.7 ± 1.5-fold ($P = 0.001$, Figure 4d) and 2.9 ± 1.8-fold ($P = 0.002$, Figure 4e) above control levels in stimulated muscle samples from sedentary and runners, respectively. The phosphorylation of both AMPKα and ACC had returned to basal control levels at 3 h after stimulation in both sedentary ($P = 0.533$ and 0.798, respectively) and runners ($P = 0.063$ and 0.539, respectively). No differences between intervention groups in the response to stimulation of either p-AMPKα (interaction $P = 0.378$, Figure 4c) or p-ACC (interaction $P = 0.229$, Figure 4f) were observed.

No effect of stimulation at either time point was observed for p-p53 and p-CREB in muscle samples from either sedentary (interaction $P = 0.083$ and 0.073, respectively) or runners (interaction $P = 0.066$ and 0.699, respectively) (Figure 4g–l).

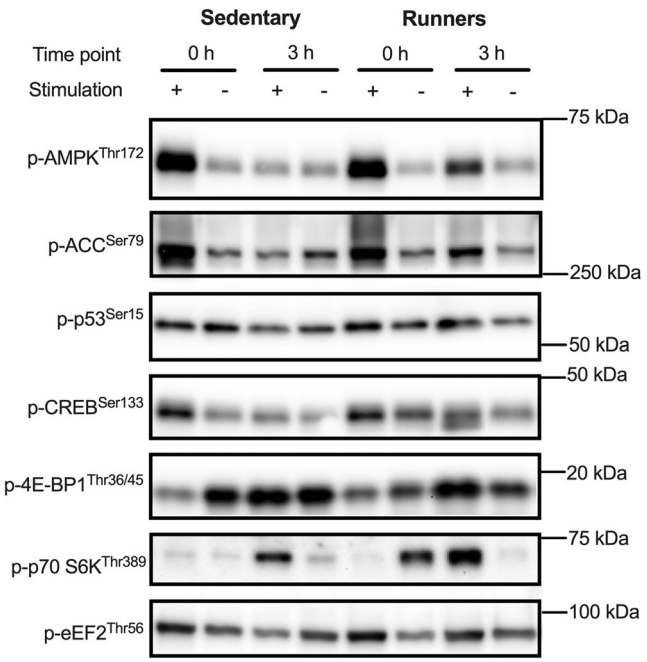

FIGURE 3 Representative blots of all target proteins for both time points and conditions in soleus muscle preparations from sedentary and runners. The displayed blots within each group are obtained from soleus muscle preparations originating from the same rat. 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1; ACC, acetyl-CoA carboxylase; AMPKα, 5′-AMP-activated protein kinase subunit α-1/2; CREB, cAMP-response element-binding protein; eEF2, eukaryotic elongation factor 2; p53, tumour suppressor protein p53; p70 S6K, p70 S6 kinase

