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ORIGINAL ARTICLE

Three weeks of sprint interval training improved high-intensity cycling performance and limited ryanodine receptor modifications in recreationally active human subjects

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Abstract

Purpose Mechanisms underlying the efficacy of sprint interval training (SIT) remain to be understood. We previously reported that an acute bout of SIT disrupts the integrity of the sarcoplasmic reticulum (SR) Ca^{2+} release channel, the ryanodine receptor 1 (RyR1), in recreationally active human subjects. We here hypothesize that in addition to improving the exercise performance of recreationally active humans, a period of repeated SIT sessions would make the RyR1 protein less vulnerable and accelerate recovery of contractile function after a SIT session.

Methods Eight recreationally active males participated in a 3-week SIT program consisting of nine sessions of four–six 30-s all-out cycling bouts with 4 min of rest between bouts.

Results Total work performed during a SIT session and maximal power (W_{max}) reached during an incremental cycling test were both increased by ~ 7.5% at the end of the training period ($P < 0.05$). Western blots performed on vastus lateralis muscle biopsies taken before, 1 h, 24 h and 72 h after SIT sessions in the untrained and trained state showed some protection against SIT-induced reduction of full-length RyR1 protein expression in the trained state. SIT-induced knee extensor force deficits were similar in the untrained and trained states, with a major reduction in voluntary and electrically evoked forces immediately and 1 h after SIT ($P < 0.05$), and recovery after 24 h.

Conclusions Three weeks of SIT improves exercise performance and provides some protection against RyR1 modification, whereas it does not accelerate recovery of contractile function.

Keywords Physical exercise · Skeletal muscle · Sprint interval training · Ryanodine receptor 1

Abbreviations

$[Ca^{2+}]_i$	Cytosolic free $[Ca^{2+}]$
DHPR	Dihydropyridine receptor
MVC	Maximal voluntary contraction
PS10	Supramaximal paired electrical stimulation pulses at 10 Hz
PS100	Supramaximal paired electrical stimulation pulses at 100 Hz
RyR1	Ryanodine receptor 1
SERCA2	Sarcoplasmic reticulum Ca^{2+} ATPase 2
SIT	Sprint interval training
SR	Sarcoplasmic reticulum
VAL	Voluntary activation level
W_{max}	Maximal power reached during incremental exercise test

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Introduction

High-intensity interval training regimes consisting of short bouts of exercise performed at maximal or supramaximal intensities alternated with recovery periods (sprint interval

training, SIT) have emerged as popular alternatives to continuous endurance training (Gibala and McGee 2008; Gibala et al. 2014). In spite of a low training volume, SIT increases cardiac output, maximal oxygen uptake, and skeletal muscle mitochondrial content to a similar or even greater extent than traditional endurance training (Hebisz et al. 2016; Astorino et al. 2017; Paquette et al. 2017; Gillen et al. 2016).

The exact mechanisms underlying muscular adaptations to SIT remain incompletely understood. An increased cytosolic free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) at rest is known to stimulate mitochondrial biogenesis in skeletal muscle (Bruton et al. 2010; Wright et al. 2007). The ryanodine receptor 1 (RyR1), a 2.3 MDa Ca^{2+} channel (Zalk et al. 2015), is the core of a multi-protein complex, which controls the release of Ca^{2+} from the sarcoplasmic reticulum (SR) to the cytosol (Zalk and Marks 2017). Altered integrity of the RyR1 protein complex has been linked to increased SR Ca^{2+} leak and elevated $[Ca^{2+}]_i$ at rest, increased mitochondrial biogenesis and hence improved fatigue resistance (Bruton et al. 2010; Ivarsson et al. 2019). We recently observed fragmentation of RyR1 leading to a decreased abundance of the full-length RyR1 protein in Western blots performed on muscle biopsies obtained from untrained individuals 24 h after a single SIT session (Place et al. 2015). Moreover, experiments on isolated mouse muscle fibres revealed a SIT-induced SR Ca^{2+} leak and, as a result, a prolonged increase in $[Ca^{2+}]_i$ at rest (Place et al. 2015). In contrast to untrained subjects, endurance athletes showed no decrease in full-length RyR1 after one SIT session (Place et al. 2015), which indicates that training protects against SIT-induced RyR1 alteration. However, due to the cross-sectional design of our previous study, it remains unclear whether the increased resistance against RyR1 fragmentation in endurance athletes was due to muscles adapting to the stress caused by repeated training sessions and/or to different baseline characteristics between the athletes and the untrained individuals.

We here report an intervention study where recreationally active males were subjected to a 3-week SIT program with measurements of cycling exercise performance, full-length RyR1 protein expression and recovery of isometric force at the start and end of the training period. We hypothesized that in addition to improving exercise performance, the 3-week period of repeated SIT sessions would induce some protection against RyR1 protein modifications and accelerate the recovery of contractile function after a SIT session.

Methods

Ethical approval

The study protocol was approved by the Research Ethics Committee of the Vaud canton (98/15) and was in agreement with the latest revision of the Declaration of Helsinki.

Participants and experimental protocol

Potential participants were recruited through paper and online advertisement at the University of Lausanne. Study subjects had to be male, healthy, between 20 and 40 years of age and physically active but not engaged in any structured training. Participants with musculoskeletal conditions or with known reactions to anaesthesia were excluded. Eight eligible, recreationally active males (for participant characteristics see Table S1) were informed of the experimental procedures and gave their written informed consent for participation.

All participants reported to the laboratory at least 48 h prior to the first SIT session for familiarization with neuromuscular testing and an exercise test for assessment of maximal cycling power (W_{max}). The exercise test was performed on a cycle ergometer (Excalibur Sport, Lode, Netherlands) starting at 0 W with increments of 1 W every 2 s until failure (for individual results see Table S1).

The 3-week training program consisted of nine SIT sessions (3 sessions/week) whereof the first and the last were part of a testing session. Each SIT session comprised a warm-up (5 min at ~ 100 W), four–six repetitions of 30-s all-out cycling bouts at 0.7 Nm/kg body weight (Wingate tests) with 4 min of rest between bouts and a cool down (according to individual preference). Sessions 1 and 7–9 were composed of six sprints; sessions 2 and 3 were composed of four sprints and sessions 4–6 were composed of five sprints (Fig. 1a). The same cycle ergometer with continuous power recording was used to quantify the amount of work produced during each session. Throughout the 3-week intervention, participants were asked to maintain their usual physical activities.

The two testing sessions started with neuromuscular testing of the knee extensors and a *vastus lateralis* muscle biopsy. After that, the participants warmed up and performed one SIT session (six sprints). Assessment of neuromuscular function was repeated immediately after finishing the last 30-s sprint and then again 1 h, 24 h and 72 h after exercise. Post-exercise muscle biopsies were collected 1 h, 24 h and 72 h after the first SIT session, and 1 h and 24 h after the last SIT session (Fig. 1b). The two testing sessions were scheduled at the same time of the day ($\pm$ 1 h) and participants were asked to refrain from caffeine intake and intense physical activity before the sessions (for 12 h and 24 h, respectively).

A second incremental exercise test was performed 72 h after the last SIT session for assessing W_{max} after training.

Neuromuscular testing of knee extensors

Neuromuscular function testing consisted of a 3–4-s maximal voluntary contractions (MVC) during which a 100-Hz