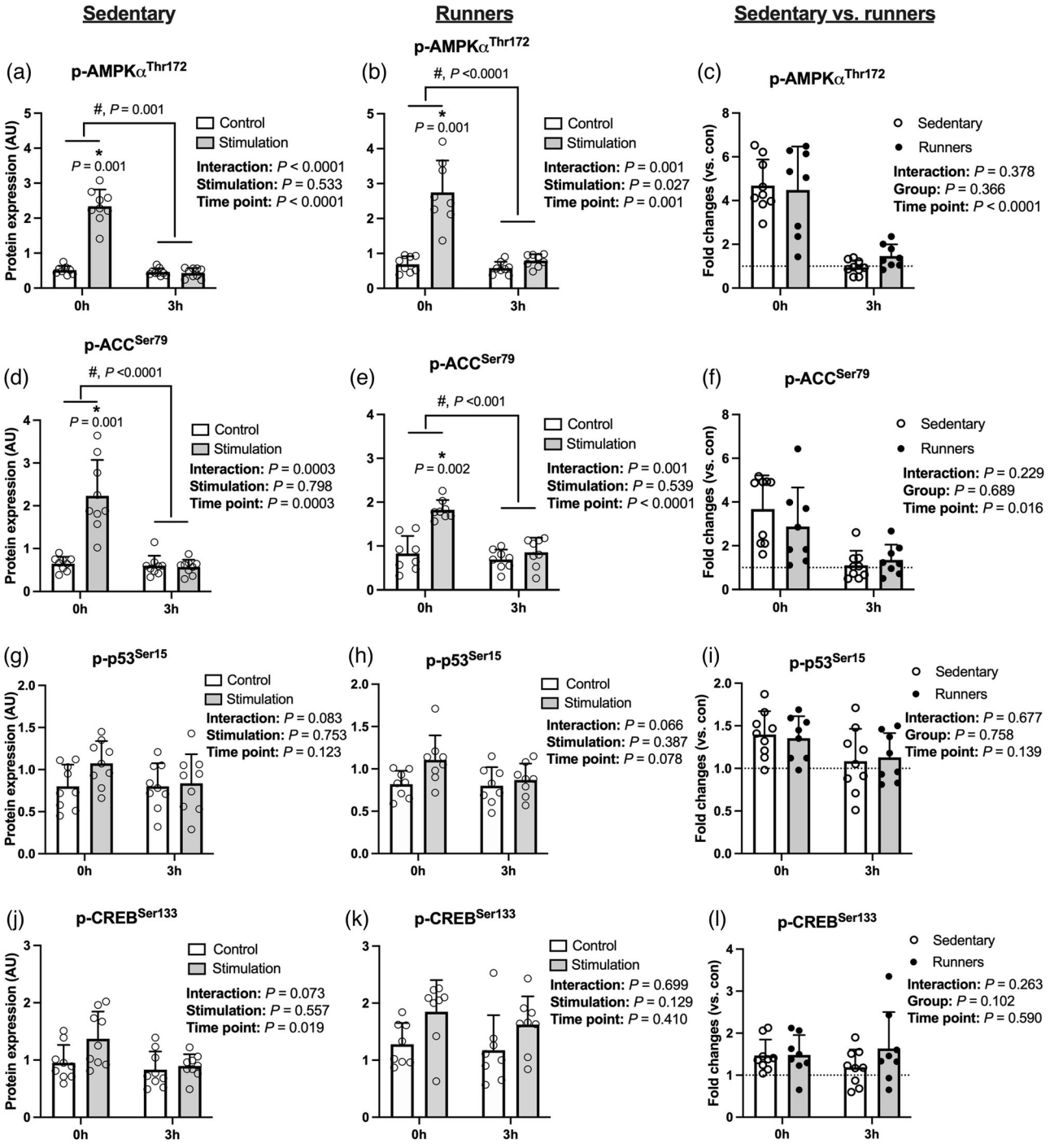

FIGURE 4 5′-AMP-activated protein kinase (AMPK)-related protein signalling. Protein expression of phosphorylated AMPKα, ACC, tumour suppressor p53, and cAMP-response element-binding protein (CREB) signalling in soleus muscle preparations of sedentary (a, d, g, j) and runners (b, e, h, k), measured immediately (0 h) or 3 h after 40 min of dynamic muscle contractions (Stimulation) or time-matched, non-stimulated control condition (Control). Comparisons between sedentary and runners for stimulated data relative to control are displayed in (c, f, i, l). Interaction and main effects derived from linear mixed-effects analysis are displayed to the right of each panel. *Difference vs. control within time point as evaluated by pairwise comparisons. #Difference in stimulated–control contrasts between time points as evaluated by linear comparison analysis. Bars represent means ± SD. $n = 9$ and 8 for each condition and time point in sedentary and running trained group, respectively