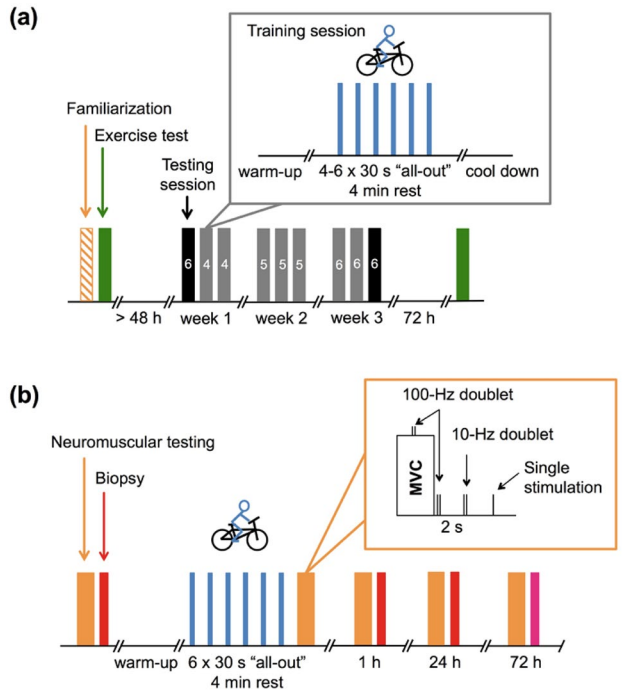

Fig. 1 Overview of the study intervention and the testing procedures. **a** Overview of all laboratory visits. Numbers in the testing and training session bars indicate the number of sprints performed during that session. **b** Illustration of the testing procedures during the first and last SIT sessions. At 72 h, biopsies were only obtained after the first SIT session. *MVC* maximal voluntary contraction

paired electrical stimulation (pulse width 1 ms) was delivered to the femoral nerve. This was followed every 2 s by supramaximal paired electrical stimulation pulses at 100 Hz (PS100) and 10 Hz (PS10), and a single supramaximal electrical stimulation pulse to measure the maximal M-wave amplitude (Fig. 1b). The experimental setup for neuromuscular testing was described previously (Place et al. 2015). Briefly, participants were seated on a custom-built chair with a knee angle of 90° and a trunk–thigh angle of 100° (180° = full extension) for force measurements and electrical stimulations. Isometric MVC force was defined as the peak force attained during each MVC. For electrical stimulations, at each testing occasion, the current intensity was set to 120% of the current required to obtain maximal twitch force and M-wave amplitude (Neyroud et al. 2014). Amplitudes of PS10 and PS100 evoked forces were quantified and the PS10/100 ratio was used to quantify low-frequency force depression. Voluntary activation level (VAL), an index of central fatigue, was calculated as (1 − superimposed PS100 evoked force/resting PS100 evoked force) × 100. A correction was applied to account for PS100 being delivered slightly prior to or after peak force (Strojnik and Komi 1998). M-wave peak-to-peak amplitude was measured from the electromyographic activity of the *vastus lateralis* muscle in response to single supramaximal electrical stimulation as previously described (Place et al. 2015). All data were stored and analyzed off-line using Acqknowledge software (Biopac Systems).

Muscle biopsies

Needle biopsies from the dominant *vastus lateralis* muscle were taken using previously described procedures (Magistris et al. 1998). Briefly, after alcohol cleaning and local anaesthesia, a 1–2-mm-long skin cut was made with a scalpel tip. A 14-gauge disposable needle was then inserted perpendicular to the muscle fibres until the fascia was pierced and three biopsy samples (~ 15 mg each) were collected with an automatic biopsy device (Bard Biopsy Instrument, Bard Radiology, Covington, USA). Samples were immediately frozen in liquid nitrogen and stored at − 80 °C until analysis.

Western blot

Biopsies were homogenized as previously described (Place et al. 2015). Samples were diluted 1:1 in loading buffer (4 M urea, 20% glycerol, 250 mM Tris, 4% SDS, 0.5 mg/100 mL bromophenol blue, 5% β-mercaptoethanol (v/v), pH 6.8), heated (5 min; 95 °C) and loaded on 4–12% precast Bis–Tris gels (NP0336, NuPAGE; 10 µg protein/well). After transfer and blocking (see (Place et al. 2015)), membranes were incubated overnight at 4 °C with 1:3000 mouse anti-RyR1 (ab2868, Abcam) and 1:500 mouse anti-L type Ca^{2+} channel dihydropyridine receptor (DHPR) alpha-2 subunit (ab2864, Abcam) primary antibodies. Washing, incubation with secondary antibody, visualization, and quantification of bands were done as described before (Place et al. 2015). Membranes were stained with Coomassie staining solution (#161-0436, Biorad) and immuno-labelled band intensities were normalized to total protein content of each lane.

Statistical analyses

Statistical analyses were performed with SigmaPlot software (v11; Systat, Chicago, USA). Power analyses were performed with desired power set to 0.9 and alpha set to 0.05. A meta-analysis of the response to a short period of repeated SIT sessions provided an expected increase in exercise performance of ~ 6% (SD 3%) (Weston et al. 2014). Place et al. (2015) showed ~ 70% (SD 10%) lower full-length RyR1 expression 24 h after a SIT session in untrained subjects than in endurance athletes; this protection is likely larger than what can be expected after only 3 weeks of training and we set the difference to be detected to 20%. Power analyses performed with these values gave a sample size of five subjects and we decided to include eight subjects in the study.

Paired *t* tests were used to test for differences in total work and W_{max}; for these measurements we also calculated the effect size as ($mean_{trained} - mean_{untrained}$)/$SD_{untrained}$). Two-way repeated measures ANOVA (training status × time) was used for the analysis of RyR1 and DHPR protein quantification, MVC, PS10 and PS100 forces, VAL, and M-wave amplitude during SIT stimulations. In case of significant main effect, post hoc analyses were performed using the Holm–Sidak method. The α level for statistical significance was set to $P < 0.05$. Data are reported as mean ± SD.

Results

Improved performance during SIT and increased W_{max} after 3 weeks of SIT

In this study, recreationally active participants were exposed to four–six all-out 30-s Wingate-cycling bouts three times per week for 3 weeks. Table S1 provides a data set at the individual level of participant characteristics and the training effect on cycling performance. To verify that this training protocol had a positive effect on sprint power (Weston et al. 2014), we quantified power output during each of the six Wingate-cycling bouts at the beginning and at the end of the training period. While power output for the first cycling bout was similar in the untrained and trained states, higher power outputs were produced during the remaining intervals in the trained state (Fig. 2a). Hence, total work performed during the last SIT session was 7.4% higher than that during the first SIT session ($P = 0.0081$; effect size = 0.86) (Fig. 2b). In addition, W_{max} reached during the incremental cycling test was 7.8% higher after the 3-week training period ($P = 0.0019$; effect size = 0.73) (Fig. 2c).

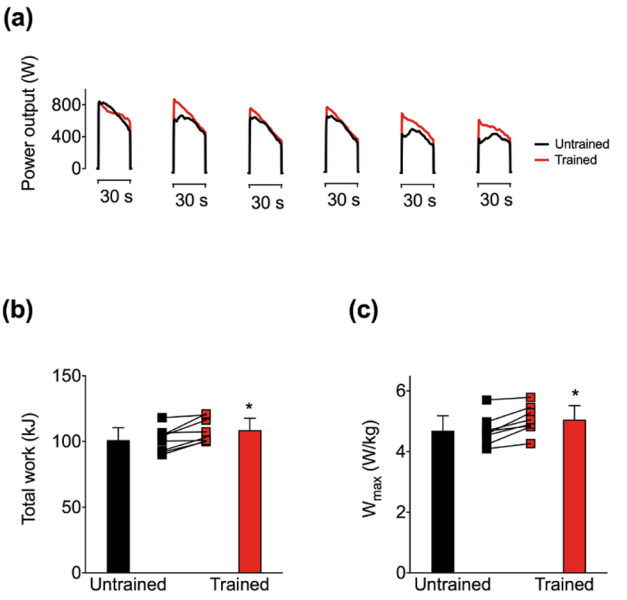

Fig. 2 Three weeks of SIT improve exercise performance in recreationally active subjects. **a** Typical power output traces during the six Wingate cycling bouts in the untrained and trained states. **b** Total work performed during the six Wingate cycling bouts of SIT sessions in the untrained and trained states. **c** Maximal power (W_{max}, normalized to body mass) reached during the incremental exercise test prior to the first SIT session and after the last SIT session. Symbols connected by lines in **b** and **c** represent data from each individual; bars represent mean ± SD ($n = 8$); *$P < 0.05$ untrained vs. trained with paired *t* test

SIT-induced RyR1 modification at the start and end of 3 weeks of SIT

We previously observed a substantial decrease in full-length RyR1 protein expression 24 h after a SIT session in untrained individuals but not in endurance-trained athletes (Place et al. 2015). Based on this finding, we here hypothesized that exposing untrained individuals to repeated SIT sessions would induce protection against RyR1 alterations. Full-length RyR1 protein expression was assessed in muscle biopsies before and after the 3-week training period. Due to limited muscle biopsy material, Western blotting could not be performed at all seven time points in all participants. The expression of full-length RyR1 was lower in the untrained than in the trained state at 24 h after the SIT session ($P = 0.018$, Fig. 3a). The expression of full-length RyR1 expression showed full recovery 72 h after the SIT session in the untrained as well as in the trained state (the latter measured before the last SIT session, i.e. corresponding to the pre point in the trained state). The expression of the t-tubular voltage sensor, the DHPR, which plays a key role in skeletal muscle excitation–contraction coupling by physically interacting with the RyR1 (Dulhunty 2006), did not differ between the untrained and trained states ($P = 0.60$, Fig. 3b).