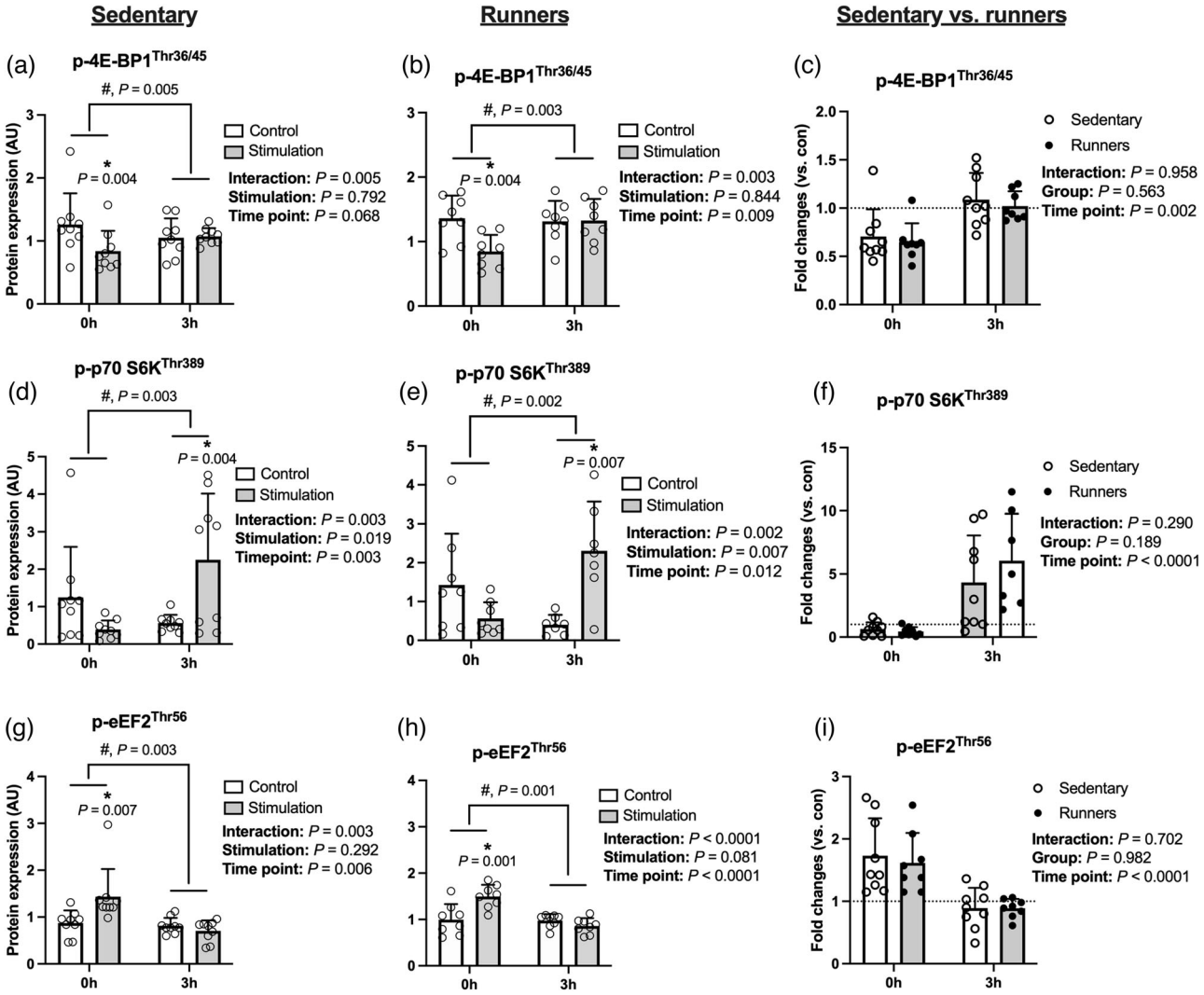

FIGURE 5 Protein signalling related to the mechanistic target of rapamycin complex 1 (mTORC1) substrates of eukaryotic initiation factor 4E-binding protein (4E-BP1) and p70 S6 kinase (p70 S6K), as well as eukaryotic elongation factor 2 (eEF2). Protein expression of phosphorylated 4E-BP1, p70 S6K and eEF2 in soleus muscle preparations of controls (a, d, g) and runners (b, e, h), measured immediately (0 h) or 3 h after 40 min of dynamic muscle contractions (Stimulated) or time-matched, non-stimulated control condition (Control). Bars represent means and lines paired control-stimulation muscle split preparations pairs in (a, b, d, e, g, h). Comparisons between sedentary and runners for stimulated data relative to control are displayed in (c, f, i). Interaction and main effects derived from linear mixed-effects analysis are displayed to the right of each panel. *Difference vs. control within time point as evaluated by pairwise comparisons. #Difference in stimulated–control contrasts between time points as evaluated by linear comparison analysis. Bars represent means ± SD. $n = 9$ and 8 for each condition and time point in sedentary and running trained group, respectively, except for $n = 7$ in p70 S6K in running trained at 3 h time point due exclusion of one outlier pair