There was a large variability in the expression of full-length RyR1 expression after SIT sessions. We, therefore, extended our analyses to the individual level with focus on 24 h after SIT sessions, where we previously observed an ~ 85% decrease in full-length RyR1 expression in untrained subjects (Place et al. 2015). At 24 h after SIT sessions, RyR1 analysis could be performed on biopsies from six participants in the untrained state and seven participants in the trained state and the results revealed two broad groups: susceptible, where the full-length RyR1 was < 60% of the pre-value (Fig. 3c), and resistant, where it was > 80% of the pre-value (Fig. 3d). In the untrained state, RyR1 was susceptible in five subjects and resistant in one subject. The opposite trend was observed in the trained state where RyR1 was susceptible in only two out of seven subjects. A striking feature was revealed when relating these RyR1 expression data at the individual level to the corresponding

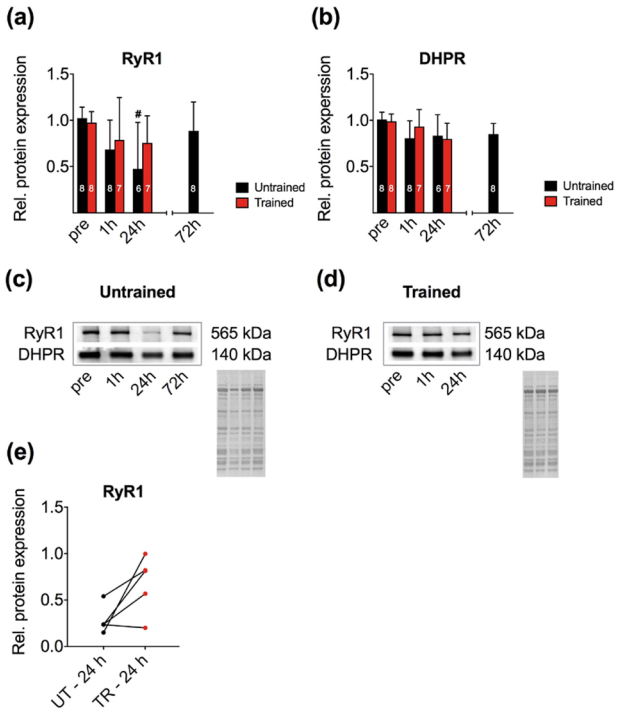

Fig. 3 Three weeks of repeated SIT sessions provide some protection against the SIT-induced reduction of full-length RyR1 protein expression. Full-length RyR1 (**a**) and DHPR (**b**) protein expressions before and at different time points after SIT in the untrained and trained states. Band intensities were normalized to total protein and expressed relative to the mean of the pre-values in each subject, which was set to 1. Data are displayed as mean ± SD, the number of subjects is indicated in each bar. $^{\#}P < 0.05$ untrained vs. trained state with two-way repeated measures ANOVA followed by Holm–Sidak post hoc test. Representative RyR1 and DHPR bands from Western blots before (pre) and 1 h, 24 h and 72 h after a SIT session in the untrained (**c**) and trained (**d**) states; inserts show Coomassie protein staining of the membranes, which were used for normalization of RyR1 and DHPR band intensities to total protein content of each lane. Note that reliable Western blots were not obtained 72 h after the SIT session in the trained state. **e** Individual data for RyR1 expression 24 h after SIT sessions in the untrained (UT) and trained (TR) states

cycling exercise performance. The two susceptible subjects in the trained state showed 12% and 16% increases in the work performed in the last compared to the first SIT session, which were markedly larger than the mean increase of 5% in the resistant subjects, and this occurred despite an average training-induced increase in the power at exhaustion in the incremental cycling test (3.8 and 4.2% vs. a mean of 5.5% in the non-responders).