3.6 | Signalling related to translation initiation and elongation

Phosphorylation of 4E-BP1 was downregulated immediately after stimulation in muscle samples from both intervention groups (Figure 5a, b, respectively). Accordingly, relative to control condition, p-4E-BP1 expression was 0.7 ± 0.3 and 0.7 ± 0.2 lower at the 0 h time point in muscle samples from sedentary and runners, respectively ($P = 0.004$ for both groups). At the 3 h time point, p-4E-BP1 expression was comparable to control levels for both sedentary ($P = 0.792$) and runners ($P = 0.844$), indicating a return to baseline levels. For p-p70 S6K, an increase corresponding to a 4.3 ± 3.7-fold ($P = 0.019$, Figure 5d) and 6.1 ± 3.7-fold ($P = 0.007$, Figure 5e) greater expression in stimulated muscle samples from sedentary and runners was observed 3 h after stimulation, respectively, when compared to control condition. No differences between intervention groups regarding the response to stimulation were observed for either p-4E-BP1 (interaction $P = 0.958$, Figure 5c) or p-p70 S6K ($P = 0.290$, Figure 5f). Phosphorylation of eEF2 increased 1.7 ± 0.6-fold and 1.6 ± 0.5-fold above control levels immediately after stimulation in muscle samples from sedentary ($P = 0.007$, Figure 5g) and runners ($P = 0.001$, Figure 5h), respectively. Three hours after stimulation, p-eEF2 had

returned to control levels in both sedentary and runner muscle samples (P = 0.291 and 0.081, respectively). No differences were observed between groups in the response to stimulation for p-eEF2 (interaction P = 0.702, Figure 5i).

4 | DISCUSSION

In the current study, we utilized a purpose-designed *ex vivo* dynamic electrostimulation model to resemble endurance exercise. This protocol was employed to evaluate myofibre protein signalling in in vivo running-trained rats as compared to age-matched sedentary control rats. The main finding was that despite the model-produced recognizable patterns in canonical signalling for metabolic gene transcription and translation initiation, prior in vivo running training did not produce major changes in contraction-induced in protein signalling.

To achieve an endurance trained state, we employed a commonly used in vivo voluntary running model, i.e., a self-paced exercise modality relying on habitual, spontaneous, rodent activity behaviour. Average daily and total running distances performed by the running group were within ranges of previous observations (Broch-Lips et al., 2011; Hyatt et al., 2019; Manabe et al., 2013; Rodnick et al., 1989). Moreover, we observed greater heart wet weight (i.e., indicative of cardiac hypertrophy), greater soleus mass and greater glycogen content in the trained rats. Collectively, these results support several previous observations that the training model produces adaptations known to assist aerobically demanding work (Broch-Lips et al., 2011; Brown et al., 1992; Henriksen & Halseth, 1994; Legerlotz et al., 2008; Rodnick et al., 1989).

To achieve an endurance exercise-mimicking single-bout exercise session that was comparable between animals, we employed a purpose-designed dynamic *ex vivo* electrostimulation protocol to attempt to resemble a stretch-shortening contraction pattern of in vivo running exercise contraction. As recently described (Jakobsgaard et al., 2021), using a combination of low-frequency electrical pulses delivered in intermittent bursts and length manipulation of the muscle–tendon unit in stretch–shortening sine waves, we attempted to reproduce the activity patterns of in vivo rodent locomotion during running (Bernabei et al., 2017). Using this approach, no between-group differences emerged for 30 s average peak forces relative to P_o and for summed force–time integral during the 40-min stimulation. Accordingly, the matching in absolute work (force–time integral) and peak force performance relative to P_o, as well as the comparable between-group relative declines in glycogen content, support that contractile performance during the 40-min of *ex vivo* stimulation was similar for the runners and the sedentary controls. Noteworthy, the average drop in P_o after stimulation was more pronounced (i.e., by ~8 percentage points) in isolated muscle preparations of the control group. This indicates that the trained muscle preparations of the running group were more fatigue resistant.

4.1 | Influence of training status on signalling for transcriptional regulation of metabolic genes

In accordance with our previous findings in younger rodent fast- and slow-twitch phenotype muscles (Jakobsgaard et al., 2021), we observed activation of AMPKα and ACC phosphorylation at 0 h followed by return to control levels at the 3 h time point. Both tumour suppressor protein p53 and CREB are putative AMPK substrates that are considered to assist transcription of genes related to skeletal muscle oxidative metabolism (Bartlett et al., 2014; McGee & Hargreaves, 2010). To our knowledge, no studies have previously examined the activation of p53 by phosphorylation in relation to *ex vivo* dynamic muscle contractions. Our analysis revealed a tendency towards an effect of stimulation on p53 phosphorylation in both groups in the present study, indicating that it is sensitive to intrinsic factors inherent of the *ex vivo* stimulation paradigm. Phosphorylation of both CREB and AMPKα has previously been demonstrated after endurance exercise in endurance exercise-accustomed humans (Vissing et al., 2013). Our analysis did not reveal a stimulation effect on CREB phosphorylation. However, when evaluating the effect of stimulation in both groups separately within each time point (see supplemental Statistical Summary table), phosphorylation increased for both CREB and p53.