Western blotting could be performed at 24 h after SIT sessions both in the untrained and trained states in five participants and these were used to specifically test our hypothesis that 3 weeks of repeated SIT sessions would make the RyR1 less vulnerable. Before the SIT sessions, Western blots showed no difference in the expression of full-length RyR1 between the untrained and trained state (relative expression 0.97 ± 0.12 vs. 1.03 ± 0.12; $P = 0.64$), i.e. the resting period between the eighth and ninth SIT sessions was long enough to allow recovery of full-length RyR1 expression. At 24 h after the SIT sessions, on the other hand, four out of five subjects showed a markedly lower expression of full length RyR1 in the untrained than in the trained state (Fig. 3e).

Recovery of neuromuscular function after a SIT session is not affected by 3 weeks of SIT

Neuromuscular function was assessed before and after SIT in the untrained and trained states. MVC force was depressed immediately (~40% decrease) and 1 h (~25% decrease) but not 24 h and 72 h after the SIT session both in the untrained and the trained states (Fig. 4a). VAL was not decreased after SIT either in the untrained or trained state (Fig. 4b). Forces induced by electrical nerve stimulation at low (10 Hz) and high (100 Hz) frequencies were significantly decreased immediately and 1 h after SIT with no difference between training states (Fig. 5a–c). Force reductions were larger at 10 Hz than at 100 Hz resulting in a reduced PS10/100 ratio, but again there was no difference between the untrained and trained states (Fig. 5d). There was no SIT-induced decrease in M-wave amplitude before or after training (Fig. 5e).

Discussion

In this study, eight recreationally active male performed 3 weeks of cycling SIT. The cycling sprint power and W_{max} in an incremental cycling test were increased at the end of this 3-week training period. Muscle biopsies from a majority of participants showed a marked decrease in full-length RyR1 protein expression 24 h after the first SIT session, whereas the majority of muscle biopsies taken after the last SIT session showed limited decrease; thus, the repeated SIT sessions provided protection against RyR1 alteration, although this protection was not complete. Conversely, the 3-week training period did not accelerate the recovery of isometric force after a SIT session.

The participants of our study performed in total 47 all-out cycling bouts, accumulating to a total sprint time of only 23.5 min. Nevertheless, this very low volume exercise training regimen increased sprint power, measured as the increase in total work performed during the last compared to the first SIT session (see Fig. 2a, b), as well as W_{max} reached during the incremental cycling test (see Fig. 2c) by approximately 7.5%. These improvements are comparable to the training effects reported in a recent meta-analysis of similar SIT protocols in sedentary individuals (Weston et al. 2014). Thus, high-intensity cycling performance can be substantially improved with a total SIT duration of only 23.5 min. From this perspective, SIT appears as a time-efficient alternative to