Post-exercise changes in myocellular phosphorylation of AMPKα, ACC and p53 have previously been observed to be attenuated following endurance training in humans, when testing with the same absolute exercise workload in relation to biopsy sampling (Benziane et al., 2008; Granata et al., 2019; Lee-Young et al., 2009; McConell et al., 2005), but not when employing the same relative workload (i.e., same percentage of $\dot{V}_{O_2peak}$) (Wilkinson et al., 2008). A common adaptation to endurance exercise training is lowered physiological responses of central and peripheral myofibre outcomes (e.g., heart rate, blood lactate, AMP/ATP ratio, PCr and glycogen utilization) to a given absolute workload (Egan & Zierath, 2013; McConell et al., 2005). Accordingly, attenuated homeostatic disturbances in myofibres could explain the attenuated signalling responses (such as AMPKα phosphorylation) to a given absolute workload following a training period. In this regard, comparisons of relative workload of in vivo settings versus *ex vivo* settings may present a challenge. Common in vivo practices involve using an intensity anchor related to whole-body metabolism (e.g., % $\dot{V}_{O_2peak}$) or absolute workload (e.g., watts or running velocity) (Jamnick et al., 2020), whereas *ex vivo* approaches in isolated muscles allow for matching of workload relating to, for example, intrinsic contractile capacity (such as % P_o) or total force output (such as force–time integral). In the present study, average contractile workload between the groups was matched in relative terms (% P_o) as well as absolute terms (summed force–time integral) during the 40-min main stimulation protocol. This may explain the lack of influence of prior training on metabolic protein signalling in response to *ex vivo* contractile activity. Support for this notion can be inferred from the observation of comparable glycogen utilization during stimulation in both groups. However, it has previously been reported

that a lack of differentiated metabolic signalling despite matched glycogen utilization can occur (McConell et al., 2005; Nordsborg et al., 2010). Further clarification on this could potentially be obtained by inclusion of a broader spectrum of metabolite measures. Noteworthy, a previous animal study using 1 week of low-frequency in vivo electrostimulation has reported attenuated AMPKα phosphorylation in white, but not red, rat tibialis anterior muscle (Ljubicic & Hood, 2009). Moreover, Durante et al. (2002) have reported greater AMPK enzyme activity in red, but not white, quadriceps muscle immediately after in vivo running exercise matched by absolute workload in endurance exercise-untrained versus trained rats. Hence, a predisposed muscle phenotype may affect protein signalling responses more than prolonged training (Jakobsgaard et al., 2021).

4.2 | Influence of training status on contraction-stimulated protein signalling related to translation initiation and elongation

We observed an increase in signalling for translation initiation after AMPK signalling had subsided. In accordance, at 0 h after stimulation, 4E-BP1 phosphorylation was suppressed in both groups, but increased back to control level at the 3 h time point concurrently with an increase in p70 S6K phosphorylation. Increased phosphorylation of p-p70 S6K^{Thr389} and p-4E-BP1$^{Thr36/45}$ is considered a putative readout of translation initiation (Figueir, 2017). Moreover, eEF2 phosphorylation was increased in both groups immediately after stimulation, indicating a suppression of elongation activity (Browne & Proud, 2002; Rose, Alsted, et al., 2009), followed by a return to control levels at 3 h. Metabolically demanding contractile work is thought to induce a transient AMPK- and/or Ca^{2+}/calmodulin-mediated suppression of myofibre mTORC1 and eEF2 elongation activity (Atherton et al., 2005; Dreyer et al., 2006; Rose, Alsted, et al., 2009; Rose, Bisiani, et al., 2009). However, some findings from in vivo exercise studies have indicated that coinciding increases in both AMPK and mTORC1 signalling can also co-exist in the recovery period following endurance exercise (Benziane et al., 2008; Camera et al., 2010; Rose, Alsted, et al., 2009).