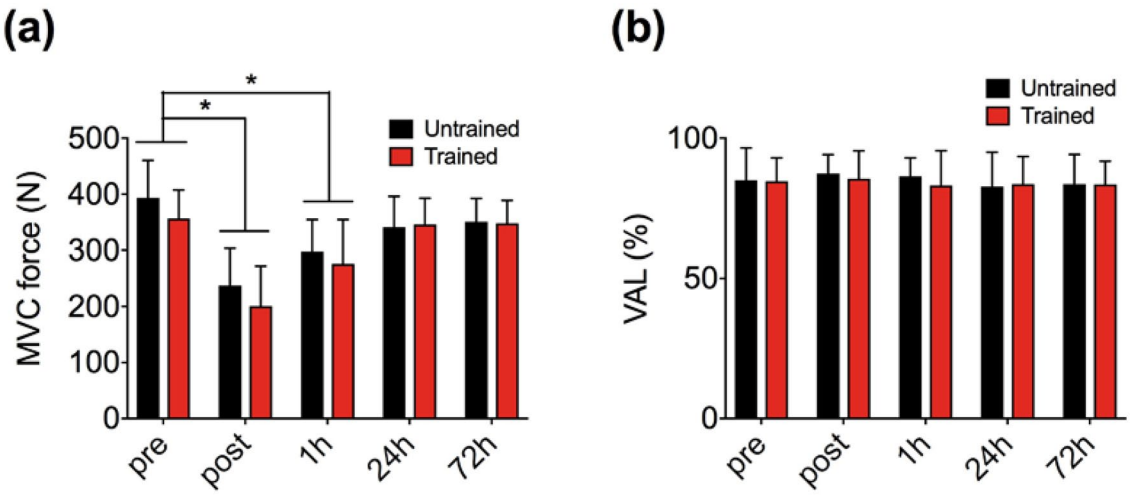

Fig. 4 The SIT induces force depression is of intramuscular origin and independent of training status. **a** Maximal voluntary contraction (MVC) force is reduced immediately and 1 h after a SIT session in the untrained and trained states. **b** Maximal voluntary activation level (VAL) is unchanged after SIT and independent of the training state. Bars represent mean ± SD ($n = 8$); *$P < 0.05$ before *vs*. after SIT session with two-way repeated measures ANOVA; no significant differences between the untrained and trained state were observed

traditional endurance training performed at lower intensities. Still, the training sessions are substantially longer than the short times of actual sprint exercise; the total time required for one SIT session with six sprints was about 35 min when taking into account that SIT sessions also involve warm-up, rest periods between the sprint bouts and cool down.

We recently showed an ~ 85% decrease in full-length RyR1 protein expression 24 h after a SIT session in recreationally active subjects (Place et al. 2015). This is in accordance with the present finding that in the untrained state, full-length RyR1 expression 24 h after the SIT session was < 60% of the pre-value in biopsies from five participants. However, in contrast to our previous study, one participant did not respond with a decrease in full-length RyR1 expression after the first SIT session. In our previous study, we did not observe any SIT-induced decline in full-length RyR1 expression in elite endurance athletes (Place et al. 2015), which indicates that endurance training provides protection against RyR1 degradation. In view of that, we here tested the hypothesis that the 3 weeks of SIT would protect against RyR1 degradation. Accordingly, in the trained state full-length RyR1 expression was > 80% of the pre-value in five out of seven of the present subjects. Moreover, in the five participants where RyR1 expression could be assessed 24 h after both the first and the last SIT sessions, four subjects showed markedly higher full-length RyR1 expression in the trained than in untrained state. Thus, our results show a clear trend for a training-induced protection of RyR1 degradation. Nevertheless, a large decrease in full-length RyR1 expression 24 h after the last SIT session was observed in two of the present participants, i.e. the proposed training-induced RyR1 protection was not absolute. Taken together, these results suggest that the stress induced during a SIT session must reach a certain threshold to initiate RyR1 degradation and that endurance training protects against exceeding this threshold. However, this suggestion is based on the results of few subjects and further studies are required for the proposal to be established or refuted.

RyR1 is a central player in excitation–contraction coupling and modifications in this protein would be expected to affect intracellular Ca^{2+} handling and subsequently force production. Intriguingly, in the untrained state, our human data show depressed MVC, 10 Hz and 100 Hz forces immediately and 1 h after the SIT session, but forces were restored 24 h after exercise when the most marked reduction of full-length RyR1 protein was observed. Moreover, forces were similarly depressed immediately and 1 h after the SIT session in the untrained and trained states. The neural drive was not decreased after SIT either in the untrained or trained state, as judged from unaltered VAL, which indicates that the reduced MVC force has an intramuscular origin. There was no SIT-induced decrease in M-wave amplitude before or after training, which would rule out impaired sarcolemmal excitability as the mechanism behind the decrease in MVC, 10 Hz and 100 Hz forces. The force depression after the SIT sessions was larger with 10 Hz than with 100 Hz stimulation, which implies that it was either due to decreased SR Ca^{2+} release or reduced myofibrillar Ca^{2+} sensitivity (Westerblad et al. 1993; Cheng et al. 2018). A major causative role of decreased SR Ca^{2+} release due to the presently observed reduction in full-length RyR1 protein expression is unlikely because forces had recovered 24 h after the SIT sessions despite remaining decreases in full-length RyR1 expression.