Evaluation of the effect of training status on activation of p70 S6K in response to endurance exercise (0–4 h after) may, similarly to AMPK, be influenced by standardization choices of either relative or absolute workload in humans (Benziane et al., 2008; Wilkinson et al., 2008). In accordance, mTORC1 downstream signalling has recently been demonstrated to closely relate to mechanical tension in a dose–response manner (Rindom & Vissing, 2016; Rindom et al., 2019). Thus, accustomization to a given magnitude of mechanical tension may lower downstream mTORC1 signalling for translation initiation. In support, it has recently been demonstrated that short-term accustomization (1–3 bouts) to high-frequency isometric in situ contractions, matched by absolute force–time integral, could reduce p70 S6K and 4E-BP1 phosphorylation (Kotani et al., 2021). Albeit assessed at a later time point, work by Ogasawara et al. (2013) also observed attenuated phosphorylation of p70 S6K, but not 4E-BP1, in rat gastrocnemius muscle post-stimulation (24 h) after 12 and 18 bouts of high-frequency in situ electrostimulation. However, in contrast to our mode of stimulation, the in situ electrostimulation protocol applied in these studies were designed to resemble the motor recruitment pattern of resistance exercise, so outcomes are likely to differ.

4.3 | Limitations and future directions

On a general note, while translational approaches using both human and rodent experimental models in synergy aid in evaluating contraction-induced molecular responses in myofibres (Knudsen et al., 2021), differential molecular responses between species may exist (Nelson et al., 2019). Hence, this should be acknowledged as a limitation, as the present application of rat skeletal muscle preparations may entail that the findings are specific to this species. Consequently, the *ex vivo* approach excludes the potential influence of some exercise-induced humeral cues on myocellular signalling, such as endocrine cues (Egan & Zierath, 2013; Gonzalez et al., 2016). Substrate availability for the muscle preparation in the buffer chamber may moreover be considered more constant than in in vivo regimens. Additionally, while the ability to standardize the contractile stress between groups is a methodological strength of the *ex vivo* contraction model, it may be argued that such strict standardization is not possible to achieve in in vivo exercise models. Collectively, the model may be considered to be more limited to evaluation of the effects of intrinsic myocellular cues with limited transferability to human in vivo conditions. On the other hand, *ex vivo* electrostimulated dynamic contractions affect, for example, ATP-turnover, Ca^{2+} oscillations and mechanical tension development (Jensen et al., 2007; Rindom et al., 2019; Wright et al., 2007), which are all considered important upstream cues for canonical AMPK and/or mTORC1 signalling (Egan & Zierath, 2013).

On a specific note, in the current study, due to the size of the soleus muscles from the 14-week-old rodents, muscles were split in two (tendon-to-tendon) to reduce the circumference of the muscle preparations. This was done to optimize diffusion conditions of oxygen and substrates in organ baths. While this approach has been utilized in *ex vivo* settings before (Broch-Lips et al., 2011), it cannot be excluded that this procedure may introduce variation in phosphorylation of proteins sensitive to mechanical stress (e.g., mTORC1-associated proteins).

Finally, our selection of signalling biomarkers may be considered rather narrow. Accordingly, future studies could conduct a more broad-spectrum evaluation of proteome responses, with evaluation at other regulatory levels (e.g., level of transcription or other epigenetic modifications), or in redundant protein signalling pathways.

5 | CONCLUSION

In summary, our findings do not support the contention that training status alters canonical myofibre protein signalling in response to intrinsic cues inherent in endurance-type *ex vivo* dynamic muscle contractions. These findings add to the current understanding of

molecular regulatory events responsible for skeletal muscle plasticity, and the extent to which blunting of molecular responses by myofibre accustomization to exercise training may be an important aspect to consider.

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COMPETING INTERESTS

The authors declare no conflicts of interests or financial interests.

AUTHOR CONTRIBUTIONS

J.E.J., F.D.P. and K.V. conceived and designed the present study. J.E.J. performed most of the animal intervention and subsequent experiments. Data interpretation was done by J.E.J. and K.V. Initial manuscript draft was written by J.E.J., with subsequent by revision by K.V. and F.D.P. All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

DATA AVAILABILITY STATEMENT

Upon reasonable requests, the analysed datasets are available from the corresponding author.

ORCID

Jesper Emil Jakobsgaard https://orcid.org/0000-0003-1818-9860
Frank Vincenzo de Paoli https://orcid.org/0000-0002-2174-2295
Kristian Vissing https://orcid.org/0000-0001-5810-5241

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SUPPORTING INFORMATION

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