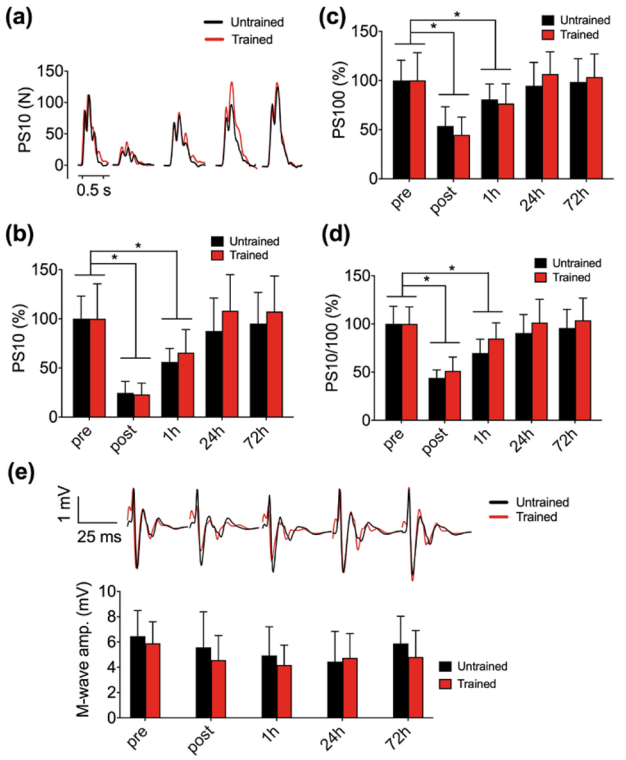

Fig. 5 The SIT-induced decrease in evoked contraction force is more marked at low stimulation frequencies and independent of the training status. **a**, **b** Typical traces and mean values for 10-Hz paired stimulation (PS10) and **c** 100-Hz paired stimulation (PS100). **d** Force depression after SIT is more marked at low stimulation frequencies, leading to a decrease in the PS10/100 ratio. **e** Original records and mean values for M-wave amplitude in the *vastus lateralis* muscle in the untrained and trained states. Original records start 5 ms after stimulation, i.e. excluding the stimulation artefact. Data obtained before (pre) and immediately (post), 1 h, 24 h and 72 h after SIT sessions. All bars represent mean ± SD ($n = 8$); $^{*}P < 0.05$ before *vs*. after SIT session with two-way repeated measures ANOVA; no significant differences between the untrained and trained states were observed

Thus, the cause(s) of the SIT-induced delayed force recovery remains to be established.

Study limitations

This study was performed without control group(s) and, in some instances, this limited our ability to draw robust conclusions. For example, the reproducibility of RyR1 modifications in the untrained state could have been assessed by having subjects only performing the first and last SIT sessions. Due to the unexpectedly large variability in RyR1 degradation, more than the present eight participants would have been required to, for instance, draw solid conclusions regarding the relation between the changes in SR Ca^{2+} handling due to decreased full-length RyR1 expression or other RyR1 modifications and the training-induced increase in cycling performance. Participants performed repeated SIT for only 3 weeks and a longer training period might be required to get full protection against RyR1 degradation, i.e. in this respect, reach a state similar to that of elite endurance athletes. Due to a limited amount of muscle tissue obtained in the present biopsies, we decided to strictly focus on RyR1 and DHPR protein expression. Thus, we did not assess mechanism(s) underlying the variation in RyR1 susceptibility between subjects.

Conclusions

We conclude that 3 weeks of SIT, with a total of only 23.5-min high-intensity exercise, can improve cycling sprint power and W_{max}. Furthermore, it provided some protection against RyR1 modifications, but this did not result in improved force recovery after SIT sessions. The mechanisms underlying the variability in SIT-induced RyR1 modifications and their relation to the physiological training response remain to be investigated.

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Author contributions MS, DN, SK, BK, HW, NP, and DCA conceived and design the study. MS, DN, CT, NZ, HW, and NP acquired, analysed, and interpreted data. MS and DN drafted the manuscript and all authors reviewed and revised it critically for important intellectual content. All authors approved the final version of the manuscript and agreed 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.

Compliance with ethical standards

Conflict of interest The authors declare that no conflict of interests exists.

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